ISOLATION AND DIFFERENTIATION OF *VIBRIO* SPECIES FROM SEAFOOD AND MOLECULAR CHARACTERISATION OF *VIBRIO PARAHAEMOLYTICUS*

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DISSERTATION SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF BIOTECHNOLOGY

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ABSTRACT

Vibrio species are significant causes of gastroenteritis in humans associated with consumption of raw or under-cooked seafood. Pathogenic vibrios are common causes of seafood-borne illness in Southeast Asian countries including Malaysia, where the temperature is optimal for their growth. The objectives of this study were to investigate the prevalence of *Vibrio* spp. in raw seafood using conventional and molecular methods; to compare these methods based on their sensitivity and discriminatory power; and to analyse pathogenicity and genetic variability of *V. parahaemolyticus* isolates.

One-hundred and fifty seafood samples, including fish, shrimps, prawns, cockles, oysters, clams and squids, were collected from retail stores and hypermarkets in Kuala Lumpur, Petaling Jaya and Seri Kembangan. Colony appearance on CHROMagar™ *Vibrio* and thiosulfate-citrate-bile salts-sucrose (TCBS), followed by conventional biochemical tests including oxidase, Triple Sugar Iron (TSI), Sulfur reduction – Indole – Motility (SIM), Methyl Red (MR) and Voges-Proskauer (VP), and salt tolerance tests were used for preliminary identification of *Vibrio* species.API 20E test strip, was applied for biochemical confirmation of vibrios. A multiplex polymerase chain reaction (PCR) targeting *gyrB* gene for detection of *Vibrio* spp., and *pntA* genes for *V. cholerae, V. parahaemolyticus* and *V. vulnificus* was further evaluated on spiked fish, shrimp and oyster, respectively. Sensitivity of the multiplex PCR on spiked seafood was 2.0×10^3 CFU ml^{-1} for *V. cholerae* and *V. parahaemolyticus,* and 9.0×10^3 CFU mL^{-1} for *V. vulnificus.* The multiplex PCR indicated 100% accuracy and 100% specificity, hence it was used for confirmation of the isolates. Based on the multiplex PCR results, 63% (93/150) of the seafood samples, purchased from the retail stores, harboured *Vibrio* spp., where the prevalence of *V. cholerae, V. parahaemolyticus* and *V. vulnificus* was 5.3% (8/150), 29% (43/150) and 8% (12/150), respectively. PCR determination of the

virulence genes in *V. parahaemolyticus* isolates showed that all of the isolates tested (n =50) contained *toxR* gene, while 4% (2/5) and 12% (6/50) contained thermostable direct hemolysin (*tdh*) gene and thermostable direct hemolysin-related (*trh*) gene, respectively. Repetitive Extragenic Palindromic PCR (REP-PCR) was performed to genetically characterize the *V. parahaemolyticus* isolates. Forty-one REP profiles were observed and *V. parahaemolyticus* isolates were categorized into 10 distinct clusters at 80% similarity.

In summary, detection of *Vibrio* spp. was done more efficiently by CHROMagar^{TM} *Vibrio* rather than TCBS. Comparison of the results of biochemical tests with multiplex PCR indicated that API 20E had higher discriminatory power (83%) to differentiate *V. cholerae, V. parahaemolyticus* and *V. vulnificus*, compared to the conventional biochemical tests (56%). The *gyrB/pntA* genes-based multiplex PCR was confirmed as an accurate and efficient screening tool for detection of *Vibrio* spp. Virulent *V. parahaemolyticus* isolates were isolated from seafood and it implies a potential risk to consumers if such seafoods were consumed uncooked. High genetic diversity of the *V. parahaemolyticus* isolates was shown by REP-PCR and it was able to distinguish the isolates with different virulotypes.

ABSTRAK

Spesies *Vibrio* adalah sebab-sebab yang ketara gastroenteritis pada manusia yang dikaitkan dengan pengambilan makanan laut mentah atau kurangmasak.Vibrios patogen merupakan punca biasa penyakit bawaan-makanan laut di negara-negara Asia Tenggara termasuk Malaysia, di mana suhu optimum diperlukan untuk pertumbuhan mereka.Objektif kajian ini adalah untuk menyiasat kejadian *Vibrio* spp. dalam makanan laut mentah menggunakan kaedah pengenalan konvensional dan molekul, untuk membandingkan kaedah-kaedah ini berdasarkan kepekaan mereka dan kuasa diskriminasi, dan menganalisis pathogenicity dan kebolehubahan genetik diasingkan *Vibrio parahaemolyticus*.

150 sampel makanan laut termasuk ikan, udang, udang, kerang, tiram, kepah dan sotong telah dikutip daripada kedai-kedai runcit dan pasar raya besar di Kuala Lumpur, Petaling Jaya dan Seri Kembangan. Penampilan Koloni pada ^{TM}*Vibrio* dan agar 'thiosulfate-citrate-bile salts-sucrose' (TCBS) diikuti oleh ujian biokimia konvensional termasuk 'oxidase', 'Triple Sugar Iron'(TSI), 'Sulfur reduction - Indole –Motility' (SIM), 'Methyl Red' (MR) dan 'Voges-Proskauer'(VP), dan ujian toleransi garam telah digunakan untuk pengenalan awal spesies *Vibrio*. API 20E ujian jalur, telah memohon untuk pengesahan biokimia vibrios. 'Multiplex Polymerase Chain Reaction' (PCR) mensasarkan gen *gyrB* untuk pengesanan *Vibrio* spp, dan gen *pntA* untuk *V. cholerae, V. parahaemolyticus* dan *V. vulnificus* terus dinilai berdasarkan 'spiked'ikan, udang dan tiram, masing-masing. Kepekaan 'multiplex' PCR pada makanan laut'spiked'adalah $2.0x10^3$ CFU mL^{-1} untuk *V. cholerae* dan *V. parahaemolyticus* dan $9.0x10^3$ CFU mL^{-1} untuk *V. vulnificus.* Multiplex PCR menunjukkan ketepatan 100% dan 100% spesifikasi, kerana ia telah digunakan untuk pengesahan yang diasingkan. Berdasarkan kepada kaedah konvensional dan molekul, 63% (93/150) sampel makanan laut melabuhkan

Vibrio spp. di mana kelaziman *V. cholerae, V. parahaemolyticus* dan *V. vulnificus* 5.3% (8/150), 29% (43/150) dan 8% (12/150), masing-masing. PCR penentuan gen 'virulence' dalam *V. Parahaemolyticus* menunjukkan bahawa semua yang diasingkan (n = 50) mengandungi gen *toxR*, manakala 4% (2/50) dan 12% (6/50) yang terkandung 'thermostable direct hemolysin gene'(*tdh*) dan 'thermostable direct hemolysin-related gene'(*trh*), masing-masing. 'Repetitive Extragenic Palindromic' PCR (REP-PCR) telah dilaksanakan untuk ciri-ciri genetik diasingkan *V. parahaemolyticus.* Empat puluh satu profil REP diperhatikan dan diasingkan *V. parahaemolyticus* dikategorikan kepada 10 kelompok yang berbeza pada 80% persamaan.

Secara ringkasnya, CHROMagar^{TM} *Vibrio* lebih effisien didalam pengesanan *Vibrio* spp. berbanding TCBS.Perbandingan keputusan ujian biokimia dan API 20E dengan 'multiplex' PCR menunjukkan bahawa API 20E mempunyai kuasa diskriminasi yang lebih tinggi (83%) untuk membezakan *V. cholerae, V. parahaemolyticus* dan *V. vulnificus*, berbanding dengan ujian biokimia (56%). Gen *gyrB/pntA* berasaskan 'multiplex' PCR telah disahkan sebagai alat saringan yang tepat dan cekap untuk pengesanan *Vibrio* spp. Semua diasingkan 'virulent'*V. parahaemolyticus* daripada makanan laut dan ia berpotensi risiko kepada pengguna jika makanan laut yangdimakan itu tidak dimasak sepenuhnya. Kepelbagaian genetik yang tinggi diasingkan *V. parahaemolyticus* telah ditunjukkan oleh REP-PCR dan ia dapat membezakan diasingkan dengan 'virulotypes' berbeza.

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LIST OF SYMBOLS AND ABBREVIATIONS

%	Percentage
et al.	Et alia
°C	Degree Celsius
spp.	Species
&	And
LB	Luria-Bertani
TCBS	Thiosulphate-citrate-bile salt sucrose
TSI	Triple Sugar Iron Agar
SIM	Sulfur reduction – Indole – Motility test
MR	Methyl Red
VP	Voges-Proskauer
API	Analytical Profile Index
UV	Ultra Violet
DNA	Deoxyribonucleic Acid
PCR	Polymerase Chain Reaction
REP	Repetitive Extragenic Palindromic
dNTP	Deoxynucleotide triphosphate
ddH_2O	Double-distilled water
$MgCl_2$	Magnesium Chloride
NaCl	Sodium Chloride
TBE	Tris-borate EDTA
Taq	*Thermus aquaticus*
Bp	Base pair
µl	microlitre
mL	millilitre
µM	micromolar

mM	millimolar
mg	milligram
g	gram
h	hour/hours
min	minute/minutes
s	seconds
DOI	Date of Isolation
POI	Place of Isolation
Vp	*Vibrio parahaemolyticus*
Vv	*Vibrio vulnificus*
Vc	*Vibrio cholera*
Vm	*Vibrio mimicus*
Va	*Vibrio alginolyticus*
Vf	*Vibrio fluvialis*
Vsp	*Vibrio* sp. (Unknown species)
tdh	Thermostable Direct Hemolysin
trh	Thermostable Direct Hemolysin-Related
Kota Dmn	Kota Damansara
UM	University of Malaya
Sec 17	Section 17
Mut Dmn	Mitiara Damansara
Trop Dmn	Tropicana Damansara
Dmn In	Damansara Intan
K/K	Slant alkaline / Butt alkaline
K/A	Slant alkaline / Butt acidic
A/A	Slant acidic / Butt acidic
K/a	Slant alkaline/ Butt slightly acidic

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CHAPTER 1

INTRODUCTION

The members of the genus *Vibrio* are plentiful in marine and coastal environments and are considered as one of the main causes of gastroenteritis in humans. The majority of infections are attributed to consumption of raw or insufficiently cooked seafood products. The number of *Vibrio* spp. classified as pathogenic strains is at least 11 (Janda *et al*., 1988; Holmberg *et al*., 1992), including *V. cholerae* as the main cause of diarrhea, *V. parahaemolyticus* as the cause of foodborne gastroenteritis (Ozer, *et al*., 2008; Pruzzo, *et al*., 2005) and *V. vulnificus* which is known to cause 95% of all deaths associated with seafood consumption (Rosche, *et al*., 2006). Other pathogenic species includes *V. alginolyticus, V. damsela, V. fluvialis, V. furnissii, V. hollisae, V. metschnikovii, V. cincinnatiensis* and *V. mimicus* (Pruzzo, *et al*., 2005). Food safety is a crucial concern (Jacxsens *et al*., 2009) and marine products as the main source of a large number of pathogenic bacteria, including *Vibrio* spp., need to be taken more into consideration.

Preliminary identification of *Vibrio* species can be performed based on colony appearance on selective media such as thiosulfate-citrate-bile salts-sucrose (TCBS) agar and CHROMagar^{TM} *Vibrio* (Di Pinto *et al*., 2011), followed by conventional biochemical tests including Oxidase, Triple Sugar Iron (TSI), Sulfur reduction – Indole – Motility (SIM), Methyl Red (MR) and Voges-Proskauer (VP), and salt tolerance tests.Media should be prepared with 2-3% sodium chloride to allow the growth of halophilic species.Biochemical confirmation can also be accomplished using commercial identification systems such as the API 20E test kit (DePaola et al., 2003; Di Pinto *et al*., 2011). However it is important to ensure that cultures are suspended in a saline medium to ensure the growth of halophilic species.

Despite a variety of conventional biochemical tests which can be used to identify presumptive bacterial colonies based on their phenotypic profiles, molecular approaches to the identification and characterization of *Vibrio* spp. has been developed and highly

utilized due to their higher sensitivity and specificity rather than the conventional methods (Di Pinto *et al*. 2005). Various Polymerase Chain Reaction (PCR) based methods have been developed and reported which either aim the identification or the characterization of different species in either monoplex or multiplex PCR. The *dnaJ* gene-based PCR developed by Nhung *et al*. (2007a,b) for the differentiation of *Vibrio* spp. , the detection of *V. Parahaemolyticus* based on *gyrB* and *toxR* genes reported by Venkateswaran *et al*. (1998) and Kim *et al*. (1999) and multiplex PCR with *vvh* and *viuB* for the detection of *Vibrio vulnificus*, with *ompU*, *toxR*, *tcpI*, and *hlyA* to detect *V. cholerae*, and with *tlh*, *tdh*, *trh*, and open reading frame 8 for *V. parahaemolyticus* developed by Panicker *et al.* (2004) are some cases in point.

A multiplex PCR targeting *gyrB* gene to detect *Vibrio* species and *pntA* gene for *V. parahaemolyticus*, *V. cholerae*, *V. vulnificus*was reported by Teh *et al.* (2010) in order to differentiate *Vibrio* spp. This multiplex PCR demonstrated high specificity in *Vibrio* species identification. However, this PCR has not been evaluated on seafood samples and it is necessary to determine the sensitivity of this multiplex PCR on seafood samples, as vibriosare widely found in seafood products (Aberoumand, 2010).

One of the most important *Vibrio* species is *V. parahaemolyticus*, a ubiquitous human pathogen which also causes gastroenteritis when consumed in a contaminated seafood (Matsumoto *et al*., 2000; Khan *et al*. 2002). 25% of foodborne diseases are caused by *V. parahaemolyticus* compared to other *Vibrio* species (Feldhusen, 2000) and it is considered as a major cause for these illnesses (DePaola *et al.* 2003; Pruzzo *et al*. 2005; Johnson *et al*. 2009).

toxR gene is present in all *V. parahaemolyticus* isolates, either pathogenic or non-pathogenic (Dileep *et al*., 2003; Sujeewa *et al*., 2009) and has been used for identification of *V. parahaemolyticus* (Sechi *et al*., 2000), because of its specificity for *V. parahaemolyticus*. Thus, the presence of *toxR* gene in a *V. parahaemolyticus* isolate

does not indicate that it is virulent.A *toxR*-based PCR assay was developed by Kim *et al.* in 1999 to determine the presence of *toxR* gene in *V. parahaemolyticus* strains.

Pathogenicity of *V. parahaemolyticus* strains cannot be indicated by the presence of *toxR* gene, as it is present in either pathogenic or non-pathogenic strains. Hence, *toxR* gene can be used for the detection of *V. parahaemolyticus* (Sechi *et al*., 2000). A *toxR*-based PCR assay has been previously developed to detect *toxR* gene in *V. parahaemolyticus* strains (Kim *et al.*, 1999).

The presence of thermostable direct hemolysin gene (*tdh*) and/or the thermostable direct hemolysin-related gene (*trh*) determines the virulence and pathogenicity of *V. parahaemolyticus* (DePaola *et al*. 2003; Robert-Pillot *et al.* 2004; Roque *et al.* 2009). PCR assays have been applied among *V. parahaemolyticus* strains for the detection of *tdh* and *trh* genes. Oligonucleotide primers have been designed for the amplification of these two genes (Lee *et al.* 1993; Tada *et al.* 1992).

Studies on the genomic variation and molecular epidemiology of *V. parahaemolyticus* are often carried out to track sources and spread of the pathogen. Repetitive Extragenic Palindromic PCR (REP-PCR) is an appropriate typing method for *V. parahaemolyticus* strains because of its reproducibility (Wong, 2003; Chakraborty & Surendran, 2009) and high discriminatory ability. It has been well utilized in order to compare strains for epidemiological surveillance (Maluping *et al*., 2008).

High prevalence of *Vibrio* spp. in coastal waters and seafood products of Southeast Asian countries has been reported by many investigators (Wong *et al*., 1999; Zulkifli *et al*., 2009a), due to the optimal condition for their growth. Moreover, the consumption of seafoods and marine products is quite high in these countries, and consequently there is a high risk of infection and diseases associated with *Vibrio* species in Malaysia and other Southeast Asian countries. Consequently, efficient methods for detection, differentiation and characterization of *Vibrio* spp. are required to be included

in screening programmes in order to prevent infections and diseases associated with the pathogenic strains.

Hence, the main aim of this research is to compare and evaluate different conventional and molecular methods for detection and differentiation of *Vibrio* species. Kuala Lumpur, Petaling Jaya and Seri Kembangan were chosen for sample collection in this study, because of their high residential populations. The seafood samples were purchased from the most popular retail stores and hypermarkets in these areas.

The objectives of this research were:

1. To further evaluate the sensitivity and specificity of the multiplex PCR targeting *gyrB* and *pntA* genes for detection and differentiation of *Vibrio* spp.
2. To isolate and identify *Vibrio* spp. by conventional methods and to confirm the isolates by the multiplex PCR.
3. To determine the toxigenicity and virulence level of *V. parahaemolyticus* isolates.
4. To genetically characterize *V. parahaemolyticus* strains by DNA fingerprinting.

CHAPTER 2

LITERATURE REVIEW

2.1 Properties and Characteristics of *Vibrio*

Vibrio species are Gram-negative bacteria with a curve rod shape and polar flagella with sheaths. All of the members are motile, oxidase positive and facultative anaerobes with no spores (Farmer, 1992). The genus *Vibrio* includes approximately 106 species, known to occur naturally in marine and estuarineaquatic ecosystems (Oliver and Kaper, 2001; Harvio-Heath *et al*., 2002; Ozer *et al*., 2008). *Vibrio* species have been frequently associated in many food poisoning outbreaks and they are considered as one of the most important pathogens associated with foodborne and waterborne diseases. Figure 1.1 shows the results of a report published by Morbidity and Mortality Weekly Report (MMWR) on incidence and trends of infection with foodborne pathogens, obtained from 10 sites in the United States, since 1996 till 2010 (Centers for Disease Control and Prevention, 2011). As it is clearly observable, the population of *Vibrio* species has experienced an upward trend through these years. Specially, in the recent years, from 2007 till 2010, a drastic growth in the prevalence of *Vibrio* species has been observed.

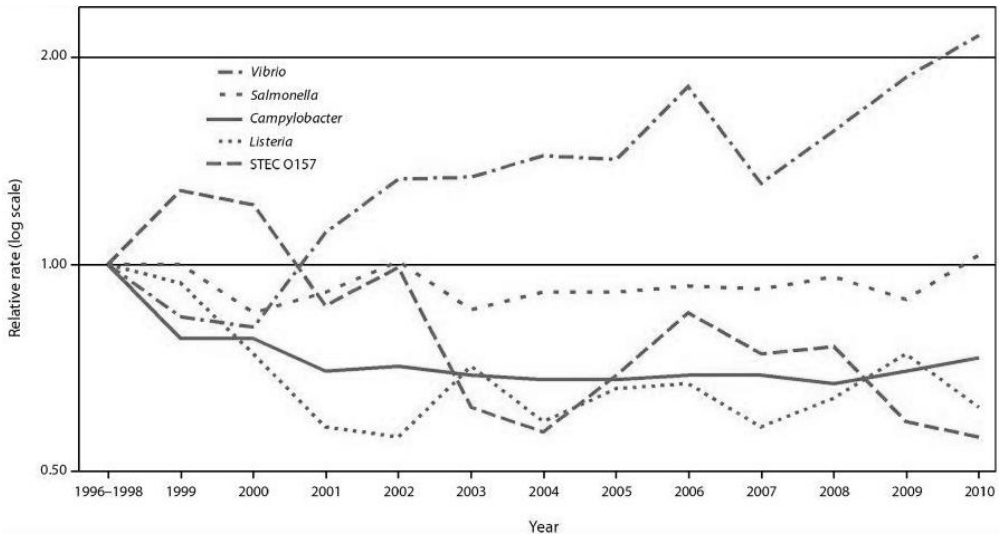


Figure 2.1.1: Relative rates of laboratory-confirmed infections with *Campylobacter*, STEC O157, *Listeria*, *Salmonella*, and *Vibrio*, compared with 1996--1998 rates, by year. Foodborne Diseases Active Surveillance Network, United States, 1996-2010 (Centers for Disease Control and Prevention, 2011).

Mostly they are tolerant to alkaline pH, but sensitive to acid pH. Because of high content of sodium chloride in the habitat, they can stand lower water activity (a_w) which is 0.980 (Madigan *et al*., 2004), but there are also some non-halophilic *Vibrio* species, based on their sodium chloride requirement. Except for the *Vibrio* species that are non-halophilic, such as *V. cholerae* and *V. mimicus*, other *Vibrio* species have the requirement of saline for their growth.

Cytochrome oxidase is produced by the majority of *Vibrio* spp. and they show positive result to oxidase test and this can be used as a characteristic in order to differentiate them from the other enteric microorganisms (Enterobacteriaceae) such as *Escherichia coli* (Madigan *et al*., 2004). Besides, *Vibrio* spp. produce catalase, and as a matter of fact in catalase test, they produce observable bubbles.

As mentioned before, *Vibrio* species are facultative anaerobes. And in both aerobic and anaerobic environments they are able to undergo respiratory and fermentative metabolism, respectively. In addition, they can ferment sugars without producing gas and hydrogen sulphide (Madigan *et al*., 2004). Hence, they can be distinguished from the *Aeromonas* group by their failure in gas production.

2.2 Host Range and Transmissions of *Vibrio*

Highly occurrence of *Vibrio* spp. in marine and aquatic environments, leads to their presence in seafood and any food of freshwater origin, especially from temperate climates around the world. Some species construct relations with aquatic animals and as a matter of fact, they have a wide variety of hosts among marine products including fish, shellfish, oyster, prawn, shrimp, squid and many more freshwater animals (Khan *et al*., 2002; Sujeewa *et al*., 2009). The abundance of *Vibrio* spp. in raw seafood and marine products, makes these sorts of food appropriate for their transmission. And it results in the association of *Vibrio* spp. studies with food safety issues.

2.3 Pathogenicity of *Vibrio* spp.

Among *Vibrio* spp., there are 12 species which have been proved, by different investigations, to be human pathogens that cause diseases associated with seafood (Janda *et al*., 1988; Holmberg, 1992; Farmer *et al*., 2003; Khaira *& Galanis,* 2007). These *Vibrio* species have been frequently reported as an important cause of gastrointestinal diseases, acute septicaemia and skin infections in humans either by consumption of contaminated seafood or by exposure to aquatic environments (Ottaviani *et al.* 2009). One of the most important species is *V. cholerae* and particularly serotypes O1 and O139, as the main cause of diarrhea. There are other pathogenic serotypes of *V. cholerae* but they cause less severe diarrhea. *V. parahaemolyticus* has been frequently known as the cause of foodborne gastroenteritis outbreaks in the world (Ozer *et al*., 2008; Pruzzo *et al*., 2005). *V. vulnificus* causes 95% of all deaths associated with the consumption of seafood products (Rosche *et al*., 2006). These three species have been known as the most frequent causes of foodborne illnesses. Other pathogenic species includes *V. alginolyticus, V. damsela, V. fluvialis, V. furnissii, V. hollisae, V. metschnikovii,* and*V. mimicus* (Pruzzo *et al*., 2005).

Vibrio species more frequently occur in warmer waters or in the seasons that the temperature of coastal waters is suitable for their growth. The risk of infection will be more when seafoods are consumed raw (Di Pinto *et al*., 2008), or in an insufficiently cooked mode and also when they are post-heat contaminated (Noorlis *et al*., 2011).

2.4 *Vibrio cholerae*

Like other vibrios, *V. cholerae* is a Gram-negative non-spore-forming, motile, curved rod (Baumann et al., 1984). It has many serogroups. The strains in 01 and 0139 are mostly associated with epidemic cholera (Singh *et al*., 2001). *V. cholerae* has a very

high sensitivity to heat and it can be easily killed by cooking at a proper temperature for a proper period of time. Improper heating or inappropriate length of cooking may not be able to kill the whole cells that are present in a food. The optimum temperature for its growth is between 30 and 37°C (Doyle & Cliver, 1990). It has a very rapid growth rate, in raw or cooked seafoods at its optimum temperature or even at room temperature. Rapid growth can be facilitated in alkaline foods. At 5-10°C it has a better survival in cooked foods. Addition of 1% sodium chloride (NaCl) can stimulate the growth of *V. cholerae*. However, one of the most important distinctions of *V. cholera* from other *Vibrio* spp. is that it is able to grow in a nutrient broth or agar with no NaCl added. Although it can grow in the pH range of 5.9 to 9.6, the optimum pH for its growth is 7.6 (Baumann et al., 1984).

V. cholerae is normally waterborne and it is transmitted mainly through water, but it can be transmitted through contaminated food supplies as well. Seafoods obtained from marine water environments are other sources of *V. cholerae* contaminations.Other types of food can also get contaminated by the pathogen and become a source of *V. cholerae*. This contamination can happen directly by the patients by the use of their feces as fertilizer for vegetables or by contaminated water. A person who suffers from the disease can also be a source of contamination, when the handling of food is done with poor personal hygiene (Dobosh *et al*., 1995; Lim, 2001).

Appropriate hygienic measures can prevent the spread of the causative bacteria. Decontamination of water can be done by boiling or chemical treatment, and it contributes to reduce the infections. Harvesting seafood from contaminated water which is found to harbor *V. cholera* should be stopped. Finally, the consumption of raw or insufficiently-cooked seafood should be inhibited. To do a proper heat treatment for a suspected food the length and the temperature of the treatment should be taken into consideration to make sure about its efficiency to kill the pathogen.

2.5 *Vibrio parahaemolyticus*

Vibrio parahaemolyticus is a Gram-negative bacteria and it is nonsporulating, motile and curved rod. It is generally oxidase and catalase positive. It grows in a medium that contains glucose with no gas production. But it cannot ferment lactose and sucrose. Although the optimum temperature for its growth is 30-37°C, it is able to grow in a temperature range of 5-42°C. The cells are able to multiply very rapidly in a medium containing 3-5% NaCI and they can tolerate up to 8% NaCl but they are sensitive to 10% NaCl. Growth is restricted at pH 5.0 or below. The optimum pH for growth is 7.8 to 8.6 within a range of 4.8 to 11. The cells are very sensitive to heating (pasteurization), drying and freezing. It can grow in the presence or absence of oxygen, but grows optimally under aerobic conditions (Desmarchelier 1997; Oliver *et al*., 1997).

V. parahaemolyticus is a ubiquitous human pathogen which may cause gastroenteritis when contaminated raw, insufficiently cooked or postheat-contaminated seafoods are consumed (Feldhusen, 2000; Ozer *et al*., 2008; Pruzzo *et al*., 2005). Since this species is highly abundant in marine products, they have become a major concern in the production and trade of seafood (DePaola *et al*., 2003). Established food safety measures are required to be conducted for marine products, as the main source of a large number of pathogenic bacteria, including *V. parahaemolyticus*.

V. parahaemolyticus is mostly found during summer in Europe and United States, when the temperature is 25ºC and above, while itcan be found all year round in Southeast Asia (Zulkifli *et al*., 2009b). In Malaysia, the probability of outbreaks for *V. parahaemolyticus* is very high, as the climate is suitable for the growth of *Vibrio* species (Khan *et al*., 2002; Elhadi *et al*., 2004). Moreover, virulent strains in raw seafood have also been reported in Malaysia (Sujeewa *et al*., 2009). Thus, it has been drawn to the attention of public health and food safety.

The following control measures should be taken to evade these infections and diseases. Consumption of raw, improper-cooked or post-heat contaminated seafoods should be strongly avoided, especially by susceptible individuals. Raw or cooked seafood products should be refrigerated or freezed and they must be consumed in a proper period of time. One problem that has to be taken into consideration is cross-contamination of the products, and it can be avoided by an appropriate way of sanitation. Last but not least, proper temperature for either cooking the products or keeping them may drastically reduce the contamination of food products. Temperature abuse may lead to irreparableresults, even if it is only for a short period of time (Mortimore & Wallace, 1994).

2.6 *Vibrio vulnificus*

Vibrio vulnificus is a lactose and salicin positive bacteria and it can be found in the estuarine environment in the coastal waters. The cells grow optimally at 37°C with the temperature range from 8 to 43°C. The pH range for growth is 5 to 10, in which the optimum is 7.8. It is phenotypically very similar to *V. parahaemolyticus* (Oliver & Kaper, 2001).

V. vulnificus is known as a lethal pathogen because it can invade the bloodstream. The infection and fatality rate caused by *V. vulnificus* is extremely high (40-60%) among the people who have liver and gastric problems and immunedeficiencies. It can also cause severe wound infections in the individuals who have skin lesions, pre-existing cuts and skin punctures. Foodborne transmissions mostly occur in the people who have had some health problems like diabetes or pre-existing liver disease (Blake *et al*., 1979; Shapiro *et al*., 1998). In other cases, when healthy individuals are infected, gastroenteritis occurs. Control measures should include methods discussed for *V. Parahaemolyticus* (Mortimore and Wallace, 1994).

2.7 Isolation of *Vibrio* spp.

2.7.1 Sample collection

As previously mentioned vibrios inhabit marine environments and are associated with aquatic animals including fish, shellfish, shrimp, oyster, squid, prawn, and other freshwater animals (Khan *et al*., 2002; Sujeewa *et al*., 2009). Hence, seafood and marine products are mostly used for isolation of *Vibrio* species. After collection, samples should be cooled immediately into about 7° C to 10° C, and then should be analyzed as soon as possible. Vibrios might be injured if they undergo a rapid cooling. It is better to avoid any direct contact of the samples with ice, in order to maximize the survival and existence of vibrios. They are able to grow fast at ambient temperatures in seafood (Cook, 1997). Extreme heat and cold may kill vibrios and inhibit their recovery but under mild refrigeration, they can survive well (Boutin et. al., 1985). In the cases that we need to have a frozen storage of the samples, it is recommended to keep the samples at -80°C (Bradshaw *et al*., 1974).

2.7.2 Behavior of vibrios on selective agar

Thiosulfate-citrate-bile salts-sucrose (TCBS) has been recommended in the standard method for the isolation of *V. cholerae* from foods. The method includes an enrichment in Alkaline Peptone Water (APW) at 35±2ºC overnight and then isolation on TCBS medium. The same method was also recommended other vibrios, such as *V. vulnificus* and *V. parahaemolyticus* as well (Elliot *et al*., 1995). Most *Vibrio* species have a considerable growth on TCBS while the growth of most non-vibrios is inhibited on this medium. However, *V. parahaemolyticus* colonies on TCBS are very difficult to distinguish visually from the colonies of other bacteria, because they might be covered

by a yellow color produced by sucrose-fermenting bacteria (Hara-Kudo *et al*., 2001). The colony colours that appear on TCBS for different *Vibrio* species have been presented in Table 2.7.1 and Figure 2.7.1

Table 2.7.1: The colony colors of *Vibrio* spp. on thiosulfate-citrate-bile salts-sucrose (TCBS) agar

SPECIES	COLONY COLOUR
V. parahaemolyticus	Green
V. vulnificus	Green
V. cholerae	Yellow
V. alginolyticus	Yellow
V. furnissii	Yellow
V. fluvialis	Yellow

Source: Hardy Diagnostics (www.catalog.hardydiagnostics.com)

CHROMagar™ *Vibrio* (CHROMagar; Paris, France) is another selective medium which allows for the detection and isolation of *V. alginolyticus*, *V. cholerae, V. parahaemolyticus,* and *V. vulnificus* by the use of chromogenic technology, resulting in colonies that can be distinguished on the basis of color development. It is more accurate and specific than TCBS (Di Pinto *et al*., 2011). The colony colors which appear onCHROMagar™ *Vibrio* for different *Vibrio* species have been shown in Table 2.7.2 and Figure 2.7.2.

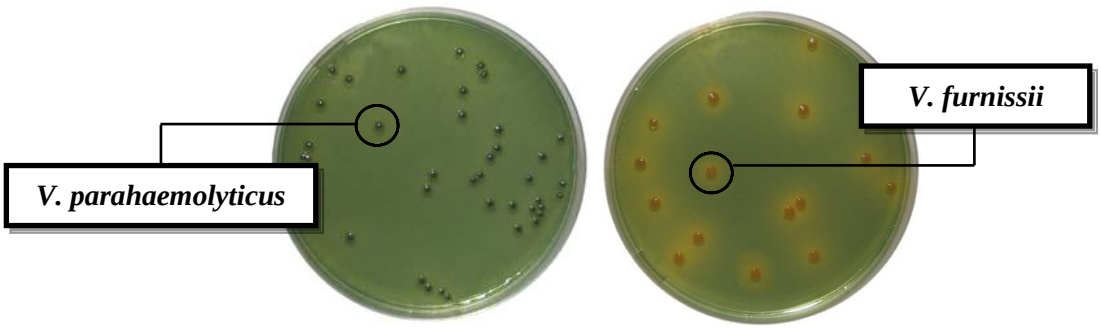


Figure 2.7.1: The colony colors of *Vibrio* spp. on TCBS (Adapted from E&O Laboratories Ltd , www.eolabs.com)

Table 2.7.2: The colony colors of *Vibrio* spp. on CHROMagar™ *Vibrio*

SPECIES	COLONY COLOUR
V. parahaemolyticus	mauve
V. vulnificus / V. cholerae	green blue to turquoise blue
V. alginolyticus	Colourless

Source:www.CHROMagar.com

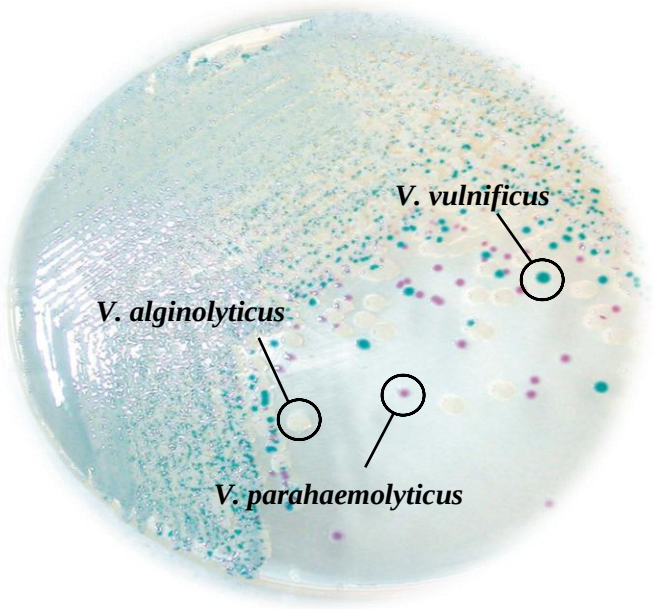


Figure 2.7.2: The colony colors of *Vibrio* spp. on CHROMagar™ *Vibrio* (Adapted from www.CHROMagar.com)

2.8 Conventional Methods for the Identification of *Vibrio* spp.

2.8.1 Biochemical tests

Some of the differential characteristics of some *Vibrio* species associated with human illness related to seafood consumption have been clearly demonstrated in Table 2.8.1.

Table 2.8.1: Biochemical characteristics of human pathogenic Vibrionaceae commonly encountered in seafood

		V. alginolyticus	*V. cholerae*	*V. fluvialis*	*V. mimicus*	*V. parahaemolyticus*	*V. vulnificus*
TCBS agar		Y	Y	Y	G	G	G
AGS		KA	Ka	KK	KA	KA	KA
Oxidase		+	+	+	+	+	+
Arginine dihydrolase		–	–	+	–	–	–
Ornithine decarboxylase		+	+	–	+	+	+
Lysine decarboxylase		+	+	–	+	+	+
Growth	0% NaCl	–	+	–	+	–	–
in (w/v):	3% NaCl	+	+	+	+	+	+
	6% NaCl	+	–	+	–	+	+
	8% NaCl	+	–	V	-	+	-
	10% NaCl	+	–	–	–	–	–
Growth at 42°C		+	+	V	+	+	+
Acid	Sucrose	+	+	+	–	–	–
from:	D-Cellobiose	–	–	+	–	V	+
	Lactose	–	–	–	–	–	+
	Arabinose	–	–	+	–	+	–
	D-Mannose	+	+	+	+	+	+
	D-Mannitol	+	+	+	+	+	V
	ONPG	–	+	+	+	–	+
	Voges-Proskauer	+	V	–	–	–	–

Adapted from Elliot et al. (1995)

Abbreviations	
TCBS : thiosulfate-citrate-bile salts-sucrose	V : variable among strains
AGS : arginine-glucose slant	KK : Slant alkaline / Butt alkaline
Y : yellow	KA : Slant alkaline /Butt acidic
G : green	Ka : Slant alkaline/ Butt slightly acidic

(a) Oxidase test

Oxidase test is an important differential procedure which should be performed on all gram-negative bacteria that are to be identified. The test reagent, N, N, N', N'-tetra-methyl-p-phenylenediamine dihydrochloride acts as an artificial electron acceptor for the enzyme oxidase.The oxidase test is able to identify the organisms in which the enzyme cytochrome oxidase is produced and it is very helpful to categorize the organisms into groups at initial stages of their identification. Cytochrome oxidase is a participant in the chain of electron transport. It transfers electrons from a donor molecule to oxygen (Isenberg, 2004).

(b) Triple Sugar Iron (TSI)

Triple Sugar Iron Agar is applied to differentiate of microorganisms based on the fermentation of glucose (dextrose), lactose, and sucrose and the production of hydrogen sulfide. Phenol red which is a pH indicator, ferrous sulfate and nutrient agar are also present. Phenol red becomes yellow below pH 6.8 and it is red above it. Triple Sugar Iron Agar is a proper test to differentiate enteric, Gram-negative bacilli obtained from dairy samples, food products, and clinical specimens (Murray *et al*., 1995). Triple Sugar Iron Agar contains three carbohydrates, including dextrose, lactose and sucrose. Those bacteria that are able to ferment any of the three sugars in the medium will produce some byproducts. The produced byproducts are mostly acids, and they will affect the color of phenol red (the red pH-sensitive dye) and will change it from red into a yellow color. Position of the color change is very important and it indicates the production of acid related to glucose fermentation when the yellow color is in the butt, or associated with the acidic byproducts from the fermentation of lactose or sucrose when the yellow color is in the slant. Enterobacteria are able to ferment sugars in the

anaerobic butt of the tubes. Some bacteria use thiosulfate anion in the role of a terminal electron acceptor, and they reduce it to sulfide. If this happens, the hydrogen sulfide (H_2S) which has been newly formed, will react with ferrous sulfate which exists in the medium in order to form ferrous sulfide, and it will be visible in the form of a black precipitate. Examples of sulfide-producing bacteria include *Salmonella*, *Proteus*, *Citrobacter* and *Edwardsiella* species but not *Vibrio* species. The blackening of the medium associated with the production of ferrous sulfide is almost always observed in the butt (bottom) of the medium.Production of gases other than hydrogen sufide is indicated either by cracks or bubbles in the media or the media being pushed away from the bottom of the tube (Difco,1984). Interpretations of TSI test associated with the observed colors and gases have been shown in Table 2.8.2.

Table 2.8.2: Interpretations of TSI test based on the observed colors and gases

Slant color	**Interpretation**
Red	Does not ferment either lactose or sucrose
Yellow	Ferments lactose and/or sucrose

Butt color / condition	**Interpretation**
Red	No fermentation, the bacterium is an obligate aerobe
Yellow	Some fermentation has occurred, acid has been produced, it is a facultative anaerobe.
Gas formed	Seen as cracks in the agar, bubbles, or the entire slant may be pushed out of the tube.
Black	H_2S has been produced

Adapted from Difco Manual (1984)

(c) Sulfur reduction – Indole – Motility (SIM) test

i. Sulfur reduction

The sulfur reduction test is very helpful in order to differentiate enteric organisms. For the organisms that are able to reduce sulfur into hydrogen sulfide, the produced hydrogen sulfide will combine with the iron in the medium and will form a black precipitate which is ferric sulfide. The positive result for this test is the reduction of sulfur, which is observable by any blackening of the medium (Difco, 1984). Positive result for sulfur reduction test indicates that the isolate which has been tested is not *Vibrio* spp.

ii. Indoleproduction

There are some microorganisms which are able to break down the amino-acid tryptophan, and it leads to the production of indole. This ability is a distinct and very important characteristic which can be used for the identification and classification of bacteria. The addition of an indole reagent to the growth medium can detect the presence of indole. Kovac's reagent is an example of indole reagent in which a pink coloration will be produced in the reagent if the microorganism is able to break down tryptophan and produce indole (MacFaddin, 1980).

iii. Motility

A hanging-drop and other wet preparations can be utilized to determine the motility of a microorganism. In this method, a sloppy agar tube is used and the organism is inoculated into the central point of the tube. The tube is then incubated and the organisms will be observed that have migrated outside the central tube. In a semisolid agar, observation of the spreading growth will be very helpful to detect the

motility of the tested organisms and it may become much more visible with the incorporation of a tetrazolium dye in the medium. The dye reduces, as the organisms grow and the colour of the medium changes. The incubation temperature is very important; most of the motile organisms are motile at lower temperatures (e.g. 15-25 °C) and may not be motile at the temperature (e.g. 37 °C) optimal for growth.

The organisms will use their flagella in order to migrate away from the stab line, when they are stabbed into the semisolid agar using a straight wire or stab. It leads to the production of turbidity throughout the medium. Non-motile organisms will grow along the stab line and they are not able to migrate away from the stab line, and as a matter of fact the will leave the surrounding medium clear (Difco, 1984).

(d) The methyl red (MR) and Voges-Proskauer (VP) tests

In this method, the media enables the discrimination of high-ratio organisms from low-ratio organisms. More hydrogen ions can be produced In this medium by low-ratio organisms in comparison with high-ratio organisms. The high quantity of hydrogen ions which has been generated by low-ratio organisms creates an acidity level and this level of acidity will inhibit their growth, whereas significantly lower level of hydrogen ions is produced by high-ratio organisms. The media allows the differentiation of the organisms based on hydrogen ions production and hence after growing in this media, the distinction between low-ratio organisms and high-ratio organisms is allowed by the final hydrogen ion concentration. pH indicators were used, as an assay for final hydrogen ion concentration. Two pH indicators have been tested including paranitrophenol and methyl red. Methyl red is used today in the methyl red (MR) test.

Various coliform organisms can be distinguished from each other by the tests. All of these organisms can vigorously ferment glucose, and as a result, the pH value of the glucose medium will fall quickly. The paired MR-VP tests were previously used in order to distinguish between the members of the family Enterobacteriaceae, but recently they are also applied to characterize other bacterial groups including Actinobacteria (Holt, 1994; Schumann *et al*., 2003).

(e) Salt Tolerance test

Most *Vibrio* species have a physiological requirement for NaCl, and salt is an important factor of the selective enrichment broths, plating and identification media for *Vibrio* spp. *V. cholerae* which is non-halophilic, does not have a salt requirement and is the least salt tolerant of the Vibrios. The medium which is most frequently recommended for selective enrichment of *V. cholerae*, alkaline peptone water, contains only 0.5-1% NaCl (FDA, 1998).

Typical *V. parahaemolyticus* is not able to grow in any media without salt but it can grow in 3, 6 and 8% salt broth or agar media. While, the maximum NaCl tolerance of *V. Vulnificus* is 5-6% (FDA, 1998; Mortimore and Wallace, 1994). The salt tolerance rates of different *Vibrio* species has been summarized in Table 2.8.3.

Table 2.8.3: Growth response of *Vibrio* species to various concentrations of NaCl

%NaCl	***Vibrio spp.***				
	V. cholerae	***V. mimicus***	***V. vulnificus***	***V. parahaemolyticus***	***V. alginolyticus***
0	+	+	-	-	-
3	+	+	+	+	+
6	-	-	+	+	+
8	-	-	-	+	+
10	-	-	-	-	+

Source: Baumann *et al.* (1984)

2.8.2 API 20E

The analytical profile index (API) is a method which is frequently used for classification of bacteria on the basis of a variety of experiments. Pierre Janin of Analytab Products, Inc. invented API in the United States in the 1970s. The API testing system is currently manufactured by bioMérieux. It has introduced the existing techniques in a miniaturized and standardized version.

The API-20E test kit gives the opportunity to identify the members of Enterobacteriaceae and also associated organisms by means of a method in which the incubation and reading of the results can be done in an easy way.

Living bacteria produce metabolites and wastes as part of the business of being a functioning cell. The reagents in the cupules in the API-20E test kit are specifically designed to test for the presence of products of bacterial metabolism specific to certain kinds of bacteria.

API-20E system has been evaluated by many investigators (Smith *et al*., 1972; Brooks *et al*., 1974; Nord *et al*., 1974) for the identification of the family Enterobacteriaceae. API 20E test kit has been also used to identify *Vibrio* species by many investigators (Overman *et al*., 1985; Demurcan & Candan, 2006; Martinez-Urtaza *et al*., 2006; Al-Mouqati *et al*., 2012; Paydar *et al*., 2013). The agreement level of the obtained results from API-20E with the results from conventional biochemical reactions and identification was quite high.

2.9 Molecular Methods to Identify and Characterize *Vibrio* spp.

There are numerous PCR-based methods which have been developed for identification of *Vibrio* species. Tarr *et al*., (2007) had proposed a multiplex PCR using species-specific primers to amplify the *rpoB* gene regions in four species (*V. cholerae, V. parahaemolyticus, V. vulnificus,* and *V. mimicus*) followed by |DNA sequence analysis.

A multiplex PCR assay using three collagenase-targeted primer pairs for the species-specific detection of *Vibrio alginolyticus, Vibrio cholerae,* and *Vibrio parahaemolyticus* was developed by Di Pinto *et al.* (2005). In the multiplex PCR developed by Neogi *et al*. (2010) species-specific PCR primers were designed based on *toxR* gene for *V. cholerae* and *V. parahaemolyticus*, and *vvhA* gene for *V. vulnificus.*

Another multiplex PCR was designed by Bauer *et al*. (2007) target the *toxR* genes of *V. parahaemolyticus, V. cholerae* and *V. vulnificus*, in which the primers were selected from the *toxR* GenBank sequences: AB029913 (position 498 to 477, *V. parahaemolyticus*), M21249 (position 1053 to 1034, *V. cholerae*) and AF170883 (position 415 to 396, *V. vulnificus*).

A *dnaJ* gene-based PCR was developed by Nhung *et al*. (2007a,b) for the differentiation of *Vibrio* spp. Other PCR assays for the detection of *V. parahaemolyticus* based on *gyrB* and *toxR* genes were reported by Venkateswaran *et al*. (1998) and Kim *et al*.(1999), respectively. Elsewhere, a multiplex PCR with *vvh* and *viuB* for the detection of *Vibrio vulnificus*, with *ompU*, *toxR*, *tcpI*, and *hlyA* to detect *V. cholerae*, and with *tlh*, *tdh*, *trh*, and open reading frame 8 for *V. parahaemolyticus* developed by Panicker*et al*. (2004).

gyrB is a highly conserved housekeeping gene, commonly found in *Vibrio* spp. (Le Roux*et al.*2004). *pntA* gene encodes transhydrogenase alpha subunit that is one ofthe housekeeping genes in most of the *Vibrio* species, *V. parahaemolyticus*, *V. cholerae*, *V. vulnificus* and other bacteria. A multiplex PCR targeting *gyrB* and *pntA* gene was previously developed in order to differentiate *V. parahaemolyticus*, *V. cholerae*, *V. vulnificus*and other *Vibrio* spp. (Teh *et al*., 2010). This multiplex PCR demonstrated high specificity in *Vibrio* species identification. However, this PCR has not been evaluated on seafood samples and it is necessary to determine the sensitivity of this multiplex PCR on seafood samples, as *V. parahaemolyticus* is widely found in seafood products (Sujeewa *et al*., 2009; Aberoumand, 2010).

2.9.2 Virulotyping of *V. parahaemolyticus*

V. parahaemolyticus causes a significant proportion of the gastroenteritis via seafood consumption in different parts of the world.

toxR gene is present in all *V. parahaemolyticus*, either pathogenic or non-pathogenic (Dileep *et al*., 2003; Sujeewa *et al*., 2009) and has been used for its identification (Sechi *et al*., 2000; Kim et al., 1999). Thus, the presence of *toxR* gene in a *V. parahaemolyticus* isolate does not indicate that it is virulent.

Only some strains of *V. parahaemolyticus* can cause gastroenteritis and those strains are often but not always positive for a thermostable direct hemolysin (*tdh*) or a thermostable direct hemolysin-related hemolysin (*trh*). Thus the pathogenicity of *V. parahaemolyticus* can be determined by the presence of virulence factors including, *tdh* gene and/or the *trh* gene (DePaola *et al*., 2003; Robert-Pillot *et al*., 2004; Roque *et al*., 2009). Majority of environmental strains are *tdh* negative and the prevalence of the *tdh* positive *V. parahaemolyticus* in coastal waters varies from 0.1 to 4% (FAO/WHO

2011). Furthermore, the incidence of pathogenic *V. parahaemolyticus* in seafood is usually low, but sometimes, based on geographic area, the percentage might be higher (e.g., 1–4% in oysters) (FAO/WHO, 2011).

There are PCR assays which have been developed using oligonucleotide primers designed to amplify the genes for detection of *tdh* and *trh* genes in order to distinguish the pathogenicity of strains (Lee & Pan, 1993; Tada *et al*., 1992).

2.9.3 Molecular typing of *V. parahaemolyticus* strains

Molecular typing methods can be applied to gain useful data on the genetic diversity of strains. The study of geographical dispersion of strains and also host distribution of them can be conducted based on their genetic relatedness (Olive and Bean, 1999). By DNA fingerprinting, it is possible to identify individuals based on DNA markers. Each of the individuals will have a unique pattern, which is different from others, except for identical twins, where the patterns are the same (Zulkifli, *et al*., 2009b). There are a variety of PCR-based methods including Random Amplified Polymorphic DNA PCR (RAPD-PCR), Repetitive Extragenic Palindromic PCR (REP-PCR) and Enterobacterial Repetitive Intergenic Consencus Sequence PCR (ERIC-PCR) (Maluping *et al*., 2008).

Studies on the genomic variation and molecular epidemiology of *V. parahaemolyticus* are often carried out to track sources and spread of the pathogen (Chakraborty & Surendran, 2009). DNA primers complementary to repetitive DNA sequencesare used inREP-PCR genomic fingerprinting. These repetitive sequences are highly conserved andthey are naturally present in multiple copies in the genomes of most Gram-negative and several Gram-positive bacteria (Lupski and Weinstock, 1992). In the investigation by Maluping *et al*. (2008), the presence of these repetitive

sequences in *V. parahaemolyticus* was suggested, as the presence of the repeatable fingerprints in REP-PCR was observed. REP-PCR is an appropriate typing method for *V. parahaemolyticus* strains because of its reproducibility (Wong, 2003; Chakraborty *&* *Surendran*, 2009) and high discriminatory ability. It has been well utilized in order to compare strains for epidemiological surveillance (Maluping *et al*., 2008).

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 *Vibrio* spp. positive controls

Bacterial cultures of of known *V. cholerae*, *V.mimicus*, *V. parahaemolyticus* and *V. vulnificus* strains, were used as positive controls for further evaluation of the multiplex PCR targeting *gyrB* and *pntA* genes. The strains were taken form the stocks available in Medical Microbiology Laboratory, Institute of Graduate studies (IPS), University of Malaya.

3.1.2 Seafood samples

Different types of raw seafood including fish, shrimps, prawns, cockles, oysters, clams and squids were purchased from retail stores and hypermarkets including Giant, Carrefour, Jusco, Jaya Grocery and Cold storage in Kuala Lumpur, Petaling Jaya and Seri Kembangan, for isolation of *Vibrio* spp.

3.1.3 Media for bacterial growth

3.1.3.1 Enrichment Media - Alkaline Peptone Water (APW)

Peptone (Oxoid, Hampshire, England)	10 g
NaCl (AnalaR, Leicestershire, England)	10 g
Distilled water	1000 mL

(Media formulated with alkaline pH; pH = 8.5 ± 0.2)

Peptone and NaCl were added to the distilled water and it was then autoclaved at 121ºC for 10-15 min.

3.1.3.2 Selective Media

a. CHROMagar™ *Vibrio* (CHROMagar, Paris, France)

The agar was suspended in the proportion of 74.7 g/L in distilled water.

The media was heated to reach 100ºC (no more than 100ºC) while swirling and stirring regularly until complete fusion of the agar.

It was then cooled down to 45-50ºC and was dispensed into plates.

b. Thiosulfate-citrate-bile salts-sucrose agar (TCBS agar)

TCBS (Oxoid, Hampshire, England)	8.9 g
Distilled water	100 mL

The media was then heated to boil at 100ºC (not more than 100ºC), with agitation until complete fusion of the agar. It was cooled down to 45-50ºC in water bath and was dispensed into plates.

3.1.3.3 Non-selective Media

a. Luria-Bertani (LB) Broth

Tryptone (Oxoid, Hampshire, England)	1.0 g
Yeast Extract (Oxoid, Hampshire, England)	0.5 g
NaCl (AnalaR, Leicestershire, England)	0.5 g
Distilled water	100 mL
Additional 2.5% NaCl (AnalaR, Leicestershire, England)	2.5 g

The media was then autoclaved for 10 min at 121ºC.

b. Luria-Bertani (LB) Agar

Bacteriological agar was added to the LB broth until a final concentration of 1.5% (w/v) was obtained, before being autoclaved. It was cooled down into 45-50ºC in water bath and was dispensed into plates and tubes.

c. Difco™ Veal Infusion Broth

DI broth powder (BD Diagnostics, Sparks, Maryland, United States)	5 g
ddH_2O water	200 mL
Additional 2.5% NaCl (AnalaR, Leicestershire, England)	5 g

The media was autoclaved for 15 min at 121ºC, then cooled and aliquoted into 1.5 mL microcentrifuge tubes. The tubes were kept in the fridge (at 4ºC).

d. Difco™ Veal Infusion Agar

DI broth powder (BD Diagnostics, Sparks, Maryland, United States)	5 g
ddH_2O water	200 mL
Bacteriological agar	3 g
Additional 2.5% NaCl (AnalaR, Leicestershire, England)	5 g

The media was autoclaved for 15 min at 121ºC, then cooled and aliquoted into 1.5 mL microcentrifuge tubes. The tubes were then left to solidify and they were kept at room temperature.

3.1.4 Conventional Methods for the Identification of *Vibrio* spp.

3.1.4.1 Biochemical Tests

a. Oxidase Test

N, N, N',N'-tetra-methyl-p-phenylenediamine dihydrochloride powder (Oxoid, Hampshire, England)

b. Triple Sugar Iron (TSI)

TSI Agar (Oxoid, Hampshire, England)	5.5 g
ddH_2O water	100 mL

TSI agar was added to distilled water and was boiled to dissolve. 10 mL of the gel was dispensed into each test tube. The tubes were autoclaved for 15 min at 121°C and they were then put slant to harden.

c. Sulfur reduction – Indole – Motility (SIM) test

SIM powder (Oxoid, Hampshire, England) was added to distilled water as recommended by the manufacturer and was boiled to dissolve. It was dispensed into sterile universal bottles and the bottles were then autoclaved at 121°C for 15 min. They were left on bench at room temperature to harden.

d. The methyl red (MR) and Voges-Proskauer (VP) tests

MRVP powder (Oxoid, Hampshire, England) was added into water following the guidelines and was boiled to dissolve, then was dispensed into test tubes (5 mL / test tube) and the tubes were autoclaved at 121°C for 15 min.

e. Salt Tolerance test

At the first step, 1% Peptone (Oxoid, Hampshire, England) Water was made and then this solution was used to prepare four different salt tolerance solutions of 0%, 3%, 6% and 10% NaCl (AnalaR, Leicestershire, England).

1% Peptone water + { 0% NaCl; 3% NaCl; 6% NaCl; 10% NaCl

They were boiled to dissolve and then were dispensed into test tubes (4 mL per test tube). Then they were autoclaved at 121°C for 15 min and were left for 24 h on bench.

3.1.4.2 API 20E

- API 20E strips (store at 2-8ºC) (bioMerieux® SA, France)
- 0.85% sterile saline
- API 20 E reagent kit or individual reagents :
  - TDA
  - JAMES
  - VP 1 + VP 2
  - NIT 1 + NIT 2
- Zn reagent
- Oxidase reagent
- Mineral oil (Ref. 70 100)
- API 20 E Analytical Profile Index

3.1.5 Solutions and reagents for polymerase chain reaction (PCR)

(Promega, Madison, United States)

a. 10X PCR buffer in storage

The PCR buffer was available as a commercial product from Promega in the composition which has been shown below:

Tris-HCL, pH 8.0	50 mM
NaCl	100 mM
EDTA	0.1 mM
DTT	1 mM
Glycerol	50 %
Triton (R) X-100	1 %

b. Magnesium Chloride ($MgCl_2$) (25mM)

c. Taq DNA polymerase (5 U/µl)

d. PCR nucleotide Mix (40 mM), pH 7.5

The PCR nucleotide mix was available as a commercial product from Promega in the composition that has been shown below:

dATP	10 mM
dCTP	10 mM
dGTP	10 mM
dTTP	10 mM

3.1.5.5 Primers (Bioneer)

The primers used in the multiplex PCR targeting *gyrB* and *pntA* genes were designed by Teh *et al*. (2010). Primer sequences used in the multiplex PCR and the size of the expected amplicons are presented in Table 3.1.1.

Table 3.1.1: The primer sequences (Bioneer) used in the *gyrB* and *pntA* gene-basedmultiplex PCR, and the size of the expected amplicons.

Oligonucleotide primer	Sequence	Target	Amplicon size (bp)
pntA 1C	5'-CAGTAAAGAAACGACCAAACTC-3'	*Vibrio cholerae*	338
pntA 2C	5'-TGCCAGTTTCGATGATGCCG-3'		
pntA 1P	5'-AGCAAGTTTCGATGATGCTG-3'	*Vibrio parahaemolyticus*	409
pntA 2P	5'-ACCAGCAACCAAAACTTTCGCT-3'		
pntA 1V	5'-CTGTAACAAGGCACCGACAA-3'	*Vibrio vulnificus*	656
pntA 2V	5'-TCACAACCGCACTGATTCCAG-3'		
gyrB 1	5'-AGCCAAACNAAAGAYAARYT-3'	*Vibrio* spp.	493
gyrB 2	5'-CGYARYTTRTCYGGRTTRTRYTC-3'		

Source: Teh *et al*. (2010)

The primers used for *toxR* gene were designed by Kim *et al.,* (1999). And the primers used for detection of *tdh* and *trh* genes were described by Tada *et al*. (1992). Primer sequences for *toxR, trh* and *tdh* genes used in the PCR amplifications and the size of the expected amplicons have been shown in Table 3.1.2. The Repetitive Extragenic Palindromic (REP)-PCR was performed using REP primer (5'-GCGCCGICATGCGGCATT-3') as reported by Navia *et al*. (1999).

Table 3.1.2: The primer sequences (Bioneer) used in the PCR amplifications targeting *toxR, trh* and *tdh* genes and the size of the expected amplicons.

Oligonucleotide primer	Sequence	Target	Amplicon size (bp)
toxR-R	5'-GTCTTCTGACGCAATCGTTG-3'	*toxR* gene	368
toxR-F	5'-ATACGAGTGGTTGCTGTCATG-3'		
tdh-R	5'-CCACTACCACTCTCATATGC-3'	*tdh* gene	251
tdh-F	5'-GGTACTAAATGGCTGACATC-3'		
trh-R	5'-GGCTCAAAATGGTTAAGCG-3'	*trh* gene	250
trh-F	5'-CATTTCCGCTCTCATATGC-3'		

Source: Kim et al. (1999) & Tada et al. (1992)

3.1.5.6 Solutions and reagents for Agarose Gel Electrophoresis

a. 10X TBE

Tris base (Sigma-Aldrich, St. Louis, Missouri, USA)	121.2 g
Boric acid (Oxoid, Hampshire, England)	61.8 g
EDTA (Sigma-Aldrich, St. Louis, Missouri, USA)	0.745 g

Distilled water was added to reach the total volume of 1000 mL. The solution was then autoclaved for 15 min at 121°C and then was stored at room temperature. The stock solution was diluted to 0.5X before using.

b. Agarose Gel (1.5%)

Agarose powder (Oxoid, Hampshire, England)	1.5 g
0.5X TBE buffer	100

c. Ethidium bromide (10 mg/mL)

Ethidium bromide (Sigma-Aldrich, St. Louis, Missouri, USA)	100 mg
Deionised water	10 mL

The solution was stored at room temperature in a dark container and it was diluted to 1.0 µg/ mL with distilled water before being used. To get the desired dilution, 10 µL of the stock solution was added to 100 mL distilled water.

3.2 Methods

Figure 3.2.1 demonstrates the outline of the steps included in this study. In brief, the collected seafood samples were used for isolation of *Vibrio* spp. using two selective media, TCBS and CHROMagar^{TM} *Vibrio*. The isolates were tested by 5 conventional biochemical tests and API20E kit, and the identification of the species was then confirmed using the multiplex PCR targeting *gyrB* and *pntA* genes. The sensitivity and specificity of the multiplex PCR was evaluated before being applied to the isolates. In the next step, *V. parahaemolyticus* isolates were further confirmed by a monoplex PCR targeting *toxR* gene. These isolates were tested for the existence of virulent genes by PCR and were genetically characterized using REP-PCR.

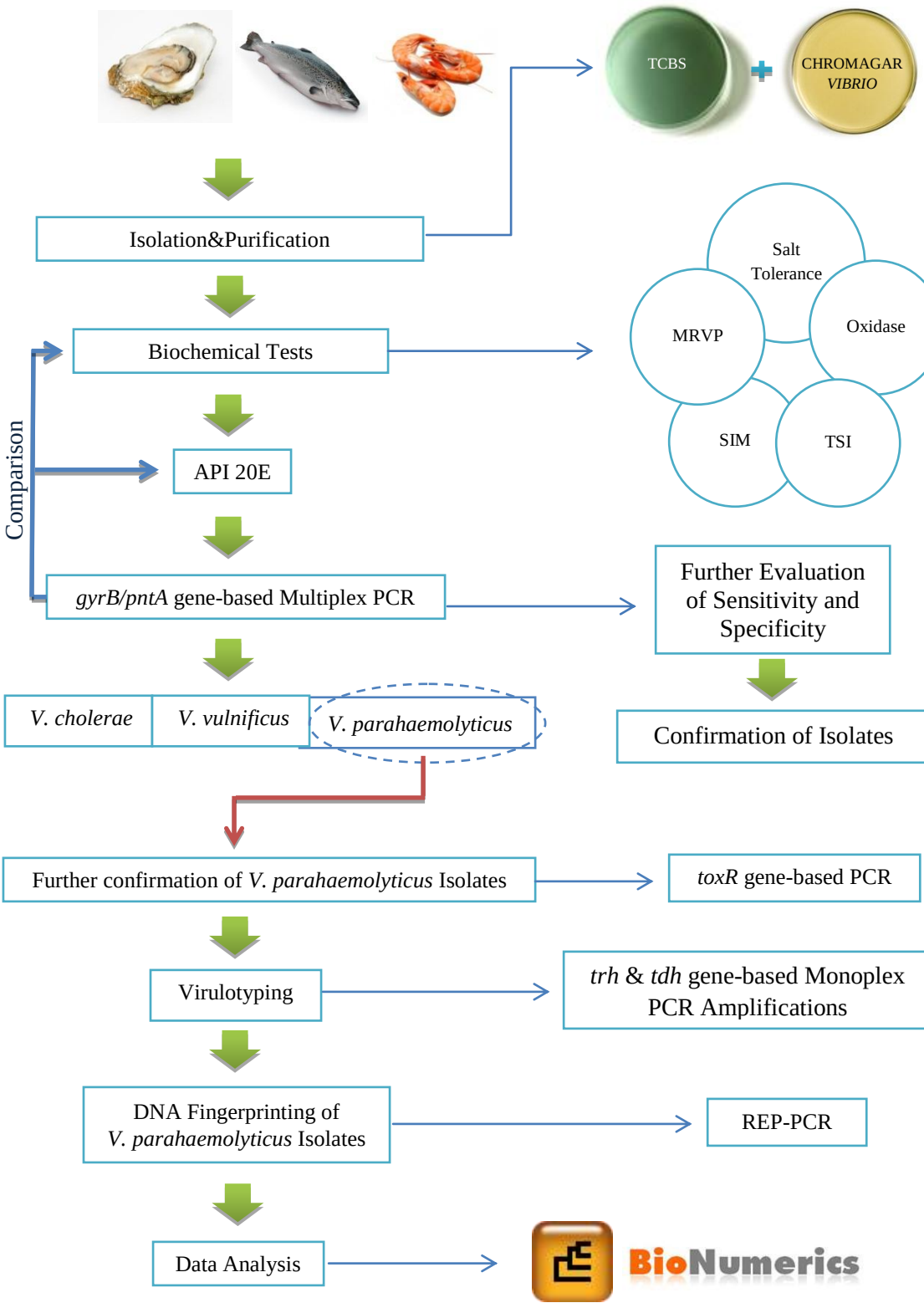


Figure 3.2.1: An outline of the steps and the experiments, conducted during the study.

3.2.1 Seafood Samples Collection

A total of one hundred and fifty raw seafood samples were collected from Jaya Grocery (Tropicana Damansara), Carrefour (Tropicana City Mall), Tesco (Mutiara Damansara), Pasar Chowkit at PWTC, Pasar Malam (Section 17), food stalls around University of Malaya, Giant (Kota Damansara), food Court(Midvalley Mega Mall), Giant and Food Court(The Mines). A variety of raw seafood samples were collected, including 46 fish, 23 shrimps, 18 prawn, 14 cockles, 23 oysters, 11 clams, and 15 squids.

3.2.2 Enrichment of the Collected Samples

Alkaline Peptone Water (APW) was used for the enrichment of the samples in order to provide a suitable environment for *Vibrio* spp. to grow and reach to a detectable level for the presumptive identification. Adilution of 10^{-1} was prepared with the samples by combining 5g of each sample with 45 mL of APW in a sterile stomacher bag. The samples were stomached for 2 min in order to homogenize the mixture. The sampls were then incubated at 37°C for 16-18 h.

3.2.3 Isolation of presumptive *Vibrio* spp.

TCBS and CHROMagar™ *Vibrio* were used as selective media to identify the presumptive *Vibrio* spp. from other microorganisms in the enriched samples. A loop full of culture from APW was streaked on the surface of agar plates and then incubated at 37°C for 16-18 h. Green (blue-green) and yellow colonies on TCBS agar plates, and mauve, green-blue and colorless colonies on CHROMagar™ *Vibrio* were presumptivelyselected as *Vibrio* spp. colonies, and transferred onto Luria-Bertani (LB) agar plates.

3.2.4 Purification of presumptive *Vibrio* spp.

Luria-Bertani (LB) agar supplemented with 2.5% NaCl was used as the non-selective medium for purification of bacteria cultures. Two to 5 colonies were selected and subcultured on LB agar plates. Purified cultures were stored in Difco^{TM} Veal Infusion agar at room temperature and also in Difco^{TM} Veal Infusion broth with 50% glycerol at -20ºC.

3.2.5 Conventional Methods for the Identification of *Vibrio* spp.

3.2.5.1 Conventional Biochemical Tests

Selected colonies which had the characteristic morphology of *Vibrio* spp. were confirmed by conventional biochemical tests. Five different biochemical tests including oxidase, TSI, SIM, MR-VP and salt tolerance tests were performed on the presumptive isolates to identify different species. The tests were conducted as described by Barrow and Feltham (1993) and Kaysner *et al*. (2004).

(a) Oxidase Test

Sterilized toothpick was used to pick bacterial colonies. The colonies were streaked on filter paper which was saturated with 0.5% N, N, N',N'-tetra-methyl-p-phenylenediamine dihydrochloride.

The oxidase reagent contains a chromogenic reducing agent, which is a compound that changes color when it becomes oxidized. If the test organism produces cytochrome oxidase, the oxidase reagent will turn blue or purple within 15 s. Hence, oxidase-positive isolates were indicated by blue or purple color which rapidly appeared and oxidase-negative isolates were indicated by lack of color change.

(b) Triple Sugar Iron (TSI)

TSI slants were prepared and after hardening, a needle loop was used to pick a colony. The needle was stabbed into the agar until close to the bottom and then it was streaked on the surface. The tubes were incubated at 37°C for 18-20 h and then the results were read. The slant and butt for each of the test tubes were examined and recorded.

(c) Sulfur reduction – Indole – Motility (SIM) Test

Sterile needle loops were used to pick colonies in order to inoculate into SIM media. The bottles were incubated at 37°C for 18-20 h. Yellowish brown color of the main media shows that there is no sulfure reduction. If the media around the line is not clear and has some dots inside, it indicates that the bacteria are motile. For Indole test, a drop of Kovac's reagent was added on the media. A layer of pink color shows that it is positive and yellow color indicates that it is negative.

(d) The methyl red (MR) and Voges-Proskauer (VP) tests

Single colonies of the isolates were inoculated into MRVP broth and the test tubes were then incubated at 37°C for 18-20 h. After 18-20 h 1 mL of the broth was aliquoted into another sterile test tube for MR test and the main one was used for VP test.

For the MR test, a drop of Methyl Red was added into the cell culture broth and the results were observed immediately. The ones turned to red and yellow, were considered as positive and negative, respectively. For those that turned to orange color,

the growth was not enough and longer incubation was required.

To do the VP test, two reagents VP1 and VP2 with the ratio, VP1:VP2 (3:1) were added into 2 mL of the cell suspension. Red color represented positive results, while brown color was considered as negative. The results were read after 10 min.

VP1: α-Naphtol 5% (α-Naphtol 50gr – ethanol 1L)

VP2: KOH 40% (KOH 400gr – ddH_2O 1L)

(e) Salt Tolerance Test

The prepared tubes were left for 24 hat room temperature and they were then inoculated using sterile loop and were incubated for 20 h at 37°C.The results were read after the incubation. The growth of bacteria was observed in the solutions that became turbid and those with no growth remained clear.

3.2.5.2 API 20E

Each of the twenty cupules in the plastic strip was inoculated with a saline suspension of a pure bacterial culture, rehydrating the dried reagent in each tube. Based on the manufacturer (bioMerieux® SA, France) instructions, some of the tubes were completely filled (VP, GEL and CIT), whereas others were topped off with mineral oil. In the cupules that were topped off with mineral oil, anaerobic reactions (reactions that occur in the absence of oxygen) can be carried out (ADH, LDC, ODC, URE and H_2S) (Figure 3.2.2).

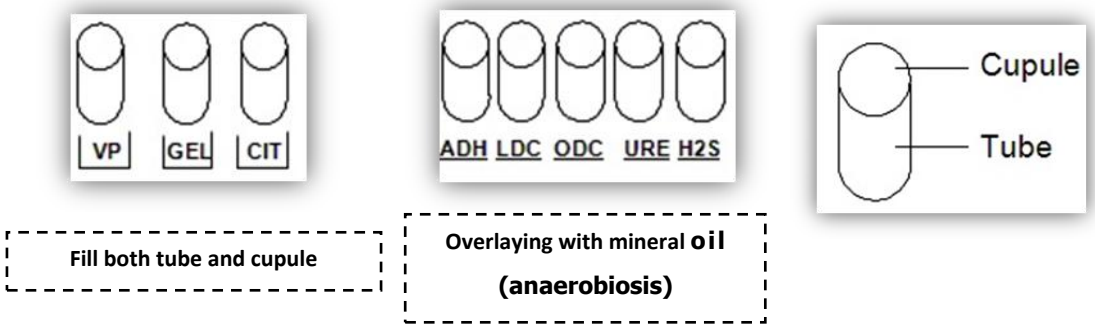


Figure 3.2.2: API 20E manufacturer (bioMerieux® SA, France) instructions on inoculation of the strip.

The strip was then incubated in a small, plastic humidity chamber for 18-24 h at 37°C. After incubation, each tube (an individual test) was assessed for a specific color change indicating the presence of a metabolic reaction that sheds light on the microbes identity. Some of the cupule contents change color due to pH differences, others contain end products that have to be identified using additional reagents.

Interpretation of the 20 reactions, in addition to the oxidase reaction (which was done separately), was converted to a seven-digit code. The code was then looked up in the manual of the names of bacterial species associated with each seven-digit string of numbers.

3.2.6 Molecular Methods for Identification and Characterization of *Vibrio* spp.

3.2.6.1 Template preparation

Bacterial cultures on Luria Bertani Agar plates with 3% NaCl were used to extract crude DNA, by the use of boiling cell extraction method. A single colony of bacterial cells was suspended in 50 µl of distilled water and vortexed shortly. The suspension was heated at 99ºC for 5 min and then was chilled in ice for 10 min. The cell debris was pelleted by centrifugation at 13,400 g for 2 min and 5 µl of clear supernatant was used as the DNA template in a PCR.

3.2.6.2 Multiplex PCR targeting *gyrB* and *pntA* genes

(a) PCR conditions and cycling parameters

The multiplex PCR was done using previously described primers specific for *gyrB*and *pntA* genes (Teh *et al*., 2010). Table 3.2.1 shows the volumes of the components used for the multiplex PCR. The PCR reaction was carried out using the cycling conditions demonstrated in Table 3.2.2 The PCR products were then separated using electrophoresis on 1.5% agarose gel, and visualisedunder UV by staining with ethidium bromide.

Table 3.2.1: The volumes of the components used for the *gyrB/pntA* gene-based multiplex PCR.

Component	Stock concentration	Reaction concentration	Volume (µl)
DNA template	--	--	5
Buffer	5 X	1 X	5
$MgCl_2$	25 mM	2 mM	2
dNTP mix	10 mM	200 µM	0.5
Primers (*gyrB*1,2)	10 µM each	0.3 µM each	1.5 each
Primers (*pntA* 1C,2C,1P,2P,1V,2V)	10 µM each	0.3 µM each	0.75 each
Taq DNA polymerase*	5 U/ µl	2 U	0.3
ddH_2O	--	--	Make to 25
Total volume	--	--	25

Source: Teh *et al*., 2010

Table 3.2.2: The conditions used for the *gyrB/pntA* gene-based multiplex PCR.

Stage	Conditions		
	Temperature	Length	
Predenaturation	95ºC	3min	
Denaturation	94 ºC	30s	35 cycles
Annealing	59 ºC	30s	
Extension	72 ºC	1min	
Final extension	72 ºC	5min	

Source: Teh *et al*., 2010

(b) Evaluation of the multiplex PCR amplification

The multiplex PCR targeting *gyrB* and *pntA* genes was performed using positive and negative controls. Ten strains from each of the species *V. cholerae, V. parahaemolyticus, V. vulnificus* were used as positive controls for *pntA* genes, 10 *V. mimicus*strains as positive controls for *gyrB* gene and six strains of *Salmonella* spp., six strains of *Listeria monocytogenes* and six strains of *Escherichia coli* were used as

negative controls, in order to determine the specificity of the multiplex PCR. The strains were taken form the stocks available in Medical Microbiology Laboratory, Institute of Graduate studies (IPS), University of Malaya. The formulas used to determine the accuracy and specificity of the multiplex PCR have been demonstrated below (Duan and Su, 2005; Di Pinto et al., 2011).

Accuracy (%) = [True positive/(True positive + False positive + False negative)] x 100

Specificity (%) = [True negative/(True negative + False positive + False negative)] x 100

The procedure described by Teh *et al*. (2008) was modified and used for sensitivity test on spiked food sample. Briefly, a single colony of each *V. cholerae, V. parahaemolyticus* and *V. vulnificus* culture was inoculated in 1 mL of Luria Bertani (LB) broth, supplemented with 2.5% NaCl, and incubated overnight at 37 °C. An aliquot of 100 µl from each of the overnight cultures of *V. cholerae, V. parahaemolyticus* and *V. vulnificus* were spiked in 5g of fish, shrimp and oyster, respectively and the spiked samples were left at room temperature for half an hour. Next, the spiked samples were homogenised with 45 mL of the alkaline peptone water (APW) for 2 min and incubated overnight at 37°C. The next day, serial 10-fold dilutions (10^{-1}-10^{-9}) of the food homogenate was carried out. The procedure of 10-fold dilution has been illustrated in Appendix 1. An aliquot of 100µl from each of the dilutions was plated on CHROMagar™ *Vibrio*, supplemented with 2.5% NaCl, for colony count.The plates of LB agar were incubated overnight at 37°C and then were used to count the colonies on the plates using a colony counter. Another 100 µl of each dilution was boiled, centrifuged and the supernatant was used for direct PCR analysis (Paydar *et al*., 2013). The procedure is demonstrated in Figure 3.2.3.

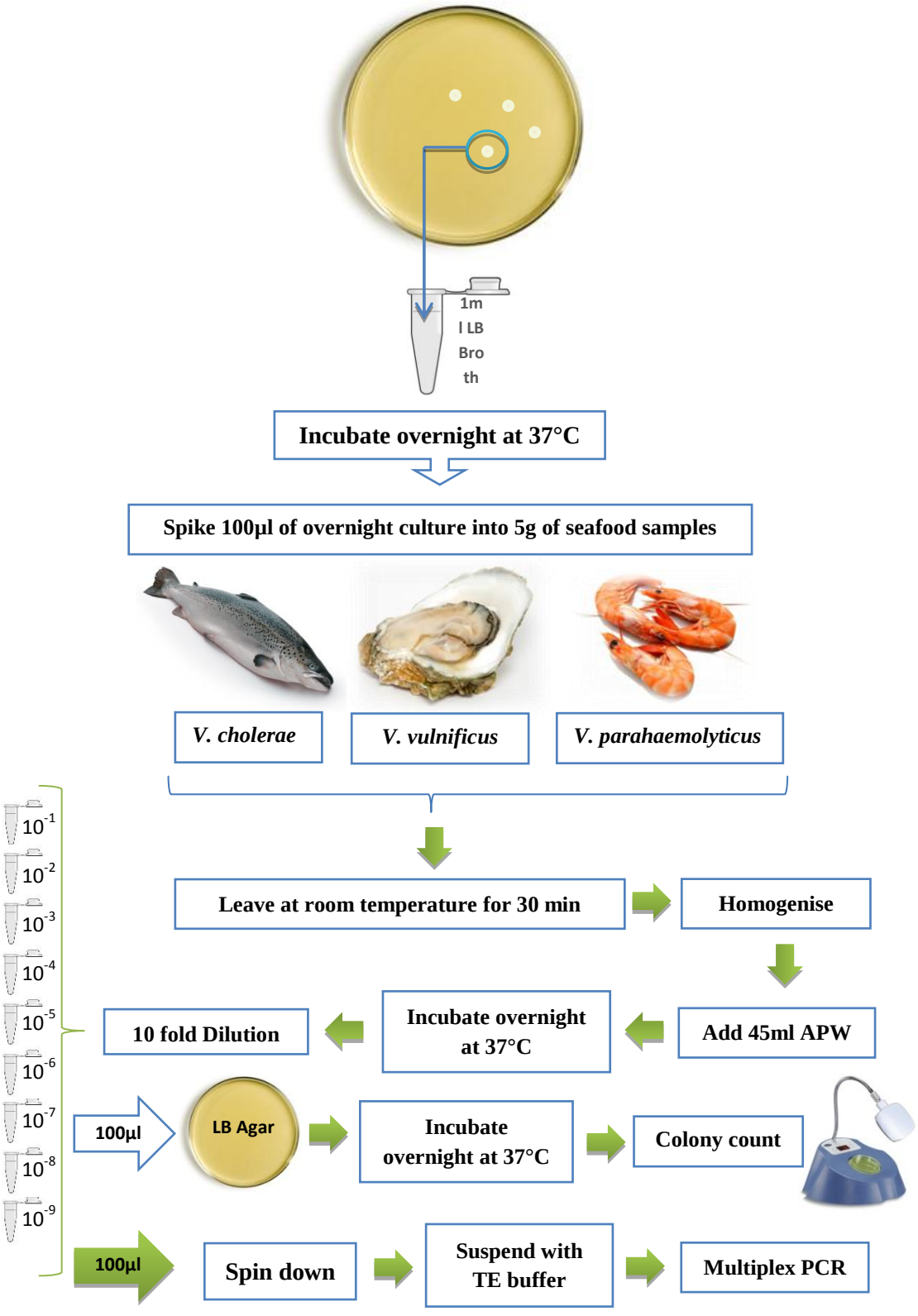


Figure 3.2.3: The procedure used for sensitivity test on spiked seafood.

(c) Confirmation of the isolates using the multiplex PCR

The isolates, identified as *Vibrio* spp. by conventional biochemical tests and API20E kit, were tested in the *gyrB/pntA* genes-based multiplex PCR to confirm the identification (as in 3.2.6.2 a).

3.2.6.3 Comparison of selectivity and sensitivity of TCBS and CHORMagar™ *Vibrio*

Sensitivity of the two selective media used in this study, including TCBS and CHORMagar™ *Vibrio*, were evaluated and compared, based on the total number of isolated colonies, by the following formula:

$$S = \frac{A}{B} \times 100$$

Where S = sensitivity of each medium; A = number of colonies isolated by the medium; B = total number of the colonies isolated by the two media.

Selectivity of these two media was also evaluated based on the results obtained from the multiplex PCR targeting *gyrB* and *pntA* genes. The following formula was used to calculate the selectivity of the two selective media:

$$T = \frac{M}{N} \times 100$$

Where T = selectivity of each medium; M = number of colonies isolated by the medium and confirmed by the multiplex PCR; N = total number of colonies isolated by the medium.

3.2.6.4 Comparison of the discriminatory power of the conventional biochemical tests with API 20E

The isolates obtained from the two selective media were tested by biochemical tests including oxidase, TSI, SIM, MR-VP, salt tolerance tests and API 20E and they were confirmed by the multiplex PCR targeting *gyrB* and *pntA* genes. The findings of the multiplex PCR was used as a reference to compare the discriminatory power of the conventional biochemical tests and API 20E to differentiate the three *Vibrio* species (*V. cholerae, V. parahaemolyticus* and *V. vulnificus*), by the following formula:

$$D = \frac{E}{F} \times 100$$

Where D = discriminatory power of the test; E = number of the isolates differentiated by the test; F = number of the isolates differentiated by the multiplex PCR.

3.2.6.5 Detection of *toxR* gene among *V. parahaemolyticus* isolates

Detection of the virulence genes in *V. parahaemolyticus* is only for determination of pathogenic isolates whereas *toxR* genes can be used to determine all isolates either pathogenic or non-pathogenic and also functions as regulator for expression of the virulence factor genes in *V. parahaemolyticus* (Sechi *et al.*, 2000). The *toxR*-based PCR assay developed by Kim *et al.* (1999) was used to determine the presence of *toxR* gene in *V. parahaemolyticus* isolates.

Detection of *toxR* gene was performed using specific primers for *toxR* gene, as described in Kim *et al.* (1999). The volumes of the components used for the PCR have been shown in Tables 3.2.5 and the conditions used have been presented in Table 3.2.6. Electrophoresis on 1.5% agarose gel was done to separate the PCR products. They were visualised by staining with ethidium bromide.

Table 3.2.3: The volumes of the components used for the monoplex PCR targeting *toxR* gene.

Component	Stock concentration	Reaction concentration	Volume (µl)
DNA template	--	--	5
Buffer	5 X	1 X	5
$MgCl_2$	25 mM	1 mM	1
dNTP mix	10 mM	120 µM	0.3
Primers (*toxR*-F,R)	10 µM each	0.8 µM each	0.2 µl each
Taq DNA polymerase*	5 U/ µl	1 U	0.2
ddH_2O	--	--	Make to 25
Total volume	--	--	25

Source: Kim *et al.*, 1999

Table 3.2.4: The conditions used for the monoplex PCR targeting *toxR* gene.

Stage	Conditions		
	Temperature	Length	
Predenaturation	96ºC	5min	
Denaturation	94 ºC	1min	20 cycles
Annealing	63 ºC	1.5min	
Extension	72 ºC	1.5min	
Final extension	72 ºC	7min	

Source: Kim *et al.*, 1999

3.2.6.6 Detection of *tdh* and *trh* gene among *V. parahaemolyticus* isolates

Detection of *tdh* and *trh* genes in *V. parahaemolyticus* isolates is important to study the distribution of pathogenic isolates especially in seafood.Detection of these two genes was done separately in two monoplex PCR amplifications using specific oligonucleotide primers, as previously described by Tada *et al*., (1992). The same conditions and cycling parameters were used for both of the PCR amplifications. The volumes of the components used for the monoplex PCR have been shown in Tables

3.2.7 and the conditions used have been presented in Table 3.2.8. The PCR products were then separated using electrophoresis on 1.5% agarose gel, stained by ethidium bromide and they were finally visualized under UV.

Table 3.2.5: The volumes of the components used for the monoplex PCR amplifications targeting *tdh* or *trh* genes.

Component	**Stock concentration**	**Reaction concentration**	**Volume (µl)**
DNA template	--	--	5
Buffer	5 X	1 X	5
$MgCl_2$	25 mM	1 mM	1
dNTP mix	10 mM	120 µM	0.3
Primers (*trh*-R,F)or(*tdh*-R,F)	10 µM each	0.8 µM each	0.2 µl each
Taq DNA polymerase*	5 U/ µl	1 U	0.2
ddH_2O	--	--	Make to 25
Total volume	--	--	25

Source: Tada *et al*., 1992

Table 3.2.6: The conditions used for the monoplex PCR amplifications targeting *tdh* or *trh* gene.

Stage	**Conditions**		
	Temperature	Length	
Predenaturation	96ºC	5min	
Denaturation	94 ºC	1min	35 cycles
Annealing	55 ºC	1min	
Extension	72 ºC	1min	
Final extension	72 ºC	7min	

Source: Tada *et al*., 1992

3.2.6.7 PCR Fingerprinting

Repetitive Extragenic Palindromic (REP)-PCR was done by the use of REP primer as described by Navia *et al*. (1999). The volumes of the components used for REP-PCR have been shown in Tables 3.2.9 and the conditions used have been presented in Table 3.2.10. Electrophoresis on 1.5% agarose gel was used to separate the PCR products. They were then stained by ethidium bromide and finally visualized under UV. The resulted representative gel photos of the REP-PCR products were used for data analysis using BioNumerics software. The steps of using BioNumerics software are presented in Figure 3.2.4.

Table 3.2.7: The volumes of the components used for REP-PCR

Component	**Stock concentration**	**Reaction concentration**	**Volume (µl)**
DNA template	--	--	5
Buffer	5 X	1 X	5
$MgCl_2$	25 mM	2.5 mM	2.5
dNTP mix	10 mM	50 µM	0.2
REP Primer	10 µM	0.6 µM	0.15
Taq DNA polymerase*	5 U/ µl	1 U	0.2
ddH_2O	--	--	Make to 25
Total volume	--	--	25

Source: Navia *et al*., 1999

Table 3.2.8: The conditions used for REP-PCR

Stage	**Conditions**		
	Temperature	Length	
Predenaturation	94ºC	4min	
Denaturation	94 ºC	1min	35 cycles
Annealing	42 ºC	1min	
Extension	68 ºC	8min	
Final extension	72 ºC	8min	

Source: Navia *et al*., 1999

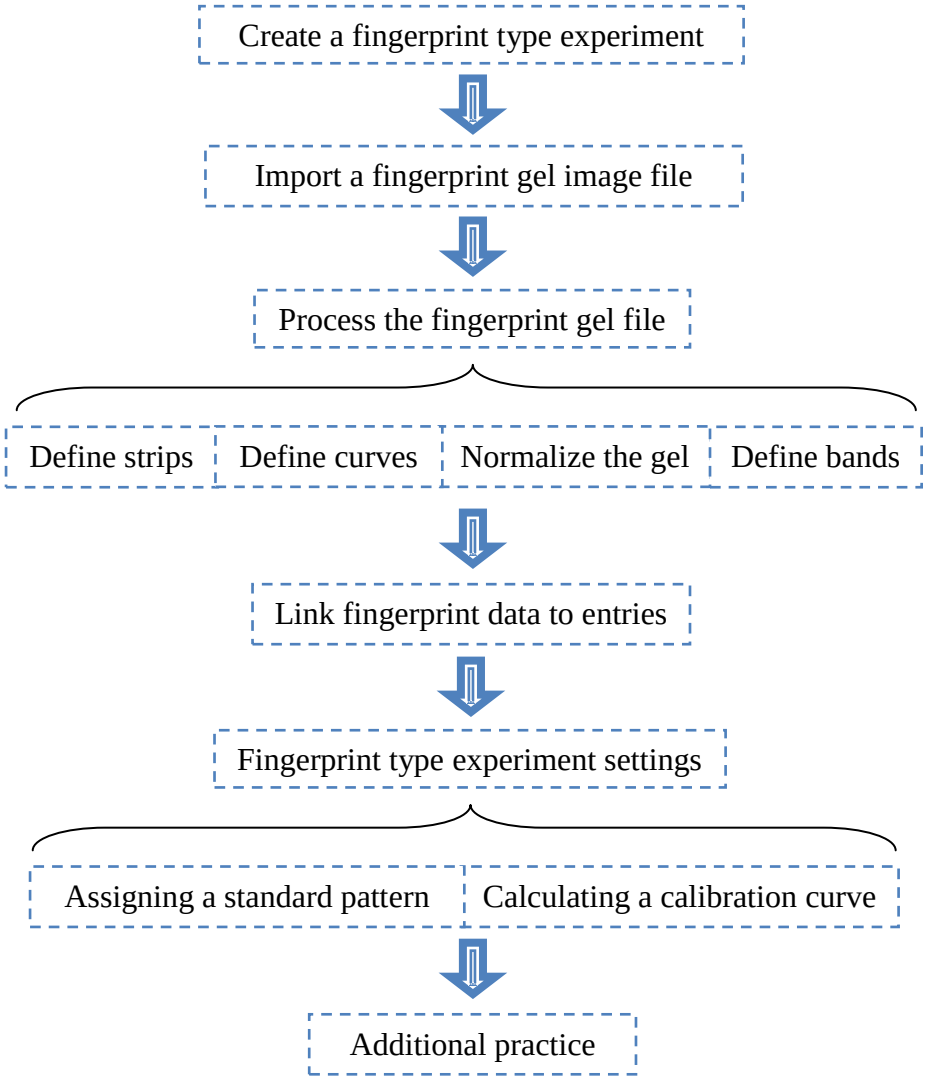


Figure 3.2.4: Steps of using BioNumerics software for fingerprint data analysis.

CHAPTER 4

RESULTS

4.1 Isolation of presumptive *Vibrio* spp.

4.1.1 Thiosulfate-citrate-bile salts-sucrose (TCBS)

Green colonies and yellow colonies were selected from TCBS cultures and were streaked on Luria Bertani Agar (LBA; with 3% NaCl supplementation). On TCBS, yellow colonies were presumptively identified as *V. alginolyticus, V. cholerae, V.fluvialis, V. metschnikovii* and *V. furnissii*. Green or blue-green colonies were assumed to be *V. parahaemolyticus, V. vulnificus* and *V. mimicus*. A total of 81 colonies were selected by TCBS, from the 150 seafood, and were plated on LBA (3% NaCl) for further confirmation.

4.1.2 CHROMagar™ *Vibrio*

V. parahaemolyticus is mauve or violet on CHROMagar™ *Vibrio*; *V. vulnificus* and *V. cholerae* are green-blue to turquoise blue; *V. alginolyticus* is colourless or milk-white. The colonies with these colors were picked and plated on LBA (3% NaCl) for further confirmation and testing.The colonies of different *Vibrio* species on CHROMagar™ *Vibrio* have been shown in Figure 4.1.1.

A total of 175 colonies were picked by CHROMagar™ *Vibrio* from the 150 seafood, and were plated on LBA (3% NaCl) for further confirmation.

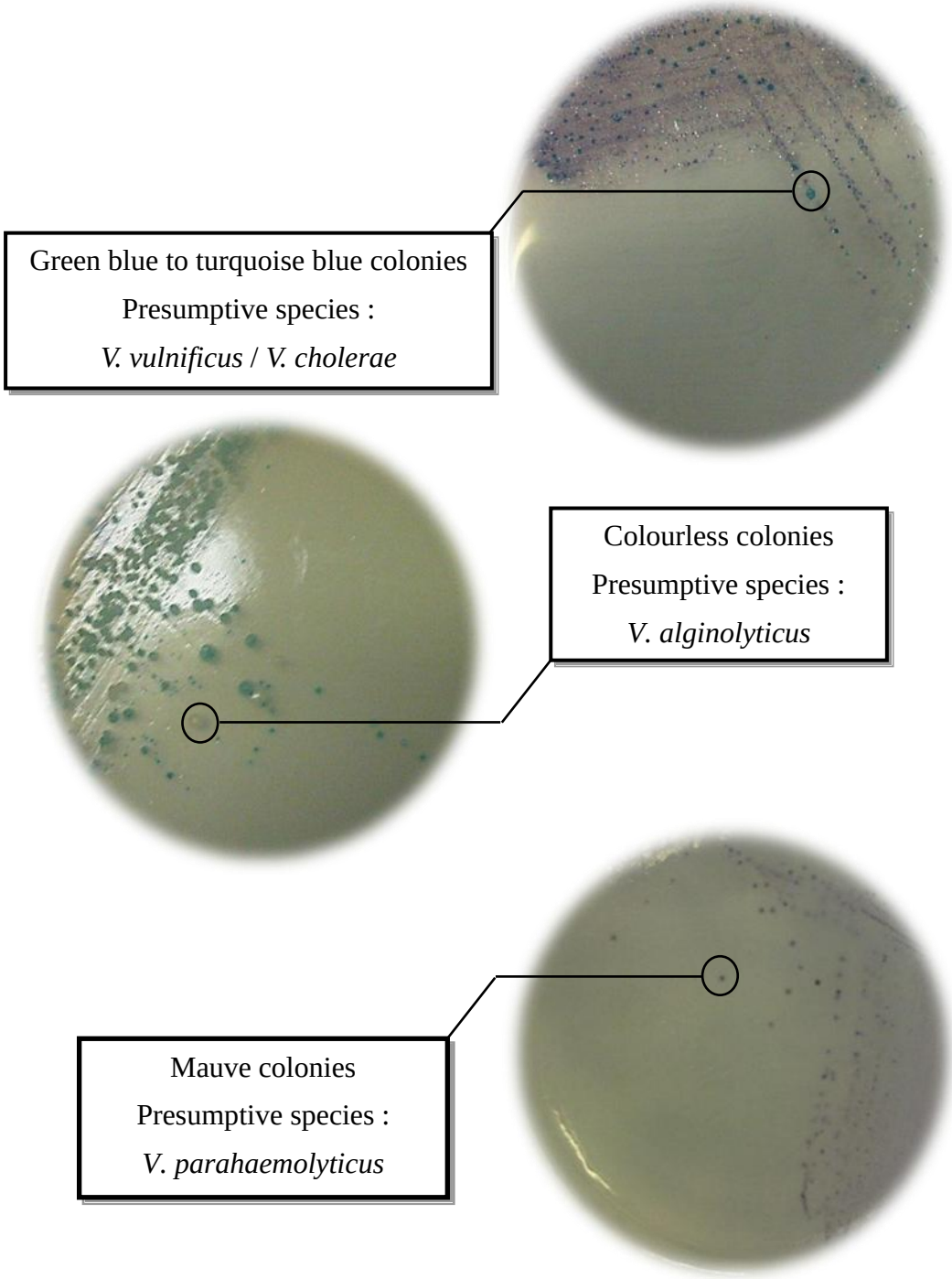


Figure 4.1.1: Colonies of various *Vibrio* species on CHROMagar^{TM} *Vibrio*

4.2 Conventional Methods for the Identification of *Vibrio* spp.

4.2.1 Biochemical Tests

In the initial stage of identification, conventional biochemical tests were conducted, to identify the presumptive *Vibrio* colonies. Oxidase test, Triple Sugar Iron (TSI), Sulfur reduction–Indole–Motility (SIM) test, Methyl Red and Voges-Proskauer (MR-VP) test and salt tolerance were performed as previously described in Barrow and Feltham (1993) and Kaysner *et al*. (2004). Some of the results of SIM, MR-VP and TSI tests have been presented in Appendices 2, 3 and 4.

The results obtained from biochemical tests and the interpretations of the results have been shown in Appendix 5. Out of 256 colonies tested, 187(73%) were presumptively identified as *Vibrio* spp., including 36*V. parahaemolyticus,* 16 *V. cholerae,* 19 *V. vulnificus*, 17 *V. alginolyticus* and18 *V. mimicus*. For 24 isolates, conventional biochemical tests were not able to distinguish *V. parahaemolyticus* from *V. vulnificus* due to the phenotypic similarities of the two species observed in the results of oxidase, TSI, SIM and MR-VP tests (Kaysner *et al*., 2004).

4.2.2 Presumptive isolates of *Vibrio* spp. confirmed by API 20E Kit

The presumptive isolates of *Vibrio* spp. were tested by the API 20E test kit. Appendices 6 and 7 indicate two examples of the results obtained by the API 20E kit for the isolates. Appendix 8 shows *Vibrio* spp. confirmed by the API 20E kit and the percentage of confirmation. All of the 187 presumptive isolates tested, were confirmed as *Vibrio* spp. including 59 (31%) *V. parahaemolyticus,* 44 (24%) *V. cholerae*, 24 (13%) *V. vulnificus,* 35 (19%) *V. alginolyticus* and10 (5%) *V. fluvialis*.

4.3 Molecular Methods to Identify and Characterize *Vibrio* spp.

4.3.1 Multiplex PCR

4.3.1.1 Further evaluation on the specificity of the multiplex PCR

In the initial stage, a multiplex PCR was conducted to confirm the specificity of *gyrB* and *pntA* primers,shown in Table 3.1.1. The resultant amplicons were 338 bp, 409 bp, 656 bp and 493 bp for *pntA*-C, *pntA*-P, *pntA*-V and *gyrB*, respectively.The PCR reaction mixture has been shown in Table 3.2.1, and the cycling parametershave been presented in Table 3.2.2 (Teh *et al*., 2010).

Ten strains from each of the species *V. cholerae*, *V. Parahaemolyticus* and *V. vulnificus* were used as positive controlsfor *pntA* genes, ten *V. mimicus*strains as positive controls for *gyrB* gene, and six strains of *Salmonella* spp., six strains of *Listeria monocutogenes* and six strains of *Escherichia coli* were used as negative controls. Positive results were obtained for all of the positive controls and the results for negative controls were negative. The accuracy and specificity of the primers used in the multiplex PCR including *gyrB* primers for *Vibrio* spp. and *pntA* primers for *V.cholerae*, *V. parahaemolyticus* and *V. vulnificus* were obtained as 100%. The analysis of the data was done according to Duan and Su (2005) (Di Pinto, 2011). Calculations of the accuracy and specificity of *gyrB* and *pntA* primers have been presented in Table 4.3.1 and Table 4.3.2, respectively.

Table 4.3.1: Calculation of the accuracy of *gyrB* and *pntA* primers

Accuracy (%) = [True positive/(True positive + False positive + False negative)] x 100	
gyrB gene	Accuracy (%) = [40/(40+0+0)] x 100 = 100%
pntA gene (*V. cholerae*)	Accuracy (%) = [10/(10+0+0)] x 100 = 100%
pntA gene (*V. parahaemolyticus*)	Accuracy (%) = [10/(10+0+0)] x 100 = 100%
pntA gene (*V. vulnificus*)	Accuracy (%) = [10/(10+0+0)] x 100 = 100%

Table 4.3.2: Calculation of the specificity of *gyrB* and *pntA* primers

Specificity (%) = [True negative/(True negative + False positive + False negative)] x 100	
gyrB gene	Specificity (%) = [18/(18+0+0)] x 100 = 100%
pntA gene (*V. cholerae*)	Specificity (%) = [48/(48+0+0)] x 100 = 100%
pntA gene (*V. parahaemolyticus*)	Specificity (%) = [48/(48+0+0)] x 100 = 100%
pntA gene (*V. vulnificus*)	Specificity (%) = [48/(48+0+0)] x 100 = 100%

The representative gel photo of the PCR has been shown in Figure 4.3.1. As it is observable, the 493bp. bands that have appeared on the gel for the *Vibrio* spp. positive controls including *V. cholerae, V. parahaemolyticus, V. vulnificus* and *V. mimicus*, indicate that the *gyrB* primers were specific to prime the target *gyrB* gene region. 338 bp. bands for *V. cholerae* positive controls, 409 bp. for *V. parahaemolyticus* and 656 bp. bands for *V. vulnificus* affirmed the specificity of the primers for the target *pntA* genes. All in all, the multiplex PCR confirmed that all of the selected primers were specific to the target *gyrB* and *pntA* genes regions.

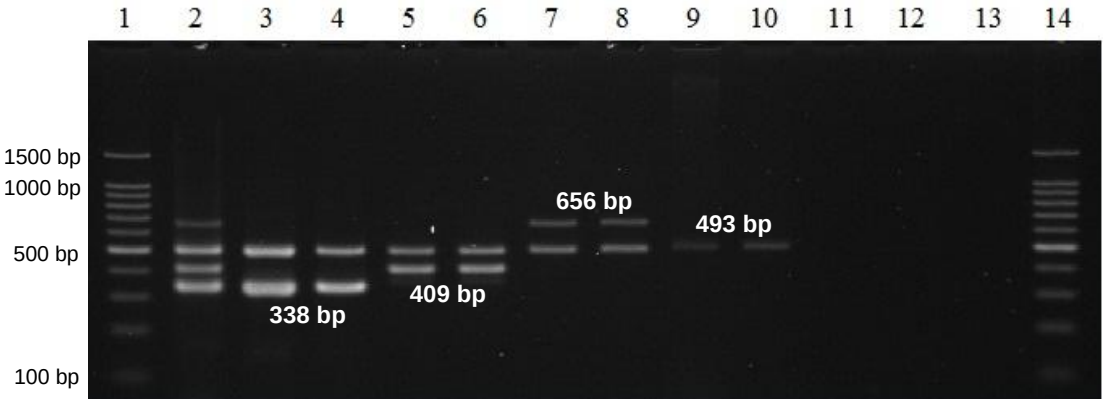


Figure 4.3.1: Multiplex PCR targeting *gyrB* and *pntA* genes to demonstrate the specificity of the PCR. Lane 1, 100 bp. DNA marker; lane 2, *gyrB* and *pntA* positive control; lanes 3 and 4, *V. cholerae* (493/338 bp); lanes 5 and 6, *V. parahaemolyticus* (493/409 bp); lanes 7 and 8, *V. vulnificus* (493/656 bp); lanes 9 and 10, *V. mimicus* (493 bp); lane 11, *Salmonella* sp.; lane 12, *Escherichia coli*; lane 13, negative control; lane 14, 100 bp. DNA marker.

4.3.1.2 Further evaluation on the sensitivity of PCR on spiked food

The sensitivity of the primers was evaluated by *V. cholerae, V. parahaemolyticus* and *V. vulnificus* isolates spiked on fish, shrimp and oyster samples, respectively. The representative gel pictures of the different dilutions of the spiked samples for *V. cholerae, V. parahaemolyticus* and *V. vulnificus* have been shown in Figure 4.3.2, Figure 4.3.3 and Figure 4.3.4, respectively. Sensitivity of the multiplex PCR was found to be 2.0 x 10^3 CFU mL^{-1} for both *V. cholerae* and *V. parahaemolyticus* and 9.0 x 10^3 CFU mL^{-1} for *V. vulnificus.* The calculations of the values have been shown in Table 4.3.3.

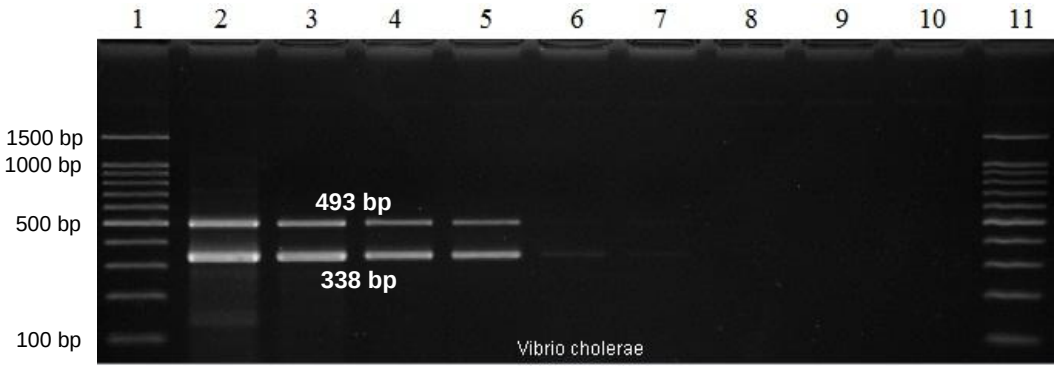


Figure 4.3.2: Multiplex PCR targeting *gyrB and pntA* geneson fish samples spiked with different dilutions of *V. cholerae* to demonstrate the sensitivity of the PCR for detection of *V. cholerae*. Lane 1, 100 bp. DNA marker; lane 2, *V. cholerae* positive control (493/338 bp); lane 3, dilution 10^{-2}; lane4, dilution 10^{-4}; lane5, dilution 10^{-5}; lane6, dilution 10^{-6}; lane 7, dilution 10^{-7}; lane 8, dilution 10^{-8}; lane 9, dilution 10^{-9};lane 10, negative control; lane 11, 100 bp. DNA marker.

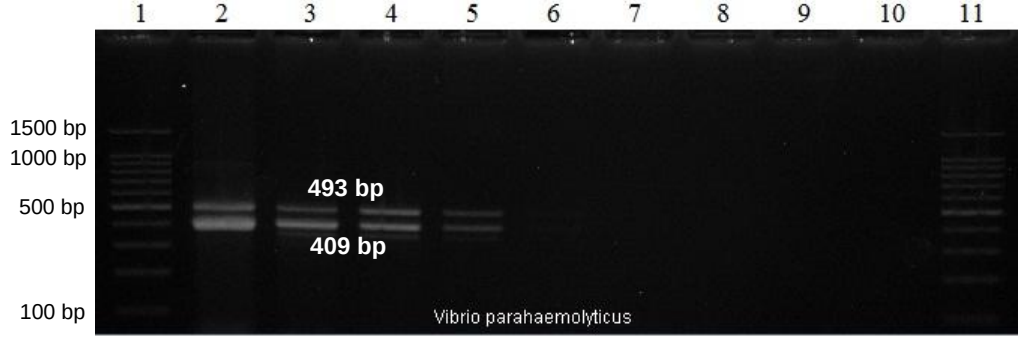


Figure 4.3.3: Multiplex PCR targeting *gyrB and pntA* geneson shrimp samples spiked with different dilutions of *V. parahaemolyticus* to demonstrate the sensitivity of the PCR for detection of *V. parahaemolyticus*. Lanes 1, 100 bp. DNA marker; lane 2, *V. parahaemolyticus* positive control (493/409 bp); lane 3, dilution 10^{-2}; lane 4, dilution 10^{-4}; lane 5, dilution 10^{-5}; lane 6, dilution 10^{-6}; lane 7, dilution 10^{-7}; lane 8, dilution 10^{-8}; lane 9, dilution 10^{-9};lane 10, negative control; lane 11, 100 bp. DNA marker.

Figure 4.3.4: Multiplex PCR targeting *gyrB and pntA* geneson oyster samples spiked with different dilutions of *V. vulnificus* to demonstrate the sensitivity of the PCR for detection of *V. vulnificus*. Lane 1, 100 bp. DNA marker; lane 2, *V. vulnificus* positive control (656/493 bp); lane 3, dilution 10^{-2}; lane 4, dilution 10^{-4}; lane 5, dilution 10^{-5}; lane 6, dilution 10^{-6}; lane 7, dilution 10^{-7}; lane 8, dilution 10^{-8}; lane 9, dilution 10^{-9};lane 10, negative control; lane 11, 100 bp. DNA marker.

Table 4.3.3: Calculation of the sensitivity of the primers for the *gyrB/pntA* genes-basedmultiplex PCR

Species	A (CFU/mL)	B	S = B x Q (CFU/ mL)
V. cholerae	192×10^8	10^{-7}	1.92×10^3
V. parahaemolyticus	184×10^7	10^{-6}	1.84×10^3
V. vulnificus	91×10^8	10^{-6}	9.1×10^3

Abbreviations
S : Sensitivity of the primers for detection of the species (CFU/mL)
A : Number of colonies per mL (no dilution)
B : The highest dilution with observable band in gel electrophoresis

4.3.1.3 Multiplex PCR for simultaneous identification of *Vibrio* spp. in raw seafood

A simultaneous detection of *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*, was performed using the *gyrB/pntA* genes-based multiplex PCR, which employs a combination of primers (*pntA* 1C, *pntA* 2C, *pntA* 1P, *pntA* 2P, *pntA* 1V, *pntA* 2V, *gyrB* 1 and*gyrB* 2) shown in Table 3.1.1. The cycling conditions (Table 3.2.2) and components volume (Table 3.2.1) described by Teh *et al.* (2010). The isolates were tested and 187 isolates were confirmed as *Vibrio* spp. and among these isolates, 50 *V. parahaemolyticus*, 12 *V. vulnificus* and 8 *V. cholerae* were detected. The results obtained

from the multiplex PCR and conventional methods have been shown in Appendix 8. Comparison of these findings unravelled some limitations of the conventional biochemical tests for identification of some *Vibrio* species. Although these biochemical tests were able to identify *Vibrio* spp. isolates, they failed to discriminate *V. parahaemolyticus* from *V. vulnificus*, due to the similarities of the two species.

4.3.1.4 Prevalence of different *Vibrio* spp. in raw seafood

Out of 150raw seafood samples, 93 (62%) were positive for *Vibrio* spp. as determined by CHROMagar™ *Vibrio*, conventional biochemical tests, API 20E kit and finally confirmed by PCR. 43 (29%) seafood samples were confirmed to contain *V. parahaemolyticus*. This amount was 12 (8%) samples for *V. vulnificus* and 8 (5.3%) for *V. cholerae*. 187 isolates were taken from the samples. Figure 4.3.5 and Table 4.3.4 clearly demonstrates the prevalence of *Vibrio* spp. in the seafood samples.

By the Multiplex PCR assay, 50 (27%) *V. parahaemolyticus*, 12 (6.4%) *V. vulnificus* and 8 (4.3%) *V. cholerae* from seafood samples were confirmed.

4.3.1.5 *Vibrio* spp. detected in different raw seafood samples

A variety of raw seafood samples were collected in order to isolate *Vibrio* spp. Table 4.3.5 shows the percentages of the samples harboured *Vibrio* spp., *V. cholera, V. parahaemolyticus* and *V. vulnificus* for each type of seafood tested. The results indicate that the majority of the samples are contaminated with *Vibrio* spp., and fish, shrimp and prawn samples have the maximum contamination of vibrios. Figure 4.3.6 clearly demonstrates the percentages of *V. cholera, V. parahaemolyticus, V. vulnificus* and other *Vibrio* spp. in the different types of seafood samples tested. As can be seen, *V. parahaemolyticus* was the most prevalent species among different types of seafood.

Table 4.3.4: Prevalence of *Vibrio* spp. in different kinds of raw seafood collected from retail stores in Kuala Lumpur and Petaling Jaya, confirmed by the *gyrB/pntA* genes-basedmultiplex PCR.

Seafood samples	**Percentage of the samples harboured *Vibrio* spp.** (Number of positive/Total samples tested x 100 %)			
	V. cholerae$^{(+)}$	*V. parahaemolyticus*$^{(+)}$	*V. vulnificus*$^{(+)}$	Total *Vibrio* spp.$^{(+)}$
Fish	4.6%	44.2%	11.6%	72.1%
Cockles	0.0%	21.4%	7.1%	57.1%
Clams	0.0%	18.2%	18.2%	54.5%
Shrimp	7.7%	34.6%	7.7%	65.4%
Squid	0.0%	26.7%	0.0%	53.3%
Prawn	11.1%	27.8%	5.6%	61.1%
Oyster	8.7%	26.1%	4.4%	52.2%

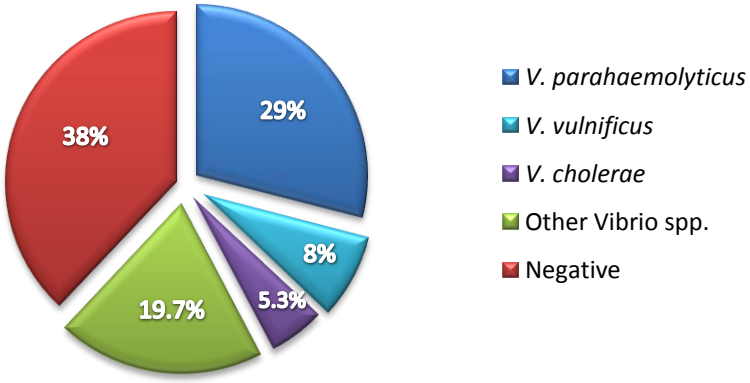


Figure 4.3.5: Prevalence (number of positive/total tested) of *Vibrio* spp. in raw seafood collected from retail stores in different locations of Kuala Lumpur, Petaling Jaya and Seri Kembangan, confirmed by the *gyrB/pntA* genes-basedmultiplex PCR.

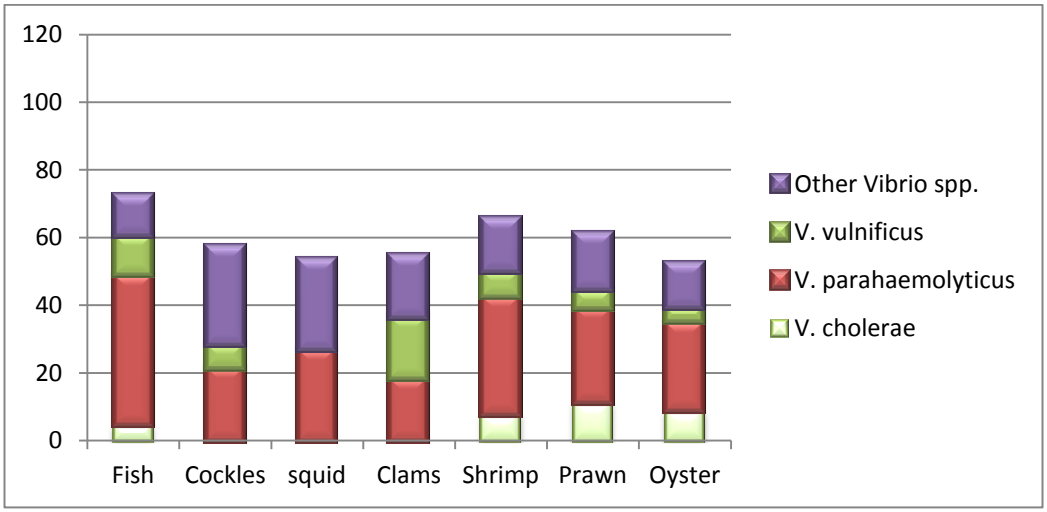


Figure 4.3.6: Prevalence of *Vibrio* spp. in raw seafood collected from retail stores in Kuala Lumpur and Petaling Jaya, confirmed by the multiplex PCR targeting *gyrB* and *pntA* genes.

4.3.2 Comparison of selectivity and sensitivity of TCBS and CHORMagar™ *Vibrio*

In this study, two types of selective agar were used in the isolation of *Vibrio* spp. from seafood. Thiosulphate-citrate-bile salt-sucrose agar (TCBS), and CHROMagar™ *Vibrio*. As the same samples were used for isolation by both of the two media, the sensitivity of the two media can be compared. Of the total 256 colonies, 81 colonies (32%) were picked from TCBS and 175 (68%) from CHROMagar™ *Vibrio*.

The isolates from each of the two media were tested by biochemical tests including oxidase, TSI, SIM, MR-VP, salt tolerance tests and API 20E and they were confirmed by the multiplex PCR targeting *gyrB* and *pntA* genes. The findings of the multiplex PCR were used to evaluate the selectivity of the two selective media. From the 81 presumptive colonies isolated by TCBS 46 (57%) isolates were confirmed as *Vibrio* spp., while 141 (81%) isolates out of 175 isolated by CHROMagar™ *Vibrio* were confirmed to be *Vibrio* spp. It affirms that CHROMagar™ *Vibrio* has a higher selectivity in comparison with TCBS media. Figure 4.3.7 clearly illustrates the higher selectivity and sensitivity of CHROMagar™ *Vibrio* compared with TCBS agar. It shows that CHROMagar™ *Vibrio* is more efficient for isolation of *Vibrio* species and it is better to use this highly selective media for *Vibrio* studies rather than TCBS. Many other bacterial species can survive on TCBS, even Gram-positive bacteria, while in fact TCBS shall inhibit their growth, due to the presence of bile-salt (Merck, 2012).

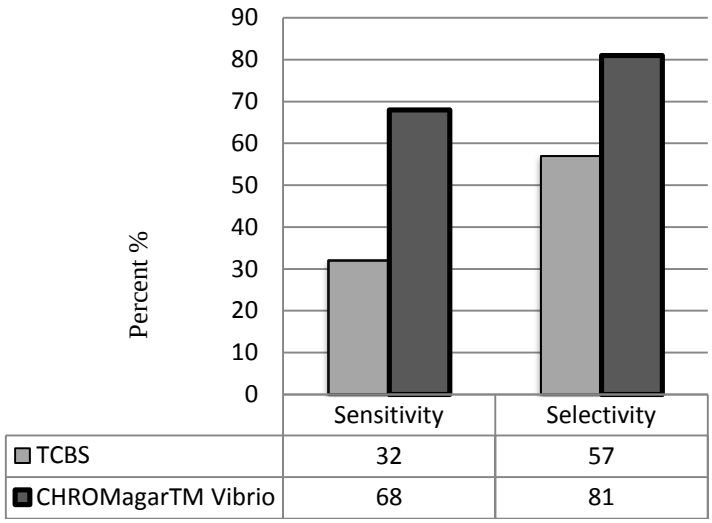


Figure 4.3.7: Comparison of efficiency of two selevtive media.

4.3.3 Comparison of the discriminatory power of the conventional biochemical tests with API 20E

The table in Appendix 8 clearly shows the isolates and a summary of the results from conventional biochemical tests, API and multiplex PCR.The results obtained from the multiplex PCR were compared with conventional biochemical tests and API 20E. Some isolates have been observed for which, biochemical tests and API 20E were not able to detect the correct species. However, the detection power of these conventional methods for identification of vibrios at genus level was quite satisfactory. Comparison of the results of API 20E and the conventional biochemical tests with the findings of the multiplex PCR indicated that the discriminatory power of API 20E for *V. cholerae, V. parahaemolyticus* and *V. vulnificus* has been higher than the conventional biochemical tests. API 20E was able to have a correct presumption for slightly more than 80% of the isolates, while the total discriminatory power of the biochemical tests was less than 60% (Table 4.3.6). Figure 4.3.8 clearly highlights the difference of the two types of methods in the detection and differentiation of *V. cholerae, V. parahaemolyticus* and *V. vulnificus* for the isolates applied in this study.

Table 4.3.5: Number of the isolates differentiated by biochemical tests and API 20E, and confirmed by the multiplex PCR targeting *gyrB* and *pntA* genes.

	Multiplex PCR	**Biochemical tests**	**API 20E**
V. cholerae	8	6 (75%)	7 (88%)
V. parahaemolyticus	50	26 (52%)	40 (80%)
V. vulnificus	12	7 (58%)	11 (92%)
Total	70	39 (56%)	58 (83%)

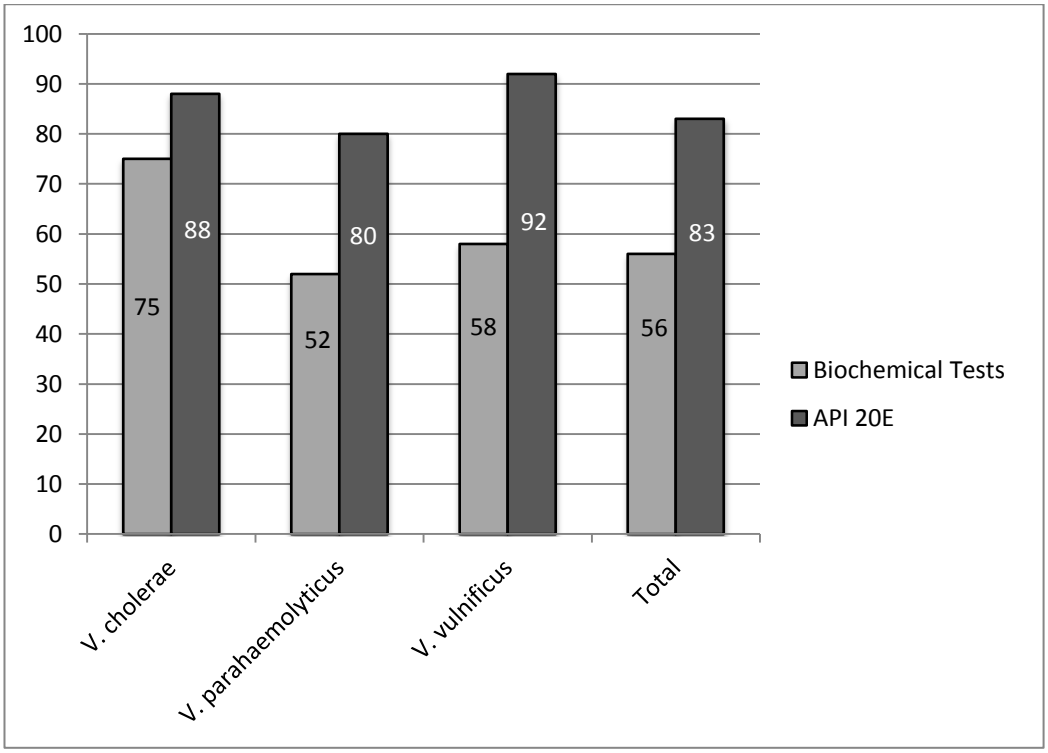


Figure 4.3.8: Comparison of discriminatory power between the applied biochemical tests and API 20E, based on the results obtained from the multiplex PCR targeting *gyrB* and *pntA* genes.

4.3.4 Detection of *toxR* gene among *V. parahaemolyticus* isolates

Detection of *toxR* genes in *V. parahaemolyticus* strains was performed in order to compare with the gyrB/pntA-based detection as *toxR* gene is present in all *V. parahaemolyticus* strains either pathogenic or non-pathogenic. The monoplex PCR targeting *toxR* genes was carried out using *toxR* primers (amplicon size 368bp) (Kim *et*

al.,1999) with the reaction conditionspresented in Table 3.2.5 and the cycling parameters described in Table 3.2.6.

The obtained results indicate that all of the *V. parahaemolyticus* isolates were positive for *toxR* gene (Figure 4.3.9).The findings of the PCR targeting *toxR* gene were in concurrence with the results obtained form the *gyrB/pntA* genes-based multiplex PCR for *V. parahaemolyticus* identification. *toxR* gene with 368base pairs size, appeared on the gel.

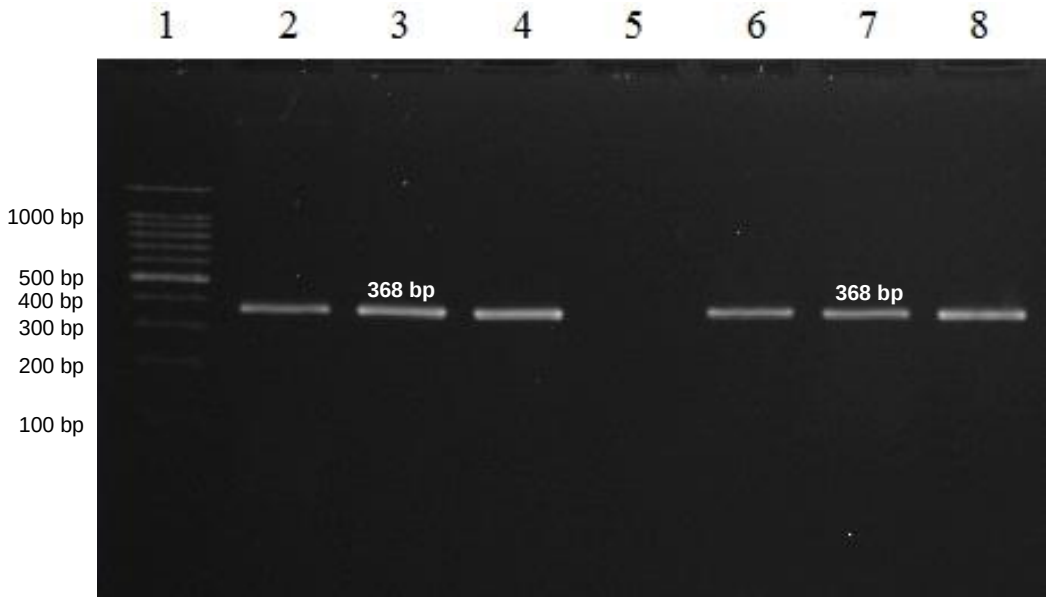


Figure 4.3.9: Monoplex PCR targeting *toxR* gene in *V. parahaemolyticus* isolates in which the size of the product is 368 bp. Lane 1, 100 bp. DNA marker; lane 2, positive control; lane 5, negative control; lane 3, A21; lane 4, A32; lane 6, B26; lane 7, B37; lane 8, D07.

4.3.5 Detection of *tdh* and *trh* genes among *V. parahaemolyticu* isolates

The monoplex PCR amplifications targeting *trh* and *tdh* genes were performed to study the distribution of pathogenic *V. parahaemolyticus* isolates. Specific oligonucleotide primers for *tdh* gene (amplicon size 251bp) and *trh* gene (amplicon size 250bp) were used (Tada *et al*., 1992). It was not possible to use the primers in a multiplex PCR for both of the genes, because the size of the two amplicons is too close

(Table 3.1.2). The components volumes and the cycling conditions of the two PCR amplifications have been presented in Table 3.2.7 and Table 3.2.8, respectively.

Out of the 50 *V. parahaemolyticus* strains 6 strains (12%) were *trh*-positive and 2 strains (4%) were *tdh*-positive. The representative gel photo for the PCR targeting *trh* and *tdh* gene has been shown in Figure 4.3.10 and Figure 4.3.11, respectively. None of the isolates was positive for both of the genes *trh* and *tdh*. *trh* and *tdh* genes with 250 and 251 base pairs size respectively, appeared on the gel.

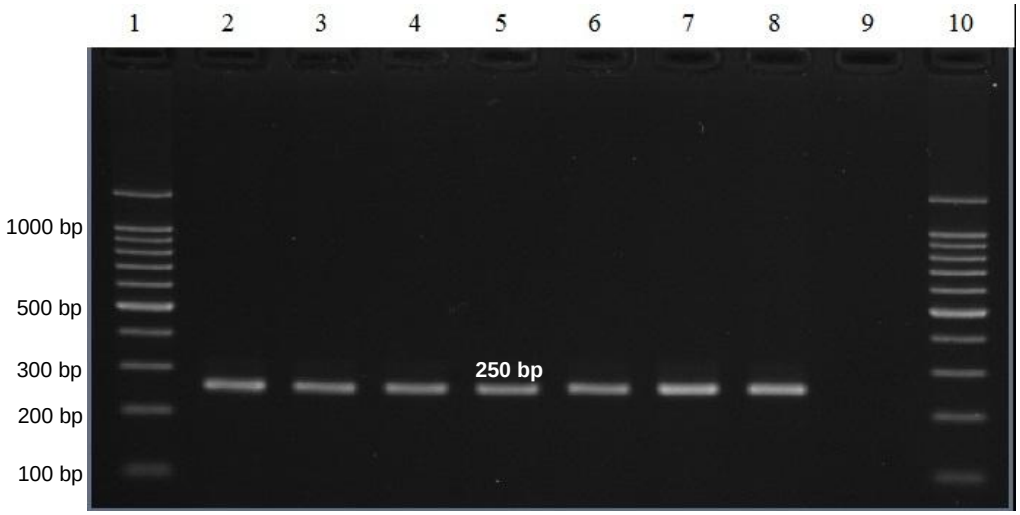


Figure 4.3.10: Monoplex PCR targeting *trh* gene in *V. parahaemolyticus* isolates. Lane 1, 100 bp. DNA marker; lane 2, positive control; lane 3, B12; lane 4, B19; lane 5, B24; lane 6, B31; lane 7, B32; lane 8, B45; lane 9, negative control; lane 10, 100 bp. DNA marker.

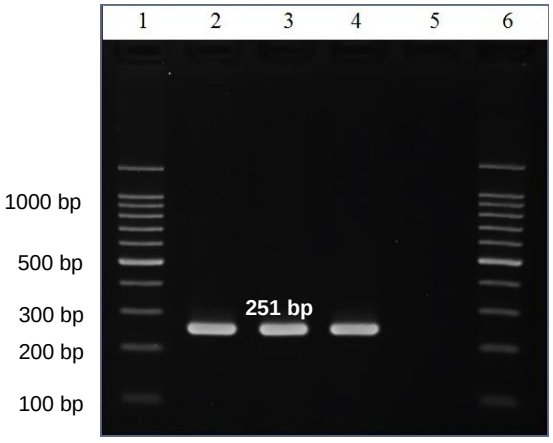


Figure 4.3.11: Monoplex PCR targeting *tdh* gene in *V. parahaemolyticus* isolates. Lane 1, 100 bp. DNA marker; lane 2, positive control; lane 3, A30; lane4,B13; lane 5, negative control; lane 6, 100 bp. DNA marker.

4.3.6 Molecular typing of *V. parahaemolyticus* isolates using REP-PCR

Molecular typing was done to differentiate the*V. parahaemolyticus* isolates isolated from seafood and also to analyse genetic variability among the isolates. Repetitive Extragenic Palindromic (REP)-PCR was performedusing REP primer(Navia *et al*., 1999).The components volumes used in REP-PCR have been shown in Table 3.2.9 and the cycling conditions have been presented in Table 3.2.10.

The representative gel photos of REP-PCR have been presented in Figure 4.3.12 and Figure 4.3.13. Data analysis was done using BioNumerics software. The dendrogram based on the profiles obtained by REP-PCR is shown in Figs 4.3.14. As it can be seen, the *V. parahaemolyticus* isolates have been well differentiated. REP-PCR subtyped the 50 isolates into 41 profiles (F= 0.35 - 1.0) consisting of 3 to 13 bands ranging from 200 to 2000 bp. Ten clusters were observed at the similarity of 80%.No clear trends were observed with any of the clusters based on source of isolation and location.Even the isolateswhich have been isolated at the same time or from the same location, are not genetically related. Low level of similarity in the REP profiles of the majority of the isolates, indicates a high genetic diversity among the *V. parahaemolyticus* isolates.The REP profiles of B42 and B48, as two cases in point, which have been isolated from a particular type of seafood, from the same location and at the same time, shared less than 40% similarity. It affirms low level of genetic relatedness among the*V. Parahahaemolyticus* isolates.

It is also observable in the dendrogram that there is a high level of similarity among the isolates with the same virulotypes. It shows that REP-PCR, as an efficient method of molecular typing for *V. parahaemolyticus* isolates, was able to differentitate the isolates with different virulotypes. The results of virulotyping of the isolates have been presented in the dendrogram shown in Figure 4.3.14.

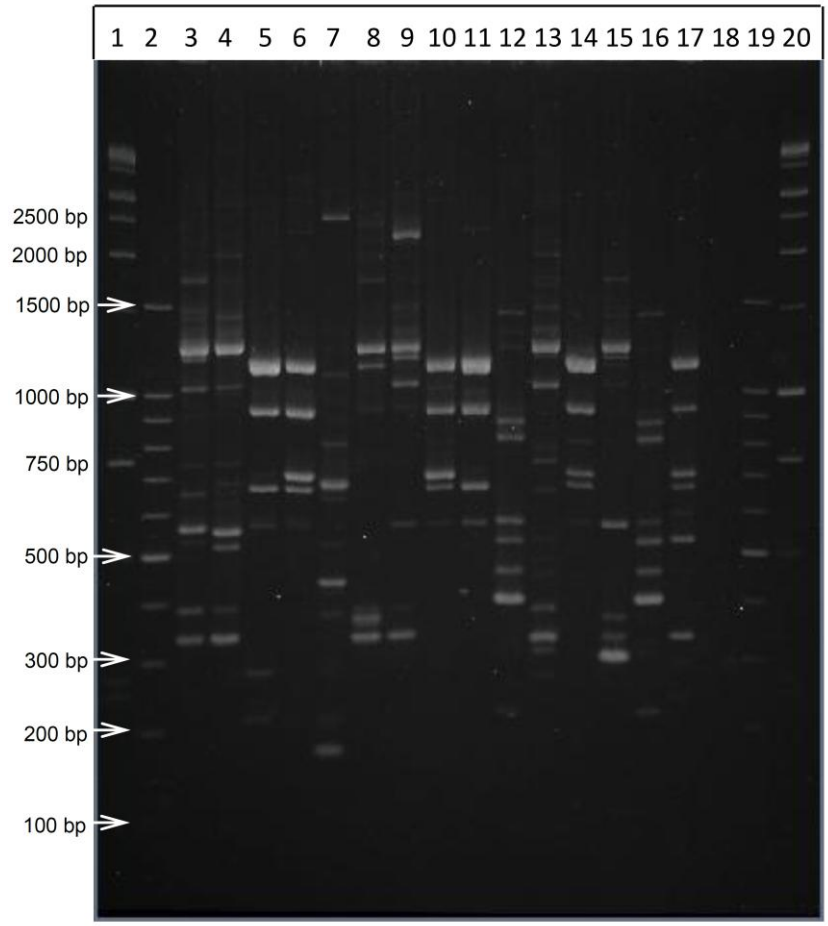


Figure 4.3.12: The first representative gel electerophoresis photo of REP-PCR for *V. parahaemolyticus* isolates from raw seafood samples. Lane 1, 1 kb. DNA marker; lane 2, 100 bp. DNA marker; lane3, D25 (positive control); lane 4, B23; lane 5, B39; lane 6, B12; lane 7, B46; lane 8, B17; lane 9, B07; lane 10, B11; lane 11, B19; lane 12, B44; lane 13, B38; lane 14, B32; lane 15, B28; lane 16, B02; lane 17, B48; lane 18, negative control; Lane 19, 100 bp. DNA marker; lane 20, 1 kb. DNA marker.

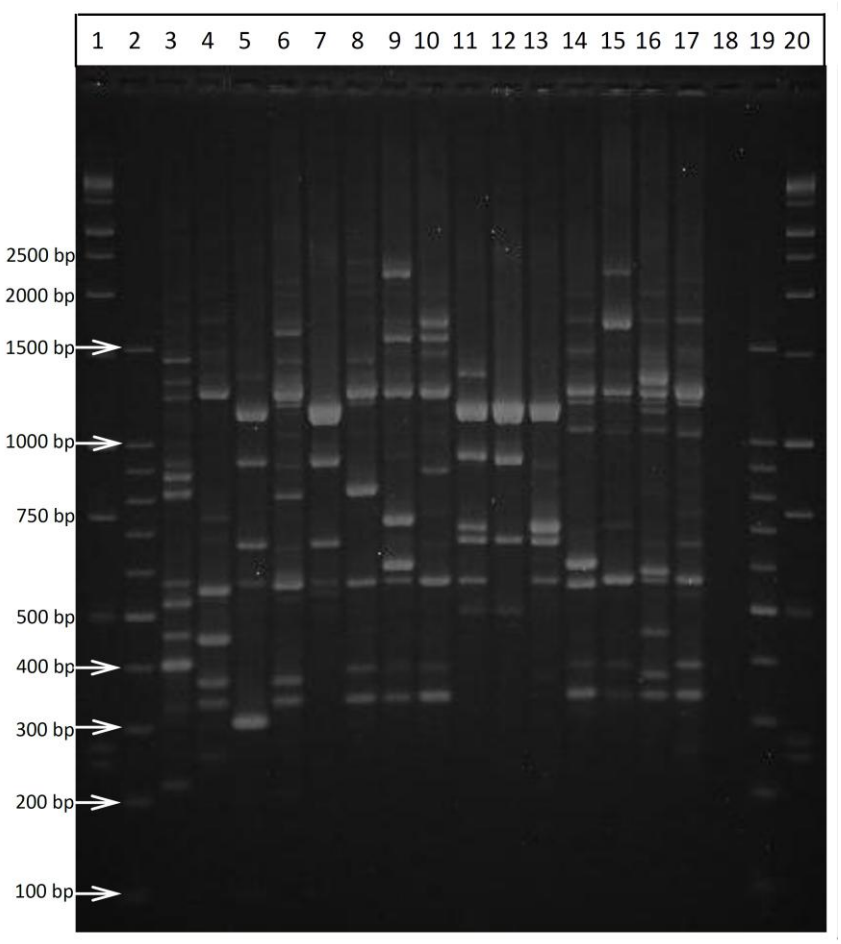
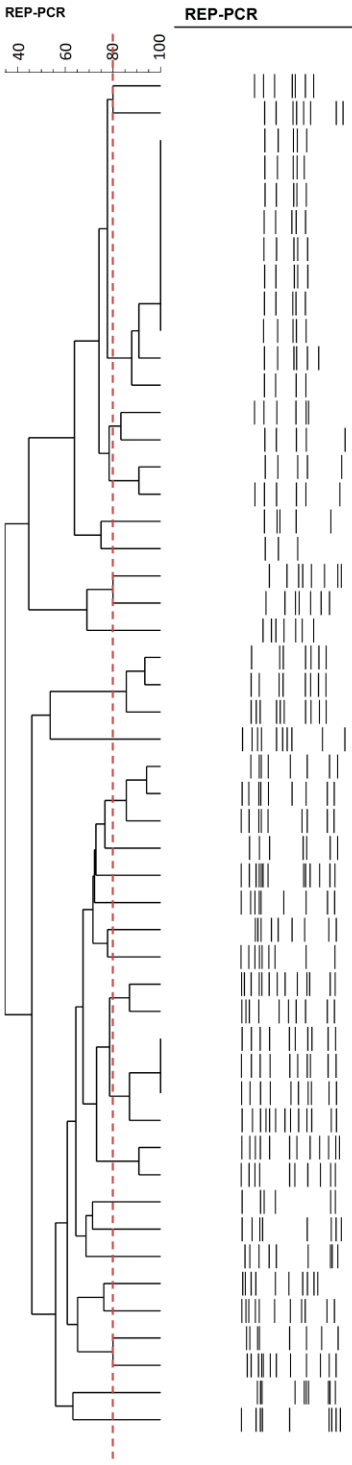


Figure 4.3.13: The second representative gel electerophoresis photo of REP-PCR for *V. parahaemolyticus* isolates from raw seafood samples. Lane 1, 1 kb. DNA marker; lane 2, 100 bp. DNA marker; lane3, B33; lane 4, B42; lane 5, B31; lane 6, B29; lane 7, B16; lane 8, B36; lane 9, B18; lane 10, B40; lane 11, B34; lane 12, B20; lane 13, B45; lane 14, B15; lane 15, B13; lane 16, B21; lane 17, D25 (positive control); lane 18, negative control; Lane 19, 100 bp. DNA marker; lane 20, 1 kb. DNA marker.



Key	Source	Date of Isolation	Location	Virulotypes	REP profile	
B34	Fish	161110	Sec. 17	toxR+	REP1	A
B48	Fish	161110	UM	toxR+	REP2	
A21	Fish	230810	Kota Dam.	toxR+	REP3	B
A03	Cockle	230810	Kota Dam.	toxR+	REP3	
B12	Shrimp	21110	Dam. In.	toxR+ , trh+	REP3	
B19	Squid	21110	Dam. In.	toxR+ , trh+	REP3	
B24	Oyster	161110	Sec. 17	toxR+ , trh+	REP3	
B03	Shrimp	21110	Mut. Dam.	toxR+ , trh+	REP3	
B32	Shrimp	161110	Sec. 17	toxR+ , trh+	REP3	
B45	Prawn	161110	Sec. 17	toxR+ , trh+	REP3	
A10	Clam	230810	Kota Dam.	toxR+	REP4	
B11	Prawn	21110	Dam. In.	toxR+	REP5	
B16	Fish	21110	PWTC	toxR+	REP6	C
B39	Oyster	161110	UM	toxR+	REP7	
B25	Fish	161110	Sec. 17	toxR+	REP8	D
B31	Clam	161110	Sec. 17	toxR+	REP9	
A02	Cockle	230810	Kota Dam.	toxR+	REP10	
B30	Prawn	161110	Sec. 17	toxR+	REP11	
B26	Fish	161110	Sec. 17	toxR+	REP12	
B46	Fish	161110	UM	toxR+	REP13	
B20	Shrimp	161110	Sec. 17	toxR+	REP14	
B02	Prawn	21110	PWTC	toxR+	REP15	E
B44	Fish	161110	UM	toxR+	REP16	
B33	Squid	161110	Sec. 17	toxR+	REP17	
B37	Clam	161110	UM	toxR+	REP18	
A30	Prawn	230810	Mut. Dam.	toxR+ , tdh+	REP19	F
B13	Oyster	21110	Dam. In.	toxR+ , tdh+	REP20	
B15	Fish	21110	PWTC	toxR+	REp21	
A31	Fish	230810	Dam. In.	toxR+	REP22	
B21	Squid	161110	Sec. 17	toxR+	REP23	
B36	Fish	161110	Sec. 17	toxR+	REP24	
A22	Fish	230810	Dam. In.	toxR+	REP25	
B07	Shrimp	21110	PWTC	toxR+	REP26	
B29	Oyster	161110	Sec. 17	toxR+	REP27	G
B40	Cockle	161110	UM	toxR+	REP28	
B23	Fish	161110	UM	toxR+	REP29	H
D25	Shrimp	131210	Midvalley	toxR+	REP29	
D07	Oyster	131210	Midvalley	toxR+	REP29	
D18	Fish	131210	Midvalley	toxR+	REP30	
B38	Oyster	161110	UM	toxR+	REP31	I
B42	Fish	161110	UM	toxR+	REP32	
B17	Fish	21110	PWTC	toxR+	REP33	
B28	Fish	161110	Sec. 17	toxR+	REP34	
A18	Shrimp	230810	Kota Dam.	toxR+	REP35	
A11	Fish	230810	Kota Dam.	toxR+	REP36	
B18	Shrimp	21110	Mut. Dam.	toxR+	REP37	
A28	Fish	230810	Dam. In.	toxR+	REP38	J
A32	Shrimp	230810	Dam. In.	toxR+	REP39	
A16	Squid	230810	Kota Dam.	toxR+	REP40	
B27	Fish	161110	Sec. 17	toxR+	REP41	

Figure 4.3.14: Dendrogram based on REP-PCR banding patterns of *V. parahaemolyticus* isolated from different samples, sources and locations (Sec 17 = Section 17; UM = University of Malaya; Kota Dam = Kota Damansara; Dam In = Damansara Intan; Mut Dam = Mutiara Damansara; PWTC = Putra World Trade Centre; Midvalley = Midvalley Megamall). The similarity scale is shown at the top of the dendrogram and 10 clusters are represented by capital letters.

In this study, five samples were identified (one shrimp and four fish samples) which were contaminated by multiple *V. parahaemolyticus* isolates of different traits based on REP profiles. The similarity shared among the isolates from the same sources ranged from 25% - 47% (Figure 4.3.15).This finding indicates that one seafoodsample could be contaminated by multiple subtypes of *V. parahaemolyticus*.

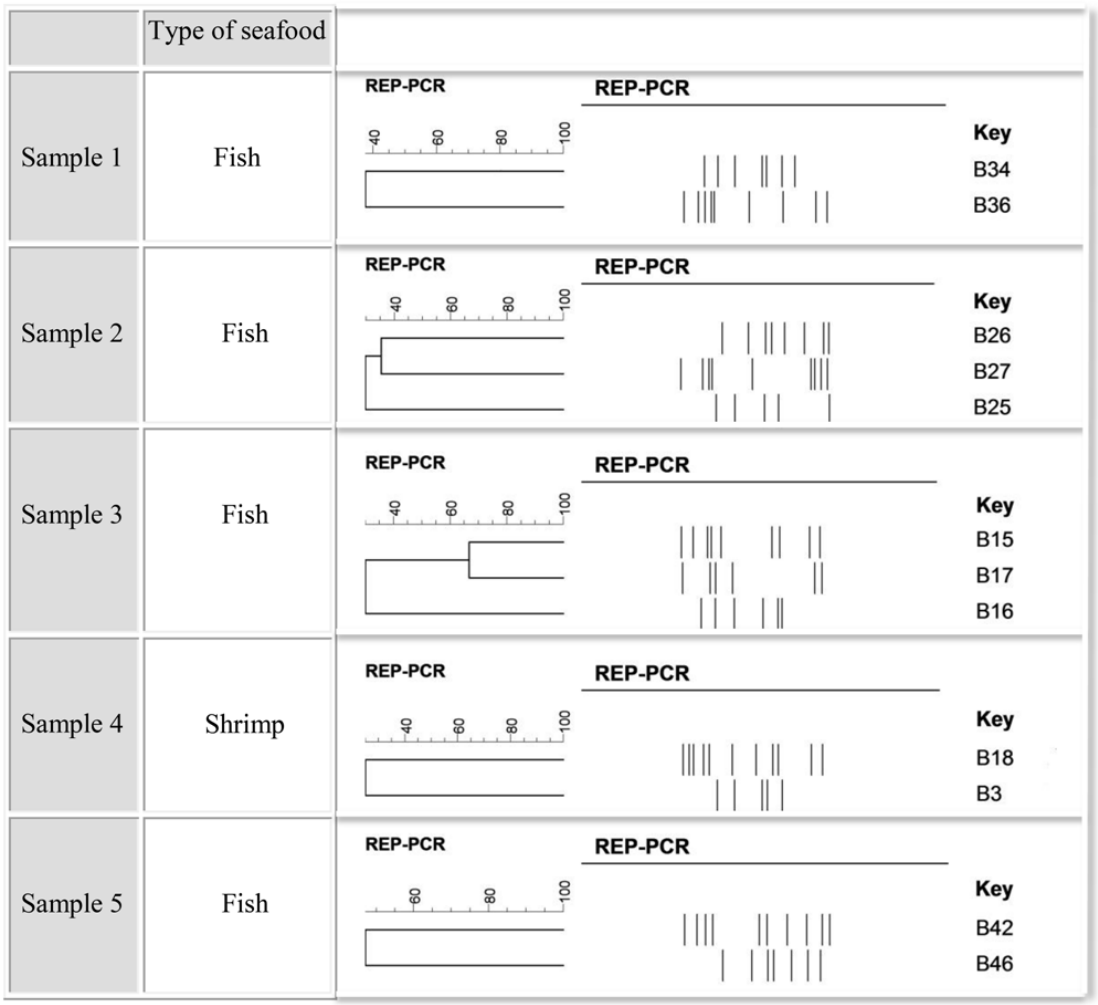


Figure 4.3.15: Illustration of the cases in which one seafood sample was contaminated by multiple subtypes of *V. parahaemolyticus*, based on REP profiles.

CHAPTER 5

DISCUSSION

A variety of methods were used in this study for identification and characterization of *Vibrio* species, and the results of these methods were compared to evaluate their discriminatory power and reliability. These methods included both conventional and molecular methods to make a comprehensive comparison of them.

Two different selective media were applied for colony appearance, as the initial step of identification of *Vibrio* species. Based on the results obtained, CHROMagar^{TM} *Vibrio* was confirmed as a more reliable medium, due to its higher efficiency and sensitivity compared with TCBS agar. Comparison of the two methodshighlighted that CHROMagar^{TM} *Vibrio* is more accurate and specific than TCBS (Figure 4.2.1) and it was in concurrence with the study by Di Pinto *et al*. (2011), where the accuracy and specificity of just 51% and 71% for TCBS compared with 88% and 95% for CHROMagar^{TM} *Vibrio* was obtained for isolation and identification of *V. parahaemolyticus* from shellfish samples.

Colony appearance on selective media was followed by conventional biochemical tests, including Oxidase, TSI, SIM, MR-VP and salt tolerance tests, for detection of *Vibrio* spp.The conventional biochemical tests were not able to distinguish *V. parahaemolyticus* from *V. vulnificus* in some cases (Appendix 5). The phenotypic similarities of the two species observed in the results of oxidase, TSI, SIM, and MR-VP tests (Kaysner *et al*., 2004) asserts that the tests which have been applied in this study were not able to efficiently differentiate these two species. Thus, for detection of the species of the isolates, the conventional biochemical tests showed low efficiency. However, the overall findings of these tests indicated that they are able to be used for detection of vibrios at genus level.

API 20E was used for confirmation of the results obtained from the biochemical tests, and the same problem was observed in the results of API 20E, where the test was unable to detect the exact species of some isolates. However, the number of these cases for the API 20E kit was much less than the biochemical tests and overall findings of API 20E indicated its higher efficiency for detection and differentiation of *V. cholerae, V. parahaemolyticus* and *V. vulnificus,*compared with conventional biochemical tests (Figure 4.3.7).

Although the majority of the results obtained from the biochemical tests and API 20E were in concurrence with the results of the multiplex PCR, the limitations of these methods in the detection and differentiationof vibrios at species level, confirms that it is required to use these conventional methods together with a molecular method for confirmation, particularly for critical studies.In the study by Croci*et al*. (2007) a comparison was made between different biochemical methods of identification and molecular method, where higher specificity of molecular methods was obtained and it indicated the need for confirmation by means of molecular methodsin order to avoid false-positive results by biochemical identifications. It was also reported by Di Pinto *et al*. (2011) where misidentifications by API 20E were observed and to avoid the erroneous biochemical identifications, confirmation of the data by molecular methods was suggested.

PCR-based detection targets the specific region of DNA, for identification of bacterial strains. Furthermore, PCR facilitates the identification of the strains that are viable but non-culturable (VNBC) (Binsztein *et al*., 2004). It is less labour intensive and much faster than conventional methods and that is the reason of its increasing application among investigators (Teh *et al*., 2008).

Multiplex PCR is a very utilizable variant of PCR which enables the performance of more reactions in a shorter time comparing to monoplex PCR and also with less effort. In this method, simultaneous amplification of two or more than two regions on DNA templates can be done (Markoulatos *et al*, 2002). The critical point in any multiplex PCR is the performance of the amplification without sacrificing the sensitivity and specificity for higher efficiency.Hence, further evaluation of the multiplex PCR targeting *gyrB* and *pntA* genes was done on spiked seafood samples to check the discriminatory power of the multiplex PCR for environmental samples and its efficiency was confirmed as a reliable detection tool in *Vibrio* spp. investigations. The *gyrB* primers amplified all *Vibrio* strains and negative results were obtained for*Salmonella* sp. and *Escherichia coli* strains. The amplification of *pntA* genes for *V. cholerae, V. parahaemolyticus* and *V. vulnificus* was successfully done and all of the *pntA* primers were specificfor the targeted species and no cross-activities happened.Thesensitivity of the primers in the multiplex PCR for detection of*V. cholera,V. parahaemolyticus* and *V. vulnificus* was evaluatedusing spiked fish, shrimp and oyster, respectively, and the values have been obtained as 2.0 x 10^3CFU mL^{-1} for both *V. cholera* and *V. parahaemolyticus* and 9.0 x 10^3CFU $mL^{-1}$for *V. vulnificus*. In the previous study by Teh *et al*. (2010), sensitivity of the *pntA* primers was evaluated based on the same multiplex PCR, in which pure cultures of positive controls for each of the *Vibrio* species were used.The values obtained by Teh et al. (2010) for *V. cholerae, V. parahaemolyticus* and *V. vulnificus* were very closeto the findings of this study. This indicates that the PCR can efficiently detect *V. parahaemolyticus, V. cholera* and *V. vulnificus* from seafood without any inhibitions, as it was able to efficiently detect the three species at a proper level of sensitivity from spiked seafood samples.

The *gyrB/pntA* genes-based multiplec PCR was applied to all of the isolates and the results at genus level were identical to those obtained from conventional identification methods (Appendix 8). High prevalence of *Vibrio spp*. (62%) among raw seafood samples in this study, was close to the findings of the previous study by Elhadi *et al.* (2004), where the prevalence of *Vibrio* spp. was found as 58% among different seafood samples includingshrimp, squid, crab, cockles, and mussels, collected from four states in Malaysia. High prevalence of *Vibrio* spp. was obtained in the study by Noorlis *et al.* (2011) where 98.7% of 150 examined fish samples collected from the hypermarkets in Selangor, Malaysia were positive for *Vibrio* spp.The presence of *Vibrio* spp. in raw seafood might be expected, as these organisms naturally occur in marine environments and aquatic systems. Especially in seafoods from regions with temperate climates, where the prevalence of *Vibrio* spp. is expected to be higher, due to the optimal conditions for their growth in those areas. However, the high level of *Vibrio* spp. in some samples of raw seafood may indicate inadequate control in storage temperature from the time of harvesting and this level is regarded as unsatisfactory by some food criteria (Aberoumand, 2010).

The findings of this study, based on the results obtained from the *gyrB/pntA* genes-based multiplex PCR and confirmed by *toxR* gene-based PCR, indicate the prevalence of *V. parahaemolyticus* in 29% of raw seafood samples (Figure 4.3.5).Other studies in Southeast Asian countriesalso reportedrelatively high prevalence of *V. parahaemolyticus* in different seafoods. 24% of the freshwater fish samples tested by Noorlis *et al.* (2011) were found to harbour *V. parahaemolyticus*. A higher percentage was also reported by Zulkifli et al. (2009a), in which 50% of cockle samples collected from Padang, Indonesia were found to harbour *V. parahaemolyticus*. Seafood samples imported from Hong Kong, Indonesia, Thailand and Vietnam were tested by Wong et al. in 1999 and 45.9% of the samples were positive for *V. parahaemolyticus*. The results

obtained from this study and other relevant investigations affirm the potential of *V. parahaemolyticus* as a major foodborne pathogens and one of the main causes of gastroenteritis outbreaks associated with seafood consumption in different parts of the world (Di Pinto *et al*., 2011) and especially in Southeast Asian countries, due to the high prevalence of *V. parahaemolyticus* and vast consumption of seafood (Wong *et al*., 1999). The high prevalence of *V. parahaemolyticus* in seafood and marine products is considered as potentially hazardous, regarding the probability of pathogenicity among the contaminant strains.

toxR gene can be used to identify *V. parahaemolyticus* isolates (Sechi *et al.*, 2000), either pathogenic or non-pathogenic (Dileep *et al.*, 2003; Sujeewa *et al.*, 2009). Hence, *toxR* gene-based PCR was used in this study, to confirm the results of *gyrB/pntA* genes-based multiplex PCR for *V. parahaemolyticus* isolates.All of the*V. parahaemolyticus* isolates were confirmed by the presence of *toxR* gene. Consequently, the identical results of *V. parahaemolyticus* detection obtained from the *gyrB/pntA* genes-based multiplex PCR and the *toxR* gene-based PCR, indicate that both of the amplifications are reliable for the identification of this species.

Different investigations reported that most of the clinical and pathogenic strains of *V. parahaemolyticus* harboured at least one of the two hemolysin genes (Shirai et al., 1990). This affirms that there is a strong correlation between the presence of hemolysin and pathogenicity.In this study, the presence of *trh* gene in six (12%) isolates washigherthanthe previous findings that only a small percentage (<8%) of environmental *V. parahaemolyticus* populations harboured *trh* (Robert-Pilot *et al.*, 2004; Wong, 2003; Hervio-Heath *et al.* 2002; Ottaviani *et al.*, 2010).The prevalence of *tdh* gene among the isolates was 4% (2/50). According toDileep *et al.* (2003) and Kanjanasopa *et al.* (2011) the prevalence of *tdh*-positive isolates from the environmental sources was as low as 1-2%. However, higher prevalence of *tdh*-positive

isolates (>10%) in the oysters collected from Alabama, United States and from the southwest coast of India was reported by Depaola *et al.* (2003) and Deepanjali *et al.* (2005), respectively.The lower percentage obtained in this study, compared with Depaola et al. (2003) and Deepanjali et al. (2005) may be due to the limitations of conventional methods, in which only a few colonies can be tested. Therefore, direct PCR (Deepanjali et al., 2005) and colony hybridization technique (Depaola et al., 2003) can help to have an accurate identification of virulent isolates.

The results obtained fromvirulotyping of *V. parahaemolyticus* isolates confirm the existence of *tdh* and *trh* genes among the isolates.The two genes haveindispensable roles in virulence. Hence, the results of this study indicate that pathogenic *V. parahaemolyticus* isolates are present in raw seafood sold in retail stores and hypermarkets in Malaysia and it confirms the high risk of infection for seafood and marine products consumers. Especially for those who consume the products in raw or insufficiently cooked conditions.

The results obtained from REP-PCR, indicated genetic diversity among *V. parahaemolyticus* isolates, as a few isolates formed clusters and most of them had unique REP profile. Moreover, the similarity value of the majority of the isolates was less than 80% (Figure 4.3.13). No clear genetic relatedness was observed among the isolates isolated from same type of seafood, from same location or at the same time. Low level of similarity was obtained even among the isolates which were isolated from the same seafood sample, and all of these confirm the heterogeneity of the isolates. High genetic diversity of *V. parahaemolyticus* strains had been previously reported in other studies (Wong *et al*. 1999; Cook *et al*. 2002; Wong *et al*. 2003). Maluping *et al.* (2008) reported 17 different REP profiles obtained from 17 *V. parahaemolyticus* isolates. In another study by Chakraborty *& Surendran* (2009) 20 different REP profiles were observed among 27 *V. parahaemolyticus* isolates. In the investigation by Sudheesh

et al.(2002) only few isolates were clustered with a similarity of more than 80%. It suggests that the genetic diversity which exists among *V. parahaemolyticus* isolates, is a natural attribute of this species (Johnson *et al*., 2009), which makes its characterization very difficult.

Similar REP profiles were obtained for the *trh*-positive isolates and the REP profiles of the *tdh*-positiveisolates shared a very high similarity. However, the isolates of different virulotypes shared less than 40% similarity based on REP-fingerprinting. Therefore, it can be hypothesized that REP-PCR might be able to distinguish strains with different virulotypes. Johnson *et al*. (2009) also reported that *tdh*-positive strains were distinctly related to *trh*-positive strains.

Based on the results obtained from the present study and other relevant studies conducted to investigate *Vibrio* spp. populations (Elhadi *et al.*, 2004; Noorlis *et al.*, 2011), it is confirmed that Vibrios are important pathogens associated with seafood. Different seafoods including fish, shrimps, prawn, cockles, oysters, clams and squids, are in high demand in Malaysia. The presence of *Vibrio* spp. in a remarkable percentage of raw seafood samples, collected from retail stores in this study, shows that they are consumed by many customers daily. And consequently, there is the risk of getting infected by consumption of contaminated food, due to the existence of pathogenic strains in seafood and marine products.

Inadequate cooking and contamination by raw seafood are of the main contribution factors for these cases. Many of the contaminated or cross-contaminated seafoods might be consumed uncooked or in an insufficiently cooked mode and it might increase the possibilities of infections. To decrease the risks of getting such infecions, it is necessary to consume well-cooked seafood and to avoid the consumption of raw or undercooked seafood products. Another problem which is associated with vibrios infections by consumption of seafood is post-heat contamination. Such risk is further

increased if the seafood is mishandled during processing where pathogens are able to multiply exponentially under favourable conditions. Hence, strict controls in storage temperature must be conducted for either uncooked or cooked products, in order to prohibit the growth of *Vibrio* spp.

There is also the probability for cross-contamination of other foods by a contaminated product which is stored or sold near them (Noorlis *et al*., 2011).The use of contaminated containers without any proper hygienic measures might be the source of contamination. In the study conducted by Yang *et al*. (2008), hygienic problems have been identified as a major cause of *V. parahaemolyticus* contaminations and infection. To assure the proper way of handling and transportation of seafoods, hygienic issues should be mandatory. It can contribute to reduce the level of contamination and cross-contamination of the foods which are kept and sold in fresh hypermarkets and retail stores.

A reliable diagnosis is the initial part of a successful monitoring and surveillance plan for vibrios investigations. Detection and differentiation of the strains with high sensitivity and specificity is desirable. Laborious, expensive and time consuming methods are not appropriate for larger scales. The results of this research indicated higher efficiency of Polymerase Chain Reaction (PCR) rather than conventional methods for identification and differentiation of different species of vibrios. Another advantage of PCR compared with conventional identification methods is the ability of this method to discriminate virulent strains and avirulent ones (Deepanjali et al., 2005). Furthermore, lots of time and energy can be saved by molecular methods. Hence, efficient molecular methods with a high discriminatory power need to be developed and evaluated to be applied in future investigations.The sensitivity and specificity of the multiplex PCR, obtained in this study for *Vibrio* spp. detection, affirms that it can be utilized in future monitoring programmes.

CHAPTER 6

CONCLUSION&RECOMMENDATION

6.1 CONCLUSION

1. CHROMagar^{TM} *Vibrio* was confirmed as a more reliable medium for detection of *Vibrio* spp., due to its higher efficiency and sensitivity compared with TCBS agar.

2. Comparison of the results obtained from biochemical tests and API 20E with the multiplex PCR findings indicated that API 20E had higher discriminatory power (83%) to differentiate*V. cholerae, V. parahaemolyticus* and *V. vulnificus*, compared with the biochemical tests (56%).

3. 100% specificity and accuracy and the sensitivityof the primers obtained from further evaluation of the *gyrB/pntA* genes-based multiplex PCR on spiked seafood samples, confirmed that it has the potential to serve as a rapid detection of *Vibrio* spp.

4. Same results were obtained for *V. parahaemolyticus* detection from the *gyrB/pntA* genes-based multiplex PCR and the PCR targeting *toxR* gene. It affirms that both of the amplifications are reliable for the identification of *V. parahaemolyticus* and either of them can be utilized in screening programmes.

5. Considerable prevalence of *V. parahaemolyticus* in different seafoods sold in fresh retail stores in Malaysia and the presence of virulent *trh*-positive and *tdh*-positiveisolates among the isolates asserts a high risk of contamination for the consumers.

6. The results obtained from REP-PCR indicated high genetic diversity among the *V. parahameolyticus*isolatesand REP-PCR was shown to be able to distinguish isolates with different virulotypes.

6.2 RECOMMENDATION

Based on the obtained results, a high risk of contamination by pathogenic vibrios is associated with seafood consumption in Malaysia.To reduce the risk of getting infected by pathogenic *Vibrio* species, it is necessary to avoid raw or insufficiently cooked seafood and marine products. Furthermore, we need to strictly control the storage temperature and hygienic handling of seafood.

With reference to the findings of this study, long-term monitoring and screening programmes for the detection of pathogenic *Vibrio* spp., and particularly, *V. parahaemolyticus* strains in seafood industry in Malaysia are required. In such programmes, application of molecular methods is strongly recommended to confirm the findings of conventional methods of identification and to differentiate pathogenic strains of *Vibrio* species.

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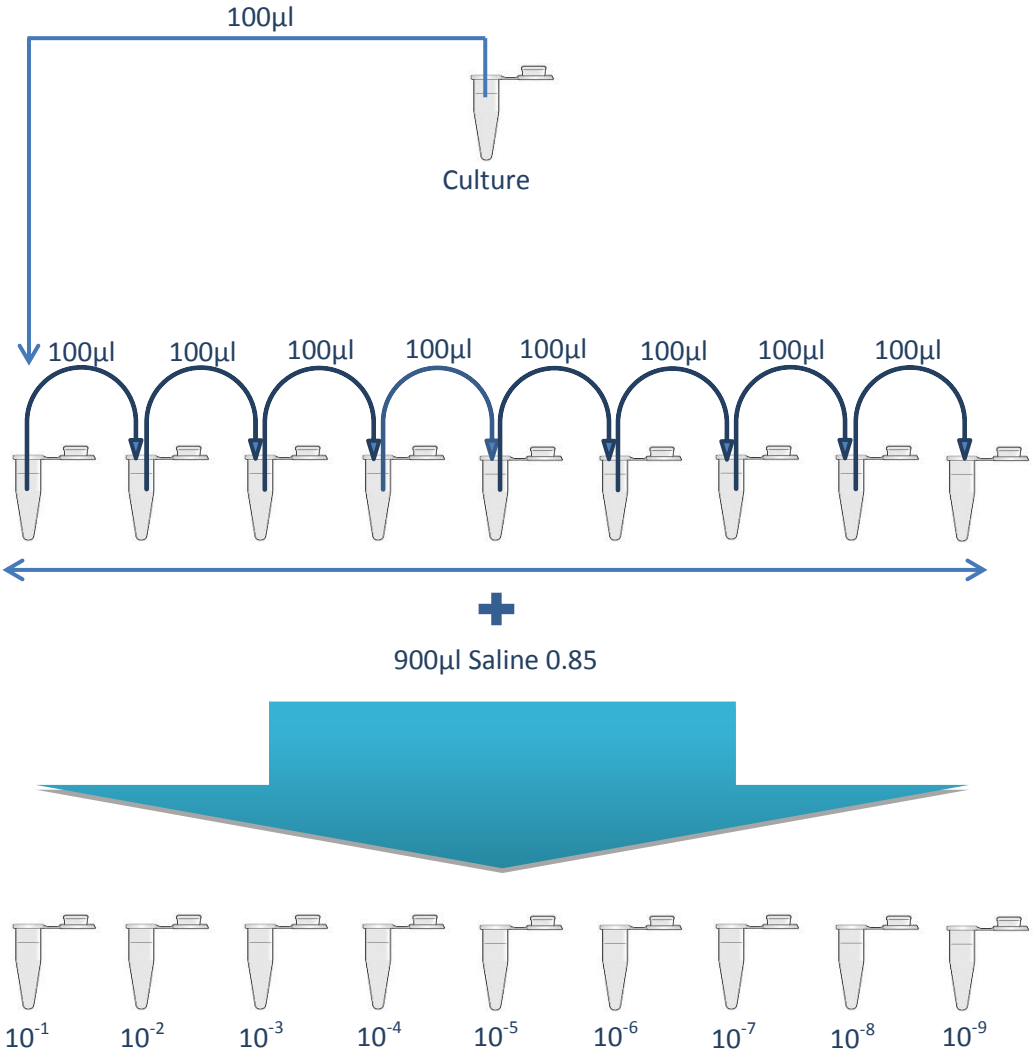
APPENDICES

APPENDIX 1

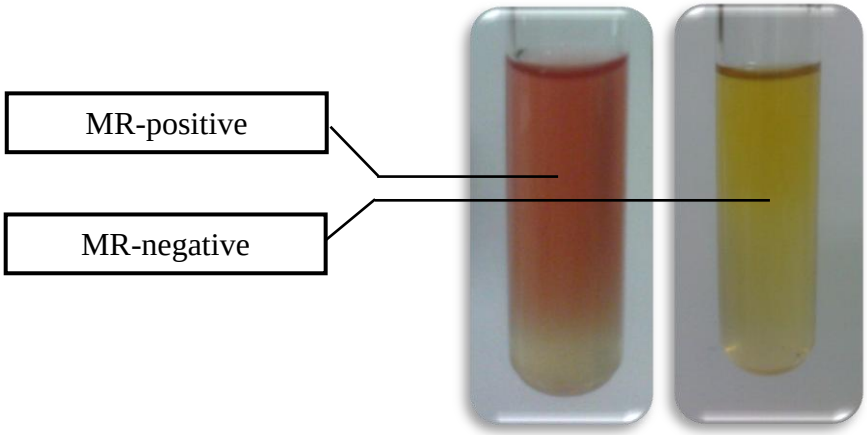
Procedure of 10-fold dilution

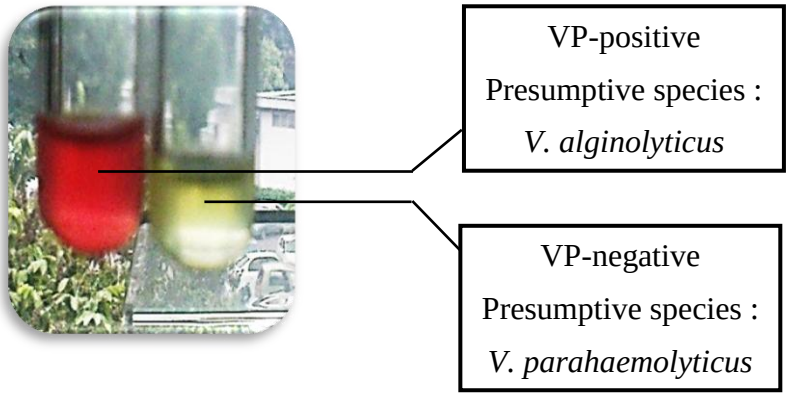


APPENDIX 2

Conventional Biochemical tests results

Methyl Red-Voges-Proskauer (MR-VP) test

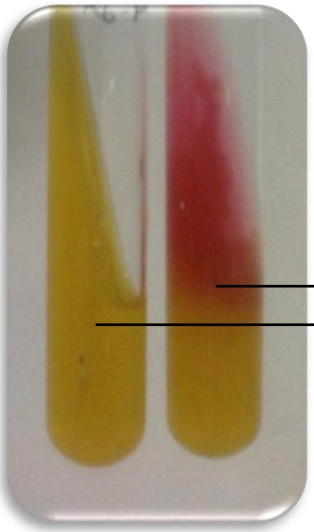




APPENDIX 3

Conventional Biochemical tests results

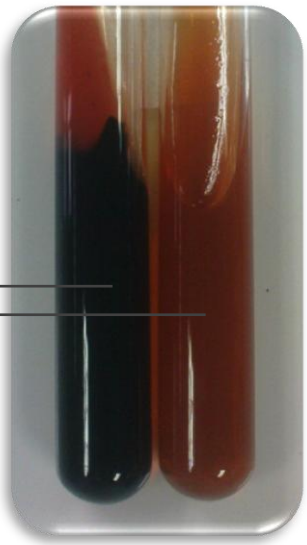
Triple Sugar Iron (TSI) test



K/A
Slant alkaline / Butt acidic

A/A
Slant acidic / Butt acidic

K/K + H_2S
Slant alkaline / Butt alkaline
+ Sulfure reduction-positive

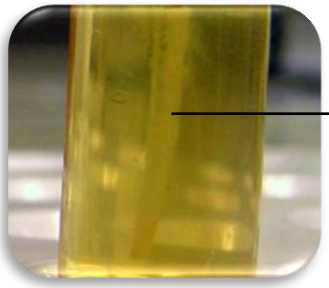


K/K
Slant alkaline / Butt alkaline
V. vulnificus
V. fluvialis

APPENDIX 4

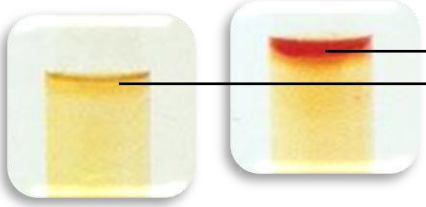
Conventional Biochemical tests results

Sulfur reduction – Indole – Motility (SIM) test



Motility-positive
Sulfur reduction-negative

Blach colour
Sulfur reduction-positive



Indole-positive

Indole-negative

APPENDIX 5

The results and presumptions of conventional biochemical tests

	Name	Oxidase	TSI	H_2S	MR	VP	Indole	Motility	Salt Tolerance				Biochemical Presumption
									0%	3%	6%	10%	
1	A01	+	K/A	-	+	-	+	+	-	+	+	-	Vv
2	A02	+	K/A	-	+	-	+	+	-	+	+	-	Vp / Vv
3	A03	+	K/A	-	+	-	-	+	-	+	+	-	Vv
4	A04	+	A/A	-	+	+	+	+	-	+	+	+	Va
5	A05	+	K/A	-	+	-	+	+	-	+	+	-	Vp
6	A06	+	A/A	-	+	-	-	+	+	+	-	-	Vc
7	A07	+	A/A	-	+	-	+	+	+	+	-	-	Vc
8	A08	+	K/A	-	+	-	+	+	-	+	+	-	Vv/Vp
9	A09	+	A/A	-	+	-	+	+	+	+	+	-	Vsp
10	A10	+	K/A	-	+	-	+	+	-	+	+	+	Vp/Vv
11	A11	+	K/A	-	+	-	+	+	-	+	+	-	Vp
12	A12	+	K/A	-	+	-	+	+	-	+	+	-	Vsp
13	A13	+	K/A	-	+	-	+	+	+	+	-	-	Vm
14	A14	+	A/A	-	+	+	+	+	+	+	-	-	Vc
15	A15	+	K/A	-	+	-	+	+	+	+	+	-	Vsp
16	A16	+	K/A	-	+	-	+	+	-	+	+	-	Vp
17	A17	+	K/A	-	+	-	+	+	-	+	+	-	Vp
18	A18	+	K/A	-	+	-	+	+	-	+	+	-	Vv
19	A19	+	A/A	-	+	-	+	+	-	+	+	+	Vsp
20	A20	+	K/A	-	+	-	+	+	-	+	+	-	Vsp
21	A21	+	K/A	-	+	-	+	+	-	+	+	-	Vv/Vp
22	A22	+	K/A	-	+	-	+	+	-	+	+	-	Vp
23	A23	+	K/A	-	+	-	+	+	-	+	+	+	Vsp
24	A24	+	A/A	-	+	+	+	+	-	+	+	+	Va
25	A25	+	K/A	-	+	-	+	+	+	+	+	-	Vsp
26	A26	+	A/A	-	+	-	+	+	-	+	+	-	Vsp
27	A27	+	K/A	-	+	-	+	+	+	+	-	-	Vm

Appendix 5 – continued.													
28	A28	+	K/A	-	+	-	+	+	-	+	+	-	Vp
29	A29	+	A/A	-	+	+	+	+	+	+	-	-	Vc
30	A30	+	K/A	-	+	-	+	+	-	+	+	+	Vp
31	A31	+	K/A	-	+	-	+	+	-	+	+	-	Vp/Vv
32	A32	+	K/A	-	+	-	+	+	-	+	+	-	Vp
33	A33	+	K/A	-	+	-	+	+	+	+	+	-	Vsp
34	A34	+	K/A	-	+	+	+	+	-	+	+	+	Va
35	A35	+	A/A	-	+	+	+	+	+	+	-	-	Vc
36	A36	+	K/A	-	+	-	+	+	-	+	+	-	Vp
37	A37	+	K/A	-	+	-	+	+	-	+	+	-	Vv / Vp
38	A38	+	K/A	-	+	-	+	+	-	+	+	+	Vsp
39	A39	+	A/A	-	+	+	+	+	-	+	+	+	Va
40	A40	+	K/A	-	+	-	+	+	+	+	-	-	Vm
41	A41	+	A/A	-	+	-	+	+	-	+	+	+	Vsp
42	B01	+	K/A	-	+	-	+	+	-	+	+	-	Vv/Vp
43	B02	+	K/A	-	+	-	+	+	-	+	+	-	Vp
44	B03	+	K/A	-	+	-	+	+	-	+	+	-	Vp
45	B04	+	A/A	-	+	+	+	+	-	+	+	+	Va
46	B05	+	A/A	-	+	-	+	+	+	+	-	-	Vc
47	B06	+	K/A	-	+	-	+	+	+	+	+	-	Vsp
48	B07	+	K/A	-	+	-	+	+	-	+	+	-	Vv
49	B08	+	A/A	-	+	+	+	+	-	+	+	+	Va
50	B09	+	A/A	-	+	+	+	+	+	+	+	+	Va
51	B10	+	K/A	-	+	-	+	+	+	+	-	-	Vm
52	B11	+	K/A	-	+	-	+	+	-	+	+	+	Vp
53	B12	+	K/A	-	+	-	+	+	-	+	+	-	Vv/Vp
54	B13	+	K/A	-	+	-	+	+	-	+	+	-	Vp
55	B14	+	K/A	-	+	-	+	+	-	+	+	-	Vsp
56	B15	+	K/A	-	+	-	+	+	-	+	+	-	Vp
57	B16	+	K/A	-	+	-	+	+	+	+	-	-	Vm
58	B17	+	K/A	-	+	-	+	+	-	+	+	-	Vp
59	B18	+	K/A	-	+	-	+	+	-	+	+	-	Vp
60	B19	+	K/A	-	+	-	+	+	-	+	+	-	Vv/Vp

Appendix 5 – continued.													
61	B20	+	K/A	-	+	-	+	+	-	+	+	-	Vp
62	B21	+	A/A	-	+	+	+	+	+	-	-	-	Vc
63	B22	+	A/A	-	+	+	+	+	+	+	+	-	Vc/Va
64	B23	+	K/A	-	+	-	+	+	-	+	+	-	Vp
65	B24	+	K/A	-	+	-	+	+	-	+	+	-	Vp
66	B25	+	K/A	-	+	-	+	+	-	+	+	-	Vv/Vp
67	B26	+	A/A	-	+	-	+	+	+	+	-	-	Vm/Vc
68	B27	+	K/A	-	+	-	+	+	-	+	+	-	Vp
69	B28	+	K/A	-	+	-	+	+	-	+	+	-	Vv
70	B29	+	K/A	-	+	-	+	+	-	+	+	-	Vv/Vp
71	B30	+	K/A	-	+	-	+	+	-	+	+	-	Vp
72	B31	+	K/A	-	+	-	+	+	-	+	+	-	Vv/Vp
73	B32	+	K/A	-	+	-	+	+	-	+	+	-	Vp
74	B33	+	K/A	-	+	-	+	+	-	+	+	-	Vm
75	B34	+	K/A	-	+	-	+	+	-	+	+	-	Vp
76	B35	+	A/A	-	+	-	+	+	+	+	-	-	Vsp
77	B36	+	K/A	-	+	-	+	+	-	+	+	-	Vv/Vp
78	B37	+	A/A	-	+	-	+	+	+	+	-	-	Vc
79	B38	+	K/A	-	+	-	+	+	-	+	+	-	Vv/Vp
80	B39	+	K/A	-	+	+	+	+	-	+	+	+	Va
81	B40	+	K/A	-	+	-	+	+	-	+	+	-	Vv
82	B41	+	K/A	-	+	-	+	+	+	+	-	-	Vm
83	B42	+	K/A	-	+	-	+	+	-	+	+	+	Vp
84	B43	+	K/A	-	+	-	+	+	+	+	-	-	Vm
85	B44	+	A/A	-	+	-	+	+	-	+	+	+	Vsp
86	B45	+	A/A	-	+	+	+	+	-	+	+	+	Va
87	B46	+	K/A	-	+	-	+	+	-	+	+	-	Vp
88	B47	+	K/A	-	+	-	+	+	+	+	-	-	Vm
89	B48	+	K/A	-	+	-	+	+	-	+	+	-	Vf
90	B49	+	A/A	-	+	-	+	+	+	+	+	-	Vsp
91	B50	+	K/A	-	+	-	+	+	-	+	+	-	Vv/Vp
92	B51	+	K/A	-	+	-	+	+	+	+	-	-	Vm
93	B52	+	K/A	-	+	-	+	+	-	+	+	-	Vv

Appendix 5 – continued.													
94	B53	+	A/A	-	+	-	+	+	+	+	-	-	Vc
95	B54	+	K/A	-	+	-	+	+	+	+	+	-	Vsp
96	B55	+	A/A	-	+	+	+	+	+	+	+	-	Vc/Va
97	B56	+	A/A	-	+	-	+	+	+	+	-	-	Vc
98	B57	+	A/A	-	+	-	-	+	-	+	+	+	Vsp
99	B58	+	K/A	-	+	-	+	+	-	+	+	-	Vp/Vv
100	B59	+	A/A	-	+	-	+	+	+	+	+	-	Vsp
101	B60	+	K/A	-	+	-	+	+	-	+	+	-	Vsp
102	B61	+	A/A	-	+	+	+	+	+	+	-	-	Vc
103	C01	+	K/A	-	+	-	+	+	-	+	+	-	Vv
104	C02	+	K/A	-	+	-	+	+	-	+	+	-	Vv/Vp
105	C03	+	K/A	-	+	-	+	+	-	+	+	-	Vv
106	C04	+	A/A	-	+	-	+	+	-	+	+	+	Vp/Vv
107	C05	+	A/A	-	+	-	+	+	-	+	+	-	Vsp
108	C06	+	A/A	-	+	+	+	+	-	+	+	+	Va
109	C07	+	K/A	-	+	-	+	+	+	+	-	-	Vm
110	C08	+	K/A	-	+	-	-	+	-	+	+	-	Vsp
111	C09	+	K/A	-	+	-	+	+	+	+	+	-	Vsp
112	C10	+	A/A	-	+	+	+	+	-	+	+	+	Va
113	C11	+	A/A	-	+	+	+	+	+	+	+	-	Vsp
114	C12	+	K/A	-	+	-	+	+	-	+	+	-	Vsp
115	C13	+	K/A	-	+	-	+	+	+	+	-	-	Vm
116	C14	+	A/A	-	+	+	+	+	-	+	+	+	Va
117	C15	+	K/A	-	+	-	+	+	+	+	+	-	Vsp
118	C16	+	K/A	-	-	-	-	+	-	+	+	+	Vsp
119	C17	+	A/A	-	+	-	+	+	+	+	-	-	Vc
120	C18	+	K/A	-	+	-	+	+	-	+	+	-	Vv
121	C19	+	A/A	-	+	+	+	+	-	+	+	+	Va
122	C20	+	K/A	-	+	-	+	+	-	+	+	-	Vsp
123	C21	+	K/A	-	+	-	+	+	-	+	+	-	Vv/Vp
124	C22	+	K/A	-	-	-	+	+	-	+	+	-	Vsp
125	C23	+	A/A	-	+	-	+	+	-	+	+	-	Vsp
126	C24	+	K/A	-	+	+	+	+	-	+	+	-	Vsp

Appendix 5 – continued.

127	C25	+	K/A	-	+	-	+	+	-	+	+	+	Vsp
128	C26	+	K/A	-	+	-	+	+	-	+	+	-	Vv
129	C27	+	K/A	-	+	-	+	+	-	+	+	-	Vsp
130	C28	+	A/A	-	+	+	+	+	+	+	-	-	Vc
131	D01	+	K/A	-	+	-	+	+	-	+	+	-	Vv
132	D02	+	K/A	-	+	-	+	+	-	+	+	-	Vp
133	D03	+	K/A	-	+	-	+	+	-	+	+	-	Vv
134	D04	+	K/A	-	+	-	+	+	-	+	+	-	Vv
135	D05	+	K/A	-	+	-	+	+	-	+	+	-	Vv/Vp
136	D06	+	K/A	-	+	-	+	+	-	+	+	-	Vp
137	D07	+	K/A	-	+	-	+	+	-	+	+	-	Vp
138	D08	+	K/A	-	+	-	+	+	-	+	+	-	Vv
139	D09	+	K/A	-	+	-	+	+	-	+	+	-	Vp
140	D10	+	K/A	-	+	-	+	+	-	+	+	+	Vp
141	D11	+	K/A	-	+	-	+	+	-	+	+	-	Vv/Vp
142	D12	+	A/A	-	+	-	+	+	+	+	-	-	Vsp
143	D13	+	K/A	-	+	-	+	+	-	+	+	+	Vsp
144	D16	+	K/A	-	+	-	+	+	-	+	+	-	Vv/Vp
145	D17	+	K/A	-	+	-	+	+	-	+	+	-	Vv/Vp
146	D18	+	K/A	-	+	-	+	+	-	+	+	-	Vp
147	D19	+	K/A	-	+	-	+	+	-	+	+	-	Vp
148	D20	+	K/A	-	+	-	+	+	+	+	-	-	Vm
149	D21	+	K/A	-	+	-	+	+	-	+	+	-	Vp
150	D22	+	K/A	-	+	-	+	+	-	+	+	-	Vp
151	D23	+	K/A	-	+	-	+	+	-	+	+	-	Vv
152	D24	+	K/A	-	+	-	+	+	-	+	+	-	Vp
153	D25	+	K/A	-	+	-	+	+	-	+	+	-	Vp
154	D26	+	K/A	-	+	-	+	+	-	+	+	-	Vv/Vp
155	D27	+	K/A	-	+	-	+	+	-	+	+	-	Vv
156	D30	+	A/A	-	+	+	+	+	+	+	+	-	Vsp
157	D31	+	K/A	-	+	-	+	+	-	+	+	+	Vsp
158	D32	+	K/A	-	+	-	+	+	-	+	+	+	Vm
159	D33	+	K/A	-	+	-	-	+	-	+	+	-	Vsp

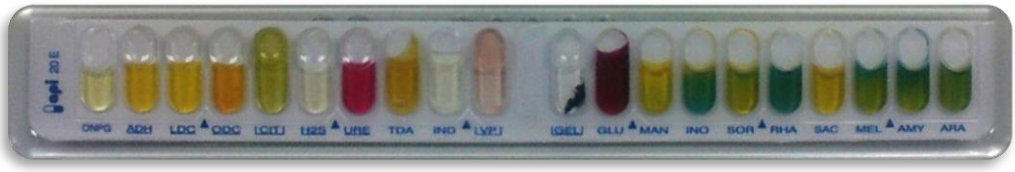
Appendix 5 – continued.

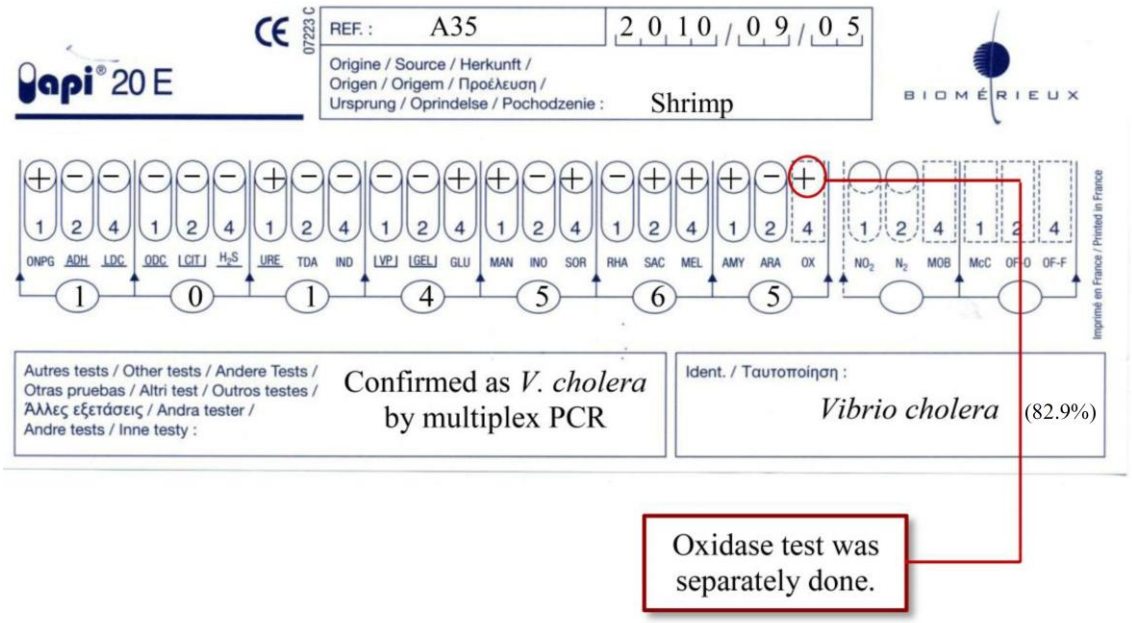
160	D34	+	A/A	-	+	+	+	+	+	+	-	-	Vc
161	D35	+	A/A	-	+	+	+	+	+	+	-	-	Vc
162	D36	+	A/A	-	+	+	+	+	-	+	+	+	Va/Vsp
163	D37	+	A/A	-	+	+	+	+	-	+	+	+	Va
164	D38	+	A/A	-	+	-	+	+	+	+	-	-	Vc
165	D39	+	K/A	-	+	-	+	+	-	+	+	-	Vsp
166	D40	+	K/A	-	+	-	+	+	-	+	+	+	Vsp
167	D41	+	K/A	-	+	-	+	+	+	+	+	-	Vsp
168	D42	+	A/A	-	+	+	+	+	-	+	+	+	Va
169	D43	+	K/A	-	+	-	+	+	-	+	+	-	Vsp
170	D44	+	K/A	-	+	-	+	+	+	+	-	-	Vm
171	D45	+	K/A	-	+	-	+	+	-	+	+	-	Vv
172	D46	+	K/A	-	+	-	+	+	-	+	+	-	Vsp
173	D47	+	K/A	-	+	-	+	+	+	+	+	-	Vsp
174	D48	+	K/A	-	+	-	+	+	+	+	-	-	Vm
175	D49	+	A/A	-	+	+	+	+	-	+	+	+	Va
176	D50	+	A/A	-	+	-	+	+	+	+	+	-	Vsp
177	D51	+	K/A	-	+	+	+	+	-	+	+	+	Vsp
178	D52	+	K/A	-	+	-	+	+	-	+	+	+	Vsp
179	D53	+	K/A	-	+	-	+	+	-	+	+	-	Vv
180	D54	+	K/A	-	+	+	+	+	-	+	+	+	Vsp
181	D55	+	K/A	-	+	-	+	+	+	+	-	-	Vm
182	D56	+	K/A	-	+	-	-	+	-	+	+	+	Vsp
183	D57	+	K/A	-	+	-	+	+	-	+	+	-	Vsp
184	D58	+	K/A	-	+	+	+	+	+	+	+	-	Vsp
185	D59	+	K/A	-	+	+	+	+	-	+	+	-	Vsp
186	D60	+	A/A	-	+	+	+	+	-	+	+	+	Va
187	D61	+	K/A	-	+	-	+	+	+	+	-	-	Vm

Abbreviations	
K/K = Slant alkaline / Butt alkaline	Vc *: Vibrio cholera*
K/A = Slant alkaline / Butt acidic	Vf : *Vibrio fluvialis*
A/A = Slant acidic / Butt acidic	Vm : *Vibrio mimicus*
Vp : *Vibrio parahaemolyticus*	Va *: Vibrio alginolyticus*
Vv *:Vibrio vulnificus*	Vsp *: Vibrio* sp. (Unknown species)

APPENDIX 6

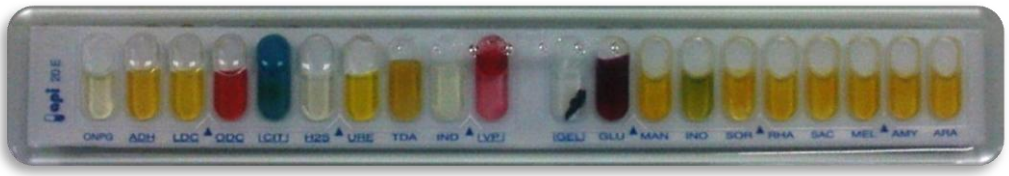
API 20E (Example 1 of test results)

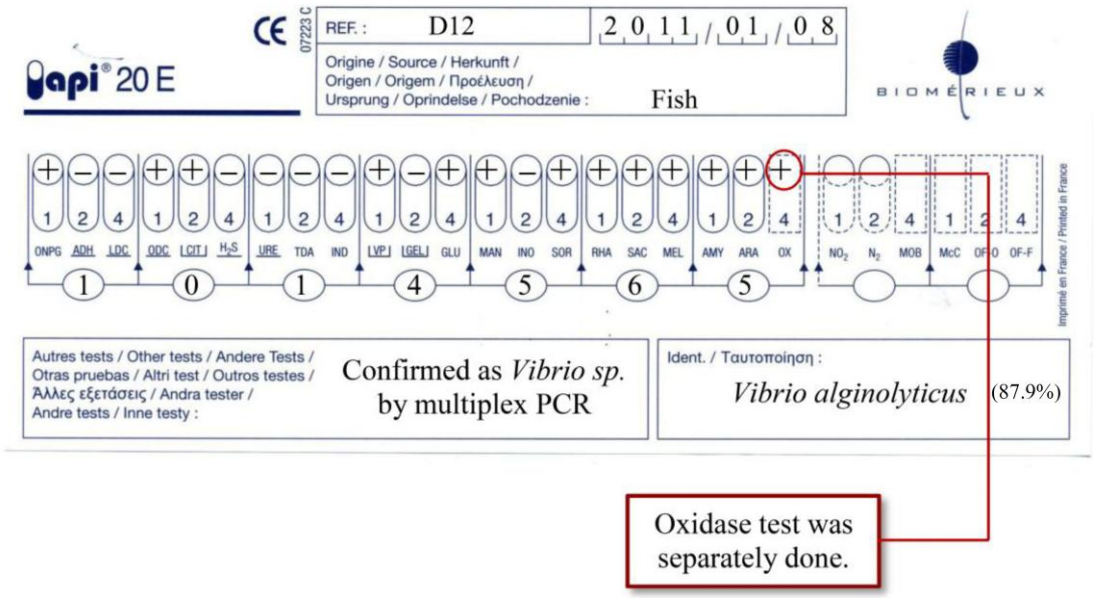




APPENDIX 7

API 20E (Example 2 of test results)





APPENDIX 8

The results obtained from conventional methods of identification and the *gyrB/pntA* genes-based multiplex PCR amplification

	Name	Sample	DOI	POI	Biochemical Presumption	API Kit confirmation	PCR Confirmation
1	A01	Fish	23/08/10	Trop Dmn	Vv	Vv (51.1%)	Vv
2	A02	Cockle	23/08/10	Trop Dmn	Vp/Vv	Vp (73.0%)	Vp
3	A03	Cockle	23/08/10	Trop Dmn	Vv	Vv (93.9%)	Vp
4	A04	Cockle	23/08/10	Trop Dmn	Va	Va (92.0%)	Vsp
5	A05	Fish	23/08/10	Trop Dmn	Vp	Vp (88.9%)	Vsp
6	A06	Fish	23/08/10	Trop Dmn	Vc	Vc (62.0%)	Vsp
7	A07	Fish	23/08/10	Trop Dmn	Vc	Va (83.2%)	Vsp
8	A08	Clam	23/08/10	Trop Dmn	Vv/Vp	Vv (73.0%)	Vv
9	A09	Clam	23/08/10	Trop Dmn	Vsp	Vv (82.5%)	Vsp
10	A10	Clam	23/08/10	Trop Dmn	Vp/Vv	Vp (99.9%)	Vp
11	A11	Fish	23/08/10	Trop Dmn	Vp	Vp (92.9%)	Vp
12	A12	Fish	23/08/10	Trop Dmn	Vsp	Va (86.0%)	Vsp
13	A13	Shrimp	23/08/10	Trop Dmn	Vm	Vc (65.3%)	Vsp
14	A14	Shrimp	23/08/10	Trop Dmn	Vc	Vc (72.5%)	Vsp
15	A15	Squid	23/08/10	Trop Dmn	Vsp	Va (82.0%)	Vsp
16	A16	Squid	23/08/10	Trop Dmn	Vp	Vp (97.7%)	Vp
17	A17	Shrimp	23/08/10	Trop Dmn	Vp	Vp (76.9%)	Vsp
18	A18	Shrimp	23/08/10	Trop Dmn	Vv	Vp (82.9%)	Vp
19	A19	Fish	23/08/10	Trop Dmn	Vsp	Va (56.1%)	Vsp
20	A20	Fish	23/08/10	Dmn In	Vsp	Vc (62.4%)	Vsp
21	A21	Fish	23/08/10	Kota Dmn	Vv/Vp	Vp (92.9%)	Vp
22	A22	Fish	23/08/10	Dmn In	Vp	Vp (99.9%)	Vp
23	A23	Fish	23/08/10	Dmn In	Vsp	Vc (57.0%)	Vsp
24	A24	Prawn	23/08/10	Dmn In	Va	Va (54.0%) Vf (12.5%)	Vsp
25	A25	Cockle	23/08/10	Dmn In	Vsp	Va (67.3%)	Vsp

Appendix 8 – continued.

26	A26	Fish	23/08/10	Dmn In	Vsp	Vc (56.0%)	Vsp
27	A27	Fish	23/08/10	Dmn In	Vm	Vc (62.4%)	Vsp
28	A28	Fish	23/08/10	Dmn In	Vp	Vp (96.6%)	Vp
29	A29	Prawn	23/08/10	Dmn In	Vc	Vc (82.9%)	Vc
30	A30	Prawn	23/08/10	Dmn In	Vp	Vp (85.4%)	Vp
31	A31	Fish	23/08/10	Dmn In	Vp/Vv	Vp (73.0%)	Vp
32	A32	Shrimo	23/08/10	Dmn In	Vp	Vp (99.9%)	Vp
33	A33	Shrimp	23/08/10	Mut Dmn	Vsp	Va (78.9%)	Vsp
34	A34	Fish	23/08/10	Mut Dmn	Va	Va (68.9%) Vf (7.7%)	Vsp
35	A35	Shrimp	23/08/10	Mut Dmn	Vc	Vc (82.9%)	Vc
36	A36	Prawn	23/08/10	Mut Dmn	Vp	Vp (93.2%)	Vsp
37	A37	Oyster	23/08/10	Mut Dmn	Vv / Vp	Vv (88.9%)	Vsp
38	A38	Oyster	23/08/10	Mut Dmn	Vsp	Vc (76.2%)	Vsp
39	A39	Fish	23/08/10	Mut Dmn	Va	Va (89.9%)	Vsp
40	A40	Fish	23/08/10	Mut Dmn	Vm	Vc (56.2%)	Vsp
41	A41	Fish	23/08/10	Mut Dmn	Vsp	Vp (73.0%)	Vsp
42	B01	Prawn	02/11/10	PWTC	Vv/Vp	Vv (73.3%)	Vv
43	B02	Prawn	02/11/10	PWTC	Vp	Vp (98.6%)	Vp
44	B03	Shrimp	02/11/10	Mut Dmn	Vp	Vp (99.9%)	Vp
45	B04	Shrimp	02/11/10	PWTC	Va	Va (88.9%)	Vsp
46	B05	Fish	02/11/10	PWTC	Vc	Vc (82.6%)	Vsp
47	B06	Clam	02/11/10	PWTC	Vsp	Vv (76.9%)	Vsp
48	B07	Shrimp	02/11/10	PWTC	Vv	Vp (88.6%)	Vp
49	B08	Shrimp	02/11/10	PWTC	Va	Va (95.8%)	Vsp
50	B9	Fish	02/11/10	PWTC	Va	Va (89.0%)	Vsp
51	B10	Prawn	02/11/10	PWTC	Vm	Vc (67.4%)	Vsp
52	B11	Prawn	02/11/10	Dmn In	Vp	Vp (98.2%)	Vp
53	B12	Shrimp	02/11/10	Dmn In	Vv/Vp	Vp (86.8%)	Vp
54	B13	Oyster	02/11/10	Dmn In	Vp	Vp (56.0%)	Vp
55	B14	Oyster	02/11/10	PWTC	Vsp	Vc (68.7%)	Vsp
56	B15	Fish	02/11/10	PWTC	Vp	Vp (98.2%)	Vp
57	B16	Fish	02/11/10	PWTC	Vm	Vp (93.0%)	Vp

Appendix 8 – continued.

58	B17	Fish	02/11/10	PWTC	Vp	Vv (73.0%)	Vp
59	B18	Shrimp	02/11/10	Mut Dmn	Vp	Vp (99.9%)	Vp
60	B19	Squid	02/11/10	Dmn In	Vv/Vp	Vp (73.0%)	Vp
61	B20	Shrimp	02/11/10	Sec 17	Vp	Vp (86.8%)	Vp
62	B21	Squid	02/11/10	Sec 17	Vc	Vc (93.0%)	Vp
63	B22	Shrimp	02/11/10	Sec 17	Vc/Va	Va (78.0%)	Vsp
64	B23	Fish	02/11/10	UM	Vp	Vp (56.0%)	Vp
65	B24	Oyster	02/11/10	Sec 17	Vp	Vp (99.9%)	Vp
66	B25	Fish	02/11/10	Sec 17	Vv/Vp	Vp (73.0%)	Vp
67	B26	Fish	02/11/10	Sec 17	Vm/Vc	Vc (88.9%)	Vp
68	B27	Fish	02/11/10	Sec 17	Vp	Vp (73.0%)	Vp
69	B28	Fish	02/11/10	Sec 17	V.vul.	Vp (98.8%)	Vp
70	B29	Oyster	02/11/10	Sec 17	Vv/Vp	Vp (99.9%)	Vp
71	B30	Prawn	02/11/10	Sec 17	Vp	Vp (99.9%)	Vp
72	B31	Clam	02/11/10	Sec 17	Vv/Vp	Vv (88.0%)	Vp
73	B32	Shrimp	02/11/10	Sec 17	Vp	Vp (98.8%)	Vp
74	B33	Squid	02/11/10	Sec 17	Vm	Vp (82.6%)	Vp
75	B34	Fish	02/11/10	Sec 17	Vp	Vp (62.9%)	Vp
76	B35	Squid	02/11/10	Sec 17	Vsp	Va (82.6%)	Vsp
77	B36	Fish	02/11/10	Sec 17	Vv/Vp	Vp (88.6%)	Vp
78	B37	Fish	02/11/10	UM	Vc	Vc (75.6%)	Vp
79	B38	Oyster	02/11/10	UM	Vv/Vp	Vp (99.9%)	Vp
80	B39	Oyster	02/11/10	UM	Va	Va (82.7%) Vf (12.5%0	Vp
81	B40	Cockle	02/11/10	UM	Vv	Vp (73.0%)	Vp
82	B41	Cockle	02/11/10	UM	Vm	Vc (84.8%)	Vsp
83	B42	Fish	02/11/10	UM	Vp	Vp (99.9%)	Vp
84	B43	Fish	02/11/10	UM	Vm	Vc (58.4%)	Vsp
85	B44	Fish	02/11/10	UM	Vsp	Va (89.9%)	Vsp
86	B45	Prawn	02/11/10	Sec 17	Va	Va (86.9%)	Vp
87	B46	Fish	02/11/10	UM	Vp	Vp (88.6%)	Vp
88	B47	Fish	02/11/10	UM	Vm	Vc (78.5%)	Vsp
89	B48	Fish	02/11/10	UM	Vf	Va (75.6%)	Vp

Appendix 8 – continued.

90	B49	Shrimp	02/11/10	UM	Vsp	Va (56.8%) Vf (35.4%)	Vsp
91	B50	Shrimp	02/11/10	UM	Vv/Vp	Vv (99.7%)	Vv
92	B51	Clam	02/11/10	UM	Vm	Vc (82.9%)	Vsp
93	B52	Clam	02/11/10	UM	Vv	Vv (99.5%)	Vv
94	B53	Clam	02/11/10	UM	Vc	Vc (82.9%)	Vsp
95	B54	Fish	02/11/10	UM	Vsp	Vv (51.1%) Vc (48.2%)	Vsp
96	B55	Fish	02/11/10	UM	Vc/Va	Vc (99.3%)	Vc
97	B56	Oyster	02/11/10	UM	Vc	Vc (48.2%) Vv (51.1%)	Vc
98	B57	Oyster	02/11/10	UM	Vsp	Va (56.2%)	Vsp
99	B58	Fish	02/11/10	UM	Vp/Vv	Vp (89.9%)	Vsp
100	B59	Fish	02/11/10	UM	Vsp	Vc (58.9%)	Vsp
101	B60	Fish	02/11/10	UM	Vsp	Va (86.0%)	Vsp
102	B61	Fish	02/11/10	UM	Vm	Vc (99.9%)	Vc
103	C01	Fish	06/12/10	Kota Dmn	Vv	Vv (97.7%)	Vv
104	C02	Shrimp	06/12/10	Kota Dmn	Vv/Vp	Vv (73.0%)	Vv
105	C03	Shrimp	06/12/10	Kota Dmn	Vv	Vp (73.0%) Vv (26.2%)	Vsp
106	C04	Fish	06/12/10	Kota Dmn	Vp/Vv	Vv (99.7%)	Vsp
107	C05	Fish	06/12/10	Kota Dmn	Vsp	Va (96.4%)	Vsp
108	C06	Oyster	06/12/10	Kota Dmn	Va	Va (97.7%)	Vsp
109	C07	Fish	06/12/10	Kota Dmn	Vm	Vc (82.9%)	Vsp
110	C08	Cockle	06/12/10	Kota Dmn	Vsp	Vf (12.8%)	Vsp
111	C09	Fish	06/12/10	Kota Dmn	Vsp	Vv (51.1%) Vc (48.2%)	Vsp
112	C10	Shrimp	06/12/10	Kota Dmn	Va	Va (86.0%) Vc (12.8%)	Vsp
113	C11	Squid	06/12/10	Kota Dmn	Vsp	Vc (76.5%)	Vsp
114	C12	Fish	06/12/10	Kota Dmn	Vsp	Vf (7.8%)	Vsp
115	C13	Fish	06/12/10	Kota Dmn	Vm	Vc (56.0%)	Vsp
116	C14	Prawn	06/12/10	Kota Dmn	Va	Va (99.5%)	Vsp
117	C15	Fish	06/12/10	Kota Dmn	Vsp	Vf (12.5%)	Vsp
118	C16	Cockle	06/12/10	Kota Dmn	Vsp	Vv (93.9%)	Vsp

Appendix 8 – continued.

119	C17	Fish	06/12/10	Kota Dmn	Vc	Vc (82.9%)	Vsp
120	C18	Fish	06/12/10	Kota Dmn	Vv	Vv (51.1%) Vc (48.2%)	Vv
121	C19	Clam	06/12/10	Kota Dmn	Va	Va (86.0%)	Vsp
122	C20	Oyster	06/12/10	Kota Dmn	Vsp	Vf (12.5%)	Vsp
123	C21	Fish	06/12/10	Kota Dmn	Vv/Vp	Vv (93.9%)	Vsp
124	C22	Fish	06/12/10	Kota Dmn	Vsp	Va (96.4%)	Vsp
125	C23	Shrimp	06/12/10	Kota Dmn	Vsp	Vc (56.6%)	Vsp
126	C24	Fish	06/12/10	Kota Dmn	Vsp	Vf (7.8%)	Vsp
127	C25	Fish	06/12/10	Kota Dmn	Vsp	Va (86.0%)	Vsp
128	C26	Cockle	06/12/10	Kota Dmn	Vv	Vp (85.0%)	Vsp
129	C27	Prawn	06/12/10	Kota Dmn	Vsp	Vv (93.9%)	Vsp
130	C28	Prawn	06/12/10	Kota Dmn	Vc	Vc (99.7%)	Vc
132	D02	Fish	13/12/10	Serdang	Vp	Vp (99.9%)	Vsp
133	D03	Fish	13/12/10	Serdang	Vv	Vv (62.0%)	Vv
134	D04	Shrimp	13/12/10	Serdang	Vv	Vv (93.9%)	Vsp
135	D05	Fish	13/12/10	Midvalley	Vv/Vp	Vp (93.0%)	Vsp
136	D06	Squid	13/12/10	Midvalley	Vp	Vp (62.8%	Vsp
137	D07	Oyster	13/12/10	Midvalley	Vp	Vv (73.0%)	Vp
138	D08	Oyster	13/12/10	Midvalley	Vv	Vv (51.1%)	Vv
139	D09	Fish	13/12/10	Midvalley	Vp	Vp (88.6%)	Vsp
140	D10	Fish	13/12/10	Midvalley	Vp	Vp (99.9%)	Vsp
141	D11	Fish	13/12/10	Midvalley	Vv/Vp	Vv (93.9%)	Vv
142	D12	Fish	13/12/10	Midvalley	Vsp	Va (87.9%)	Vsp
143	D13	Shrimp	13/12/10	Midvalley	Vsp	Vc (65.7%)	Vsp
144	D16	Fish	13/12/10	Serdang	Vv/Vp	Vp (99.8%)	Vsp
145	D17	Cockle	13/12/10	Midvalley	Vv/Vp	Vp (62.8%)	Vv
146	D18	Fish	13/12/10	Midvalley	Vp	Vp (99.9%)	Vp
147	D19	Prawn	13/12/10	Midvalley	Vp	Vp (73.0%)	Vsp
148	D20	Fish	13/12/10	Midvalley	Vm	Vp (52.7%)	Vsp
149	D21	Fish	13/12/10	Midvalley	Vp	Vp (99.9%)	Vsp
150	D22	Fish	13/12/10	Mut Dmn	Vp	Vp (88.6%)	Vsp
151	D23	Oyster	13/12/10	Mut Dmn	Vv	Vv (82.0%)	Vsp

Appendix 8 – continued.

152	D24	Fish	13/12/10	Mut Dmn	Vp	Vp (98.9%)	Vsp
153	D25	Shrimp	13/12/10	Midvalley	Vp	Vp (76.2%)	Vp
154	D26	Fish	13/12/10	Midvalley	Vv/Vp	Vp (99.5%)	Vsp
155	D27	Fish	13/12/10	Midvalley	Vv	Vp (73.0%)	Vsp
156	D30	Shrimp	13/12/10	Mut Dmn	Vsp	Va (76.8%)	Vsp
157	D31	Fish	13/12/10	Mut Dmn	Vsp	Vc (68.9%)	Vsp
158	D32	Fish	13/12/10	Mut Dmn	Vm	Vc (82.9%)	Vsp
159	D33	Oyster	13/12/10	Mut Dmn	Vsp	Vf (12.5%)	Vsp
160	D34	Oyster	13/12/10	Mut Dmn	Vc	Vc (82.9%)	Vc
161	D35	Shrimp	13/12/10	Serdang	Vc	Vc (99.3%)	Vc
162	D36	Shrimp	13/12/10	Serdang	Va/Vsp	Va (97.7%)	Vsp
163	D37	Fish	13/12/10	Serdang	Va	Va (69.0%)	Vsp
164	D38	Fish	13/12/10	Serdang	Vc	Vc (12.8%) Va (86.0%)	Vsp
165	D39	Fish	13/12/10	Serdang	Vsp	Va (89.9%)	Vsp
166	D40	Squid	13/12/10	Serdang	Vsp	Vf (12.8%)	Vsp
167	D41	Fish	13/12/10	Serdang	Vsp	Vc (68.9%)	Vsp
168	D42	Cockle	13/12/10	Serdang	Va	Va (97.8%)	Vsp
169	D43	Oyster	13/12/10	Serdang	Vsp	Vf (7.8%)	Vsp
170	D44	Prawn	13/12/10	Serdang	Vm	Vc (89.0%)	Vsp
171	D45	Fish	13/12/10	Serdang	Vv	Vv (93.9%)	Vsp
172	D46	Fish	13/12/10	Serdang	Vsp	Va (89.0%) Vf (7.0%)	Vsp
173	D47	Fish	13/12/10	Serdang	Vsp	Vc (78.9%)	Vsp
174	D48	Prawn	13/12/10	Serdang	Vm	Vc (67.5%)	Vsp
175	D49	Fish	13/12/10	Serdang	Va	Va (99.8%)	Vsp
176	D50	Oyster	13/12/10	Serdang	Vsp	Vf (12.8%)	Vsp
177	D51	Cockle	13/12/10	Serdang	Vsp	Vc 56.9%)	Vsp
178	D52	Fish	13/12/10	Serdang	Vsp	Va (89.0%)	Vsp
179	D53	Fish	13/12/10	Serdang	Vv	Vp (73.0%) Vv (26.2%)	Vsp
180	D54	Shrimp	13/12/10	Serdang	Vsp	Vf (78.0%)	Vsp
181	D55	Fish	13/12/10	Serdang	Vm	Vc (89.6%)	Vsp
182	D56	Squid	13/12/10	Serdang	Vsp	Va (98.9%)	Vsp

Appendix 8 – continued.							
183	D57	Fish	13/12/10	Serdang	Vsp	Vc (67.8%)	Vsp
184	D58	Fish	13/12/10	Serdang	Vsp	Va (96.4%)	Vsp
185	D59	Fish	13/12/10	Serdang	Vsp	Va (92.6) Vc (7.1%)	Vsp
186	D60	Clam	13/12/10	Serdang	Va	Va (97.7%)	Vsp
187	D61	Fish	13/12/10	Serdang	Vm	Vc (82.9%)	Vsp

Abbreviations		
DOI : Date of Isolation	Vv :*Vibrio vulnificus*	Vc : *Vibrio cholerae*
POI : Place of Isolation	Vf : *Vibrio fluvialis*	Vp : *Vibrio parahaemolyticus*
Va : Vibrio alginolyticus	Vm : *Vibrio mimicus*	Vsp : *Vibrio* sp. (Unknown species)