

**INVESTIGATING THE OCCURRENCE OF
Vibrio parahaemolyticus IN SHRIMP CONSUMED
IN THE TURKISH REPUBLIC OF NORTHERN
CYPRUS**

**A THESIS SUBMITTED TO THE GRADUATE
SCHOOL OF APPLIED SCIENCES
OF
NEAR EAST UNIVERSITY**

**By
GRACE CHARLES ZEBERE**

**In Partial Fulfillment of the Requirements for
the Degree of Master of Science
in
Food Engineering**

NICOSIA, 2017

**GRACE CHARLES
ZEBERE**

**INVESTIGATING THE OCCURRENCE OF *Vibrio parahaemolyticus* IN SHRIMP
CONSUMED IN THE TURKISH REPUBLIC OF NORTHERN CYPRUS**

**NEU
2017**

**INVESTIGATING THE OCCURRENCE OF
Vibrio parahaemolyticus IN SHRIMP CONSUMED
IN THE TURKISH REPUBLIC OF NORTHERN
CYPRUS**

**A THESIS SUBMITTED TO THE GRADUATE
SCHOOL OF APPLIED SCIENCES
OF
NEAR EAST UNIVERSITY**

**By
GRACE CHARLES ZEBERE**

**In Partial Fulfillment of the Requirements for
the Degree of Master of Science
in
Food Engineering**

NICOSIA, 2017

Grace Charles ZEBERE: INVESTIGATING THE OCCURRENCE OF *Vibrio parahaemolyticus* IN SHRIMP CONSUMED IN THE TURKISH REPUBLIC OF NORTHERN CYPRUS

**Approval of Director of Graduate School of
Applied Sciences**

Prof. Dr. Nadire ÇAVUŞ

**We certify this thesis is satisfactory for the award of the degree of Master of Science in Food
Engineering**

Examining Committee in Charge:

Assoc. Prof. Dr. Kaya Süer

Committee Chairman, Department of Clinical
Microbiology and Infectious Diseases, NEU

Assist. Prof. Dr. Serdar Sussever

Department of Food and Nutrition Science, NEU

Dr. Perihan Adun

Supervisor, Department of Food Engineering, NEU

I hereby declare that, all the information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Name, Last Name:

Signature:

Date:

To the Almighty God...

ACKNOWLEDGEMENTS

My utmost appreciation first goes to my thesis Supervisor Dr. Perihan Adun of the Faculty of Engineering at Near East University. The door to Dr. Perihan's office was always open whenever I ran into a trouble spot or had a question about my research. She consistently allowed this paper to be my own work, but steered me in right the direction whenever she thought I needed it.

I would also like to thank the experts who were involved in the microbiological analysis for this research project: Assoc. Prof. Dr. Kaya SÜER, Assist. Prof. Dr. Meryem Güvenir, Hafizu Ibrahim Kademi who participated and contributed. Without their passionate participation and input, the laboratory analysis could not have been successfully conducted.

I also like to acknowledge Assist. Prof. Dr. Serdar Sussever of the Department of Food and Nutrition sciences as the second reader of this thesis, and I am gratefully indebted to him for his very valuable comments on this thesis.

I am grateful to Mr. Williams Ndifreke, Mr and Mrs. Gbolahan Olowu, and to all of those with whom I have had the pleasure to work with during this research work.

This acknowledgement will be incomplete without me also acknowledging Mr. Buğra Demircioğlu the coordinator of Food Engineering Department for his tremendous counseling and mentorship. Nobody has been more important to me in the pursuit of this project than the members of my family. They are the ultimate role models. Most importantly, I wish to thank my loving and supportive husband Charles and my three wonderful children, Joseph, Elvie and Geovanna, my sister Comfort who provide unending inspiration. My Mother and siblings for their prayers.

To my wonderful course mates, friends and well-wishers, I am grateful.

ABSTRACT

This study investigates the presence of pathogenic *Vibrio parahaemolyticus* consumed marine shrimp in the Turkish Republic of North Cyprus (TRNC). Ninety (90) samples of shrimp taken from Famagusta, Kyrenia, Nicosia and Morphou. A traditional culture technique was used to identify bacteria. Enrichment of this pathogen was nutrient salt of Thiosulfate Citrate Bile Sucrose-Salts Agar (TCBS) after treatment of isolation of different marine (*Vibrio parahaemolyticus*). The identity of the bacteria was verified using BD Phoenix Instrument.

Vibrio parahaemolyticus could not be detected in shrimp samples taken from different regions of TRNC implying that *Vibrio parahaemolyticus* is not present in shrimps consumed in Turkish Republic of North Cyprus

Keywords: *Vibrio*; *Vibrio parahaemolyticus*; shellfish; seafood; shrimp; isolation; identification

ÖZET

Bu çalışmada Kuzey Kıbrıs Türk Cumhuriyeti'nde (KKTC) tüketilen karideslerde patojenik *Vibrio parahaemolyticus* varlığı araştırılmıştır. Girne, Güzelyurt, Magusa ve Lefkoşa'daki balık ve balık ürünleri satan marketlerden toplam 90 karides numunesi satın alınmıştır. Bakterinin izolasyonu ve identifikasyonu klasik kültür tekniği ile gerçekleştirilmiştir. İzolasyonda *Vibrio* türlerine özgü olan Thiosulfate Citrate Bile Sucrose (TCBS) agar besiyeri kullanılmış ve şüpheli kolonilerin doğrulanması BD Phoenix cihazı ile yapılmıştır. KKTC'nin değişik bölgelerinden toplanan karides örneklerinde *Vibrio parahaemolyticus*'a rastlanmamıştır.

Anahtar Kelimeler: *Vibrio*; *Vibrio parahaemolyticus*; karides; deniz ürünleri; izolasyon; identifikasyon

TABLE OF CONTENTS

ACKNOWLEDGEMENTS.....	i
ABSTRACT	ii
ÖZET	iii
LIST OF FIGURES.....	vii
LIST OF TABLES.....	viii
LIST OF ABBREVIATION	ix
CHAPTER 1: INTRODUCTION	1
1.1 Background Information	1
1.2 <i>Vibrio parahaemolyticus</i> Infection in Respect.....	2
1.3 Seafood Safety and Risk Assessment of <i>Vibrio parahaemolyticus</i>	4
1.4 Characteristics of <i>Vibrio parahaemolyticus</i>	4
1.4.1 Historical Background <i>Vibrio parahaemolyticus</i>	5
1.4.2 Classification and Taxonomy	5
1.4.3 Cell and Colony Morphology	7
1.4.4 Virulence Factors of <i>V. parahaemolyticus</i>	8
1.4.4.1 Haemolysins.....	9
1.4.4.2 Pathogenicity Islands (PIs)	10
1.4.4.3 Type Three Secretion Systems (TTSSs)	11
1.5 Disease Caused by <i>Vibrio parahaemolyticus</i>	13
1.5.1 Epidemiology of <i>Vibrio parahaemolyticus</i>	14
1.5.1.1 Asia	14
1.5.1.2 Europe	15
1.5.1.3 The Americas	16
1.6 Overview of Shellfish	17
1.6.1 Protein and Other Vital Nutrients of Human Importance in Shrimp.....	17
1.6.2 Shrimp Consumption in Relation to Recommended Daily Allowance	18
CHAPTER 2: THEORETICAL FRAMEWORK	19

2.1 Significance of Microbiological Investigations	19
2.2 Properties and Characteristics of <i>Vibrio</i>	20
2.2.1 Host Range and Transmissions of <i>Vibrio</i>	21
2.2.2 Pathogenicity of <i>Vibrio</i> species.....	22
2.2.3 <i>Vibrio parahaemolyticus</i>	22
2.3 Isolation of <i>Vibrio</i> spp.	23
2.3.1 Sample Collection	23
2.3.2 Behavior of <i>Vibrios</i> on Selective Agar.....	24
2.4 Conventional Methods for the Identification of <i>Vibrio</i> spp.....	26
2.4.1 BIOCHEMICAL TESTS	26
2.4.1.1 Oxidase Test.....	26
2.4.1.2 Triple Sugar Iron (TSI)	28
2.4.1.3 The Methyl Red (MR) and Voges-Proskauer (VP) Tests	30
2.4.1.4 Salt Tolerance Test	31
2.4.2 API 20E.....	32
2.5 Molecular Methods to Identify and Characterize <i>Vibrio</i> spp.....	32
2.5.1 Virulotyping of <i>Vibrio parahaemolyticus</i>	33
2.5.2 Molecular Typing of <i>Vibrio parahaemolyticus</i> Strains.....	33
2.5.3 Thermostable Direct Haemolysin (TDH).....	34
2.5.4 Thermostable Direct Haemolysin-Related Haemolysin (TRH)	35
2.5.5 Lecithin-Dependent Haemolysin (LDH).....	36
2.5.6 Heat-Stable Haemolysin (B-VPH)	36
CHAPTER 3: RELATED RESEARCH	38
CHAPTER 4: MATERIALS AND METHOD	44
4.1 Study Area	44
4.2 Sampling	44
4.3 Analytical sample preparation	47
4.4 Media, Test Kits and Equipment used	48
4.4.1 Preparation of Enrichment Media	49

4.4.2 TCBS Agar	49
4.5 Bacteriological Analysis	50
4.5.1 Reculture of Control <i>Vibrio parahaemolyticus</i> ATCC 17802	50
4.5.2 Isolation and Identification of <i>Vibrio parahaemolyticus</i>	50
4.5.2.1 Gram Staining.....	50
4.5.2.2 Catalase test.....	51
4.5.2.3 Oxidase test	51
4.6 Confirmation	51
4.6.1 Preparation of Colony Suspensions in Phoenix Inoculum Broth	51
CHAPTER 5: RESULTS AND DISCUSSION	53
5.1 <i>Vibrio parahaemolyticus</i> ATCC 17802 Growth on TCBS agar.....	53
5.2 Results.....	53
5.3 Discussion	55
CHAPTER 6: CONCLUSION AND RECOMMENDATIONS	58
REFERENCES.....	59

LIST OF FIGURES

Figure 1.1: Global dissemination of <i>Vibrio parahaemolyticus</i>	14
Figure 2.1: Relative rates of laboratory-confirmed infections with <i>Campylobacter</i> ,	21
Figure 2.2: The colony colors of <i>Vibrio</i> spp. on TCBS.....	25
Figure 2.3: The colony colors of <i>Vibrio</i> spp. on CHROMAgar™ <i>Vibrio</i>	25
Figure 2 4: Possession of virulence genes of clinical KP-positive and KP-negative <i>Vibrio</i> <i>parahaemolyticus</i>	37
Figure 4.1: Map of Cyprus showing the study area in TRNC (KKTC).....	45
Figure 4.2: Sample Preparation Processes.....	48
Figure 4.3: Steps for cultural detection of <i>Vibrio parahaemolyticus</i> in shrimp samples	52
Figure 5.1: <i>Vibrio parahaemolyticus</i> ATCC17802 growth on TCBS agar plate.....	55
Figure 5.2: The suspected TCBS agar plates.....	54

LIST OF TABLES

Table 1.1: Composition of the genus <i>Vibrio</i>	7
Table 1.2: Comparison of TDH and TRH toxins of <i>Vibrio parahaemolyticus</i>	10
Table 2.1: Colors of colonies appearing on TCBS	24
Table 2.2: The colony colors of <i>Vibrio</i> spp. on CHROMAgar™ <i>Vibrio</i>	25
Table 2.3: Biochemical characteristics of human pathogenic <i>Vibrionaceae</i>	27
Table 2.4: TSI test interpretation	28
Table 2.5: TSI test interpretation associated with the observed colors and gas.....	29
Table 2.6: Growth response of <i>Vibrio</i> species to various concentrations of NaCl	31
Table 4.1: TRNC shrimp sampling location	46
Table 4.2: TCBS agar selective isolation media composition	49
Table 5.1: Result showing the distribution of <i>Vibrio parahaemolyticus</i>	55

LIST OF ABBREVIATION

AGE	Agarose gel electrophoresis
µm	Micromolar
ABC	ATP-binding cassette
AFLP	Amplified fragment length polymorphisms
API	Analytical profile
APS	Ammonium persulfate
APW	Alkaline Peptone Water
ARDRA	Amplified DNA restriction
BoTN	Botulinum neurotoxin
Bp	Base pairs
CFU	Colony forming unit
ChiRP	Chitin-regulated pilus
CoC	Code of conduct
CPS	Capsula polysaccharide
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
ESI	Electrospray ionization
EU	European Union
FAO	Food and Agriculture Organization of United Nations
FISH	Fluorescent in-situ hybridization
FOOD	Foodbome Outbreaks Online Database
G	Gram
g⁻¹	Per gram
GAP	Good practice aquatic products
H₂S	Hydrogen sulphide
HACCP	Hazard Analysis Critical Control Point
IDSC	Infection Disease Surveillance Centre
Kb	Kilobase

kDa	Kilodalton
Kg	Kilogram
KP-positive	Kanagawa phenomenon positive
LAB	Lactic acid bacteria
LDH	Lecithin dependent haemolysin
MCMBB	Markov's beta-barrel model chains
Mg	Milligram
ml	Millilitre
MLEE	Multifocal electrophoresis enzyme
mM	Millimolar
MPN	Most Probable Number
MSHA	Mannose-sensitive hemagglutinin
NaCl	Sodium Chloride
°C	Degree Celcius
OMP	Outer membrane proteomics
OP	Opaque
PCR	Polymerase Chain Reaction
PFGE	Pulse field gel electrophoresis
PI	Pathogenicity Island
RAPD	Random polymorphous amplified DNA
RDA	Recommended daily requirement
REP	Repetitive extracellular palindromic
Sp	Species
spp	Species
ST	Sequence type
TBE	Tris borate EDT
TDH	Thermostable direct hemolysin
<i>tdh</i>	Thermostable direct hemolysin
TDH	Thermostable direct haemolysin
TeBS	Thiosulphate citrate bile salt sucrose agar

<i>toxR</i>	Toxin operon gene
TR	Translucent
TRH	TDH related hemolysin
<i>trh</i>	TDH related hemolysin
TRH	Thermostable direct haemolysin-related haemolysin
TRNC	Turkish Republic of North Cyprus
TSI	Triple Sugar Iron
TSS	Total Suspended Solid
TTSSs	Type three secretion systems
UK	United Kingdom
US	United State
USD	US dollar
UV	Ultraviolet
V	<i>Vibrio</i>
VBNC	Viable but non culturable
%	Percentage

CHAPTER 1

INTRODUCTION

1.1 Background Information

In regards to the Code of Conduct on Risk Management Strategies for *Vibrio* spp. in seafood, the main possible causes of infection by *Vibrio parahaemolyticus* have been identified as the absorption of pathogens of fish and shellfish from the environmental waters, exposure of bacteria at harvest time, and poor post-harvest conditions (Codex, 2003).

As shrimp aquaculture, *Vibrio parahaemolyticus* has been accepted as natural microflora in the aquatic environment (Ngo and Ravi, 2010) and there are many species of *Vibrio*, the *Vibrio* genus may be opportunistic pathogens suppression. According to the United Nations Food and Agriculture Organization (2011), one of the major diseases according to the organization suffers from *Vibrios* is disease which is caused by the species *Vibrio Penaeus vannamei*.

The *Vibrio* genus is abundant in marine and coastal environments and is considered one of the main causes of gastroenteritis in humans. Most infections are caused by eating raw or cooked seafood. *Vibrio* spp are the main cause of diarrhea, including *Vibrio cholerae*, *Vibrio parahaemolyticus* is the cause of food gastroenteritis (Pruzzo et al., 2005) while pathogenic strains were at least 11 (Janda et al., 1988) and shellfish consumption *Vibrio vulnificus*, known to cause 95% of all deaths (Rosche et al., 2006).

Vibrio parahaemolyticus resulting in continuous treatment with morbidity and mortality of the diseases caused by the loss of shrimp damage and *Vibrio parahaemolyticus*, which has a more pronounced and occasionally geographical area than otherwise *Vibrios* are often the most abundant. This is because the relationship with fish, this shrimp is a critical issue for business and public health organizations. Because of individual marine situation, human exercises are extreme with physical and chemical pollution. It is evident that shrimp in this way have some pathogens gained from the sea or ocean environment (João, 2010).

The increased consumption of shrimp and increased levels of cross contamination caused by the oceans of the pathogens inspired to investigate the truth of the TRNC shrimp *Vibrio*

parahaemolyticus.

The aim of this study is to investigate the presence of *Vibrio parahaemolyticus* in Shrimp consumed in the TRNC.

1.2 *Vibrio parahaemolyticus* Infection in Respect to Shrimp Aquaculture and the Supply Chain

The critical factors that affect *Vibrio parahaemolyticus* density at pre-harvest and harvest are, water temperature and salinity, air temperatures, tides and plankton (Codex, 2003, Kumazawa et al., 1999; Sarkar et al., 1985). Because this bacterium is the most abundant in the area with hot water temperatures, geographical location and seasonal parameters can be indicative of *Vibrio parahaemolyticus* level at harvest. The seasonal frequency of *Vibrio parahaemolyticus* are not considerably different in tropical countries, including Turkey as such temperature control during transport will probably be a key factor affecting the growth of *Vibrio parahaemolyticus* Turkey's aquaculture production chain (Adelaide et al., 2009).

Intervention strategies such as the minimization of the period between harvest and chilling is required after harvest to reduce the level and prevent the growth of ion can help to freeze level and reduce growth of *Vibrio parahaemolyticus*. Likewise, the harvesting methods used in diverse fishing areas can also influence the level of *Vibrio parahaemolyticus* after harvest (FDA, 2005). For example, the US Gulf Coast State of Louisiana the disease counts has predicted higher numbers of sickness when compared to other States in the region.

Vibrio parahaemolyticus contains pathogenic and non-pathogenic strains, so when assessing risks, it is necessary to highlight the levels of the pathogenic stains of *Vibrio parahaemolyticus* because this is the real cause of the disease by the bacteria. Example is the incidence of Pacific Northwest which indicated that pathogenic *Vibrio parahaemolyticus* is higher than that of the coast of the US, so harvest control standard based on total *Vibrio parahaemolyticus* in the pacific Northwest must be very rigid than those from the gulf coast (FDA, 2005). The level of *Vibrio parahaemolyticus* at the point of consumption has been assessed for oysters in the US by the level of pathogenic strain related with the characteristic serving portions (FDA, 2005). However, this assessment may differ depending on the species of seafood, consumer culture and the size of serving in every

individual area is vital.

In a study by Assavanig et al. (2008) *Vibrio parahaemolyticus* was detected in healthy workers who work in shrimp farm in the south of Thailand and also in workers at a seafood processing plant in the center of Bangkok (Athajari, 2004) which revealed that virulence genes (tdh TRH) of *V. parahaemolyticus* isolates were detected by multiple PCR. Two genes, TSS and TRH (TFR, + / + TRH) isolates were found in 4.8%, 25.3% Only TSS (TSS + / trH-) contained 4.8%, only TRH (tdh-positive / TRH +) and 65.1% had no disease-inducing genes (tdh positive / trh-). These results show that potential virulent strains were discovered from healthy carriers who had no signs of gastroenteritis. A further study of this exploitive condition of pathogenic *Vibrio parahaemolyticus* in these carriers is necessary to demonstrate if factors such as human immunization and other pathogenesis forms can play a role in the survival of *Vibrio parahaemolyticus* to holders.

Nevertheless, the research conducted indicates the likelihood that human carriers may be a source of bacterial transmission both between and from shrimp seafood farm sites. Farmers in locally-run shrimp farm may be at more danger of acquiring *Vibrio parahaemolyticus* infection than those in the large scale commercial farm due to the innovative equipment in the farms which allows the farmers to manage the shrimp culture system without having much human contact with the environment, while farmers in locally-run farms have more chance of handling cultivated shrimps directly which increases the danger of contamination (Iwamoto et al., 2010).

According to Codex (2003) paper discussion on risk management strategies for *Vibrio* spp. in seafood gave further information required in seafood transportation to develop additional food microbial risk strategies. Example is the study on the growth and survival of pathogenic *Vibrio parahaemolyticus* in shrimps at different temperatures which can be used to determine the critical control points for shrimp transportation. In addition to examining samples for bacteria Shrimp in the production process, two new / different steps, such as frozen shrimp Meat, water stool samples from shrimp farms and seafaring workers Plants (carriers of stem bacteria) and molecular diversity are important in the study of strain variation and molecular epidemiology of *Vibrio parahaemolyticus* in the shrimp production chain. Bridging these data gaps can improve quality control systems.

1.3 Seafood Safety and Risk Assessment of *Vibrio parahaemolyticus*

A good practice aquatic product (GAP) is a minimum requirement for shrimp farm management. Under the GAP program the farms are evaluated from the point of hygienic applications, regulation of antibiotics utilization and legislation on environmental practice (Aquaculture Department, Thailand, 2007). Also the Code of Conduct (CoC) is tailored to the GAP application, also covering all social activities, stakeholder's involvement in the production line and full product tracking.

Compliance to relevance Code of Conduct Management of agricultural enterprises is a compulsory obligation for farm management, harvesting and processing for high quality seafood. In 2007, some shrimp farms functioning in Thailand were approved by GAP, while only 274 farms (1-2%), were approved by the GAP and CoC (Anonymous, 2007).

After harvest handling, seafood processing requires a Good Manufacturing Practice (GMP) system which is applied to maintain product quality control. In addition, the Hazard Analysis Critical Control Point (HACCP) is an effective method for food safety inspection including bacteria evaluation and public health protection. But, microbiological evaluations of seafood products vary depending on buyers. For example, the European Union (EU) needs maximum recommended count for *Vibrio parahaemolyticus* of 10^3 MPN per gram (g^{-1}) most probably number in cooked molluscs and shellfish, while the US requires up to 10^4 MPN g^{-1} maximum for cooked crustacean products (Anonymous, 2009). Japan, a country where raw seafood is widely consumed determined zero MPN g^{-1} *Vibrio parahaemolyticus* in raw seafood products including crustaceans, fish, molluscs, bivalves etc (Anonymous, 2009). Also international food safety control was considered by the Codex Alimentarius Commission, abbreviated to Codex. The system started by Codex needs sterility practice to be implemented by all in the production line such that seafood need to be stored 10 degree all through the supply and additionally, shellfish should be washed with disinfected drinking water (Codex, 2003).

1.4 Characteristics of *Vibrio parahaemolyticus*

The infection of *Vibrio parahaemolyticus* from contaminated poorly cooked seafood has been a public health problem (Iwamoto et al., 2010) and this brought about understanding the

origin of *Vibrio parahaemolyticus* as indispensable for the study of epidemiological and molecular evolutionary of this organism. The comprehensive features which include historical background, classification and taxonomy, colony morphology and virulence factors of *Vibrio parahaemolyticus* are discussed. In specific the properties and functions of the most important major virulence factors and a better knowledge of the virulence mechanism of *Vibrio parahaemolyticus* (Coburn et al., 2007).

1.4.1 Historical Background *Vibrio parahaemolyticus*

Vibrio parahaemolyticus was first noticed in 1950 from patients with gastroenteritis in Osaka, Japan. The sickness was caused as a result of the consumption of poorly cooked salted sardines, called Shirasu (Fujino et al., 1953).

In 1953, the bacterium was also isolated as a mixed infection with *Proteus morganii* from stools and intestinal contents of patients. It was from this isolation it was first named *Pasteurella parahaemolyticus*. Consequently, in 1958, this bacterium was isolated from the stool samples of patients with food poisoning in an outbreak at Yokohama National Hospital (Takikawa, 1958). Glucose and halophilic fermentative bacterium, *Oceanomonas parahaemolyticus*, was isolated from humans and also from the marine environment in 1960, But the Japanese Ministry of Health and Welfare specified that *Pasteurella parahaemolyticus* and *Oceanomonas parahaemolyticus* are the same organism according to morphological, cultural and chemical investigations. The organism was regrouped into the genus *Vibrio* and named *Vibrio parahaemolyticus* from the report of the International Symposium of *Vibrio parahaemolyticus*, Tokyo in 1974, (Fujino et al., 1974).

1.4.2 Classification and Taxonomy

Vibrionaceae family was first described in 1965 (Janda et al., 1988). The organisms found in this family generally have a similar appearance to rod-shaped and other Gram-negative bacteria found in all water habitats. The genus *Vibrio*, an all water habitats are considered the largest species of living Gram-negative bacteria.

Vibrio parahaemolyticus, proteobacteria Class Gamma and proteobacteria belong to *Vibrio*,

Family *Vibrio*, Genus in the class of *Vibrionales* in Phylum. From the *Vibrio* genus of 34 major dimensions described by Janda et al. (1988), as such one third of these species are identified as human pathogens (Table 1.1).

Some pathogenic species outside of humans such as *Vibrio anguillarum*, *Vibrio fischeri* and *Vibrio harveyi*, are pathogens of sea fish and shellfish species (Thompson et al., 2004). The phylogenetic relationship of the genus *Vibrio* bacteria is determined in different systems. Tian et al. (2008) suggested that the *gyrB* gene is most suitable for sequencing the 16S rRNA gene and determining the *Vibrios* phylogenetic relationships of *Vibrios* and associated species according to maximum likelihood technique using polyclonal nucleotide sequences including *ftsZ*, *GyrB*, *mReb*, *pyrH*, *recA*, *rpoA* and was analyzed by Thompson et al. (2004).

The authors evaluated the phylogenetic polyclonal nucleotide sequences and 16S rRNA, including the average amino acid identity of the genomic signatures and genome BLAST atlas genome, and the combination of different bioinformatics tools suggested the *Vibrio* genus (Genomic classification) to provide more accurate identification and understanding of the genomic taxonomy of *Vibrio* species (Thompson et al., 2004).

Table 1.1: Composition of the genus *Vibrio* (Source: Janda et al., 1988)

Human Pathogens	Non-Human Pathogens
<i>Vibrio alginolyticus</i>	<i>Vibrio aestuarianus</i>
<i>Vibrio cholerae</i>	<i>Vibrio anguillarum</i>
<i>Vibrio cincinnatiensis</i>	<i>Vibrio campbellii</i>
<i>Vibrio damsela</i>	<i>Vibrio carchariae</i>
<i>Vibrio fluvialis</i>	<i>Vibrio costicola</i>
<i>Vibrio furnissii</i>	<i>Vibrio diazotrophicus</i>
<i>Vibrio hollisae</i>	<i>Vibrio fischeri</i>
<i>Vibrio metschnikovii</i>	<i>Vibrio gazogenes</i>
<i>Vibrio mimicus</i>	<i>Vibrio harveyi</i>
<i>Vibrio parahaemolyticus</i>	<i>Vibrio logei</i>
<i>Vibrio vulnificus</i>	<i>Vibrio marinus</i>
	<i>Vibrio mediterranei</i>
	<i>Vibrio natriegens</i>
	<i>Vibrio nereis</i>
	<i>Vibrio nigripulchritudo</i>
	<i>Vibrio ordalii</i>
	<i>Vibrio orientalis</i>
	<i>Vibrio Pelagius</i>
	<i>Vibrio proteolyticus</i>
	<i>Vibrio psychroerythrus</i>
	<i>Vibrio salmonicida</i>
	<i>Vibrio splendidus</i>
	<i>Vibrio tubiashii</i>

1.4.3 Cell and Colony Morphology

Vibrio parahaemolyticus, Gram-negative rod-shaped bacterium 0.5-0.8 x 1.4-2.6 Wm in size is halophilic. The ideal growth conditions of *Vibrio parahaemolyticus* are 35-37°C, pH 7.5-8.0 and approximately 0.5M NaCl (Joseph et al., 1982). The colony morphology of *Vibrio parahaemolyticus* is flexible. Several colonies of morphotypes can occur in one isolated

colony of offspring. In addition, the types of colony can alternately be reversible from translucent (TR) to opaque (OP). It is believed to be sensitive to the specific environmental conditions of the switching mechanism (McCarter, 1999). Biofilm structure, TP, and the biofilm formation in *Vibrio parahaemolyticus* is extremely competent in biofilm formation but the biofilm structures are made differently in TR and OP strains (Enos-berlage et al., 2005). In biofilms of TR strains, long columns are columns vaguely sprinkled with open channels whereas the biofilms of OP strains are more uniform, dense and lack such channels. Biofilm formation of *Vibrio parahaemolyticus* is regulated by the chitin-regulated pilus (ChiRP) and mannose-sensitive hemagglutinin pilus (MSHA) (Shime-Hattori et al., 2006).

Vibrio parahaemolyticus have several types of cells in adaptation to life under different conditions. A fluid medium that has unique free floating organisms called floaters cells exist as a single polar flagellum. Growth on the surface or a viscous medium induces the differentiation of immuno competent cells into swarmer cells. Swarm tumor cells possess good peritrichous flagellum which acts to produce movement in a very viscous medium (McCarter, 1999). The metabolic adaptation of *Vibrio parahaemolyticus* allows the organism to survive under demanding circumstances after one week of starvation at 3.5°C found that the morphology of *Vibrio parahaemolyticus* changes from rod-shaped to nodular form. These cells are preserved but were unable to grow in growth media, and therefore labeled as viable but non-culturable (VBNC) cells. The authors proposed suitable conditions of the VBNC cells when it raises the return temperature or the favorable conditions occur.

1.4.4 Virulence Factors of *V. parahaemolyticus*

Vibrio Parahaemolytic virulence factors include virulence genes in Pathogenicity Island (PI), hemolysin type three secretion systems (TTSSs), colonizing external membrane agents and proteins (OaMP). Hemolysis genes host cells is related with colonizing factors such as capsular polysaccharide (CPS) (Nakasone and Iwanaga, 1990), and OMPs (Hsieh et al., 2003).

Among the *Vibrio spp.*, *Vibrio parahaemolyticus*, *Vibrio mimicus*, has been regarded as a food pathogen, except for *Vibrio vulnificus* (Reham and Amani, 2012). *Vibrio* outbreaks were observed which were caused by *Vibrio parahaemolyticus* in August 2010, based on the

Food borne Outbreak Online (FOOD) database data (2010), an effect held in Washington, USA. This is the main cause of gastroenteritis associated with consumption of fresh or not cooked seafood (Pina et al., 2005). On the basis of Reham and Amani (2012), sepsis can cause immunity to people with immune deficiency and long-term use of steroids. The presence of TDH, TRH, or both virulent strains has been implicated in the thermogen direct encoding hemolysin gene encoding the market thermo direct hemolysin associations and the presence of the Bako gene pathogenicity *Vibrio parahaemolyticus* (Pina et al., 2005).

Experimental samples of the Bako shrimp aquaculture site have been studied to identify *Vibrio parahaemolyticus* by polymerase chain reaction (PCR) for regulatory gene detection, and (for example, the *toxR* gene), as it is relatively easy to elucidate in a shorter time than phenotypic methods. In this study, the experienced expert *Vibrio* spp is used to confirm operon gene (toxicology R), which is the regulatory gene, the presence of operons (Zulkifli et al., 2009). In particular, the front pair of primers and the rear pair can be used to detect *Vibrio* spp. Kim et al., (1999) also showed that the genome of genus *Vibrio parahaemolyticus toxR* 50% G / C detection in the forward and in the market is specific to the reverse primers used for confirming the pathogenicity of *tdh*, *Vibrio parahaemolyticus* isolates. In addition, this method is a list of the target organisms in the most probable number.

1.4.4.1 Haemolysins

The isolated strains from diarrhea faeces of patients with gastroenteritis are mostly haemolytic, meanwhile the environmental isolates are usually nonhaemolytic. Haemolysis of *Vibrio parahaemolyticus* is pictured by the lysis of human or rabbit erythrocytes on Wagatsuma agar (Chun et al., 1975). This haemolysis is named the ‘Kanagawa Phenomenon’ after the original discoverers, the Kanagawa Prefectural Public Health Laboratory, Japan. The Kanagawa Phenomenon positive strains (KP-positive) produce a thermostable direct haemolysin (TDH). Thermostable direct haemolysin-related haemolysin (TRH), which is another type of haemolysin, has been found in clinical Kanagawa negative strains (KP-negative) (Honda et al., 1988; Janda et al., 1988; Miyamoto et al., 1969). A comparison of the properties of TDH and TRH is shown in Table 1.2. Even though TDH and TRH are the most researched haemolysins of *Vibrio parahaemolyticus*, a thermolabile or lecithin

dependent haemolysin (LDH) and a heat-stable haemolysin (E-VPH) have also been described in this organism (Taniguchi et al., 1986, 1990).

Table 1.2: Comparison of TDH and TRH toxins of *Vibrio parahaemolyticus* (Taniguchi et al., 1990)

Property	TDH	TRH
Molecular weight:		
- Holo Toxin	46,000	47,000
- Subunit	23,000	23,000
PI	4.9	4.6
Heat stability	Stable at 100°C	Labile at 60°C
Antigenicity	Related but not identical to that of TRH	Related but not identical to that of TDH
Amino acid sequence acid	67% homology to amino acid sequence of TRH	67% homology to amino acid sequence of TDH
Biological activity:		
- Haemolytic activity	Rabbit, human > calf, sheep > horse	Calf, sheep > rabbit, human > horse
-Lethal activity (mouse)	cardiotoxicity	Cardiotoxicity
-Fluid accumulation in rabbit ileal loop (RIL)	250Wg/loop	100Wg/loop

1.4.4.2 Pathogenicity Islands (PIs)

A mobile genetic element which can be transmitted through bacterial strain or species is called genomic island. Infectious genes-linked from genomic islands with some antibiotic resistance genes regrouped as pathogenic islands (PIs). A PI can be used as an indicator for the identification of pathogenic bacteria in molecular diagnostics (Oelschlaeg and Hacker, 2004). PIs further have an important function to play in the development of bacteria virulence through the process of horizontal gene transfer (HGT) (Dobrindt et al., 2004).

Seven PIs in *Vibrio parahaemolyticus* such as VpaI1 - VpaI7 with size ranging from 10 kb to 81 kb, was identified in the *Vibrio parahaemolyticus* genome using the bioinformatic method (Hurley et al., 2006). Study of these VPals in 235 *Vibrio parahaemolyticus* isolates from China showed that VpaI-1 and VpaI-5 genes were precisely linked with pandemic O3:K6 strains, whereas VpaI-7 and TTSS2 were related with tdh-positive strain (Chao et al., 2009).

Using comparative genomic analyzes, the microarray identified genes that were specifically present in pandemic and non-pandemic environmental *Vibrio parahaemolyticus* pandemic strains (Izutsu et al., 2008). These genes contain 65 genes found in chromosome from 11 regions which was suggested by the authors that pandemic strains evolved from multiple genetic events, including the importation of different genetic clusters into the evolution. Furthermore, in this study, a comparison of genomes of pathogen and non-pathogenic strains showed that KP-positive strains retained the nucleotide sequences of 80 kb pathogenic localized nucleotides, but in negative KP lines. This result showed a strong correlation between the island 80 kb pathogenic and pathogenic *Vibrio parahaemolyticus* field.

1.4.4.3 Type Three Secretion Systems (TTSSs)

A type three secretion system (TTSS), a series of about 20 genes coded together in a PI region. Gram-negative bacteria secrete and inject virulence-related proteins into eukaryotic host cells through a needle-like structure by the help of TTSS mechanism. TTSS has been discovered in a variety of pathogens Gram-negative bacteria including *Yersinia spp.*, *Shigella spp.*, *Salmonella spp.*, *Vibrio spp.*, *Pseudomonas aeruginosa*, and *E. coli enteropathogens* (EPEC) (Hueck, 1998).

Makino et al. (2003) first made the discovery of two type III secretion systems, type three secretion system 1 (TTSS1) and type three secretion system 2 (TTSS2), in *Vibrio parahaemolyticus*.

Vibrio parahaemolyticus genome consisting of two circular chromosomes 3288558 and 1877212 bp bp. The entire genome contains an open reading frame 4832 (ORF). TTSS2 operon is an 80-kb chromosome 2 sequence and TTSS1 is a fragment of a PI located on the corporation of genes encoding genes that are more similar to those of *Yersinia* and gene TTSS2. However, TTSS2 *Vibrio parahaemolyticus*-associated region in *Vibrio*

parahaemolyticus consists of numerous virulence-related genes including homologues of the *E. coli* cytotoxic necrotising factor agent, the *Pseudomonas* exoenzymes T and genes present in the PI of *Vibrio parahaemolyticus*.

According to analysis of TTSSs from several strains of *Vibrio parahaemolyticus* by Makino et al. (2003), TTSS1 was identified in all tested strains however TTSS2 was discovered only in clinical KP-positive strains. The G+C content of the *Vibrio parahaemolyticus* PI is lesser (39.8%) than the average G+C content of the genome (45.4%), demonstrating that recent lateral transmission may have taken place in this region.

The functional characterization of the considered *Vibrio parahaemolyticus* TTSS1 and 2 was determined by a TTSS1 disorder including genes, *vcrD1*, *vscC1* and *vscN1* and TTSS2 behaviors, *vcrD2*, *vscC2* and *vscN2* (Park et al., 2004). The results revealed that the genes are associated with TTSS1 cytotoxicity, while TTSS2 are related to the enterotoxigenic host cell. In addition, VopD a protein associated with infectivity is encoded in TTSS1 with YOPD homology in *Yersinia* spp. Both vopp, a protein encoded by TTSS2 with YOPP homology in *Yersinia* spp., were identified respectively as secreted by *Vibrio parahaemolyticus* and TTSS1 TTSS2 (Park et al., 2004). The results show that two *Vibrio parahaemolyticus* secretory devices is responsible for the secretion of discrete proteins. However, possession of TTSS2 cannot be associated with pandemic strains of *Vibrio parahaemolyticus* since it can also be detected in *tdh*-negative strains. In contrast, some *tdh*-positive strains did not wear TTSS2. The authors suggested that TTSS2 can be obtained without the surrounding IP containing two copies of the *tdh* genes, or *tdh* genes lost or mobile IP. The roles of TTSS1 cytotoxicity and TTSS2 in enterotoxigenicity are also shown by Hiyoshi et al. (2010). In this study, bacterial pathogenesis provided by TTSS1 and TTSS2 in the *tdh* role was determined and suggested that TTSS1 HRT may have a cumulative effect on mice toxicity. Only TTSS2 but not TTSS1 and TOH, is an important factor in *Vibrio parahaemolyticus*-induced enterotoxigenicity in a rabbit model. In addition, THO secretion independently of TTSS1 and TTSS2 is also demonstrated in this study. The microarray analysis of a TTSS1 deletion mutant also reported that apoptosis required a functional TTSS1 and showed that the translocon TTSS1 dependent protein had been associated with the death of the host cell (Bhattacharjee et al., 2005).

Characterization and functional analysis of *Vibrio parahaemolyticus* TTSS1- and TTSS2-associated proteins have been extensively studied in recent years (Bhattacharjee et al., 2006).

Pseudomonas aeruginosa is secreted by both effector proteins that have a similar effect and exos of ExoT protein ADP-domain. According to Park et al., (2004) TTSS21 is also linked with host cell cytotoxicity, the results of this study revealed that the host cells is partly responsible for cytotoxicity in the host cell. Okada et al., (2009) identified a new TTSS2 in *tdh* positive strain identified as *Vibrio parahaemolyticus*.

Genes found in the TTSS2 Italy region, including *vscC2*, *vopP* and *Vopa / P*, *VPaI*, *VOPC* and *VPA1376*, which *Vibrio parahaemolyticus* was detected and isolated (Caburlotto et al., 2009). It was found that *VscC2* and *VOPP* suggest that they may occur together or separately even two genes can be obtained independently in the same medium. Then, potential infected carrying genes including *Vopt* and *vOPB2* other genes involved in *VPaI* (Caburlotto et al., 2010) have been investigated. These strains may cause adherence to human cells and cell disorders, and loss of membrane integrity. These results show that there is a threat of Pathogenic *Vibrio parahaemolyticus* in a common environment, which constitute a public health concern.

1.5 Disease Caused by *Vibrio parahaemolyticus*

Vibrio parahaemolyticus is a bacterial pathogen that is transmitted to the sea and is the main cause of gastroenteritis worldwide. The disease is caused as a result of the digestion of contaminated poorly cooked seafood especially in shellfish. The incubation period of *Vibrio parahaemolyticus* interval between 13 and 23 hours (Barker et al., 1974). Clinical signs usually start 10-15 hours after infection with diarrhea and abdominal pain. Diarrhea stools are usually hydrated and slimy, patients may also have a fever, vomiting, nausea, abdominal cramps, psychosis and general fatigue. The frequency of diarrhea is usually less than 10 times a day. *Vibrio parahaemolyticus* infection in many clinical situations, such as self-limiting, diarrhea will melt spontaneously 9-10 days. *Vibrio parahaemolyticus* infective dose ranges from 10¹⁰ colony forming units (CFU) (g⁻¹) per 105 grams. Nevertheless, it has been found that the infection is associated with the desired infectious components and the pathogenesis of infectious strains (Joseph et al., 1982). Hemolytic strains 3 x 10⁷ CFU at least 2 x 10⁵ consumed *Vibrio parahaemolyticus* by volunteer quickly developed symptoms

of gastroenteritis, although individuals who received from 4×10^9 to 1.6×10^{10} CFU of non-haemolytic strains of *Vibrio parahaemolyticus* did not show diarrhea symptoms.

1.5.1 Epidemiology of *Vibrio parahaemolyticus*

The frequency of *Vibrio parahaemolyticus* has sporadic cases of diarrhea worldwide, including Asia, Europe and the United States. Pandemic serotype O3 outbreaks: K6 appeared in many countries in Asia and later spread to other parts of the world. Geographical distribution of virulent *Vibrio parahaemolyticus* as shown in Figure 1.1.

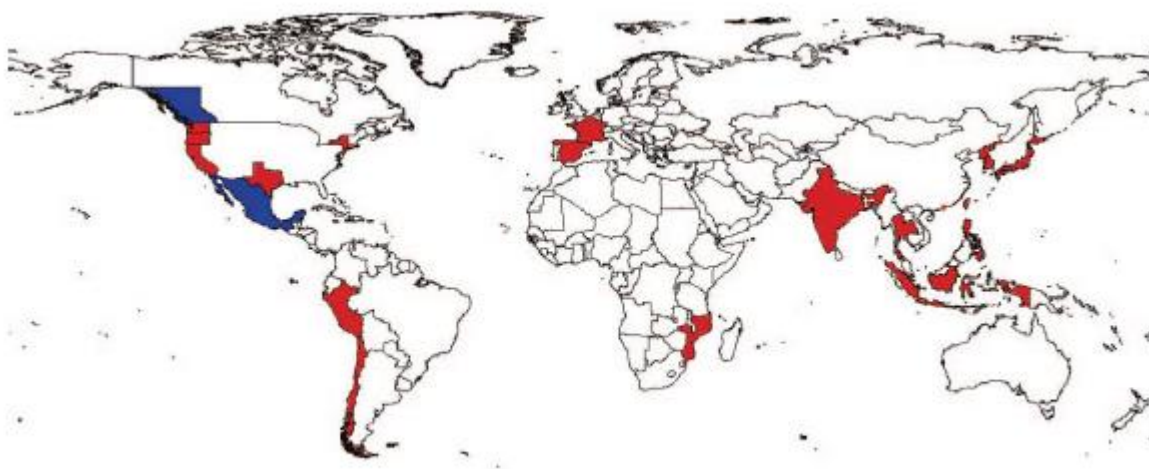


Figure 1.1: Global dissemination of *Vibrio parahaemolyticus*

Where: Red represents area where the pandemic *Vibrio parahaemolyticus* strain has spread. Dark blue represents areas where outbreaks of *Vibrio parahaemolyticus* have occurred or presence in the environment but the pandemic status of strains remains unclear. Figure adapted from Nair et al. (2007).

1.5.1.1 Asia

Vibrio parahaemolyticus was first isolated and described as a food poisoning bacteria in Japan in 1950 (Fujino et al., 1953). According to the Infection Disease Surveillance Center (IDSC, Japan) during the period 1996-1998 (Su & Liu, 2007), food poisoning in Japan was the number one cause of *Vibrio parahaemolyticus*. From 1992 onwards, the disease caused

by this organism caused food was reported in many Asian countries, including India, Bangladesh, China, Taiwan, Korea, Vietnam and Thailand. Pandemic O3: K6 1996 Origin in Calcutta, India (Okuda et al., 1997).

TDH was possessed by all pandemic O3:K6 strains from this study accounting for 50-80 % of the strains isolated from gastroenteritis patients from February-August in 1996 in Calcutta. Because it had not been formerly identified during *Vibrio parahaemolyticus* surveillance in Calcutta, it was identified as a new pandemic clone. Collective data received show that the outbreak in Calcutta was believed to be the epidemiological origin of the O3:K6 pandemic strain (Nair et al., 2007).

1.5.1.2 Europe

Random outbreaks of diarrhea due to *Vibrio parahaemolyticus* have been reported in several European countries particularly in France, Spain and Italy. Quilici et al., (2005) reported the occurrence of the pandemic serovar O3:K6 from coastal areas during 1997-2004 in France. Moreover, a severe outbreak associated with the ingestion of shrimps imported to France from Asia in 1997 (Su & Liu, 2007). Quilici et al. (2005) suggested that the clone causing the outbreak might have been brought to France in ballast water discharged from cargo ships entering the European coastal area.

In Spain, tdh-positive strains of *Vibrio parahaemolyticus* were detected in stool specimens of gastroenteritis patients. The disease is associated with raw oyster consumption between August and September 1999 (Lozano-Leon et al., 2003). The results of this study have shown that raw oysters and other shellfish are tools for the transmission of *V. parahaemolyticus* tdh positive strains in molluscs harvested from European water. During the summer of 2007, pandemic *Vibrio parahaemolyticus* O3:K6 strains were identified in faecal samples of diarrhoeal patients in Italy (Ottaviani et al., 2008). The authors also reported the presence of pathogenic *Vibrio parahaemolyticus*, soft-tissue susceptibility to TDH positive strains collected from European waters. In stool specimens of patients with diarrhea in Italy, in the summer of 2007, *Vibrio parahaemolyticus* O3 pandemic strains were detected (Ottaviani et al., 2008). Also in this study, another toxigenic *Vibrio parahaemolyticus* serovar O1:KUT and other potential pandemic strains were also isolated from local shellfish and seawater

from the Adriatic Sea, and it was proposed that the infection was as a result of the ingestion of fresh shellfish from local vendors.

In the United Kingdom (UK), *Vibrio parahaemolyticus* routine shellfish and river mouths are also found at low levels (30%) in environmental samples, including rivers water (Wagley et al., 2008). Despite the fact that more than 10% of these environmental isolates were tdh-positive, pulse field gel electrophoresis (PFGE) investigation revealed that none of the isolates from shellfish were clonally connected to clinically-derived strains or the pandemic O3:K6 serovar. But, the authors discovered that clinical isolates from the UK share close clonal resemblance with the pandemic O3:K6 strain responsible for the outbreaks in Asia.

1.5.1.3 The Americas

The geographical distribution of *Vibrio parahaemolyticus* has been reported on the West Coast, the Gulf Coast, and the Pacific coast of the US and British Columbia (Canada) (Anonymous 1997, Barker et al., 1974, Daniels et al., 2000). The first events took place along the east coast and the Gulf, including Maryland, Louisiana and the Gulf of Mexico. The pandemic region was later expanded to Canada in Washington, Oregon and California, and Pacific Northwest, including British Columbia.

Vibrio parahaemolyticus gastroenteritis first documented outbreaks in the USA were reported in Maryland in 1971 (Molenda et al., 1972). Strains of serotypes O4:K11 and O3:K30 were isolated from the stool samples of the infected patients. Steamed crab and crab salad prepared from canned crabmeat were alleged as the reason of the disease in these outbreaks. The case studies of the 1972 Louisiana outbreak and the outbreaks on two Caribbean cruise ships during 1974-1975, showed that they were due to poor shrimp boiling process and to seafood infection from the internal seawater system (Barker et al., 1974; Lawrence et al., 1979).

However, epidemic strains isolated from cruise ships were not the same. This prevalence is due to the fact that cruise ships across the various territorial waters, which contain various regional types, and that such local microorganisms (some of which are pathogenic strains) may contaminate the cruise water system (Lawrence et al., 1979). Of course, cases of

gastroenteritis have also been recorded in the Northwest of the Pacific in the late summer of 1981 (Nolan et al., 1984).

1.6 Overview of Shellfish

There is a non-taxonomic term given for shellfish in seafood consumption. Shellfish group encompasses crustaceans and molluscs representing a significant market niche of marine species with commercial interest. The classification of crustaceans as arthropods has to do with over 50,000 living species like shrimp, prawns, lobster, crayfish and barnacles. This species are eaten or consumed as raw or cooked/processed while Molluscs can be subdivided into some different classes like bivalves, gastropods and cephalopods with up to 100,000 divers species like mussels, oysters, abalone and squids which has a nutritional value and intrinsic organoleptic characteristics making molluscs very appreciative and accepted worldwide majorly in the coastal regions (Kamath et al., 2014; Lopata et al., 2010; Mao et al., 2013).

Fresh and clean shrimp can be served with sauce cooked or uncooked. From a nutritional point of view, shrimp low calories, very rich in protein and has a neutral taste. Due to these features, shrimp salad, pasta, curry, soup make natural additives and pan dishes. Shrimp have also been identified as a rich source of vitamin B12, selenium and astaxanthin, a potent natural antioxidant (Venugopal 2009). Despite the relatively high cholesterol (Robinson, 1954) dietitians and health professionals, as well as reluctance among consumers, due to which dietary parameters based on shrimp, can be seen as a healthy diet. In a clinical trial, shrimp showed moderate consumption in normolipidemic patients that would not adversely affect the overall lipoprotein profile and could be included in "healthy heart" dietary guidelines (De Oliveira et al., 1996).

1.6.1 Protein and Other Vital Nutrients of Human Importance in Shrimp

Similar to any animal meat, shrimp diet is a perfect source of protein. The shrimp are three quarters of the edible part of the water area. The remainder (dry matter) contains approximately 80% protein. The average protein content of fresh shrimp is 19.4 g / 100 g and

contributes 87% of the total energy. Our bodies cannot synthesize some amino acids and must be taken by diet; these are called basic amino acids. Some digestibility corrected amino acid protein food proteins are capable of providing the necessary amino acids according to the actual digestible amino acid content (PDCAAS) and regulation. PDCAAS of shrimp, showing superior quality protein, is 1. The load factor is 3.3 in the range of 0-5 indicating that it provides more nutrients per calorie and can be considered as a healthy food such as fish (Simopoulos, 2008).

Analysis of lipid levels of shrimp has been around 1.15 g / 100 g. You can demand no other meaty food lipids at a low level like fresh shrimp. The ingredient shrimp lipid composition comprises 65-70% phospholipids, 10-20% cholesterol and 15-20% of total acyl glycerols. The phospholipid-rich lipid shrimp frequency shows nutritional values that are an integral part of cell membranes and transport lipoproteins. As a result of epidemiological studies, it is associated with a reduction in risk of coronary heart diseases. Seafood consumption such as shrimp is rich in omega-3 PUFAs (Kris-Etherton et al., 2002; Mozaffarian and Wu, 2011; Murphy et al., 2012).

1.6.2 Shrimp Consumption in Relation to Recommended Daily Allowance (RDA) of Nutrients

The recommended daily requirement (RDA) increases the quantitative composition of nutrients, in general, to remain healthy for humans. Such children may be different for adult men and women and for different categories. The Indian Council for Medical Research (ICMR) favors UN/WHO/FAO guidelines for framing the RDA guidelines, with slight modifications. RDA for a nutrient is a standard unit that helps layperson to easily calculate their requirements depending on body weight and / or basal metabolic rate. RDA can be used to calculate the daily value of food (% DV). One hundred grams of shrimp are taken to calculate the % of meat hanging in the recommended DV section. For example, weighing 0.8 g / kg of body weight as measured by protein intake (kg / kg body weight) (Enser et al., 1996).

CHAPTER 2

THEORETICAL FRAMEWORK

2.1 Significance of Microbiological Investigations

Investigation of microbial pathogens in food is recognized as one of the most important control measures in the prevention of foodborne diseases (Velusamy et al., 2010). Estimation of bacterial populations in foods is vital in assessing the presumptive microbial safety of foods. This involves sampling, microbial examinations and evaluation of results.

Microbiological analysis constitutes essential part of food safety programme. It is irreplaceable during compliance testing for defined microbiological criteria and in assessing management commitments for overall quality. Microbiological analyses have various roles to play including monitoring of food production processes, verification and validation of HACCP systems and establishing guidelines and policies for domestic and international trade (FAO, 2005; FSSAI, 2012), and also in settling dispute among food production firms, regulatory bodies and consumers (Jarvis et al., 2007).

The quantities and species of microorganisms present in foods signify adherence to good hygiene and safety practices (Jarvis et al., 2007). Moreover, European Commission Regulation acknowledged that epidemiological studies should be performed based on standard culture techniques for isolating pathogens in foods (EC 2073/2005).

The inner parts of live fish do not support bacterial growth due to the role of body immune system. However, when the fish die, the bodies remain inactive in which the pathogenic and spoilage bacteria gain entry and multiply easily (Huss et al., 2003).

Shellfish employed filter feeding mechanism to obtain food and water necessary for their survival, and in this mechanism they accumulate pathogenic bacteria like *Vibrio parahaemolyticus* to doses even higher than those obtained from the surrounding water (Yeung and Boor, 2004).

2.2 Properties and Characteristics of *Vibrio*

Gram-negative bacteria, are the *Vibrio* species with a curved bar shape and polar flagella with sheaths with polar flagella. All members are mobile, optional, optional anaerobic oxidants and without controversy (Farmer, 1992). *Vibrio* genus includes about 106 species recognized in nature in estuaries and marine ecosystems. Types of *Vibrio* are often associated with many outbreaks of food poisoning and are considered one of the most important pathogens associated with food and waterborne illnesses.

Figure 2.1 displays the results of a report published by a weekly report on morbidity and mortality (MMWR) on the incidence and trends of pathogenic infections of food-borne infections obtained from 10 sites in the United States from 1996 to 2010 (Centers for Control and Prevention 2011 Disease). As can be seen, the population of the *Vibrio* species has been upward over the years. In particular, in recent years, 2007-2010 there has been a sharp increase in the prevalence of *Vibrio* species.

Basically, they are alkaline pH tolerant but are sensitive to acid pH. Because of the high sodium chloride content in the environment they can withstand less water activity (a_w), which is 0.980 (Madigan et al., 2004), but there are some types of *Vibrio*, depending on their sodium chloride needs. With the exception of *Vibrio* species, which are not halophilic, such as *Vibrio cholerae* and *Vibrio mimicus*, in other species of *Vibrio* there is a need for a saline solution for their growth.

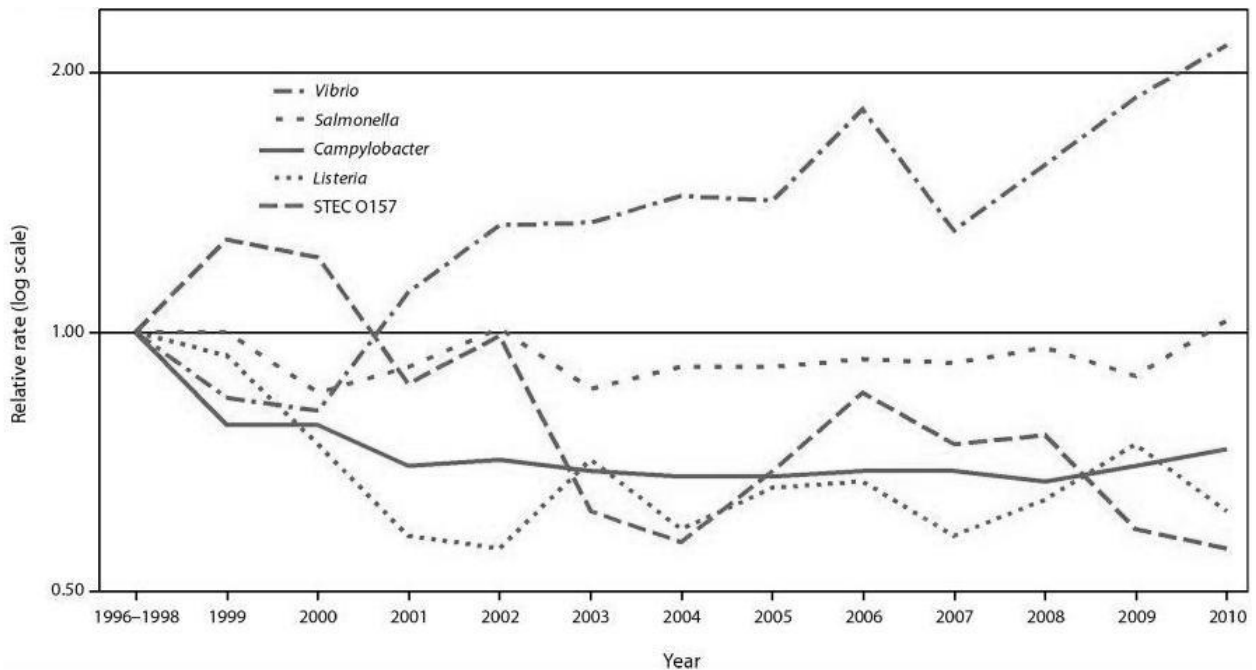


Figure 2.1: Relative rates of laboratory-confirmed infections with Campylobacter,

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).

2.2.1 Host Range and Transmissions of *Vibrio*

High incidence of *Vibrio* spp. in marine and aquatic environments, leading to their presence in seafood and all of freshwater food, especially in temperate climates all over the world. Some species build relationships with aquatic animals and, in fact, have a wide range of guests including seafood, including fish, crustaceans, oysters, shrimp, shrimp, calamari and many other freshwater animals (Sujeewa et al., 2009). The abundance of *Vibrio species* raw fish and crustaceans, making these kinds of foods appropriate for their transfer and leading to the connection of *Vibrio species* studies on food safety problems.

2.2.2 Pathogenicity of *Vibrio* species.

Among the species of *Vibrio* there are 12 species that, for various studies, have shown that they are human pathogens that cause diseases associated with seafood (Khaira & Galanis, 2007). These *Vibrio* species are often reported as an important cause of gastrointestinal tract disease, severe septicemia and skin infections in humans or contaminated fish or if exposed to an aqueous environment (Ottaviani et al., 2009).

One of the most significant species is *Vibrio cholerae* and especially serotypes O1 and O139, as the main cause of diarrhea. There are other pathogenic viruses of *Vibrio cholerae*, but cause less pronounced diarrhea. *Vibrio parahaemolyticus* is often known as the cause of food gastroenteritis epidemics in the world (Pruzzo et al, 2005). *Vibrio vulnificus* causes 95% of all deaths associated with seafood consumption (Rosche et al., 2006). These three species were known as the most common causes of food transmission disorders. Other pathogenic species include *Vibrio alginolyticus*, *Vibrio damsela*, *Vibrio fluvialis*, *Vibrio furnisii*, *Vibrio hollisae*, *Vibrio metschnikovii* and *Vibrio mimicus* (Pruzzo et al., 2005).

Types of *Vibrio* are more common in warmer waters or seasons when the coastal water temperature for their growth. The risk of infection is greater when raw seafood is consumed raw or insufficiently cooked mode as well as when they are contaminated after heating (Noorlis et al., 2011).

2.2.3 *Vibrio parahaemolyticus*

Vibrio parahaemolyticus is a Gram-negative bacterium, and it is no spore, mobile and arched rods. This is usually good oxidase and catalase. It grows in a medium containing glucose, without the production of gas. But he could not roam lactose and sucrose. Although the optimum growth temperature is 30-37 ° C, it can grow in the range of 5-42 ° C. The cells have been able to proliferate very rapidly in a medium containing 3-5% NaCl, and can carry up to 8% NaCl but are sensitive to 10% NaCl. A growth rate limited to pH 5.0 or below. The optimum pH for growth ranges from 7.8 to 8.6 in the range of 4.8 to 11. The cells are very sensitive to heat (pasteurization) and lyophilized. It can grow in presence or absence of oxygen, but it grows optimally in aerobic conditions (Oliver and Kaper, 1997).

Vibrio parahaemolyticus is a human ubiquitous pathogen that can cause gastroenteritis during consumption of contaminated unmanufactured raw or contaminated potash fish (Pruzzo et al, 2005). Since this species is very common in marine products, it has become a serious problem in fish production and marketing (DePaola et al., 2003). This requires the adoption of marine food safety measures, which are the main source of a large number of pathogenic bacteria, including *Vibrio parahaemolyticus*.

Vibrio parahaemolyticus is most common in summer in Europe and the United States, when the temperature is 25 ° C and higher, while it can be detected throughout the year in Southeast Asia (Zulkifli et al., 2009b). In Malaysia, the probability of *Vibrio parahaemolyticus* outbreaks is very high, as the climate is suitable for growth of *Vibrio* species (Elhadi et al., 2004). In addition, virulent strains of raw fish have also been reported in Malaysia (Sujeewa et al., 2009). Thus, it has been brought to the attention of public health and food safety.

2.3 Isolation of *Vibrio* spp.

2.3.1 Sample Collection

As mentioned above, *vibrios* live in the marine environment and are associated with aquatic animals, including fish, crustaceans, shrimp, oysters, squid, shrimp and other freshwater animals (Sujeewa et al., 2009). As a result, fish and marine products are mainly used for the isolation of *Vibrio* species.

After collection of samples, it must be immediately cooled to a temperature of 7° C to 10° C and must be analyzed as soon as possible. *vibrios* can be injured if they are subjected to rapid cooling.

It is best to avoid direct contact with ice samples in order to maximize the survival and existence of vibration. They are able to grow rapidly at room temperature in seafood (Cook, 1997). The extreme heat and cold can kill *vibrios* and prevent their recovery, but with moderate cooling they can survive well (Boutin et al., 1985).

2.3.2 Behavior of *Vibrios* on Selective Agar

Thiosulfate-Citrate Bile Sucrose Salts (TCBS) is a recommended standard method for isolating *Vibrio cholerae* to be fed. The method comprises enrichment in alkaline peptonate water (PMU) at $35 \pm 2^{\circ}\text{C}$ overnight and then selected on TCBS medium. The same method has been recommended for other *Vibrio*, such as *Vibrio vulnificus* and *Vibrio parahaemolyticus* (Elliot et al., 1995). Most of the *Vibrio* species are of significant growth in TCBS, while the growth of most *Vibrio* is inhibited on this growing culture. However colonies on TCBS *Vibrio parahaemolyticus* very difficult to visually distinguish colonies of other bacteria, because they can be coated with a yellow color produced by bacteria, sucrose enzymes (Hara-Kudo et al., 2001). Colors of colonies appearing on TCBS for different types of *Vibrio* are presented in Table 2.1

Table 2.1: Colors of colonies appearing on TCBS

Species	Colony Colour
<i>V. parahaemolyticus</i>	Green
<i>V. parahaemolyticus</i>	Green
<i>V. cholera</i>	Yellow
<i>V. alginolyticus</i>	Yellow
<i>V. furnissii</i>	Yellow
<i>V. fluvialis</i>	Yellow

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

CHROMAgarTM *Vibrio* (CHROMagar, Paris, France) is a more selective means to identify and isolate *Vibrio alginolyticus*, *Vibrio cholerae*, *Vibrio parahaemolyticus* and *Vibrio vulnificus* using chromogenic technology, resulting in colonies that may be different for development of color. It is more precise and specific than TCBS (Di Pinto et al., 2011). Colonies that appear on CHROMAgarTM *Vibrio* for different types of *Vibrio* are shown in Table 2.2 and Figure 2.3.



Figure 2.2: The colony colors of *Vibrio* spp. on TCBS (Adapted from E&O Laboratories Ltd, www.eolabs.com)

Table 2.2: The colony colors of *Vibrio* spp. on CHROMAgar™ *Vibrio*

Species	Colony Colour
<i>Vibrio parahaemolyticus</i>	Mauve
<i>Vibrio vulnificus</i> / <i>Vibrio cholera</i>	Green blue to turquoise blue
<i>Vibrio alginolyticus</i>	Colourless

Source: www.CHROMAgar.com

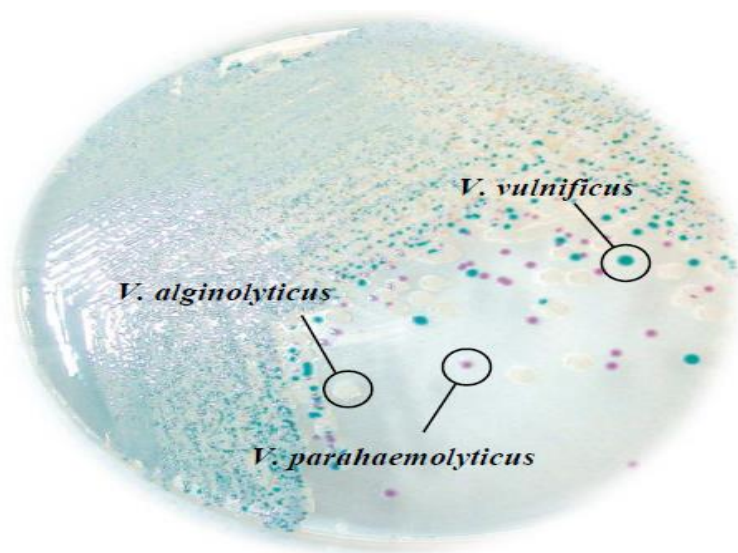


Figure 2.3: The colony colors of *Vibrio* spp. on CHROMAgar™ *Vibrio* (Adapted from www.CHROMAgar.com)

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

2.4.1 Biochemical Tests

Some of the disparity in characteristics of some *Vibrio* species linked with human disease related to seafood consumption have been clearly described in Table 2.3.

2.4.1.1 Oxidase Test

Oxidase test is a major differential process that must be done for all gram-negative bacteria to be identified. The test reagent is N, N, N', N'-tetramethyl-a-p-phenylenediaminindigydrochloride as an artificial electron acceptor for oxidase. Oxidase test is able to identify the organisms that produced cytochrome oxidase and is very useful for classifying organisms in groups at the initial stages of their identification. Cytochrome oxidase is a member of the electron transport chain. It transmits electrons from oxygen donating molecules (Isenberg, 2004).

Table 2.3: Biochemical characteristics of human pathogenic *Vibrionaceae* commonly encountered in seafood

	<i>Vibrio alginolyticus</i>	<i>Vibriocholerae</i>	<i>Vibrio fluvialis</i>	<i>Vibrio mimicus</i>	<i>Vibrio parahaemolyticus</i>	<i>Vibrio vulnificus</i>
TCBS agar	Yellow	Yellow	Yellow	Green	Green	Green
AGS	KA ^a	Ka ^b	KK ^c	KA ^a	KA ^a	KA ^a
Oxidase	+	+	+	+	+	+
Arginine dihydrolase	-	-	+	-	-	-
Ornithine decarboxylase	+	+	-	+	+	+
Lysine decarboxylase	+	+	-	+	+	+
Growth 0% NaCl	-	+	-	+	-	-
in (W/V): 3% NaCl	+	+	+	+	+	+
6% NaCl	+	-	+	-	+	+
8% NaCl	+	-	V ^d	.	+	.
10% NaCl	+	-	-	-	-	-
Growth at 42°C	+	+	V ^d	+	+	+
Acid Sucrose	+	+	+	-	-	-
from: D-Cellobiose	-	-	+	-	V ^d	+
Lactose	-	-	-	-	-	+
Arabinose	-	-	+	-	+	-
D-Mannose	+	+	+	+	+	+
D-Mannitol	+	+	+	+	+	V ^d
ONPG	+	V ^d	-	-	-	-
Voges-Proskauer	-	-	+	-	+	-

Adapted from Elliot et al. (1995)

^a Slant alkaline /Butt acidic,

^b Slant alkaline/ Butt slightly acidic,

^c Slant alkaline / Butt alkaline,

^d variable among strains

2.4.1.2 Triple Sugar Iron (TSI)

Triple iron sugar agar is used for the differentiation of microorganisms by fermentation of glucose (dextrose), lactose and sucrose, and hydrogen sulfide. Also present are red phenol, which is a pH indicator, ferric sulphate, and nutrient agar. Red phenol becomes yellow pH below 6.8, and red appears on it. Triple iron sugar agar is correctly tested for the differentiation of gram-negative intestinal bacteria derived from samples of dairy products, foodstuffs and clinical specimens (Murray et al., 1995).

Certain bacteria use thiosulfate anion as terminal electron acceptor, and they report to sulphide. If this happens, hydrogen sulfide (H_2S), which has been reformed, reacts with iron sulphate, which exists in a medium to form iron sulphide, and will be visible as a black precipitate. Examples of sulphide-producing bacteria are the types of *Salmonella*, *Proteus*, *Citrobacter* and *Edwardsiella*, but not *Vibrio*'s. Ground ingot because of iron sulphide formation is almost always observed in the (lower) end of the environment. Gas production other than hydrogen sulphide is indicated as medium or environment cracks or bubbles, pushed from the bottom of the test tube (Difco, 1984). TSI test interpretation associated with the observed colors and gas are shown in Table 2.4 and 2.5.

Table 2.4: TSI Test Interpretation

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

Table 2.5: TSI test interpretation associated with the observed colors and gas

Ferments	Interpretation Lactose and/or Sucrose
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 ₂ S has been produced

Adapted from Difco Manual (1984)

2.4.1.3 Sulfur Reduction – Indole – Motility (SIM) Test

i. Sulfur Reduction

Sulfur-reducing test is very valuable for the differentiation of enteric organisms. For sulfur-reducing bodies in hydrogen sulphide, formaldehyde hydrogen is linked to iron in the medium and forms a black precipitate that is iron sulphide. A positive result of this test is to reduce the sulfur content, which can be followed by any blackening of the soil (Difco, 1984). A positive result for reducing the sulfur content indicates that the test is not a *Vibrio species* isolated.

ii. Indole production

There are some microorganisms that are able to degrade a tryptophan, and this leads to indole formation. This ability is a distinctive and very important feature that can be used to identify and classify bacteria. The addition of indole reagent to the medium for cultivation can detect the presence of indole. Kovacs reagent is an example of the indole reagent, in which the reagent is formed in pink if a microorganism can degrade tryptophan to produce indole (MacFaddin, 1980).

iii. Motility

Drops and other damp products can be used to determine the mobility of the microorganism. This method uses the sloppy agar tube, and the organism is inoculated at the center of the tube. The tube is then incubated and organisms migrating outside the observed central tube. Semisolid agar growth spread monitoring will be very helpful in detecting the mobility of test organisms, and can become much more visible by inserting a tetrazole dye. Dye decreases with organisms growing and changing the color of the environment. Incubation temperature is very important; most mobile bodies are mobile at lower temperatures (eg 15-25 °C) and cannot be mobile at temperatures (eg 37° C) for optimal growth.

Organism uses their flagella to migrate from row shots when they hit the knife in semi-solid agar, using a straight wire or a knife. This leads to turbidity formation during the media. Still more organisms will grow along the lines of strikes, and will not be able to migrate from the beat, and will leave a clear environment (Difco, 1984).

2.4.1.3 The Methyl Red (MR) and Voges-Proskauer (VP) Tests

The media in this system allows distinguishing between organisms with a high ratio of organisms with low ratios. In this context, it is possible to receive a higher number of hydrogen ion organisms with low ratios compared to organisms with a high ratio. A large amount of hydrogen ions that are produced with low ratio organisms creates acidity, and this level of acidity inhibits their growth, while a significantly high level of hydrogen ions produced high-appearance organisms.

The media differentiate organisms through the production of hydrogen ions and, therefore, after cultivation in this medium, the difference between organisms with low aspect ratio and high elongation organisms allowed the final concentration of hydrogen ions. As the final concentration of the hydrogen ion dosage, the pH indicators used. Two pH indicators were tested, including p-nitrophenol and methyl red (Barry and Feeney, 1967).

Several coliforms can be differentiated from each other by means of trials. All of these organisms can quickly ferment glucose, and as a result, the pH value of the average glucose drops rapidly. MR-VP paired tests had previously been used to distinguish between members

of the Enterobacteriaceae family, but have been used to characterize other bacterial groups, including Actinobacteria (Holt, 1994; Schumann et al, 2003).

2.4.1.4 Salt Tolerance Test

Most of the *Vibrio* species have physiological needs of NaCl, and salt is an important factor in the selective enrichment broth, coatings and means of identification of *Vibrio species*, *Vibrio cholerae*, who is not alofila, does not need salt and a minimum tolerant of vibration salt. The medium most often recommended for the selective enrichment of *Vibrio cholerae*, alkaline peptonate water contains only 0.5-1% NaCl (FDA, 1998).

Typical *Vibrio parahaemolyticus* is not able to grow in any saltless environment, but can grow up to 3, 6 and 8% salt broth or agar while the maximum tolerance for *Vibrio vulnificus* is 5-6% NaCl (FDA, 1998; Mortimore and Wallace, 1994). Salt tolerance of various species of *Vibrio* has been summarized in Table 2.6.

Table 2.6: Growth Response of *Vibrio* Species to Various Concentrations of NaCl

<i>Vibrio spp.</i>					
%NaCl	<i>V. cholera</i>	<i>V. mimicus</i>	<i>V. vulnificus</i>	<i>V. parahaemolyticus</i>	<i>V. alginolyticus</i>
0	+	+	-	-	-
3	+	+	+	+	+
6	-	-	+	+	+
8	-	-	-	+	+
10	-	-	-	-	+

Source: Baumann *et al.* (1984)

2.4.2 API 20E

Analytical Profile Index (API) - a technique that is often used to classify bacteria based on a series of experiments. For Yanin by Products Analytab, Inc. API invented in the US in 1970. Currently, the API test system made up of bioMérieux. It presented the existing miniature methods and a standardized version.

Test Kit API-20E allows you to identify Enterobacteriaceae members and related organisms by a method where incubation and reading of results can be performed easily.

Live bacteria produce metabolites and waste as part of the business to be a functioning cell. Dome reagents in the API-20E kit are designed to test for the presence of bacterial metabolic products that are specific to certain types of bacteria.

API-20E system has been assessed by many researchers for the identification of the Enterobacteriaceae family. Kit 20E test API was also used to identify *Vibrio* species many researchers (Martinez-Urtaza et al., 2006; Al-Mouqati et al., 2012; Al 2013). The level of agreement of results with the API-20E with the results of traditional biochemical reactions and the identification was fairly high.

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

There are a variety of PCR-based methods developed to identify *Vibrio* species. Tarr et al., (2007) suggested a multiplex PCR using species-specific primers to amplify *rpoB* gene regions in four species (*Vibrio cholerae*, *Vibrio parahaemolyticus*, *Vibrio vulnificus* and *Vibrio mimicus*) followed by DNA sequence analysis.

PCR multiplex analysis using three pairs of primers for the species-specific detection of collagenase *Vibrio alginolyticus*, *Vibrio cholerae* and *Vibrio parahaemolyticus* was developed by Di Pinto et al. (2008). The multiplex PCR was developed by Neogi et al. (2010), species-specific PCR primers were designed based on the *ToxR* gene of *Vibrio cholerae* and *V. parahaemolyticus*, and *vvhA* gene for *Vibrio vulnificus*.

2.5.1 Virulotyping of *Vibrio parahaemolyticus*

Vibrio parahaemolyticus causes a significant proportion of gastroenteritis through ingesting of seafood in diverse parts of the world.

The *toxR* gene is found in all *Vibrio parahaemolyticus*, both pathogenic and non-pathogenic (Sujeewa et al., 2009) and was used for its identification (Sechi et al., 2000). Thus, the presence of *toxR* gene in *Vibrio parahaemolyticus* isolate does not show its virulence.

Only a few *Vibrio parahaemolyticus* strains can cause gastroenteritis, and these strains are often, but not always, positive for direct thermostable hemolysis (tdh) and related thermostable hemolytic embolism (TRH). Thus, *Vibrio parahaemolyticus* pathogens can be determined by the presence of virulence factors, including the *tdh* gene and / or TRH gene (Roque et al., 2009). Most environmental strains are negative, and the prevalence of *tdh* *Vibrio parahaemolyticus* positive in coastal waters varies from 0.1 to 4% (FAO / WHO 2011).

2.5.2 Molecular Typing of *Vibrio parahaemolyticus* Strains

Molecular typing methods can be used to obtain useful data on genetic diversity of strains. Study of geographic dispersion strains, as well as their distribution on hosts can be made on the basis of their genetic relationship. With digital fingerprint DNA can identify individuals based on DNA markers. Each of the individuals will have a unique structure that is different from the others, with the exception of identical twins, where the same patterns (Zulkifli, et al., 2009).

There are many PCR-based methods, including PCR, random amplified polymorph DNA (RAPD-PCR), and new intergene consensus sequence (ERIC-PCR) enterobacteria (Maluping et al., 2008).

Research on genetic variability and the molecular epidemiology of *Vibrio parahaemolyticus* are often conducted to trace the origin and spread of the pathogen (Chakraborty & Surendran, 2009). Primer of DNA complementary to repeated DNA sequences used in RERE-PCR DNA fingerprinting. These repeated sequences are highly conserved and are naturally present in

multiple copies in the genes of more gram-negative and some gram-positive bacteria (Lupski and Weinstock, 1992).

2.5.3 Thermostable Direct Haemolysin (TDH)

TDH is a haemolysin formed by KP-positive strains. The purification and characterization of TDH from *Vibrio parahaemolyticus* cultures was performed which the haemolysin was seen as a thermostable form because it refused to be deactivated after heating at 100°C for 10 min. The purified TDH was almost completely destroyed by proteinases such as pepsin, trypsin, alpha chymotrypsin and nagarse was alleged to be a protein. Gel filtration analysis showed that TDH has a molecular mass of nearly 118 kilodalton (kDa). TDH cause a comparatively low isoelectric point of pH 4-5 because it contains a large number of acidic amino acids. This haemolysin showed high haemolytic action on erythrocytes of rats, dogs, mice, and monkeys, enough haemolytic activity on erythrocytes of humans, rabbits, guinea pigs and chickens, slight haemolytic activity on erythrocytes of sheep and no haemolytic action on erythrocytes of horses (Zen-Yoji et al., 1971). Crystal structure determination and functional representation of TDH showed that attachment of the TDH molecule, via the phospholipid bilayer of the targeted cell membrane, allows water molecules to pass through the cell freely through the centre pore of the TDH structure

Nashibushi & Kaper (1990) identified two TDH chromosomal gene copies, *tdh1* and *tdh2*, from clinical KP-positive *Vibrio parahaemolyticus* strains. These two genes were categorized and allotted as TDHS (*tdh1*) and *tdhA* (*tdh2*)

Purification and classification of these two genes indicates that *tdhA* is the structural gene for TDH and is mainly responsible for the haemolytic phenotype however *tdhS* contributes reasonably little to extracellular TDH production (Nishibuchi & Kaper, 1990). Moreover, Nishibushi & Kaper (1990) also found another two *tdh* gene copies, *tdh3*, a chromosomal-borne gene, and *tdh4*, a plasmid-borne gene, from clinical KP-negative *Vibrio parahaemolyticus*.

The genes *tdh3* and *tdh4* are likely to be structural genes encoding new haemolysins, TDH/I and TDH/II, individually, and these haemolysins were narrowly related, but not identical to, TDH (Honda et al., 1991; Nagayama et al., 1995).

Though still certain to express *tdh3* and *tdh4* at the RNA transcriptional level, TDH/I and TDH/II were able to induce fluid accumulation in ligated rabbit intestine, indicating that TDH/I and TDH/II partake in enterotoxicity in KP-negative *Vibrio parahaemolyticus* (Honda et al., 1991; Nagayama et al., 1995). Because *tdhA* is the structural gene for TDH, most authors have used *tdhA* primers for *tdh* gene detection in *Vibrio parahaemolyticus* isolates. *Tdh* expression is controlled by the virulence gene regulator protein ToxRS.

2.5.4 Thermostable Direct Haemolysin-Related Haemolysin (TRH)

Vibrio parahaemolyticus, a KP-negative strain first isolated from travelers in the Republic of Maldives are capable of causing gastroenteritis (Honda et al., 1988). A new hemolysin characterization similar to that of TDH but different in some physical properties was performed by Honda et al. (1988). This new toxin is called thermoregulated direct hemolysin-associated hemolysin (TRH). The demonstrated TRH TDH is a protein with an isoelectric point of pH 4.6 and immunological similarity.

However, unlike TDH, TRH was unstable to heat treatment at 60 ° C for 10 minutes and showed different lytic activity against erythrocytes from different animals compared to activity-induced TDH. The disparity in sequence of *trh* in different *Vibrio parahaemolyticus* strains was studied by Kishishita et al. (1992) which identified that haemolysin gene (*trh2*) shared 84% sequence likeness to *trh* (subsequently named *trh1*). Amplification primers used for *trh1* have usually been used for *trh* detection in *Vibrio parahaemolyticus* isolates (Honda et al., 1991; Kishishita et al., 1992).

The presence of urease activity was linked with the TRH gene. Four hundred and ninety-eight clinical isolates of patients with diarrhea in Thailand were tested for the presence of TRH and urease activity and these authors discovered that all of the urease gene positive strains are possessed with TRH gene. In contrast, negative urease strains do not comprise the *trh* gene; this urease-positive phenotype, indicating that the preselected *Vibrio parahaemolyticus* may be an indicator of infectivity. However, *Vibrio parahaemolyticus* cluster of urease genes, THO and TRH are not involved in regulation and expression (Nakaguch et al., 2003) and clinically KP-positive and KP-negative *Vibrio parahaemolyticus* possession of the infectivity genes as seen in Figure 2.4.

2.5.5 Lecithin-Dependent Haemolysin (LDH)

All of the *Vibrio parahaemolyticus* has lecithin dependent hemolysin gene (LDH) and is actually used as a species-specific marker for *Vibrio parahaemolyticus*. LDH nucleotide sequence has no homology to *tdh*, and is thermolabile with a nucleotide sequence of 1.5 kilobase (kb) in the length (Taniguchi et al., 1986). Protoprotein and matured protein comprises of 418 and 398 amino acids respectively and a molecular weight of 47.5 kDa and 45.3 KDa, respectively. GC and *ldh* GC content is 47.6%, almost the same as the *Vibrio parahaemolyticus* genome.

2.5.6 Heat-Stable Haemolysin (B-VPH)

Taniguchi et al. (1990) discovered a further thermostable haemolysin (E-VPH) (Figure 2.4) gene from a KP-negative *Vibrio parahaemolyticus* strain, the nucleotide and amino acid sequences of which had no homology as those of *tdh* and *ldh* of *Vibrio parahaemolyticus*. The encoding E-VPH gene in *Vibrio parahaemolyticus* and associated species was found to exist in all examined *Vibrio parahaemolyticus* strains, and also in one strain of *Vibrio damsela* (Taniguchi et al., 1990).

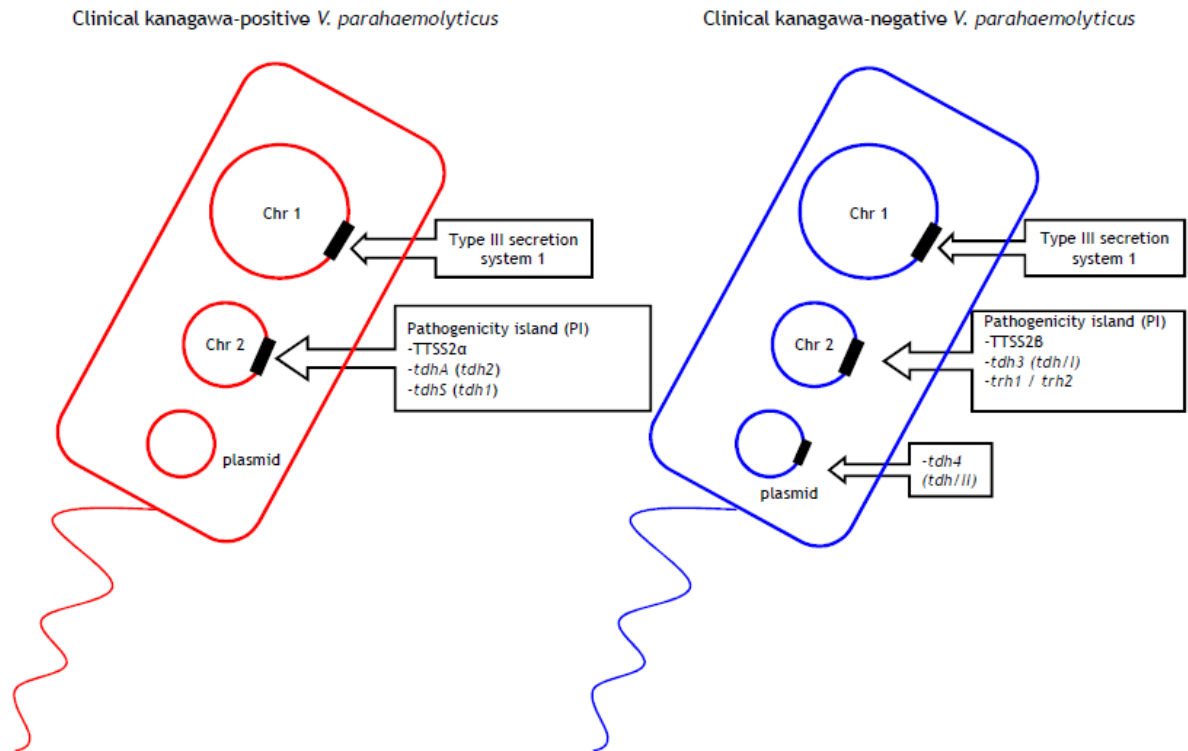


Figure 2 4: Possession of virulence genes of clinical KP-positive and KP-negative *Vibrio parahaemolyticus*.

Clinical KP-positive strains contain genes associated with type III secretion system 1 in chromosome 1, genes associated with type III secretion system 2 and two copies of *tdh* genes, *tdhA* (*tdh2*) and *tdhS* (*tdh1*), in chromosome 2. Clinical KP-negative strains contain genes associated with type III secretion system 1 in chromosome 1, genes associated with type III secretion system 2, *tdh3* (*tdh/I*), *trh1* or *trh2* in chromosome 2, and *tdh4* (*tdh/II*) in the plasmid.

CHAPTER 3

RELATED RESEARCH

Hassan et al., (2012) carried out a study to ascertain the dominance of *Vibrio parahaemolyticus* in seafood samples in The Netherlands. 200 seafood samples in total, including shrimp, fish, oyster and mussel, purchased from the market in The Netherlands were studied for the existence of *Vibrio parahaemolyticus* using both a traditional and a direct PCR-based technique. Two separate selective media, Thiosulfate Citrate Salt Agar (TCBS) and CHROMagar *Vibrio* (CV) were assessed for their effectiveness to isolate *Vibrio parahaemolyticus* from seafood samples. The results revealed that there was not dissimilarity between two media to isolate *Vibrio parahaemolyticus* out of all the seafood samples ($P > 0.05$). By means of the traditional method, *Vibrio parahaemolyticus* was isolated from 16 (8%) and 27 (13.5%) samples respectively. The samples mussels and oysters all showed positive signs. Out of the 43 isolates of *Vibrio parahaemolyticus* (on TCBS and CV) gotten, none of the isolates was positive enough for the genes *tdh* or *trh*. The PCR-based technique was carried out at 0 (t=0), 6 (t=6), and 18 (t=18) hours after the augmentation stage and allowed the discovery of *V. parahaemolyticus* in 22 (11%) and 38 (19%) samples, with the DNA extracts prepared from the first enrichment (t=6 h) and the second enrichment (t=18 h) respectively. When the DNA extracts were prepared from the sample homogenate before the augmentation stage, (t=0 h), none of the samples were detected to be *Vibrio parahaemolyticus*-positive.

The occurrence of the hemolysin genes, *tdh* and *trh*, in *Vibrio parahaemolyticus* strains isolated from ecological samples and collected in two French coastal areas, clinical samples, and seafood products imported into France was investigated.

The hemolysin genes were detected by Polymerase chain reaction (PCR) with two sets of primers. Many of the clinical isolates (91%) and 1.5% of the isolates gotten from seafood contained the hemolysin genes. Depending on the geographical location, three and fifteen percent, respectively, of the two groups of environmental strains possessed the hemolysin genes. The *tdh* and *trh* genes performs an important roles in virulence with the results showing that pathogenic *Vibrio parahaemolyticus* isolates are existing in French shore areas

and in seafood brought into France (Annick et al., 2004).

PCR processes directly used to augment broth cultures were evaluated with a traditional method based on the ISO reference for detection of *Vibrio parahaemolyticus* in 57 regular bivalve mollusc samples. Comparisons were carried out on diverse primer pairs precisely targeting the *V. parahaemolyticus*-specific *toxR* gene (Vp-toxR) and pR72H fragment, including *tdh* and *trh* hemolysin genes. The PCR technique using these different types of primer pairs and the culture technique were also studied for their limits of detection (LOD). The LODs ranged from 7–24 pg of purified DNA for each reaction tube (RT) for primer pair Vp-toxR, but for primer pair pR72H, highly differs due to the *Vibrio parahaemolyticus* strains used (0.7 pg–10.6 ng/RT). The Vp-toxR and pR72H primers permitted the detection of *Vibrio parahaemolyticus* in 25 and 8 out of the 57 samples, respectively, while only 3 *Vibrio parahaemolyticus* positive samples were gotten by the culture technique. The active presence of *Vibrio parahaemolyticus* in the Vp-toxR-positive samples was determined by sequencing the PCR products. The *trh* and Vp-toxR genes were concurrently detected in 14% of the samples, which were therefore considered as presumptively polluted with pathogenic *Vibrio parahaemolyticus*. The results highlight the reason why an effective survey of both the total and pathogenic *Vibrio parahaemolyticus* found in seafood in France is needed. The PCR procedure targeting Vp-toxR followed by *tdh* and *trh* genes is an effective and dependable technique used for the detection of total and presumptively pathogenic *Vibrio parahaemolyticus* in bivalve molluscs (Rosec et al., 2009).

Vibrio parahaemolyticus, a natural microflora of marine and coastal water bodies is linked with death of larval shrimp in penaeid shrimp in ponds. Bacteriophages take place almost in all places where their hosts are present. In this study, total circulation of *Vibrio parahaemolyticus* and its phages were studied in shrimp ponds, seawater, estuary, animal surface, and tissues. The total number of *Vibrio* in sediments of two ponds was found to be 2.6×10^3 and 5.6×10^3 cfu/g. Prevalence of *Vibrio parahaemolyticus* in the ponds was close, whereas it was obviously higher in the animal surface and tissue samples. Eight strains of *Vibrio parahaemolyticus* (V1, V3–V6, V9, V11, and V12) biochemically identified were taken for additional infection analysis with bacteriophage. Five bacteriophages capable of infecting *Vibrio parahaemolyticus* MTCC-451 strain were totally isolated from all the samples. One of the isolated bacteriophage Vp1 from estuary was able to lyse all the isolated

Vibrio parahaemolyticus strains used. Consequently, the morphology of Vp1 was estimated in TEM. Vp1 phage head measuring roughly about 50–60 nm diameter with icosahedral outline and a contractile tails of diameter 7 nm and length 100 nm and was also identified as Myoviridae (Alagappan et al., 2010).

The occurrence of *Vibrio parahaemolyticus* in Malaysian export shrimps products processing industry was studied through the processes from the catch to that of the cooked, peeled and frozen product. The organism is usually found in freshly caught and landed shrimp, and could be discovered by enrichment culture at all stages of processing. The quantities of *Vibrio parahaemolyticus* in shrimp differs from 0 to 4×10^4 , and 19 of the 50 serotypes in the current antigenic scheme were found, with O1-K38 and O1-K32 occurring frequently. All the isolates were Kanagawa-negative; but one strain was a sucrose-positive variant. The study showed that the stipulations of 10^2 g^{-1} for *Vibrio parahaemolyticus* in raw tropical shellfish are too harsh but that the Malaysian shrimp industry should be able to meet this conditions for cooked shrimp (Cann & Lesley, 1981).

Mus et al., (2014) however said that pathogenic *Vibrio* spp., *S. aureus* and *Salmonella* are present in 100 seafood samples purchased from retail outlets in Bursa city (Turkey). While some of the samples studied including fish, mussel and shrimp, 67% were found to be contaminated with *Vibrio*.

Alleged *Vibrio* spp. was identified by discovered biochemical tests, and was further confirmed by API 20E system. Discovered *Vibrio* spp. were *Vibrio parahaemolyticus* (28%), *Vibrio vulnificus* (1%) and *Vibrio cholerae* (1%), with the most predominant being *Vibrio alginolyticus* (37%). Six (6%) of the samples examined were positive for *S. aureus*. But no contaminations were noticed in the samples with *Salmonella*. The results indicated that seafood from retail outlets can be a possible means for infections with *Vibrio* spp. and *S. aureus*.

Samples of isolated and characterized pathogenic *Vibrio parahaemolyticus* from crustacean shellfishes (crab and shrimp) commonly sold in shore areas of eastern India were processed by bacteriological isolation which followed by biochemical characterization in Kaper's medium. Supposedly recognized isolates were confirmed by species-specific Vp-toxR polymerase chain reaction (PCR) assay. Virulence and pandemic characteristics of the

confirmed *Vibrio parahaemolyticus* isolates were examined by particular PCR assays. On screening of 167 samples containing crabs (n=82) and shrimps (n=85) by the standard bacteriological traditional method, *Vibrio parahaemolyticus* was presumptively discovered in 86.6% (71/82) and 82.3% (70/85) of respective samples. Among these, 46 (56%) and 66 (77.6%) isolates from crab and shrimp, respectively, were confirmed as *Vibrio parahaemolyticus* by biochemical characterization (Kaper's reaction) followed by specific Vp-toxR PCR assay. Approximately 10 isolate each from crab and shrimp was found to carry the virulence gene (*tdh*). This indicates that 12.2% of crab and 11.7% of shrimp in the study area are sheltering the pathogenic *Vibrio parahaemolyticus*. Such *tdh*+ isolates (n=20) were carried out for screening of pandemic genotype by pandemic group specific (PGS) - PCR (PGS-PCR) and GS-PCR (*toxRS* gene) where 11 (6.5%) isolates showed that the pandemic determining amplicon (235 bp) in PGS-PCR and belonged to crab (7.3%) and shrimp (6%) samples; though, 2 (2.4%) isolates were positive in GS-PCR and belonged to crab samples only. These two GS-PCR+ isolates from crab were also positive in PGS-PCR. The results of the study decisively showed that a significant percentage of crab and shrimp in these areas were sheltering pathogenic and pandemic *Vibrio parahaemolyticus* which poses a danger to public health in the consumption of poorly processed such shellfishes. Cross contamination of other marine and fresh water market fishes by such shellfishes in these areas may provide scope for spreading this pathogen in community food chain.

105 samples (each 35 of fresh water fish, sea fish and mutton) were used to estimate the levels of total and pathogenic *Vibrio parahaemolyticus* using traditional and polymerase chain reaction (PCR) methods by amplification of *toxR* gene for total *Vibrio parahaemolyticus*, *tdh* and *trh* genes for pathogenic *Vibrio parahaemolyticus*. 30 (28.6%), 39 (37.1%) samples out of 105 samples, presented positive results for total *Vibrio parahaemolyticus* by traditional and PCR methods respectively. Of the 39 samples positive for PCR 6 (5.7%), 28 (26.6%) and 3 (8.6%) were positive for *tdh*, *trh* and both *tdh* and *trh* respectively.

Vibrio parahaemolyticus was isolated from water and oysters taken from seven different sampling stations in the Great Bay and Little Bay estuarine areas of New Hampshire. The morphological and biochemical characteristics of 50 isolates fitted in general to those described for this organism in the research. All isolates produced hemolysis on blood-agar.

However, there have been no reports of *Vibrio parahaemolyticus* food poisoning outbreaks today is due to the consumption of fish or shellfish harvested from this estuarine region.

Vibrio parahaemolyticus is the major seafood pathogen connected with human gastroenteritis. Samples were taken from Vellar estuary, shrimp ponds and shrimp for characterization of *Vibrio parahaemolyticus*. A total of 26 blue green centre (BG) *Vibrio* strains were isolated and characterized through biochemical tests, *toxR* gene and 16S rRNA gene sequencing. Six strains were determined as pathogenic *Vibrio parahaemolyticus* based on pathogenic characteristics. This report suggests that preventive means must be taken before consumption of fish and shrimp (Alagappan et al., 2013).

According to Abd-Elqahny & Sallam (2010) 120 shellfish samples (40 each of shrimp, crab and cockle) were collected from different fish shops in Mansoura city, Egypt, and tested for the presence of potentially pathogenic strains of *Vibrio parahaemolyticus*. The conventional technique as shown by biochemical means showed that 40 (33.3%) of samples were positive for *Vibrio parahaemolyticus*. Molecular means represented by PCR, however, proved that only 20 (16.7%) shellfish samples were positive for *Vibrio parahaemolyticus*. These 20 positive samples were distributed as 9, 8 and 3 of shrimp, crab and cockle, respectively. Biochemical analyses of the recovered 143 presumptive *Vibrio parahaemolyticus* colonies showed that 89 isolates were discovered as *Vibrio parahaemolyticus*. PCR analysis, for the presence of the species-specific *toxR* gene, showed that out of these 89 isolates, only 27 (30.34%) were verified as *Vibrio parahaemolyticus*. These molecularly proven 27 strains were distributed as 14, 10 and 3 isolates from the tested shrimp, crab and cockle samples, respectively. Out of the 27 molecularly identified isolates, three (11%) strains were proven to be potentially pathogenic due to detection of *tdh* and/or *trh* virulent genes. One of these 3 strains derived from shrimp was positive for both *tdh* and *trh*. The second strain (derived from cockle) was positive for *tdh* only, while the third one (derived from shrimp) was positive for *trh* only. The two strains that were positive for *tdh*, were also verified to be positive for Kanagawa reaction and concluded that the tested shellfish may contain potential human health risk associated with the presence of pathogenic *Vibrio parahaemolyticus* and that the dependable molecular detection methods should be included in the routine seafood inspection in addition to the conventional bacteriological methods.

The high demand by consumers for shell fish has led to the need for larger scale, reliable shellfish supply through aquaculture or shellfish farming. However, bacterial diseases which can spread quickly among shellfish carry a huge risk the industry. Shellfish farmers therefore frequently choose to use antibiotics extensively, both prophylactically and therapeutically, in order to protect their stocks. The extensive utilization of antibiotics in aquaculture has been claimed to signify a major causative factor in the rising cases of antimicrobial resistant pathogenic bacteria in shellfish.

This study aimed to investigate the occurrence of pathogenic *Vibrio parahaemolyticus* and determine the antibiotic resistance profile as well as to perform plasmid curing in order to determine the antibiotic resistance intervention. Based on colony morphology, all 450 samples tested were positive for *Vibrio* sp; but, *tox-R* analysis revealed that only 44.4% (200/450) of these were *Vibrio parahaemolyticus* out of these 200 samples, 6.5%(13/200) were *trh*-positive while none were *tdh*-positive. Antibiotic resistance was determined for all *Vibrio parahaemolyticus* identified against 14 frequently used antibiotics and the multiple antibiotic resistance index (MAR) was calculated. The isolates showed high resistance to numerous antibiotics tested – including second and third-line antibiotics –with 88% resistant to ampicillin, 81% toamikacin, 70.5% to kanamycin, 73% to cefotaxime, and 51.5% to ceftazidime. The MAR index ranged from 0.00 to 0.79 while most of the samples have an index of 0.36 (resistant to five antibiotics). Among the 13 *trh*-positive strains, almost 70% (9/13) demonstrated resistance to 4 or more antibiotics. Plasmid profiling for all *Vibrio parahaemolyticus* isolates showed that 86.5% (173/200) have plasmids- ranging from 1 to 7 plasmids with DNA band sizes ranging from 1.2kb to greater than 10kb. 6/13 of the pathogenic *Vibrio parahaemolyticus* strains contained plasmid. After plasmid curing, the plasmid containing pathogenic strains isolated having chromosomally mediated ampicillin resistance while the rest of the resistance phenotypes are plasmid mediated. The overall results showed that while the incidence of pathogenic *Vibrio parahaemolyticus* in shellfish in Selangor still seems a fairly reassuring level, antibiotic resistance is a real worry and deserves ongoing surveillance (Letchumanan et al., 2015).

CHAPTER 4

MATERIALS AND METHOD

4.1 Study Area

In the Mediterranean, Cyprus is known as the third largest island when compared to Korsica and Crete but smaller than Sicily. Cyprus is located North-eastern part of Mediterranean and covers 3,298 square kilometer area while the island is 9,251 square kilometer (Guryay *et al.*, 2005). The Census conducted in 2011 shows population of 286,257 with 60.3% the rural settlers mainly engaged in agriculture.

The climatic condition of North Cyprus is Mediterranean having a mild and rainy winter from December to May and a hot and dry from June to September. The Landmark is a small island and it has a little natural resources and small labour force and having some restrictions in their economy (Guncavdi and Kucukcifici, 2008).

The major source of economy of North Cyprus is tourism, education, industry and the agriculture sectors. The major reason developing countries put their focus on agricultural sector is because it is considerable part of national income globally (Ergene, 2009). It can be seen that agriculture supports all parts of other sectors because of its importance.

In the TRNC, Shrimp are consumed in significant quantities and can be a source of *Vibriosis*. Accordingly, ensuring the safety of seafood by investigating the pathogenic *Vibrio parahaemolyticus* is crucial (Ward & Bej, 2006).

4.2 Sampling

Shrimp samples were taken from major seafood outlets of Nicosia (Lefkoşa), Famagusta (Mağusa), Kyrenia (Girne) and Morphou (Güzelyurt) (Figure 4.1). These representative samples were drawn in accordance with standardized procedures for fresh fish (Shrimp) sampling (ICMFS, 1986; CFIA, 2013).

Even though shrimp may contain a lot of pathogenic microorganisms, this study is aimed to determine the presence of medically important *Vibrio parahaemolyticus* in various shrimp

consumed in the TRNC.



Figure 4.1: Map of Cyprus showing the study area in TRNC (KKTC)

The samples were collected in accordance with the American Public Health Association (APHA) guidelines as indicated in publications of (Ramón et al., 2013; Samir and Khalid 2013; Qianqian et al., 2016).

Major seafood shops were selected during the sample collection.

10 randomly selected shrimps were taken from daily sold shrimp market's which was approximately 1kg. The seafood shops selected are listed in Table 4.9 below and 10 samples were collected for each (Table 4.1).

Table 4.1: TRNC shrimp sampling location

Locations		Number of samples
Girne (Kyrenia)	Lapta	10
	City Centre	10
Lefkosa (Nicosia)	Lefkosa I	10
	Lefkosa II	10
	Lefkosa III	10
Guzelyurt (Morphou)	Guzelyurt I	10
	Guzelyurt II	10
Lefke	Lefke	10
Magusa (Famagusta)	Magusa I	10
Total		90

Total of 90 individual shrimps collected for the analysis.

Shrimp samples were packed into a clean polyethylene bag then labeled and transferred into icebox and then taken directly to the laboratory of Microbiology Department, Faculty of Medicine, Near East University for bacteriological analyses. Direct contact of the Shrimp samples with storage medium was avoided to ensure maximal survival and recovery of *Vibrio parahaemolyticus*.

All samples were processed immediately upon their arrivals to the laboratory. Additionally, aseptic procedures were strictly adhered to during collection, transportation, and analysis of the shrimp samples.

4.3 Analytical sample preparation

The samples collected were washed thoroughly under running water and shucked for sampling according to Cook et al. (2002). Samples were weighed taking about 10 individual shrimps and washed, comminute with a hand blender. It was then homogenized to get a puree representative sample of the whole sample location, 25g puree was taken and then 225ml of Alkaline Peptone water (APW) and homogenate it. The homogenates were transferred into sterile polythene stomacher bags and stored in an incubator (Thermo Scientific) at 37 °C for 24 hr.

After incubation period (24 hr.), one ml of each homogenate were taken aseptically using a sterile wooden cotton applicator stick and streaked onto sterile surface dried TCBS agar plates. The plates were then incubated at 37 °C for 24 hr. Figure 4.2 summarizes the distinctive steps involved in cultural detection of *Vibrio parahaemolyticus* from seafood samples.



(a) Cleaning the Shrimp



(b) Shrimp Samples



(c) Comminuted Sample



(d) Homogenized Shrimp

Figure 4.2: Sample Preparation Processes

4.4 Media, Test Kits and Equipment used

Media and test kits: Alkaline Peptone Water (APW), TCBS Agar plates (20), TCBS Agar (100), *Vibrio Parahaemolyticus* ATTC (17802), Sodium Chloride (NaCl), Phoenix NMIC/ID, Phoenix ID Broth, Phoenix AST Broth and Phoenix AST Indicator.

Equipment: BD Phoenix 100×100, NMIC (1 KUT), Incubator (36±20C), autoclave, pH meter, sterile forceps, pipettes, bottles, jars and glass wares.

4.4.1 Preparation of Enrichment Media

Alkaline peptone water was prepared by weighing 20 g of APW powder (Liofichem srl, Italy) on a digital balance (Shimadzu, Japan) and transferred into 1000 ml distilled water volumetric container and dissolved in distilled water by shaking (according to manufacturer's instructions). The solutions were autoclaved at 121°C for 15 minutes.

4.4.2 TCBS Agar

Thiosulphate citrate bile-salts sucrose (TCBS) agar is a selective medium generally used for plating, propagating and isolating *Vibrios* from seafood (Table 4.2). In the study, ready-to use plates were used (Liofichem srl, Italy).

Table 4.2: TCBS agar selective isolation media composition (FDA/BAM, 2003)

Components	gram/liter
Yeast extract	5
Peptone	10
Sucrose	20
Sodium thiosulfate 5H ₂ O	10
Sodium citrate 2H ₂ O	10
Sodium cholate	3
Oxgall	5
NaCl	10
Ferric citrate	1
Bromthymol blue	0.04
Thymol blue	0.04
Agar	15
Distilled water	1 liter

Plates were stored in the dark at 2.8°C avoiding freezing and overheating and minimizing exposure to light prior to use. The medium is allowed to warm to room temperature before inoculation.

4.5 Bacteriological Analysis

The isolation and identification of *Vibrio parahaemolyticus* by conventional culture technique has been done in accordance with Food and Drug Administration/Bacteriological Analyses Manual.

4.5.1 Reculture of Control *Vibrio parahaemolyticus* ATCC 17802

Vibrio parahaemolyticus ATCC 17802 was used as control. It was lyophilized pellet. One pellet was dissolved in 500 µl brain heart infusion broth and then inoculated onto TCBS agar at 37 °C for 24 hours. These control colonies were used for the detection of presumptive *Vibrio parahaemolyticus* colonies in the shrimp samples.

4.5.2 Isolation and Identification of *Vibrio parahaemolyticus*

Following plate incubation, TCBS plates were checked for suspect colonies which are sucrose non-fermenting with green or bluish green color and dark blue or green center about 3-5 mm in length indicating the presence of *Vibrio parahaemolyticus* and carefully selected. The suspect colonies were purified and further characterized by carrying out catalase and Gram staining tests. Suspect isolates which are positive for catalase and Gram staining tests were selected for biochemical identification and confirmation.

4.5.2.1 Gram Staining

Crystal violet stain was placed on the smear in the glass slide for 30 - 60 s and then placed under tap water. Gram iodine mordant was added to the slide for another 30 - 60 s where it was decolorized by using ethanol and washed again under tap water. The smear was then

covered with safranin for 60 s and tap water washing was repeated and finally the slide was air dried and placed in the microscope under oil immersion

4.5.2.2 Catalase test

Hydrogen peroxide H_2O_2 was added to the culture where oxygen gas (O_2^{\uparrow}) bubbles evolved indicating positive catalase test and these were done by the use of a clean glass slide and a drop of H_2O_2 was placed on the center of the glass slide. A loop full of *Vibrio spp* culture was placed on the H_2O_2 drop where the appearance of bubbles was noticed.

4.5.2.3 Oxidase test

A clean petri dish was used and a piece of filter paper was placed on it and a freshly prepared oxidase was added in 2 to 3 drops where the colony was smeared on the filtered paper as indole-positive bacteria.

4.6 Confirmation

After counting, suspect isolates with varying morphological features were screened by Phoenix automated and rapid identification biochemical test kits (Becton Dickinson, USA).

4.6.1 Preparation of Colony Suspensions in Phoenix Inoculum Broth

Phoenix Inoculum Broth was used to make the initial McFarland suspension of microorganisms when utilizing Phoenix MIC panels (PMIC, NMIC, SMIC).

The Inoculum Density window enables us to see the default McFarland concentration for inoculum. The default density is 0.5 for Gram Negative and Gram Positive panel types. In our study, 0.5 was also selected (acceptable density is 0.50-0.60) for Gram Positive and Gram Negative panels (Strep panels use only 0.5). The Phoenix Inoculum Broth was used to reach the correct inoculum density.

For NMIC panels used in our study, one drop of the Phoenix AST Indicator was added to

each AST-S broth tube. And then 25 µl of the Phonenix Inoculum broth suspension was transferred to the Phonenix AST tubes, capped and gently inverted. Then the prepared panels were placed into BD Phoneix Instrument for 24 hours for bacterial identification.

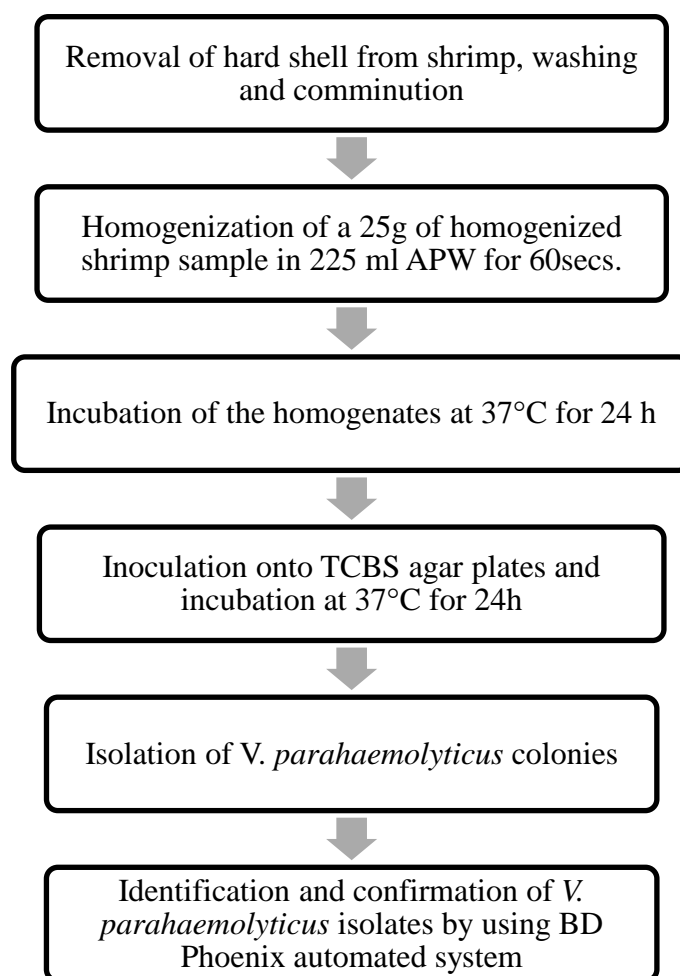


Figure 4.4: Steps for cultural detection of *Vibrio parahaemolyticus* in shrimp samples

CHAPTER 5

RESULTS AND DISCUSSION

Results obtained in this study were presented in this chapter. Findings related to our study were explored and compared, taking into consideration the various studies that investigate *Vibrio parahaemolyticus* from different shrimp and fish varieties.

5.1 *Vibrio parahaemolyticus* ATCC 17802 Growth on TCBS agar

Vibrio parahaemolyticus ATCC17802 was used to control TCBS agar activity and for the detection of presumptive *Vibrio parahaemolyticus* colonies in the Shrimp samples. TCBS agar ready-to-use plates were found to be active for the cultivation and isolation of *Vibrio parahaemolyticus* colonies (Figure 5.1).



Figure 5.1: *Vibrio parahaemolyticus* ATCC17802 growth on TCBS agar plate

5.2 Results

Apathogenic and pathogenic microorganisms are widely distributed in aquatic environment and of course in shrimp harvested from such environment. Specifically, the present study

investigated the occurrence of *Vibrio parahaemolyticus* in shrimp marketed in six major cities of TRNC and the result indicated that *Vibrio parahaemolyticus* could not be found in any of the examined shrimp samples from the six (4) locations of TRNC. Results for shrimp, locations and pathogens are presented in Table 5.1 and suspected bacterial colonies on TCBS agar in Figure 5.2.

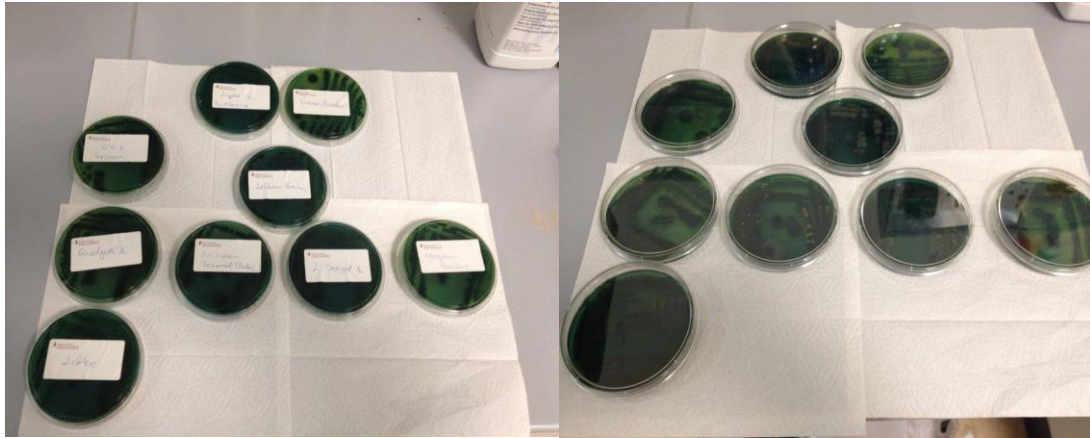


Figure 5.2: The suspected TCBS agar plates

Table 5.1: Result showing the distribution of *Vibrio parahaemolyticus* in the various retailed shrimp samples collected in TRNC

Locations		Number of samples analyzed	Number of positive <i>Vibrio parahaemolyticus</i>
Girne (Kyrenia)	Lapta	10	0
	City Centre	10	0
Lefkosa (Nicosia)	Lefkosa I	10	0
	Lefkosa II	10	0
	Lefkosa III	10	0
Guzelyurt (Morphou)	Guzelyurt I	10	0
	Guzelyurt II	10	0
Lefke	Lefke	10	0
Magusa (Famagusta)	Magusa I	10	0
Total		90	0

5.3 Discussion

Surveying, monitoring and detection of pathogens in foods are the most important approaches for reducing, controlling or preventing foodborne bacterial infections (Zhao et al., 2014). Bacterial infections mostly due to consumption of fish and shellfish have been attributed to pathogenic *Vibrio* (Ronholm et al., 2016). *Vibrio cholerae*, *Vibrio parahaemolyticus* and *Vibrio vulnificus* are the most debilitating of all *Vibrios* in humans.

Vibrio parahaemolyticus, a potential aquatic and seafood pathogen, happens to be the principal cause of seafood borne diseases throughout the planet Earth. The debilitating effects of *Vibrios parahaemolyticus* is due to the presence of virulence genes (*tdh* and *trh*),

type III secretion systems (T3SS1 and T3SS2), clonal serotypes (O3:K6 and its serovariants) and extracellular proteases (Okuda et al 1997; Makino et al., 2003; Drake et al., 2007; Caburlotto et al., 2010).

To our knowledge, occurrence of *Vibrio parahaemolyticus* in shrimp consumed in TRNC has never been investigated, although occurrence of *Vibrio parahaemolyticus* were investigated in some fish varieties consumed in Northern Cyprus. The present study examined the occurrence of the total *Vibrio parahaemolyticus* in fresh shrimp marketed in six major cities of the TRNC.

Fortunately, *Vibrio parahaemolyticus* was not found in any shrimp sampled in our study, which is similar to the study of Kademi, (2016) on the occurrence of *vibrio parahaemolyticus* in various seafood consumed in the Turkish Republic of Northern Cyprus, although some studies from the United States reported the elevations in the number of *Vibrio* infections associated with seafood (Iwamoto et al., 2010), meanwhile in Europe, *Vibrio parahaemolyticus* has been considered as emerging foodborne pathogen responsible for most of the recent sporadic and epidemic seafood borne infections, a study showing that pathogenic *Vibrios parahaemolyticus* isolates are existing in French shore areas and in seafood brought into France (Annick et al., 2004) indicates that the prevalence of *Vibrio parahaemolyticus* exist in Mediterranean region like France though it differs from this study.

The results of the study decisively showed that a significant percentage of crab and shrimp in these areas were sheltering pathogenic and pandemic *V. parahaemolyticus* which poses a danger to public health in the consumption of poorly processed such shellfishes. Cross contamination of other marine and fresh water market fishes by such shellfishes in these areas may provide scope for spreading this pathogen in community food chain (Parthasarathy et al., 2016).

However, our results are in agreement with previous study conducted in some European countries where Fish (shrimp) samples sourced from France and Great Britain contain no *V. parahaemolyticus*.

A study in Egypt by Samir and Khalid, (2013) exemplified by biochemical means indicated that 40 (33.3%) of samples were positive for *Vibrio parahaemolyticus* and 20 (16.7%) shellfish samples were positive for *Vibrio parahaemolyticus* were these 20 positive samples

were distributed as 9, 8 and 3 of shrimp, crab and cockle, respectively.

Vibrio parahaemolyticus was found in 4.0% of the winter samples, 13.3% of the spring samples, 18.6% of the summer samples and 8% of the autumn samples from the South-western part of Iran and a significant proportion of shrimps marketed and consumed in Morocco caught in the coastal region of the city of Agadir contains *Vibrio parahaemolyticus* (Kriem et al., 2015). In China *Vibrio parahaemolyticus* was detected in 103 (37.7%) of 273 samples of shrimps and this was the first report on *Vibrio parahaemolyticus* prevalence in retail shrimp in China (Xiaoke et al., 2014). All the previous studies shows that there is need to assess the safety of seafood for the general public due to the possibilities of the presence of *Vibrio parahaemolyticus* in other to proffer a suitable safety method to be used by marketers and fishermen dealing with different seafood.

CHAPTER 6

CONCLUSION AND RECOMMENDATIONS

Based on the results of our study, we could not detect *Vibrio parahaemolyticus* in shrimp samples taken from different regions of the TRNC which is one of the most important seafood borne pathogens. Concerning *Vibrio parahaemolyticus*, it is important to pay attention to temperature control during harvesting and post-harvest handling. It is also required to prevent cross-contamination during handling and preparation of shrimps and adequate cooking before consumption.

Though from this study *Vibrio parahaemolyticus* was not found in shrimps consumed in Turkish Republic of North Cyprus, it is highly recommended to investigate the occurrence of *Photobacterium damsela* (formerly *Vibrio damsela*) and *Providencia rettgeri* in various seafood including shrimps consumed in the TRNC.

REFERENCES

- Aberoumand, A. (2010). Occurrence and Characterization of Potentially Pathogenic *Vibrio* Species in Seafood Products and Mari culture Systems. *World Journal of Fish and Marine Science*, 2(5), 376-382.
- Adelaide, A., Ângela, C., Newton, C.M., Gomes, E. A., Liliana, C and Faustino, M.A.F (2009). Phage Therapy and Photodynamic Therapy: Low Environmental Impact Approaches to Inactivate Microorganisms in Fish Farming Plants: Review. *Marine Drugs*, 7, 268-313.
- Alagappan, K. M., Deivasigamani, B., Somasundaram, S. T & S. Kumaran (2010). Occurrence of *Vibrio parahaemolyticus* and Its Specific Phages from Shrimp Ponds in East Coast of India. *Current Microbiology*, 61, 235–240.
- Al-Mouqati, S., Azad, I.S., Al-Baijan, D., & Benhaji, A. (2012). *Vibrio* detection in market Seafood samples of Kuwait by biochemical (API 20E) strips and its evaluation against 16s rDNA-based molecular methods. *Research Journal of Biotechnology*, 7(3), 63-69
- Annick Robert-Pillot, Alain Gue'nole', Jean Lesne, Re'gis Delesmont, Jean-Michel Fournier, Marie-Laure Quilici (2004). Occurrence of the tdh and trh genes in *Vibrio parahaemolyticus* isolates from waters and raw shellfish collected in two French coastal areas and from seafood imported into France. *International Journal of Food Microbiology*, 91, 319– 325.
- Anonymous (1997). Outbreak of *Vibrio parahaemolyticus* related to raw oyster in British Columbia. *Can Communicable Diseases*, 23(19), 145–148.
- Anonymous (2007). Quality reference criteria, Division of fish inspection and quality control, Department of Fisheries, Thailand.
- Anonymous (2009). Quality reference criteria, Division of fish inspection and quality control, Department of Fisheries, Thailand. Retrieved on 26th April, 2017 from <http://www.fisheries.go.th/quality/std%20micro.html>.
- Assavanig, A., Suthienkul, O., Lertsiri, S., A, B., Utrarachkij, F. & Komolprasert, V. (2008).

Report of diagnosis and surveillance of enteropathogens and toxic chemicals in white shrimp (*Penaeus vannamei*) in north Andaman provinces (DSECS) (Phase I - Phang-nga). Faculty of Public Health, Mahidol University, Thailand.

Athajariya, T. (2004). The study of serotypes and virulence genes of *Vibrio parahaemolyticus* in healthy carriers at frozen seafood plants. *Master's Thesis, Mahidol University, Thailand.*

Atthasampunnpa, P. (1974). *Vibrio parahaemolyticus* food poisoning in Thailand. In International Symposium on *Vibrio parahaemolyticus*, 21–26. Edited by T. Fujino, G. Sakaguchi, R. Sakazaki & T. Y. Tokyo: Saigon publishing.

Barker, W. H., Mackowiak, P. A, Fishbein, M., Morris, G. K., D'Alfonso, J. A, Hauser, G. H. & Felsenfeld, O. (1974). *Vibrio parahaemolyticus* gastroenteritis outbreak in Covington, Louisiana, in August 1972. *American Journal of Epidemiology*, 100, 316-323.

Barry, A. L. and Feeney, K. L. (1967) Two Quick Methods for Voges-Proskauer Test. *Applied Microbiology*, 15(5), 1138-1141.

Baslé, A., Rummel, G., Storici, P., Rosenbusch, J. P. & Schirmer, T. (2006). Crystal structure of osmoporin OmpC from *E. coli* at 2.0 *American Journal of Molecular Biology*, 36(2), 933–942.

Baumann, P., Furniss, A. L., & Lee, J. V. (1984). Genus 1, *Vibrio*. In P. N. R. Krieg & J. G. Halt (Eds.), *Bergey's manual of systematic bacteriology. 1*, 518–538. Baltimore, MD: Williams & Wilkins.

Bhattacharjee, R. N., Park, K. S., Okada, K., Kumagai, Y., Uematsu, S., Takeuchi, O., Akira, S., Iida, T. & Honda, T. (2005). Microarray analysis identifies apoptosis regulatory gene expression in HCT116 cells infected with thermostable direct hemolysin-deletion mutant of *Vibrio parahaemolyticus*. *Biochemical and Biophysical Research Communications*. 335, 328–334.

Bhattacharjee, R. N., Park, K.-S., Kumagai, Y., Okada, K., Yamamoto, M., Uematsu, S.,

- Matsui, K., Kumar, H., Kawai, T., & other authors. (2006). VP1686, a *Vibrio* type III secretion protein, induces toll-like receptorindependent apoptosis in macrophage through NF-kappaB inhibition. *Journal of Biological Chemistry*, 281, 36897–36904.
- Bos, M. P., Robert, V. & Tommassen, J. (2007). Biogenesis of the Gramnegative bacterial outer membrane. *Annual Review Microbiology*, 61, 191–214.
- Boutin, B. K., Reyes, A. L., Peeler, J.T., & Twedt, R.M. (1985). Effect of temperature and suspending vehicle on survival of *Vibrio parahaemolyticus* and *Vibrio vulnificus*. *Journal of Food Protection*, 48, 875-878.
- Büttner, D. & Bonas, U. (2002). Port of entry-the type III secretion translocon. *Trends Microbiology*, 10, 186–192.
- Caburlotto, G., Gennari, M., Ghidini, V., Tafi, M. & Lleo, M. M. (2009). Presence of T3SS2 and other virulence-related genes in *tdh*-negative *Vibrio parahaemolyticus* environmental strains isolated from marine samples in the area of the Venetian Lagoon, Italy. *FEMS Microbiology Ecology*, 70, 506–514.
- Caburlotto, G., Lleò, M. M., Hilton, T., Huq, A., Colwell, R. R. & Kaper, J. B. (2010). Effect on human cells of environmental *Vibrio parahaemolyticus* strains carrying type III secretion system 2. *Infectious Immunology*. 78, 3280–3287.
- Cann, D. C., Lesley Y. Taylor. (1981). A study of the incidence of *Vibrio parahaemolyticus* in Malaysian shrimp undergoing processing for export. *Journal of Hygiene*, 87,485-491
- Centers for Disease Control and Prevention. (2011). Incidence and Trends of Infection with Pathogens Transmitted Commonly Through Food. Foodborne Diseases Active Surveillance Network, 10 U.S. Sites, 1996-2010. *Centers for Disease Control and Prevention, Atlanta, GA. Morbidity and Mortality Weekly Report (MMWR)*, 60(22), 749-755.
- Chakraborty, R. D., & Surendran, P. K. (2009). Incidence and Molecular Typing of *Vibrio parahaemolyticus* from Tiger Shrimp Culture Environments along the Southwest

Coast of India. *Food Biotechnology*, 23, 284–311.

- Chao, G., Jiao, X., Zhou, X., Yang, Z., Pan, Z., Huang, J., Zhou, L. & Qian, X. (2009). Systematic functional pandemic strain-specific genes, three genomic islands, two T3SSs in foodborne, and clinical *Vibrio parahaemolyticus* isolates in China. *Foodborne Pathogens and Diseases*, 6, 689–698.
- Chiou, S. H. & Wu, S. H. (1999). Evaluation of commonly used electrophoretic methods for the analysis of proteins and peptides and their application to biotechnology. *Analytica Chimica Acta* 383, 47–60.
- Chowdhury, N. R., Chakraborty, S. & Ramamurthy, T. (2000). Molecular evidence of clonal *Vibrio parahaemolyticus* pandemic strains. *Emerging Infectious Diseases*, 6, 631–636.
- CHROMagar. (2010). CHROMagar *Vibrio*. Retrieved January 10, 2012, from <http://www.chromagar.com/food-water-chromagar-Vibrio-focus-on-Vibrio-species-23.html>.
- Chun, D., Chung, J. K., Tak, R. & Seol, S. Y. (1975). Nature of Kanagawa phenomenon of *Vibrio parahaemolyticus*. *Infection and Immunity*, 12, 81–87.
- Clarke, T. E., Tari, L. W. & Vogel, H. J. (2001). Structural biology of bacterial iron uptake systems. *Current Topics in Medicinal Chemistry*, 1, 7–30.
- Coburn, B., Sekirov, I. & Finlay, B. B. (2007). Type III Secretion Systems and Disease. *Clinical Microbiology Reviews*, 20(4), 535–549.
- Codex. (2003). Codex alimentarius commission discussion paper on risk management strategies for *Vibrio* spp. in seafood. Orlando.
- Cook, D. W. (1997). Refrigeration of oyster shellstock: Conditions which minimize the outgrowth of *Vibrio vulnificus*. *Journal of Food Protection*, 60(4), 349–352.
- Cook, D.W., O'Leary, P., Hunsucker, J.C., Sloan, E.M., Bowers, J.C., Blodgett, R.J. and Depaola, A. (2002). *Vibrio vulnificus* and *Vibrio parahaemolyticus* in U.S. Retail Shell Oysters: A National Survey from June 1998 to July 1999. *Journal of Food Protection*,

1(5-237), 79-87.

- Cowan, S. W., Schirmer, T., Rummel, G., Steiert, M., Ghosh, R., Pauptit, A., Jansonius, J. N. & Rosenbusch, J. P. (1992). Crystal structures explain functional properties of two *E. coli* porins. *Nature*, 358, 727–733.
- Daniels, N. A., Mackinnon, L., Bishop, R., Altekruse, S., Ray, B., Hammond, R. M., Thompson, S., Wilson, S., Bean, N. H. & Griffin, P. M. (2000). *Vibrio parahaemolyticus* Infections in the United States, 1973 – 1998. *Journal of Infectious Disease*, 181, 1661–1666.
- De Haan, C. P., Kivistö, R. I., Hakkinen, M., Corander, J. & Hänninen, M. L. (2010). Multilocus sequence types of Finnish bovine *Campylobacter jejuni* isolates and their attribution to human infections. *BMC Microbiology*, 10, 200.
- De Oliveira, Silva, E. R., Seidman, C. E., Tian, J. J., Hudgins, L. C., Sacks, F. M. and Breslow, J. L., (1996). Effects of shrimp consumption on plasma lipoproteins. *American Journal of Clinical Nutrition*, 64, 712– 717.
- DePaola, A., Nordstrom, J. L., Bowersm, J. C., Wells, J. G., & Cook, D. W. (2003). Seasonal abundance of total and pathogenic *Vibrio parahaemolyticus* in Alabama oysters. *Applied and Environmental Microbiology*, 69, 1521–1526.
- Di Pinto, A., Ciccarese, G. De Carota, R., Novello, L. & Terio, V. (2008). Detection of pathogenic *Vibrio parahaemolyticus* in southern Italian shellfish. *Food Control*, 19, 1037-1041.
- Di Pinto, A., Terio, V., Novello, L., & Tantillo, G. (2011). Comparison between thiosulphate-citrate-bile salt sucrose (TCBS) agar and CHROMAgar™ *Vibrio* for isolating *Vibrio parahaemolyticus*. *Food Control*, 22(1), 124–127.
- Difco Laboratories, Inc. (1984). *Difco manual*, (9th ed.) (166-168). Detroit, MI: Difco Laboratories Inc.
- Dobrindt, U., Hochhut, B., Hentschel, U. & Hacker, J. (2004). Genomic islands in pathogenic

and environmental microorganisms. *Nature Reviews. Microbiology*, 2, 414–424.

Donovan, T.J. and Netten, P.V. (1995). Culture media for the isolation and enumeration of pathogenic *Vibrio* species in foods and environmental samples. *International Journal of Food Microbiology*, 26(1), 77-91.

Elhadi, N., Radu, S., Chen, C. H., & Nishibuchi, M. (2004). Prevalence of potentially pathogenic *Vibrio* species in the seafood marketed in Malaysia. *Journal of Food Protection*, 67, 1469-1475.

Elliot, E. L., Kaysner, C. A., Jackson, L., & Tamplin, M. L. (1995). *Vibrio cholerae*, *V. parahaemolyticus*, *V. vulnificus* and other *Vibrio* spp. In *FDA Bacteriological Analytical Manual*, (8th ed.) (9.01-9.27). Gaithersburg, MD, AOAC International.

Enos-berlage, J. L., Guvener, Z. T., Keenan, C. E. & Mccarter, L. L. (2005). Genetic determinants of biofilm development of opaque and translucent *Vibrio parahaemolyticus*. *Molecular Microbiology*, 55, 1160–1182.

Enser, M., Hallet, K., Hewitt, B., Fursey, G. A. J. and Wood, J. D. (1996). Fatty acid content and composition of English beef, lamb and pork at retail. *Food Chemistry*, 42, 443–456.

Ergene, N (2009). Foreword. Article in the Agricultural Structure and Production Book.

FAO/WHO, Food and Agricultural Organization of the United Nations / World Health Organization (2011). Fisheries and Aquaculture Report, Rome, World Health Organization and Food and Agriculture Organization of the United Nations, Report of the Joint FAO/WHO Expert Consultation on the Risks and Benefits of Fish Consumption.

FAO/WHO, Food and Agricultural Organization of the United Nations / World Health Organization (2011). Fisheries and Aquaculture Report, Rome, World Health Organization and Food and Agriculture Organization of the United Nations, Report of the Joint FAO/WHO Expert Consultation on the Risks and Benefits of Fish Consumption.

- Farmer, J. J. (1992). The family *Vibrionaceae*. In A. Balows, H. G. Trüper, M. Dworkin, W. Harder, & K. H. Schleifer (ed.), *The prokaryotes. A handbook on the biology of bacteria: ecophysiology, isolation, identification, and applications*, (2nd ed.), (2938–2951) Berlin, Germany: Springer-Verlag.
- FDA (2005). Quantitative risk assessment on the public health impact of pathogenic *Vibrio parahaemolyticus* in raw oysters. Food and Drug Administration, US Department of Health and Human Services, 142.
- Food and Agriculture Organization of the United Nations, FAO (2005). *Perspectives and guidelines on food legislation, with a new model food law*. Rome. Retrieved on 17th April, 2016 from <http://www.fao.org/3/a-a0274e.pdf>
- Food Safety and Standards Authority of India (FSSAI), (2012). *Manual on Guidelines on Sampling. Ministry of Health and Family Welfare Government of India, New Delhi*. Retrieved on 20th June, 2016 from <http://www.fssai.gov.in/Portals/0/Pdf/15Manuals/SAMPLING.pdf>
- Fujino, T., Okuno, Y., Nakada, D., Aoyama, A., Fukai, K., Mukai, T. & Ueho, T. (1953). On the bacteriological examination of shirasu food poisoning. *Medical Journal Osaka University*, 4, 299–304.
- Guncavdi, O and Kucukciftci, S (2008). Economic growth under embargoes in North Cyprus: an input-output analysis. Economic and Social Research Centre. Istanbul Technical University, Faculty of Management Research Papers, 1-30
- Guryay, E., Safakli, O.V and Tuzel, B (2005). The comparative analysis of agricultural sector productivity in North Cyprus and the European Union. *Journal of Mediterranean University*, Faculty of Agriculture, 18(1), 51-61
- Hagan, C. L., Silhavy, T. J. & Kahne, D. (2011). G-Barrel membrane protein assembly by the Bam complex. *Annual Review of Biochemistry*, 80, 189–210.
- Hara-Kudo, Y., Nishina, T., Nakagawa, H., Konuma, H., Hasegawa, J., & Kumagai, S. (2001). Improved method for detection of *Vibrio parahaemolyticus* in seafood.

Applied and Environmental Microbiology, 67, 5819-5823.

Hardy Diagnostics. (2010). TCBS Agar. Retrieved December 24, 2011, from www.catalog.hardydiagnostics.com/cd_prod/content/hugo/TCBSAgar.htm.

Hassan, Z. H., Zwartkruis-Nahuis, J.T.M. and de Boer, E. (2012) Occurrence of *Vibrio parahaemolyticus* in retailed seafood in The Netherlands. *International Food Research Journal* 19(1), 39-43.

Hiyoshi, H., Kodama, T., Iida, T. & Honda, T. (2010). Contribution of *Vibrio parahaemolyticus* virulence factors to cytotoxicity, enterotoxicity, and lethality in mice. *Infection and Immunity*, 78, 1772–1780.

Holt, J. H. (1994). *Bergey's manual of determinative bacteriology* (9th ed.). Philadelphia, PA: Lippincott Williams & Wilkins.

Honda, T., Abad-lapuebla, M. A., Ni, Y., Yamamoto, K. & Miwatani, T. (1991). Characterization of a new thermostable direct haemolysin produced by a Kanagawa-phenomenon-negative clinical isolate of *Vibrio parahaemolyticus*. *Journal of General Microbiology*, 137, 253–259.

Honda, T., Ni, Y. X. & Miwatani, T. (1988). Purification and characterization of a hemolysin produced by a clinical isolate of Kanagawa phenomenon negative *Vibrio parahaemolyticus* and related to the thermostable direct hemolysin. *Infection and Immunity*, 56, 961–965.

Hsieh, Y., Liang, S., Tsai, W., Chen, Y., Liu, T. & Liang, C. (2003). Study of capsular polysaccharide from *Vibrio parahaemolyticus*. *Infection and Immunity*, 71, 3329–3336.

Hueck, C. J. (1998). Type III protein secretion systems in bacterial pathogens of animals and plants. *Microbiology of Molecular Biology Reviews*, 62, 379–433.

Hurley, C. C., Quirke, A., Reen, F. J. & Boyd, E. F. (2006). Four genomic islands that mark post-1995 pandemic *Vibrio parahaemolyticus* isolates. *BMC genomics*, 7, 104.

- Huss, H.H. (2003): *Assessment and Management of Seafood Safety and Quality*. FAO Fisheries Technical Paper, No 444. Food and Agriculture Organization of the United Nations, Rome. Retrieved on 13th April, 2017, from <http://www.fao.org/docrep/006/y4743e/y4743e00.htm>
- Isenberg, H. D. (2004). *Clinical microbiology procedures handbook*. American Society for Microbiology, Washington, DC. United States.
- Iwamoto, M., Ayers, T., Mahon, B.M & Swerdlow, D.L. (2010). Epidemiology of Seafood-Associated Infections in the United States. *Clinical Microbiology Reviews*, 23(2), 399–411
- Izutsu, K., Kurokawa, K., Tashiro, K., Kuhara, S., Hayashi, T., Honda, T. & Iida, T. (2008). Comparative genomic analysis using microarray demonstrates a strong correlation between the presence of the 80-kilobase Pathogenicity Island and pathogenicity in kanagawa phenomenon-positive *Vibrio parahaemolyticus* strains. *Infection and Immunity*, 76, 1016–1023.
- Jain, S. & Goldberg, M. B. (2007). Requirement for YaeT in the outer membrane assembly of autotransporter proteins. *Journal of Bacteriology*. 189, 5393–5398.
- Janda, J. M., Powers, C., Bryant, R. G., & Abbott S. (1988). Current perspectives on the epidemiology and pathogenesis of clinically significant *Vibrio* spp. *Clinical Microbiology Reviews*, 1, 245-267.
- Jarvis, B. Hedges, A. J. and Corry, J. E. L. (2007). Assessment of measurement uncertainty for quantitative methods of analysis: Comparative assessment of the precision (uncertainty) of bacterial colony counts. *International Journal of Food Microbiology*, 116, 44–51.
- João P. S. Cabral. (2010). Water Microbiology. Bacterial Pathogens and Water: Review. *International Journal of Environmental Research and Public Health*, 7, 3657-3703.
- Joseph, S. W., Colwell, R. R. & Kaper, J. B. (1982). *Vibrio parahaemolyticus* and related halophilic *Vibrios*. *Critical Review in Microbiology*, 10, 77–124.

- Kademi, H. I. (2016). Investigating the occurrence of *Vibrio parahaemolyticus* in various seafoods consumed in the Turkish Republic of Northern Cyprus. *Master Thesis, Department of Food Engineering, Near East University, Lefkosa, TRNC.*
- Kamath, S. D., Rahman, A. M. A., Voskamp, A., Komoda, T., Rolland, J. M., O’Hehir, R. E., & Lopata, A. L. (2014). Effect of heat processing on antibody reactivity to allergen variants and fragments of black tiger prawn: A comprehensive allergenomic approach. *Molecular Nutrition & Food Research*, 58, 1144–1155.
- Khaira, G., & Galanis, E. (2007). Descriptive Epidemiology of *Vibrio Parahaemolyticus* and Other *Vibrio* Species Infections in British Columbia: 2001-2006. *Canada Communicable Disease Report*, 33(11), 67-78.
- Kim, Y. B., Okuda, J., Matsumoto, C., Takahashi, N., Hashimoto, S., & Nishibuchi, M. (1999). Identification of *Vibrio parahaemolyticus* strains at the species level by PCR targeted to the *toxR* gene. *Journal of Clinical Microbiology*, 37, 1173–1177.
- Kishishita, M., Matsouka, N., Kumagai, K., Yamasaki, S., Takeda, Y. & Nishibuchi, M. (1992). Sequence variation in the thermostable direct hemolysin-related hemolysin (*trh*) gene in *Vibrio parahaemolyticus*. *Applied Environmental Microbiology*, 58, 2449–2457.
- Koebnik, R., Locher, K. P. & Van Gelder, P. (2000). Structure and function of bacterial outer membrane proteins: barrels in a nutshell. *Molecular Microbiology*, 37, 239–253.
- Kriem, M.R., Banni, B., Bouchtaoui, H. El, Hamama, A., Marrakchi, A. El, Chaouqy, N., Robert-Pillot, A. and Quilici, M.L. (2015). Prevalence of *Vibrio* spp. in raw shrimps (*Parapenaeus longirostris*) and performance of a chromogenic medium for the isolation of *Vibrio* strains. *Letters in Applied Microbiology*, 61, 224—230
- Kris-Etherton, P. M., Harris, W. S. and Appel, L. J., (2002). Fish consumption, fish oil, omega-3 fatty acids and cardiovascular disease. *Circulation*, 106, 2747–2757.
- Kumazawa, N. H., Hori, K., Fujimori, K., Iwade, Y. & Sugiyama, A. (1999). Geographical features of estuaries for neritid gastropods including *clithon retropictus* to preserve

- thermostable direct hemolysin-producing *Vibrio parahaemolyticus*. *Journal of Veterinary Medical Science*, 61, 721–724.
- Laohaprertthisan, V., Chowdhury, A., Kongmuang, U., Kalnauwakul, S., Ishibashi, M., Matsumoto, C. & Nishibuchi, M. (2003). Prevalence and serodiversity of the pandemic clone among the clinical strains of *Vibrio parahaemolyticus* isolated in southern Thailand. *Epidemiology Infection*. 130, 395–406.
- Lawrence, D. N., Blake, P. a, Yashuk, J. C., Wells, J. G., Creech, W. B. & Hughes, J. H. (1979). *Vibrio parahaemolyticus* gastroenteritis outbreaks aboard two cruise ships. *Am Journal of Epidemiology*, 109, 71-80.
- Le Roux, F., Gay, M., Lambert, C., Nicolas, J. L., Gouy, M., & Berthe, F. (2004). Phylogenetic study and identification of *Vibrio splendidus*- related strains based ongyrB gene sequences. *Diseases of Aquatic Organisms*, 58, 143–150.
- Lee, C. Y., & Pan, S. F. (1993). Rapid and specific detection of the thermostable direct haemolysin gene in *Vibrio parahaemolyticus* by the polymerase chain reaction. *Journal of General Microbiology*, 139, 3225–3231.
- Lee, S. W., Wendy W (2012): Characterization of *Vibrio alginolyticus* isolated from white leg shrimp (*Litopenaeus vannamei*) with emphasis on its antibiogram and heavy metal resistance pattern. *Veterinary Archive*, 82, 221-227.
- Letchumanan V, Pusparajah P, Tan LT-H, Yin W-F, Lee L-H and Chan K-G (2015) Occurrence and Antibiotic Resistance of *Vibrio parahaemolyticus* from Shellfish in Selangor, Malaysia. *Frontier in Microbiology*, 6:14-17.
- Linda N. Ward and Asim K. Bej (2006). Detection of *Vibrio parahaemolyticus* in Shellfish by Use of Multiplexed Real-Time PCR with TaqMan Fluorescent Probes. *Applied and Environmental Microbiology*, 72(3), 2031–2042
- Lopata, A. L., O’Hehir, R. E., & Lehrer, S. B. (2010). Shellfish allergy. *Clinical and Experimental Allergy*, 40, 850–858.

- Lozano-Leon, A., Torres, J., Osorio, C. R. & Martinez-Urtaza, J. (2003). Identification of tdh-positive *Vibrio parahaemolyticus* from an outbreak associated with raw oyster consumption in Spain. *FEMS Microbiology Letter*, 226, 281–284.
- Lupski, J. R. & Weinstock, G. M. (1992). Short, interspersed repetitive DNA sequences in prokaryotic genomes. *Journal of Bacteriology*, 174, 4525–4529.
- MacFaddin, J. F. (1980). *Biochemical Tests for Identification of Medical Bacteria*, pp. 173–183. Baltimore, MD: Williams & Wilkins.
- Madigan, M. T., Martinko, J. M., & Parker, J. (2004). *Brock Biology of Microorganisms*. Upper Saddle River, NJ: Prentice Hall.
- Maiden, M. C. J., Bygraves, J. A., Feil, E., Morelli, G., Russell, J. E., Urwin, R., Zhang, Q., Zhou, J., Zurth, K., & other authors. (1998). Multilocus sequence typing- A portable approach to the identification of clones within populations of pathogenic microorganisms. *Microbiology*, 95, 3140–3145.
- Makino, K., Oshima, K., Kurokawa, K., Yokoyama, K., Uda, T., Tagomori, K. & Iijima, Y. (2003). Genome sequence of *Vibrio parahaemolyticus*: a pathogenic mechanism distinct from that of *V. cholerae*. *Lancet*, 361, 743–749.
- Maluping, R. P., Lavilla-Pitogo, C. R., Romalde, J. L., & Krovacek, K. (2008). Molecular typing and antimicrobial susceptibility of *Vibrio parahaemolyticus* strains. *Diseases in Asian Aquaculture*, 6, 451–468.
- Mao, H. Y., Cao, M. J., Maleki, S. J., Cai, Q. F., Su, W. J., Yang, Y., & Liu, G. M. (2013). Structural characterization and IgE epitope analysis of arginine kinase from *Scylla paramamosain*. *Molecular Immunology*, 56, 463–470.
- Martinez-Urtaza, J., Lozano-Leon, A., Viña-Feas, A., de Nova, J., Garcia-Martin, O. (2006). Differences in the API 20E biochemical patterns of clinical and environmental *Vibrio parahaemolyticus* isolates. *FEMS Microbiology Letters*, 255(1), 75–81.
- Matsumoto, C., Okuda, J., Ishibashi, M., Iwanaga, M., Garg, P., Rammamurthy, T., Wong,

- H. C., Depaola, A., Kim, Y. B., & other authors. (2000). Pandemic spread of an O3: K6 clone of *Vibrio parahaemolyticus* and emergence of related strains evidenced by arbitrarily primed PCR and toxRS sequence analyses. *Journal of Clinical Microbiology*, 38, 578–585.
- McCarter, L. L. (1999). The multiple identities of *Vibrio parahaemolyticus*. *Journal of Molecular Microbiology and Biotechnology*, 1, 1–7.
- Mehdi Zarei, Mahdi Pourmahdi Borujeni, Amirhesam Jamnejad, Marjan Khezzzadeh (2012). Seasonal prevalence of *Vibrio* species in retail shrimps with an emphasis on *Vibrio Parahaemolyticus*. *Food Control*, 25, 107-109
- Miyamoto, Y., Kato, T., Obara, Y., Akiyama, S., Takizawa, K. & Yamai, S. (1969). In vitro hemolytic characteristic of *Vibrio parahaemolyticus*: Its close correlation with human pathogenicity. *Journal of Bacteriology*, 100, 1147–1149.
- Moeck, G. S. & Coulton, J. W. (1998). TonB-dependent iron acquisition: mechanisms of siderophore-mediated active transport. *Molecular Microbiology*, 28, 675–681.
- Molenda, J. R., Johnson, W. G., Fishbein, M., Wentz, B., Mehlman, I. J., Jr, D. & T.a. (1972). *Vibrio parahaemolyticus* gastroenteritis in Maryland: laboratory aspects. *Applied Microbiology*, 24, 444–448.
- Mortimore, S., & Wallace, C. (Eds.) (1994). HACCP.A practical approach. London, England: Chapman and Hall.
- Mozaffarian, D. and Wu, J. H., (2011). Omega-3 fatty acids and cardiovascular disease: effects on risk factors, molecular pathways and clinical events. *Journal of the American College Cardiology*, 58, 2047–2067.
- Murphy, R. A., Mourtzakis, M. and Mazurak, V. C., (2012). n – 3 Polyunsaturated fatty acids: the potential role for supplementation in cancer. *Current Opinion in Clinical Nutrition and Metabolic Care*, 15, 246–251.
- Murray, P. R., Baron, E. J., Pfaller, M. A., Tenover, F. C., & Tenover, R. H. (Eds.)(1995).

Manual of clinical microbiology (6th ed.). Washington, DC: American Society for Microbiology.

- Nagayama, K., Yamamoto, K., Mitawani, T. & Honda, T. (1995). Characterization of a haemolysin related to Vp-TDH produced by a Kanagawa phenomenon-negative clinical isolate of *Vibrio parahaemolyticus*. *Journal of Medical Microbiology*, 42, 83–90.
- Nair, G. B., Ramamurthy, T., Bhattacharya, S. K., Dutta, B., Takeda, Y. & Sack, D. A. (2007). Global dissemination of *Vibrio parahaemolyticus* serotype O3:K6 and its serovariants. *Clinical Microbiology Reviews*, 20, 39–48.
- Nakaguchi, Y., Okuda, J., Iida, T. & Nishibuchi, M. (2003). The urease gene cluster of *Vibrio parahaemolyticus* does not influence the expression of the thermostable direct hemolysin (TDH) gene or the TDH-related hemolysin gene. *Microbiology and Immunology*, 47, 233–239.
- Nakasone, N. & Iwanaga, M. (1990). Pili of a *Vibrio parahaemolyticus* strain as a possible colonization factor. *Infectious Immunology*, 58, 61–69.
- Neogi, S. B., Chowdhury, N., Asakura, M., Hinenoya, A., Haldar, S., Saidi, S. M., Yamasaki, S. (2010). A highly sensitive and specific multiplex PCR assay for simultaneous detection of *Vibrio cholerae*, *Vibrio parahaemolyticus* and *Vibrio vulnificus*. *Letters in Applied Microbiology*, 51(3), 293-300.
- Ngo, van H & Ravi, F (2010). A Review of Probiotics in Shrimp Aquaculture, *Journal of Applied Aquaculture*, 22(3), 251-266.
- Nishibuchi, M. & Kaper, J. B. (1990). Duplication and variation of the thermostable direct haemolysin (*tdh*) gene in *Vibrio parahaemolyticus*. *Molecular Microbiology*, 4, 87–99.
- Nolan, C. M., Ballard, J., Kaysner, C. A., Lilja, J. L., Williams, L. P., Jr & Tenover, F. C. (1984). *Vibrio parahaemolyticus* gastroenteritis: an outbreak associated with raw oysters in the Pacific Northwest. *Diagnostic Microbiology Infectious Diseases*. 2, 119–128.

- Noorlis, A., Ghazali, F. M., Cheah, Y. K., Zainazor, T. C. T., Ponniah, J., Tunung, R., et al. (2011). Prevalence and quantification of *Vibrio* species and *Vibrio parahaemolyticus* in freshwater fish at hypermarket level. *International Food Research Journal*, 18, 689-695.
- Nord, C. E., Lindberg, A. A., & Dahlback, A. (1974). Evaluation of five test kits API, Auxo Tab, Enterotube, Patho Tech and R/B for identification of Enterobacteriaceae. *Medical Microbiology and Immunology*, 159, 211-220.
- Norrakiah Abdullah Sani, Sujeewa Ariyawansa, Abdul Salam Babji, Jamal Khair Hashim (2013). The risk assessment of *Vibrio parahaemolyticus* in cooked black tiger shrimps (*Penaeus monodon*) in Malaysia. *Food Control*, 31, 546-552
- Oelschlaeger, T. A & Hacker, J. (2004). Impact of pathogenicity islands in bacterial diagnostics. *APMIS*, 112, 930–936.
- Okada, N., Iida, T., Park, K., Goto, N., Yasunaga, T., Hiyoshi, H., Matsuda, S., Kodama, T. & Honda, T. (2009). Identification and characterization of a novel type III secretion system in trh-positive *Vibrio parahaemolyticus* strain TH3996 reveal genetic lineage and diversity of pathogenic machinery beyond the species Level. *Infection and Immunology*, 77, 904–913.
- Okuda, J., Ishibashi, M., Hayakawa, E., Nishino, T., Takeda, Y., Mukhopadhyay, A. K., Garg, S., Bhattacharya, S. K., Nair, G. B. & Nishibuchi, M. (1997). Emergence of a unique O3:K6 clone of *Vibrio parahaemolyticus* in Calcutta, India, and isolation of strains from the same clonal group from Southeast Asian travelers arriving in Japan. *Journal of Clinical Microbiology*, 35, 3150–3155.
- Oliver, J. D., & Kaper, J. B. (1997). *Vibrio* species. In M. P. Doyle, L. R. Beuchat, & T. D. Montville (Eds.). *Food Microbiology: fundamentals and frontiers* (pp. 228-264). Washington, DC: ASM Press.
- Ottaviani, D., Leoni, F., Rocchegiani, E., Santarelli, S., Canonico, C., Masini, L., Ditrani, V. & Carraturo, A. (2008). First clinical report of pandemic *Vibrio parahaemolyticus* O3:K6 infection in Italy. *Journal of Clinical Microbiology*, 46, 2144–2145.

- Ottaviani, D., Leoni, F., Rocchegiani, E., Santarelli, S., Masini, L., Trani, V.D., et al. (2009). Prevalence and virulence properties of non-O1 non-O139 *Vibrio cholerae* strains from seafood and clinical samples collected in Italy. *International Journal of Food Microbiology*, 132, 47–53.
- Özer, S., Aslan, G., Tezcan, S., Bulduklu, P. S., Serin, M. S., & Emekdas, G. (2008). Genetic heterogeneity and antibiotic susceptibility of *Vibrio alginolyticus* strains isolated from Horse-Mackerel (*Trachurus trachurus* L., 1758). *Turkish Journal of Veterinary and Animal Sciences*, 32(2), 107-112.
- Panicker, G., Call, D. R., Kruug, M. J., & Bej, A. K. (2004). Detection of pathogenic *Vibrio* spp. in shellfish by using multiplex PCR and DNA microarrays. *Applied and Environmental Microbiology*, 70, 7436–7444.
- Park, K. S., Ono, T., Rokuda, M., Jang, M. H., Okada, K., Iida, T. & Honda, T. (2004). Functional characterization of two type III secretion systems of *Vibrio parahaemolyticus*. *Infection and Immunology*, 72, 6659–6665.
- Paydar, M., The, C.S.J., & Thong, K.L. (2013). Prevalence and characterisation of potentially virulent *Vibrio parahaemolyticus* in seafood in Malaysia using conventional methods, PCR and REP-PCR. *Food Control*, 32(1), 13-18.
- Pina C, Pinto F, Feijo JA, Becker JD. (2005). Gene family analysis of the Arabidopsis pollen transcriptome reveals biological implications for cell growth, division control, and gene expression regulation. *Plant Physiology*, 138, 744–756.
- Priest, F. G., Barker, M., Baillie, L. W. J., Holmes, E. C. & Maiden, M. C. J. (2004). Population structure and evolution of the *Bacillus cereus* group. *Journal of Bacteriology*, 186, 7959–7970.
- Pruzzo, C., Huq, A., Colwell, R. R., & Donelli, G. (2005). Pathogenic *Vibrio* species in the marine and estuarine environment. In S. Belkin, & R. R. Colwell (Eds.). *Oceans and health: Pathogens in the marine environment* (pp. 217–251). New York, NY: Springer.
- Qianqian Yu, Mengya Niu, Mengquan Yu, Yanhong Liu, Dapeng Wang, Xianming Shi

- (2016). Prevalence and antimicrobial susceptibility of *Vibrio parahaemolyticus* isolated from retail shellfish in Shanghai. *Food Control*, 60, 263-268
- Quilici, M.-L., Robert-Pillot, A., Picart, J. & Fournier, J.-M. (2005). Pandemic *Vibrio parahaemolyticus* O3:K6 spread, France. *Emerging Infectious Disease*, 11, 1148–1149.
- Ramón H. Barraza-Guardado, José A. Arreola-Lizárraga, Marco A. López-Torres, Ramón Casillas-Hernández, Anselmo Miranda-Baeza, Francisco Magallón-Barrajas, and Cuauhtemoc Ibarra-Gámez (2013). Effluents of Shrimp Farms and Its Influence on the Coastal Ecosystems of Bahía de Kino, Mexico. *The Scientific World Journal* Volume, 1-8 Retrieved on 24th April, 2017 from <http://dx.doi.org/10.1155/2013/306370>
- Reham A.A., Amani M.S. 2012. Specific detection of pathogenic *Vibrio* species in shellfish by using multiplex polymerase chain reaction. *Global Veterinaria*, 8(5): 525-531.
- Robinson, C. H. (1954). The low cholesterol, low fat diet. *American Journal of Clinical Nutrition*, 2, 353–355.
- Roque, A., Lopez-Joven, C., Lacuesta, B., Elandalousi, L., Wagley, S., Furones, M. D. (2009). Detection and Identification of tdh- and trh-Positive *Vibrio parahaemolyticus* Strains from Four Species of Cultured Bivalve Molluscs on the Spanish Mediterranean Coast. *Applied and Environmental Microbiology*, 75(23), 7574-7577.
- Rosec, J.P Simon, M. Causse V. Boudjemaa M. (2009). Detection of total and pathogenic *Vibrio parahaemolyticus* in shellfish: Comparison of PCR protocols using pR72H or toxR targets with a culture method. *International Journal of Food Microbiology*, 129, 136–145.
- Rosche, T. M., Smith, B., & Oliver, J. D. (2006). Evidence for an intermediate colony morphology of *Vibrio vulnificus*. *Applied and Environmental Microbiology*, 72, 4356-4359.
- Samir Mohammed Abd-Elghany, Khalid Ibrahim Sallam (2013). Occurrence and molecular

- identification of *Vibrio parahaemolyticus* in retail shellfish in Mansoura, Egypt. *Food Control*, 33, 399-405.
- Sarkar, B. L., Nair, G. B., Banerjee, A. K. & Pal, S. C. (1985). Seasonal distribution of *Vibrio parahaemolyticus* in freshwater environs and in association with freshwater fishes in Calcutta. *Applied Environmental Microbiology*, 49, 132–136.
- Schumann, P., Behrendt, U., Ulrich, A., & Suzuki, K. (2003). Reclassification of *Subtercola pratensis* Behrendt et al. 2002 as *Agreia pratensis* comb. *International Journal of Systematic and Evolutionary Microbiology*, 53, 2041–2044.
- Sechi, L. A., Dupre, I., Deriu, A., Fadda, G., & Zanetti, S. (2000). Distribution of *Vibrio cholerae* virulence genes among different *Vibrio* species isolated in Sardinia, Italy. *Journal of Applied Microbiology*, 88, 475-481.
- Serichantalergs, O., Bhuiyan, N. A., Nair, G. B., Chivaratanond, O., Srijan, A., Bodhidatta, L., Anuras, S. & Mason, C. J. (2007). The dominance of pandemic serovars of *Vibrio parahaemolyticus* in expatriates and sporadic cases of diarrhoea in Thailand, and a new emergent serovar (O3:K46) with pandemic traits. *Journal of Medicinal Microbiology*, 56, 608–613.
- Shime-Hattori, A., Iida, T., Arita, M., Park, K. S., Kodama, T. & Honda, T. (2006). Two type IV pili of *Vibrio parahaemolyticus* play different roles in biofilm formation. *FEMS Microbiology Letter*, 264, 89–97.
- Simopoulos, A. P., (2008). The importance of the omega-6/omega-3 fatty acids ratio in cardiovascular disease and other chronic diseases. *Experimental Biology and Medicine*, 233, 674–688
- Singh, D. V., Matee, M. H., Jiang, S., Sabenna, F., Shukla, B. S., & Sanial, S. C. (2001). Molecular analysis of *Vibrio cholerae* O1, O139, non-O1, and non-O139 strains: clonal relationships between clinical and environmental isolates. *Journal of Clinical Microbiology*, 67, 910-21.
- Smith, J. M., Feil, E. J. & Smith, N. H. (2000). Population structure and evolutionary

dynamics of pathogenic bacteria. *BioEssays*, 22, 1115–1122.

- Smyth, D. S., Feil, E. J., Meaney, W. J., Hartigan, P. J., Tollersrud, T., Fitzgerald, J. R., Enright, M. C. & Smyth, C. J. (2009). Molecular genetic typing reveals further insights into the diversity of animal-associated *Staphylococcus aureus*. *Journal of Medicinal Microbiology*, 58, 1343–1353.
- Sriratanaban, A. & Reinprayoon, S. (1982). *Vibrio parahaemolyticus*: a major cause of travellers' diarrhea in Bangkok. *ASTMH*, 34, 128 – 130.
- Su, Y.C & Liu,C.(2007). *Vibrio parahaemolyticus*: a concern of food safety. *Food Microbiology*, 24(6), 547-558
- Sujeewa, A. K. W., Norrakiah, A. S., & Laina, M. (2009).Prevalence of toxic genes of *Vibrio parahaemolyticus* in shrimps (*Penaeus monodon*) and culture environment. *International Food Research Journal*, 16, 89-95.
- Takikawa, I. (1958). Studies on pathogenic halophillic bacteria. *Yokohama Medical Bulletin*, 9, 313–322.
- Taniguchi, H., Hirano, H., Kubomura, S., Higashi, K. & Mizuguchi, Y. (1986). Comparison of the nucleotide sequences of the genes for the thermostable direct hemolysin and the thermolabile hemolysin from *Vibrio parahaemolyticus*. *Microbiology and Pathogens*, 1, 425–432.
- Taniguchi, H., Kubomura, S., Hirano, H., Mizue, K., Ogawa, M. & Mizugushi, Y. (1990). Cloning and characterization of a gene encoding a new thermostable hemolysin from *Vibrio parahaemolyticus*. *FEMS Microbiological Letter*, 67, 339–346.
- Tarr, C. L., Patel, J. S., Puhr, N. D., Sowers, E. G., Bopp, C., & Strockbine, N. A. (2007) Identification of *Vibrio* isolates by a multiplex PCR assay and *rpoB* sequence determination. *Journal of Clinical Microbiology*, 45, 134–140.
- Tassin, M. G., Siebeling, R. J., Roberts, N. C. & Larson, A. D. (1983). Presumptive identification of *Vibrio* species with H antiserum. *Journal of Clinical Microbiology*,

18, 400–407.

Thanassi, D. G. & Hultgren, S. J. (2000). Multiple pathways allow protein secretion across the bacterial outer membrane. *Current Opinion in Cellular Biology*, 12, 420–430.

Thompson, F. L., Iida, T. & Swings, J. (2004). Biodiversity of *Vibrios*. *Microbiology and Molecular Biology Reviews*, 68, 403–431.

Tulay Elal Mus, Figen Cetinkaya, Umut Celik (2014). Occurrence of *Vibrio*, *Salmonella* and *Staphylococcus aureus* in retail fresh fish, mussel and shrimp. *Acta Veterinaria Brno*, 83, 75–78.

Velusamy, V.K., Arshak, O., Korastynska, K., Oliwa V. and Adley, C. (2010). An overview of foodborne pathogen detection in the perspective of Biosensors, Research review paper. *Journal of Biotechnology Advances*, 28, 232-254.

Venkateswaran, K., Dohmoto, N., & Harayama, S. (1998). Cloning and nucleotide sequence of the *gyrB* gene of *Vibrio parahaemolyticus* and its application in detection of this pathogen in shrimp. *Applied and Environmental Microbiology*, 64, 681–687.

Venugopal, V. (ed.), (2009). In *Marine Products for Healthcare: Functional and Bioactive Nutraceutical Compounds from the Ocean*, CRC Press, London. 221–239.

Wagley, S., Koofhethile, K., Wing, J. B. & Rangdale, R. (2008). Comparison of *Vibrio parahaemolyticus* isolated from seafoods cases of gastrointestinal disease in the UK. *International Journal of Environmental Health Research*, 18, 283–293.

WHO & FAO. (2011). Risk assesement of *Vibrio parahaemolyticus* in seafood. World Health Organization/Food and Agriculture Organization microbiological risk assessment series 16, 193pp. Rome.

Wooldridge, K. G. & Williams, P. H. (1993). Iron uptake mechanisms of pathogenic bacteria. *FEMS Microbiological Review*, 12, 325–348.

Xiaoke Xu, Qingping Wu, Jumei Zhang, Jianheng Cheng, Shuhong Zhang, Kui Wu (2014) Prevalence, pathogenicity, and serotypes of *Vibrio parahaemolyticus* in shrimp from

Chinese retail markets. *Food Control*. 46, 81-85

- Yamamoto, A., Vuddhakul, V., Charernjiratragul, W., Vose, D., Osaka, K., Shigematsu, M., Toyofuku, H., Yamamoto, S. & Nishibuchi, M. (2008). Quantitative modeling for risk assessment of *Vibrio parahaemolyticus* in bloody clams in southern Thailand. *International Journal of Food Microbiology*, 124, 70–78.
- Yanagihara, I., Nakahira, K., Yamane, T., Kaieda, S., Mayanagi, K., Hamada, D., Fukui, T., Ohnishi, K., Kajiyama, S., & other authors. (2010).
- Yang, L., Zhan, L., Han, H., Gao, H., Guo, Z., Qin, C., Yang, R., Liu, X. & Zhou, D. (2010). The low-salt stimulon in *Vibrio parahaemolyticus*. *International Journal of Food Microbiology*, 137, 49–54.
- Yeung, P.S. and Boor, K.J. (2004). Epidemiology, pathogenesis, & prevention of foodborne *Vibrio parahaemolyticus* infections. *Journal of Foodborne Pathogens and Diseases*, 1, 74-88.
- Zen-Yoji, H., Hitokoto, H., Morozumi, S. & Le Clair, R. a. (1971). Purification and characterization of a hemolysin produced by *Vibrio parahaemolyticus*. *Journal of Infectious Diseases*, 123, 665–667.
- Zulkifli, Y., Alitheen, N. B., Raha, A. R., Yeap, S. K., Marlina, Son, R. and Nishibuchi, M. 2009. Antibiotic resistance and plasmid profiling of *Vibrio parahaemolyticus* isolated from cockles in Padang, Indonesia. *International Journal of Food Research*, 16, 53-58.
- Zulkifli, Y., Alitheen, N. B., Son, R., Raha, A. R, Samuel, L., Yeap, S. K., & Nishibuchi, M. (2009b). Random amplified polymorphic DNA-PCR and ERIC PCR analysis on *Vibrio parahaemolyticus* isolated from cockles in Padang, Indonesia. *International Food Research Journal*, 16, 141-150.