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

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

By
HAFIZU IBRAHIM KADEMI

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

NICOSIA, 2016

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: Hafizu Ibrahim Kademi

Signature:

Date: 08/09/2016

Hafizu Ibrahim KADEMI: INVESTIGATING THE OCCURRENCE OF *Vibrio parahaemolyticus* IN VARIOUS SEAFOOD CONSUMED IN THE TURKISH REPUBLIC OF NORTHERN CYPRUS

Approval of Director of Graduate School of Applied Sciences

Prof. Dr. İlkay SALİHOĞLU

We certify that, 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	Faculty of Medicine, NEU
Dr. Perihan Adun	Supervisor, Faculty of Engineering, Department of Food Engineering, NEU
Assist. Prof. Dr. Serdar Susever	Faculty of Health Sciences, NEU

NEU JURY REPORT

DEPARTMENT OF
FOOD ENGINEERING

Academic Year: 2015-2016

STUDENT INFORMATION

Full Name	Hafizu Ibrahim KADEMI		
Undergraduate degree	B. Tech.	Date Received	Spring 11 April 2012
University	KUST, WUDIL NIG.	CGPA	3.81

THESIS

Title: INVESTIGATING THE OCCURRENCE OF *Vibrio parahaemolyticus* IN VARIOUS SEAFOOD CONSUMED IN THE TURKISH REPUBLIC OF NORTHERN CYPRUS

METHOD **Description:** This study investigates the presence of pathogenic *Vibrio parahaemolyticus* in seafood consumed in the Turkish Republic of Northern Cyprus (TRNC). Thiosulfate Citrate Bile Sucrose-Salts Agar (TCBS) medium was employed in the isolation of this pathogen (*V. Parahaemolyticus*) from different seafood. Sixty samples of fish were obtained from major seafood outlets and sea coasts in Nicosia, Famagusta, Kyrenia and Morphou. Conventional culture technique was employed for the bacterial identification. The identity of the bacteria were confirmed by BD Phoenix.

Supervisor: Dr Perihan Adun **Food Engineering Department**

The jury has decided to (accept) ~~editing and minor revision / reject the student's thesis.~~
The decision was taken unanimously / ~~by majority.~~

COMMITTEE MEMBERS

Number Attending	3	Date	25/08/2016

Name	Signature
Assoc. Prof. Dr. Kaya Süer, Chairman of the Jury	
Dr. Perihan Adun (Supervisor), Member of the Jury	
Assist. Prof. Dr. Serdar Susever, Member of the Jury	

APPROVALS

Date	Head of Food Engineering Department
25/08/2016	(a) Büşra Demirözü

DEPARTMENT OF FOOD ENGINEERING
DEPARTMENTAL DECISION

Date: 25/08/2016

Subject: Completion of M.Sc. Thesis

Participants: Assoc. Prof. Dr. Kaya Suer, Dr. Perihan Adun, Assist. Prof. Dr. Serdar Susever

DECISION

We certify that the student whose number and name are given below, has fulfilled all the requirements for a M.Sc. degree in Food Engineering.

		CGPA
20145840	Hafizu Ibrahim KADEMI	4.00

Assoc. Prof. Dr. Kaya Süer Committee Chairman, Faculty of Medicine, NEU.

Assist. Prof. Dr. Serdar Susever, Committee Member, Faculty of Health Sciences, NEU.

Dr. Perihan Adun, (Supervisor), Committee Member, Faculty of Engineering, NEU.

Head of Food Engineering Department

(a) Bülent Demiröğlu

ACKNOWLEDGEMENTS

My gratitude is endless to the One and only One that makes impossible to become possible.

Distinctively, I would like to express my appreciation to all people who contributed in one way or the other in my educational pursuit.

At first, I would like to express my deepest gratitude to my supervisor Dr. Perihan Aysal ADUN, for her generosity and unwavering support as well as open-minded approach, without whom this study would not have been completed. No amount of inks and papers are enough to transcribe my appreciation. I would like to thanks all the chairs in my jury for their scholarly recommendations.

I would like to also use this opportunity to credit Mr. Buğra Demircioğlu, the coordinator of Food Engineering Department for his tremendous counselling and mentorship. I am also grateful to all the lecturers of Food Engineering Department of Near East University for their support and encouragement.

It is a great pleasure to acknowledge my lecturers at Kust Wudil, particularly Malam Munir Abba Dandago (my academic father and a role model); your teachings, guidance and support are indelible in my mind.

I am highly grateful to Kano state government under the leadership of Engr. (Dr) Rabiu Musa Kwankwaso for sponsoring my master's program, may Allah (S.W.T) rewards him abundantly. Accordingly, I wish to acknowledge Center of Excellence of Near East University for sponsoring this research.

Above ground, I am indebted to my parents Malam Ibrahim Muhammad and Malama Halima Ibrahim for giving me their all to live an examplanary life, I am indeed grateful.

Finally, I am thankful to all my colleagues, friends and relatives whose names are numerous to mention.

To the entire ummah

ABSTRACT

This study investigates the presence of pathogenic *Vibrio parahaemolyticus* in seafood consumed in the Turkish Republic of Northern Cyprus (TRNC). Sixty samples of fish were obtained from major seafood outlets and sea costs of Famagusta, Kyrenia, Nicosia and Morphou. Conventional culture technique was employed for the bacterial identification. After having been enriched, isolation of this pathogen (*V. Parahaemolyticus*) from different seafood was performed on Thiosulfate Citrate Bile Sucrose-Salts Agar (TCBS) medium. The identity of the bacteria were confirmed by using BD Phoenix Instrument.
We could not find *Vibrio parahaemolyticus* in fish samples taken from different regions of TRNC which is one of the most important seafoodborne pathogens. However seafood consumed in TRNC might be a source of other bacterial pathogens like *Photobacterium damselae* (formerly *Vibrio damsela)* and *Providencia rettgeri* species, since the concentrations of these bacteria were found to be greater than 10^5 cfu/ml (minimum infective dose) in sea bass and sea bream fishes from Kyrenia and from Morphou regions respectively.

Keywords: Isolation; *V. Parahaemolyticus*; TCBS; culture method; Seafood; investigating; food safety; TRNC

ÖZET

Bu çalışmada Kuzey Kıbrıs Türk Cumhuriyeti'nde (KKTC) tüketilen deniz ürünlerindeki patojen bir bakteri olan *Vibrio parahaemolyticus*'un olası varlığı araştırılmıştır. KKTC'nin Mağusa, Girne, Lefkoşa ve Güzelyurt bölgelerindeki deniz ürünleri satan marketlerden ve balıkçılardan 60 balık örneği toplanmıştır. Balıkların solungaç ve iç organları ayrıldıktan sonra alkali peptonlu suda ayrı ayrı homojenize edilip zenginleştirilmiş ve Thiosulphate Citrate Bile Salt Sucrose (TCBS) Agarda izole edilmiştir. TCBS agarda üreyen şüpheli koloniler BD Phoenix cihazı kullanılarak tanımlanmışlardır.

Kültüre alınan örneklerin hiçbirinde *Vibrio parahaemolyticus*'a rastlanmamıştır. Girne'den alınan levrek örneklerinden bir balığın iç organlarında patojen *Providencia rettgeri* ve Güzelyurt'tan alınan çipura örneklerinden bir balığın yine iç organlarında patojen *Photobacterium damsalae* (önceki adıyla *Vibrio damsela*) bulunmuştur.

Balık örneklerinde *Vibrio parahaemolyticus* bulunmaması halk sağlığı açısından sevindirici bir sonuç olmakla beraber KKTC'de yaygın şekilde tüketilen balık örneklerinden bazılarında 10^5 cfu/ml (minimum infektif doz) düzeyinde rastlanılan *Providencia rettgeri* ve *Photobacterium damsalae* patojen bakterilerinin varlığının araştırılması önerilmektedir.

Anahtar Kelimeler: İzolasyon ve identifikasyon; verifikasyon; *Vibrio parahaemolyticus*; TCBS; deniz ürünleri; balık, gıda güvenliği; KKTC

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS AND SYMBOLS

API:	Analytical profile index
APS:	Alternative protein source
APW:	Alkaline peptone water
a_w:	Water activity
BAM:	Bacteriological analyses manual
BD:	Becton Dickinson
CAC:	Codex Alimentarius Commission
CDC:	Centers for Disease Control and Prevention
CFU:	Colony forming unit
D-value:	Decimal reduction time/dose
DHA:	Docosahexaenoic acid
EC:	European Commission
EPA:	Eicosapentaenoic acid
FAO:	Food and Agriculture Organization of the United Nations
FDA:	Food and Drug Administration
G:	Gram
GAP:	Good Aquaculture Practice
GHP:	Good Hygiene Practice
GMP:	Good Manufacturing Practice
GST:	Glucose salt teepol
HACCP:	Hazard Analysis Critical Control Points
Hr:	Hour
IAEA:	International Atomic Energy Agency
ICMFS:	International Commission on Microbiological Specifications for Foods
Kg:	Kilogram
KGy:	Kilogray
KP:	Kanagawa phenomenon
LAMP:	Loop-mediated amplification assay
LOD:	Limit of detection

M:	Meter
MC:	Microbiological criteria
MID:	Minimum infective dose
ML:	Milliliter
Min:	Minute
MPN:	Most probable number
NaCl:	Sodium Chloride
Na+:	Sodium ion
NGO:	Non-Governmental Organization
pH:	Hydrogen ion concentration
PCR:	Polymerase chain reaction
SPB:	Salt polymyxin broth
SCB:	Salt colistin broth
ST:	Sodium taurocholate
STS:	Salt tripticase soy broth
TCBS Agar:	Thiosulphate citrate bile salts sucrose agar
TCI:	Thiosulphate chloride-iodide
TDH:	Thermostable direct hemolysin
***Tlh*:**	Thermolabile hemolysin
TRH:	TDH- related hemolysin
TRNC:	Turkish Republic of Northern Cyprus
TSA:	Tryptone soy broth
T3SS:	Type three secretion systems
WHO:	World Health Organization
°C:	Degree Celsius
%:	Percent

CHAPTER 1
INTRODUCTION

1.1 Background Information

Foodborne infections caused by microorganisms are the most persistent non-communicable infections all over the world and are the most frequent, costly and yet preventable public health problems. Foodborne gastrointestinal infections cause significant morbidity and mortality globally, and despite the huge resources spent for the control programs, these infections continue to implicate public health and economy (Helms et al., 2006). Seafood is implicated in a number of these infections throughout the world; with United States having 10-19%, Australia 20%, European Union 42.5%, Canada 62% and Japan 87% (Butt et al., 2004; FAO, 2016a).

Seafood is consumed globally because of its significant contributions in nutrition and well-being of the consumers. However, despite its significance, seafood contain a number of deleterious microbial loads such as bacteria, viruses such as norovirus and microparasites such as flukes.

The relevance of microorganisms associated with seafood after harvest depends on two major factors: environmental conditions and microbial state of the harvesting water; water temperature, degree of saltiness, proximity of harvesting ground to polluted areas, feeding mechanism of seafood, method of harvest and preservation techniques employed (Feldhusen, 2000).

The bacterial biota of seawater is mostly Gram-negative; although, Gram-positive bacteria exist there basically as ephemerals (Jay, 2000). Pathogenic bacteria associated with seafood could be divided into three major groups: the **indigenous bacteria** (*Vibrionaceae* spp., *Listeria monocytogenes*, and *Clostridium botulinum*), **enteric bacteria** which occur due to faecal contamination (*Salmonella* spp., *Yersinia enterocolitica, Escherichia coli,* amongst others) and **those encountered in the course of processing** (*Bacillus* spp., *Clostridium perfringens* and *Staphylococcus aureus*) (Feldhusen, 2000).

Vibrionaceae is a family of Proteobacteria inhabiting aquatic systems and seafood harvested from such systems. This includes the genus *Photobacterium*, *Vibrios*, *Aeromonas* and *Plesiomonas* (Colakoğlu et al., 2006).

Occurrence of *Vibrio* species have been reported in seafood harvested from **contaminated waters**, or which have been **mishandled improperly** after harvesting (Baffone et al., 2000). They play significant role in seafood associated infections (Huss, 1997).

Nevertheless, not all *vibrios* pose dangers to humans. In all the 65 species of the genus, only 12 are known as human pathogens (Nair et al., 2006), and 8 species regarded as agents of food poisoning (Oliver and Kaper, 2007). Most importantly, three species including *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* are responsible for the pathogenicity in food by food contamination (DePaola et al., 2010).

Vibrios associated with seafood gained more attention as they are an important cause of food poisoning in humans (Quintoil et al., 2007 and DePaola et al., 2010). *V. parahaemolyticus* is the leading causative agent of acute gastroenteritis in human after ingestion of contaminated raw, undercooked, or mishandled marine food products (Letchumanan et al., 2014).

V. parahaemolyticus are enteropathogenic bacteria responsible for many seafoodborne illnesses as a result of ingestion of contaminated seafood such as raw fish or shellfish. The organism manifests through nausea and vomiting, abdominal cramps, fever and subsequent watery to bloody diarrhea after a short period of time following ingestion of the food. Although the mechanism of illness is not clear yet; fecal leukocytes are usually observed. The disease occurs throughout the world with highest prevalence in areas where uncooked seafood is used (Jawetz et al., 1995).

V. parahaemolyticus are the classical agents of seafood-associated gastroenteritis in the U.S and many Asian countries (Mead et al., 1999), although rare cases have been reported in European countries (Robert-Pillot et al., 2004). *V. parahaemolyticus* is frequently isolated in seafood everywhere in the world (Martinez-Urtaza et al., 2005; Colakoğlu et al., 2006; Fluenzalida et al., 2007; Iwamoto et al., 2010; Adebayo-Tayo et al., 2011; Francis et al., 2012).

The growing consumption of seafood, the increase prevalence, and the elevated levels of cross contamination caused by aquatic pathogenic microbes motivated us to investigate the occurrence of *Vibrio parahaemolyticus* in seafood in the TRNC. Infections due to ingestion of seafood contaminated with *V. parahaemolyticus* result in frequent hospitalizations with morbidity and mortality. *V. parahaemolyticus* has a greater seasonal and geographic range than other *Vibrios* and it is generally more abundant year round. Because of its association with seafood, this agent is a significant concern to the seafood

industry and public health agencies. *V. parahaemolyticus* can readily be detected and enumerated with available facilities in the Near East University Laboratories. Seafood took significant portion in the diet of people in the Turkish Republic of Northern Cyprus (TRNC) and that there are no or less adequate information regarding the safety of seafood.

It is very unfortunate that nearly almost all marine environments have been polluted with biological and chemical pollutants as a result of human activities. It is, therefore, obvious seafood harvested from marine or aquatic environments contain some pathogenic microorganisms.

Most of fish species consumed in the TRNC are imported from different countries around the world, however, due to its significance, many attempts have been made to grow commercial seafood in the TRNC. In addition to two established farms, another project aimed at producing Sea bass and Sea bream has been planned to provide 29 tons in 2003 with hope of increasing in the subsequent years. In TRNC, the estimated demand for finfish, in particular Sea bass and Sea bream is above 1100 tons per year and is increasing continously (Anonymous, 2012).

The aim of this study is to investigate the presence of *Vibrio parahaemolyticus* in various types of seafood consumed in the TRNC. Objectives include:

I. To assess the safety of some seafood varieties in TRNC in terms of potentially pathogenic *Vibrio parahaemolyticus.*

II. To acquire epidemiological and analytical data for risk assessment of *V. parahaemolyticus* for seafood of the TRNC.

III. To evaluate the frequency of occurrence of this pathogen among various types of seafood.

In terms of area, our research is limited to Turkish Republic of Northern Cyprus (TRNC). Sampling area includes major seafood outlets of Nicosia (Lefkoşa), Famagusta (Mağusa), Kyrenia (Girne) and Morphou (Güzelyurt). In the context of our research, seafood is limited to finfish species. Even though seafood may contain a lot of pathogenic microorganisms, this study is aimed to determine the presence of medically important *V. parahaemolyticus* in various finfish varieties consumed in the TRNC.

1.2 Overview on Seafood

Potter and Hotchkiss (2007) defined seafood as a food originated from salt water only, while foods originated from all aquatic environments either fresh or salt water are referred to as marine foods. This shows that seafood are subclasses of marine foods or that marine foods are the general nomenclature of all foods originated from aquatic environments.

Accordingly, Venugopal (2006) and Ronholm et al (2016) defined seafood as a vast group of biologically diverse animals and their products; comprising of fish, whether of marine, freshwater, or estuarine habitat, and shellfish, consisting crustacean and mollusks. The crustacean consist of crab, lobster, crayfish and shrimp, while the mollusks comprises subgroups of bivalves such as oyster, mussel, and scallop, univalve creatures which include snail, conch and abalone, and cephalopods comprising cuttlefish, octopus and squid. By extension, seafood refers to all edible forms of aquatic life either from marine or fresh water habitat. Seafood comprises all flora and fauna found in aquatic habitat, the prominent one being fish and shellfish.

Seafood comprises of other animals and plants such as seaweed and sea cucumber. Seafood can also be in form of manufactured or processed foods usually frozen or canned. They include precooked, battered, breaded, and frozen fillets, shrimps, fish sticks, canned tuna, sardines and salmon. Moreover, fish are often pickled, salted, smoked or dried (Potter and Hotchkiss, 2007).

Seafood is an excellent substrate for the survival of microorganisms in aquatic environments. This is because of the soft texture of their flesh and similar living habits with these microbes in the same ecological habitat, obviously these bacteria become part of microflora of seafood. Consequently, inappropriate packaging, shipment and preservation of the seafood harvested from contaminated aquatic environments give room for these pathogens to multiply rapidly and cause life threatening foodborne illnesses to people who consume this contaminated seafood (Colakoğlu et al., 2006). Seafood harvested from tropical and subtropical or from temperate regions usually accommodates significant doses of *V. parahaemolyticus*. Routine analysis for *V. parahaemolyticus* indicates the presence of both pathogenic and enteropathogenic strains.

1.2.1 Proximate composition and nutrition of seafood

Seafood serves as an important source of proteins and other nutrients in the diets of many people and it is adding to food security of the growing world population. Proper attention

in post–harvest handling, processing and transportation of seafood are the cornerstone of ensuring better quality and safety. Maintaining the nutritional value of the seafood, preserving the benefits of its rich composition and avoiding costly and debilitating effect of seafood-borne illnesses could not be overemphasized (FAO, 2015). Significant number of people throughout the globe depend on seafood as a primary source of valuable nutrients particularly protein, poly unsaturated fatty acids (PUFAs), vitamins and minerals (Francis et al., 2012). Virtually, the nutritional value of seafood, fish in particular, led to its worldwide acceptance and excessive consumption. The low fat nature of some seafood and the availability of essential fatty acids in some fishes which are vital in tackling the risks of coronary heart problems, have increased the public awareness of dietary and health significance of seafood consumption (Amusan et al., 2010).

The chemical composition and nutritional attributes of a healthy fish of a given species vary considerably with respect to the season of the year and maturity index (Potter and Hotchkiss, 2007), and artificial diet of aquacultured fish (Onwuka, 2014). For instance, the fat content in muscle of herring may vary from about 8% to 20% depending on the period of the year and availability of food. The average compositions of most fish are: 18-35% total solids, 14-20% protein, 0.2-20% fat, meanwhile 1.0-1.8% is ash (Potter and Hotchkiss, 2007).

Nutritionally, finfish provide high quality protein compared to some categories of shellfish especially mollusks, partly due to their high water content (Onwuka, 2014).

Proteins of finfish are highly digestible and are as good as red meat proteins in terms of essential amino acids. Accordingly, the most essential role of finfish in the diet is the provision of high quality proteins (Potter and Hotchkiss, 2007). In another statement, Onwuka (2014) highlighted that fish proteins are basically similar to other animals' proteins, meaning they contain sarcoplasmic proteins (containing enzymes and myoglobin), myofibrillar or contractile proteins (such as chitin and myosin) and the connective tissue proteins (i.e. collagen).

The fats present in fish are easily digestible and mostly liquid at room temperature because they contain fewer amounts of saturated fatty acids. Seafood oil contains the omega-3-polyunsaturated fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) which have been reportedly vital in preventing many diseases including coronary disease in humans (Onwuka, 2014).

Seafood is a good source of important micronutrients (required in small amounts) like vitamins and minerals. The fat of fish is an excellent source of the fat-soluble vitamins; A, D, E and K and B-vitamins (thiamine, riboflavin and niacin). This is the rationale behind giving cod liver oil to small children (Potter and Hotchkiss, 2007; Onwuka, 2014).

Seafood is an excellent source of essential mineral elements particularly Iodine (Potter and Hotchkiss, 2007). Other minerals include Iron, Magnesium, Calcium and Phosphorus (Onwuka, 2014).

1.2.2 Seafood and foodborne pathogens

The Food and Agriculture Organization of the United Nations (1994) declared that fish provides about 60% of the world's supply of protein and that 60% of the developing world gains more than 30% of their protein from fish annually (Amusan et al., 2010).

Seafood is one of the most rapid growing sources of food. Since ancient times, seafood played a significant role in the diet and served as main supply of animal protein worldwide (Amusan et al., 2010). Significant number of people throughout the globe depend on seafood as a primary source of valuable nutrients particularly protein, poly unsaturated fatty acids (PUFAs), vitamins and minerals (Francis et al., 2012). Virtually, the nutritional value of seafood, fish in particular, led to its worldwide acceptance and excessive consumption. The low fat nature of some seafood and the availability of essential fatty acids in some fishes which are vital in tackling the risks of coronary heart problems, have increased the public awareness of dietary and health significance of seafood consumption (Amusan et al., 2010).

With increased seafood consumption; foodborne illnesses associated with seafood is also increasing. Seafood is being responsible for significant figures of foodborne diseases throughout the globe (Francis et al., 2012).

According to Donnenberg (2005) raw fish has become the most vulnerable of all food to microbial spoilage as microbes such as bacteria, fungi and viruses are commonly associated with fresh fish as such may pose dangers to public health. Raw clams and oysters are known to cause infectious diseases such as hepatitis and gastroenteritis (Potter and Hotchkiss, 2007).

It is very unfortunate that nearly almost all marine environments have been polluted with biological and chemical pollutants as a result of human activities. Therefore, it is obvious that seafood harvested from marine or aquatic environments contain some pathogenic

microorganism. Consumption of seafood that has been infected with microbes can result in respiratory irritation in man (Potter and Hotchkiss, 2007).

More widely, the World Health Organization (WHO) stated that raw or undercooked seafood provides good medium for several prevalence of food-borne diseases (WHO, 2002).

The possibility of contamination of raw foods by dangerous microorganisms is equally applicable to seafood when compared to any other food possibly due to their soft texture. Effects of processing, preservation factors and storage conditions affect the frequency or level of contamination (Huss, 2003).

Vibrios and other pathogenic microorganisms may accumulate in molluscan bivalves through filter feeding in the aquatic environments. Moreover, molluscan bivalves are usually developed and harvested in shallow and near-shore estuarine habitat, so, they are susceptible to contain large number of pathogens including Vibrios. They create a substantial health risk to the consumers (Gram and Huss, 2000).

1.2.3 Prevalence, occurrence and distribution of *V. parahaemolyticus* in seafood

Naturally, *V. parahaemolyticus* occurs in aquatic environments and seafood harvested from such environments. However, the occurrence of *V. parahaemolyticus* in seafood depends on several factors including; the type of aquatic environment, seasonal temperature, degree of contamination of the surrounding water and type or species of seafood. A number of studies from various regions around the world justified the variations in occurrence, prevalence and distribution of the total and pathogenic *V. parahaemolyticus* in seafood.

Generally, shellfish (fig. 1.2) contain high number of *V. parahaemolyticus* than finfish (Jones et al., 2014; Odeyemi, 2016). Moreover, even among shellfish, oysters have the highest number of occurrence of *V. parahaemolyticus* (Odeyemi, 2016).

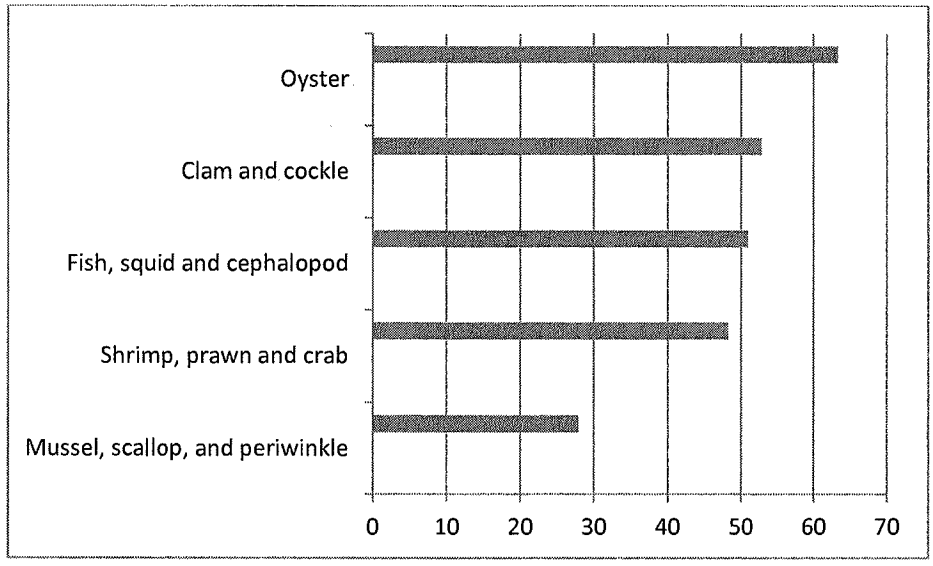

Figure 1.1: Occurrence, prevalence and distribution of *V. parahaemolyticus* in seafood

1.2.4 Microbiological criteria of seafood

"A microbiological criterion (MC) has been define by the Codex Alimentarius Commission as a risk management metric which indicates the acceptability of a food, or the performance of either a process, or a food safety control system following the outcome of sampling and testing for microorganisms, their toxins/metabolites or markers associated with pathogenicity or other traits at a specified point of the food chain" (CAC, 1997).

Seafood must comply with microbiological criteria (MC) that are relevance to seafood in order to meet public health interest. MC are prepared to determine the effectiveness of Good Hygiene Practices and Hazard Analysis Critical Control Point (HACCP).

MC are usually established based on international agreed principles as in Codex Alimentarius. MC are established standards used in assessing the safety and quality of foods. The Commission Regulation (EC) No. 2073/2005 on microbiological criteria for foodstuffs maintained that developing reliable methods for detecting potentially pathogenic *V. parahaemolyticus* is prerequisite for establishing effective microbiological criteria of seafood which will subsequently help to implement good sanitary plan.

Additionally, because of its widespread distribution in marine environments, short generation and fast replication times and low infectious doses of the pathogenic strains of *V. parahaemolyticus* in humans (Kaysner & DePaola, 2000), intensive and continuous monitoring and evaluation are highly needed in order to assess the potential health risk arising from seafood consumption.

1.3 Fish

Fish or finfish have been described as aquatic vertebrates, ectothermic in nature (having streamlined body), covered with scales, with two sets of paired fins and several unpaired fins (Onwuka, 2014). More generally, the term "fish" is used to described any non-tetrapod chordate (animal with backbone), with respiratory gills and limbs in form of fins (Nelson, 2006).

In TRNC, like other Mediterranean countries, the most important finfish consumed are Sea bream (*Sparus aurata* L.) and European Sea bass (*Dicentrarchus labrax*). According to a report released by the Food and Agriculture Organization of the United Nations, Mediterranean seafood production has been increased in the previous decades as a result of large production of Sea bream and Sea bass (FAO, 2011).

1.3.1 Sea bream (*Sparus aurata* L.)

Sea bream (*Sparus aurata* L.) also known as gilthead sea bream (Çipura in Turkish) is a protandrous fish species, hermaphrodite in nature which is commonly found in the Mediterranean Sea, the coasts of Atlantic Sea and rarely in the Black Sea (Figure 1.2).

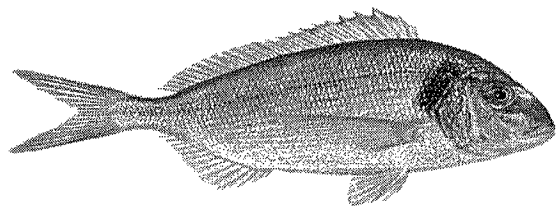

Figure 1.2: Gilthead Sea bream (*Sparus aurata* L.)
(http://ec.europa.eu/fisheries/marine_species/farmed_fish_)

Due to euryhaline and eurythermal nature of this species, it is usually farmed in an extensive system in coastal lagoons and ponds, until 1980s when intensive farming systems were developed. Around 1981-82, genetic modification was successfully carried out leading to massive production. This fish species added largely to aquaculture production in the Mediterranean region due its high adaptability to intensive farming conditions which is capable of attaining high market value in just 18-24 months after hatching.

The production capacity of Sea bream farming industry is increasing in the last few decades like that of salmon farming industry. In 2014, the world aquaculture production of gilthead Sea bream is about 158,389 tonnes and in the EU, it is one of the three main farmed fish species after rainbow trout (*Onchorynchus mykiss*) and Atlantic salmon (*Salmon salar*) (FAO, 2014b).

Mediterranean countries are the major producers, Greece being the largest producer, with production capacity of (51.50%), seconded by Turkey (15.00%) and Spain (14.60%). Additionally, considerable production occurs in Cyprus, and other neighboring countries along the coast of Mediterranean Sea.

However, infections caused by pathogenic bacteria associated with seafood result in huge economic loss to the aquaculture industries (Balebona et al., 1998), and *V. parahaemolyticus* is among the pathogenic bacteria of public health interest that is frequently isolated from Sea bream (Kusuda et al., 1979; Li et al., 1999; Zorilla et al., 2003; Yildiz and Visick, 2009; Austin, 2010; Li et al., 2013).

It is therefore imperative to investigate this fish species for the occurrence of *V. parahaemolyticus* in order to meet local and international trade requirements.

1.3.2 European Sea bass (*Dicentrarchus labrax*)

European Sea bass (*Dicentrarchus labrax*) (Turkish name 'Levrek') is a marine fish species from Moronidae family. It is found mostly in and around Mediterranean regions up to Northeastern Atlantic Ocean (through Norway to Senegal), and also in the Black Sea coasts. European Sea bass is abundantly distributed in coastal waters, lagoons, estuaries and rivers.

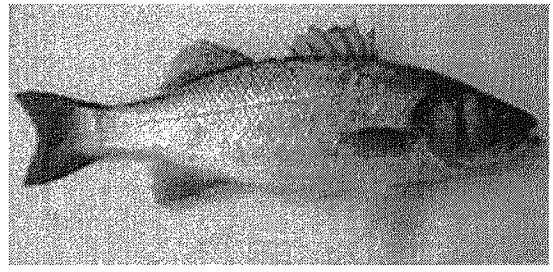

Figure 1.3: European Sea bass (*Dicentrarchus labrax*) (http://www.fishbase.org/summary/63)

European Sea bass was named *Dicentrarchus* because of the presence of two dorsal fins (Figure 1.3). Morphologically, it possesses silver sides and white belly, sterrated and

spinned operculum, can be as long as 1m in length and 15kg in weight (Froese et al., 2006).

The European Sea bass were traditionally farmed in coastal lagoons and tidal reservoirs before the need to develop mass-production of juveniles started in the 1960s. It was during this time, France and Italy developed reliable mass-production techniques for this fish species and by the late 1970s, these techniques reached most of the Mediterranean countries. The European Sea bass became the first cultured non-salmonid species in Europe and it is widely cultured in most Mediterranean regions, with Greece, Turkey, Italy, and Spain as major producers' followed by Croatia and Egypt, and considerable productions in other Mediterranean countries (FAO, 2016b).

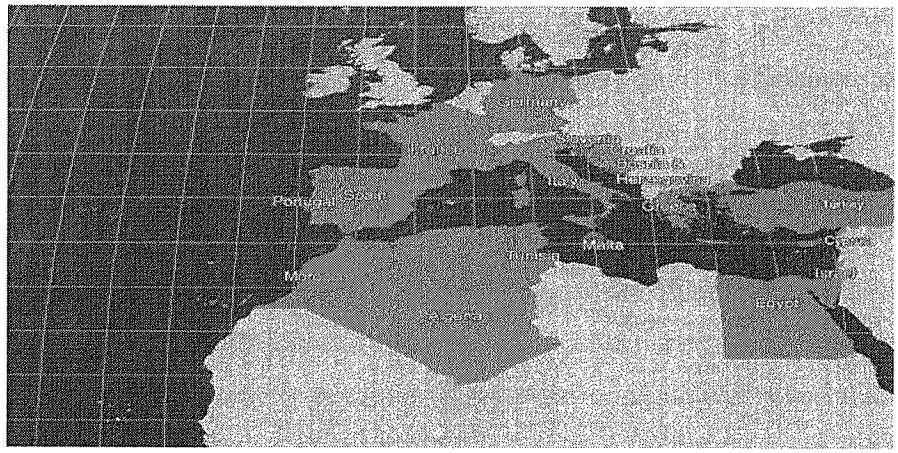

Figure 1.4: Main producer countries of *Dicentrarchus labrax* (FAO Fishery Statistics 2006)

1.4 Historical Background and Classification of *Vibrios*

The microorganisms of genus *Vibrio* derived their names from Italian scientist Filippo Pacini (1854) who first isolated them in clinical specimens from cholera patients in Florence, Italy. However, his findings were not widely considered due to the prevalence of non-pathogenic *Vibrios* in the environment (Adams and Moss, 2000). Eventually, Robert Koch (1843-1910) established the cause and effect relationship between *V. cholerae* and outbreak of cholera (Adams and Moss, 2000).

Another historic backup for the occurrence of vibrios is the isolation and identification of *V. cholerae* biotypes by Gotschlich in 1906 at the El Tor quarantine station for pilgrims in

the city of Sinai, Egypt. This is responsible for the seventh pandemic of *V. cholerae* throughout the world (Adams and Moss, 2000).

Vibrios and other members of the same family (*Vibrionaceae*) *Aeromonas, Campylobacter, Helicobacter,* and *Plesiomonas* species are gram-negative rods that are widely found in nature. The *vibrios* are dominantly found in marine and surface waters (Jawetz et al., 1995). Their cellular arrangements may be linked end to end producing S shapes and spirals. They used single polar-flagellum for movements, classified as oxidase-positive, non-spore-formers and withstand both aerobic and anaerobic conditions (Nafees et al., 2010). They are also known to metabolize through fermentation (Michael and John, 2006). Mckane and Kandel (1996) described Vibrios as comma-shaped bacilli that are responsible for the frequent and deadly epidemics of gastrointestinal diseases all over the world especially in developing countries.

Different species of vibrio (Table 1.1) (see Appendix 1) have been named as agent of diseases, causing different health irregularities such as cholera, gastrointestinal problems, wound and ear infections and septicemia. In Japan, about 50-70% of the first foodborne gastroenteritis outbreak has been linked to enteropathogenic *V. parahaemolyticus*. *V. fluvialis* has been randomly isolated from various cases of diarrhea especially in warm countries. *V. vulnificus* causes severe extra-intestinal infections such as septicemia often without diarrhoea. This normally occurs on disease-suffering individuals who ate seafood, particularly shellfish (Adams and Moss, 2000).

All vibrios species, with exception of *V. cholerae* and *V. mimicus* require sodium chloride (NaCl) media for their growth (Drake et al., 2007). The optimal growth of enteropathogenic *Vibrios* is around 37°C and the general temperature range is between 5-43°C. Despite, approximately 10°C is considered minimum in natural habitats. In favorable conditions *Vibrios* can multiply rapidly in generation times of as little as 11min and 9min for *Vibrio parahaemolyticus* and other non-pathogenic marine *Vibrios* such as *V. natringens* respectively (Adams and Moss, 2000). The minimum *aw*, for growth of *V. parahaemolyticus* varies between 0.937 and 0.986 depending on the solute used.

There are about sixty five (65) species in the genus *vibrio*; fortunately, twelve (12) are regarded as disease-causing to humans (Nair et al., 2006). These include *V. cholerae*, *V. mimicus*, *V. parahaemolyticus*, *V. alginolyticus*, *V. cincinnatiensis*, *V. hollisae*, *V. vulnificus*, *V. furnissii*, *V. fluvialis*, *V. damsela*, *V. metshnikovii*, and *V. carchariae* (Drake et al., 2007). However, eight (8) species are usually observed in food (Oliver and Kaper,

2007). Some Vibrio species and their associated infections are given in Table 1.1 (see Appendix 1).

Nonetheless, among all the extant species of the genus *Vibrio*, only three species including *V. cholerae*, and other two non-cholera Vibrios (*V. parahaemolyticus*, and *V. vulnificus*) are the most significant and responsible for epidemic associated with food (DePaola et al., 2010).

1.4.1 Factors affecting growth and biogenesis of *Vibrios*

Many factors influence the growth and biogenesis of *Vibrios* either singly or in combination. Among these factors include:

I. Temperature: Water temperature can greatly influence the availability of *Vibrios* in seafood. *Vibrios* can grow rapidly between 20 and 40°C. Optimum temperature (37°C) can increase the rate of growth and generation times of 9 to 10 minutes have been found (ICMFS, 1996a). The minimum and maximum growth temperatures of these organisms range from 5°C to 43°C respectively (Adams and Moss, 2000). All *Vibrios* are heat-sensitive. In seafood especially shellfish, heating to internal temperature of at least 60°C for some minutes is sufficient to destroy the pathogenic *vibrios* (Dalsgaard et al., 2001). Lower temperatures can critically control or prevent the growth of *Vibrios*. It is well documented that *V. parahaemolyticus* is positively correlated with increased in temperature (Mudoh et al., 2014). Accordingly, one study indicated that *V. parahaemolyticus* can survive at higher temperatures of between 15 to 44°C and died at -20 to 10°C (Boonyawantang et al., 2012).

II. Effect of pH and other factors: All *Vibrios* can survive in acidic condition, yet grow best at pH values slightly above neutrality, i.e. 7.5 to 8.5. They can also survive in drying condition. More strongly, *V. parahaemolyticus* has an absolute Na+ ion requirement and grows optimally at about 2 to 4% NaCl. Freshwater incapacitates this organism (Adams and Moss, 2000).

1.5 Control of *Vibrios* in Seafood

As already been discussed in the literature, seafood support the economies of various countries besides its role in nutrition. Despite, seafood may contain a number of

pathogenic microorganisms either from aquatic environment such as *Vibrios*, *Aeromonas* or from the general environment after catch such as *C. botulinum* and *L. monocytogenes.*

The environments where seafood lived also determined the type of pathogenic bacteria they contain and the hazards encountered. The pathogenic bacteria can be found on both live and raw fish material. Some of the common pathogenic bacteria associated with seafood include *Vibrio spp.*, *Aeromonas*, and *Clostridium botulinum* type E (naturally found in aquatic environment) and *Salmonella* spp., *Listeria monocytogenes*, *C. perfringens* and *C. botulinum* type A and B (present in the general environment). Although, the occurrence of later organisms does not draw much attention since they occur in numbers insignificant to cause disease, but accumulation of large numbers of *Vibrio spp.* in filter-feeding mollusks poses public concern especially when they are consumed in raw form (Huss, et al 2000). The Minimum Infective Dose (MID) of these pathogenic bacteria is almost ($>10^5$-10^6 cells) (Twedt, 1989).

Vibrios are among the inherent pathogens in seafood causing many outbreaks, a lot of control measures should be put in place to eliminate or reduce these pathogens from seafood. (Huss, et al 2000) suggested that monitoring seafood raw material on-board fishing containers should be included in seafood safety preventive control programs.

In general, control of pathogenic microorganisms in seafood varied across the types of seafood, shellfish accommodate more pathogens than finfish. Among the shellfish molluscan bivalve are the major concern, for example the European Union Regulations have established guidelines with respect to control of live bivalve mollusks. This is based on classifying growing waters and examining the faecal contamination, test for *Salmonella* and toxic algae in the final product. Nonetheless, there is still doubt on the effectiveness of controlling indigenous pathogenic bacteria in raw or lightly steamed seafood (EU Regulation, 1991 as cited in Huss, 1997).

Nowadays, various emerging technologies can be used to reduce, suppress, or destroy pathogenic *vibrios* in seafood without changing the organoleptic and sensory properties of the product. Technologies like high pressure preservation, preservation with natural compounds of plant origin, phage lysis and irradiation were found effective in controlling pathogenic *vibrios* in seafood (Ronholm et al., 2016).

It is well documented that *Vibrios* spp. are sensitive to irradiation. Many irradiation processes can destroy *Vibrios* and prevent decontamination of seafood. Because of their sensitivity to radiation, 1 kGy dose may destroy them in raw seafood (IAEA, 2001).

A number of studies reported that ionizing radiation can effectively decontaminate fish and seafood from life-threatening pathogens. Doses of 1.0-2.0 kGy can completely eliminate *V. parahaemolyticus* from seafood without damaging the products (Matches and Liston, 1971; Molins et al., 2001).

The response of *V. parahaemolyticus* to ionizing radiation was examined in alkaline phosphate saline and frozen shrimp homogenate. The D_{10} values were found to be 0.03 to 0.05 kGy and 0.04 to 0.06 kGy respectively. The study indicated that 0.90 kGy would be enough to decontaminate the frozen shrimp from all pathogenic bacteria without changing the nutritional quality and sensory attributes (Bandekar et al., 1987). The D_{10} value of *V. parahaemolyticus* was further reaffirmed by Ito and others (1989) to be 0.03 kGy in NaCl+ 0.067 M phosphate buffer, while the equivalent value in raw and cooked shrimp was 0.38 kGy.

Other studies conducted by Rashid et al (1992) and Ito et al (1993) reported that 3.0 kGy and 3.50 kGy doses can reduce the numbers of *Vibrionaceae* and *Listeria monocytogenes/Salmonella* spp. respectively from frozen shrimp. *V. cholerae* and *V. vulnificus* can be completely eliminated from crabmeat at doses of 1.0 kGy and 0.35 kGy respectively (Grodner and Hinton, 1986 and Grodner and Watson, 1990).

Additionally, from farm to fork, the control of *Vibrios* and other pathogenic bacteria associated with seafood can be achieved by effective and efficient adoption of Good Aquaculture Practices (GAPs), Good Manufacturing Practices (GMPs) and Hazard Analysis and Critical Control Points (HACCP) food safety programmes.

Recently, food industry, organization of producers, governments and Non-governmental organizations (NGOs) have collectively developed GAP codes, standards and regulations aimed at codify agricultural practices at farm level. The objectives include realization of trade and regulatory requirements (food safety and quality), capturing new market demands, improving natural resources utilization and many more (FAO, 2008).

In Turkey, Fisheries Regulation No 22223 is concerned with legislation pertaining food safety issues in fisheries and aquaculture. It entails procurement of operating licenses by the firm, sanitary requirements of facilities, technical requirements for the processing of fresh seafood, frozen fishery products and processed seafood products and characteristics of fresh seafood intended for human consumption (FAO/Turkey, 2016).

While HACCP-based safety programmes are routinely implemented in the manufacture of seafood products, the practice of such programmes at farm levels is at an early stage.

Although, not only seafood sector and few animal husbandry sectors were lag behind in terms of efficient implementation of HACCP-based food safety programmes at farm levels, judiciously attributed to inadequate scientific data pertaining the quality of on-farm control of pathogenic microorganisms (FAO, 1998). The introduction of HACCP-based food safety programmes from farm levels to point of consumption might reduce the risk of pathogenic *Vibrios*.

Moreover, indigenous bacteria can be controlled by the application of probiotic technology particularly in aquaculture production system. Selected bacterial species can be introduced to change the microbial composition of the growing waters. Probiotic strains of *Bacillus* species could be added into water bodies to displace pathogenic *Vibrios* (David, 1999).

Eradicating these bacteria from seafood is somehow not possible, though strategies could be developed in favor of the growth of some and inhibits others through optimizing the presence of probiotics and other potential vectors. Additionally, tools that may reduce the number of *Vibrios* at any stages of seafood production could be useful in reducing the occurrence of these pathogens in seafood.

CHAPTER 2
THEORETICAL FRAMEWORK

2.1 Significance of Microbiological Investigations

Investigation of microbial pathogens in food is recognised 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). This depends on the commitments of the authorities concern along the food chain (Jasson et al., 2010). Qualitative analysis is usually performed for the detection of pathogenic *Vibrios* (Denovan and Netten, 1995). Although, quantitative analysis can also be performed rarely (Kaysner et al., 1989; Cook et al., 2002; Su and Liu, 2007; Blanco-Abad et al., 2009). Moreover, European Commission Regulation acknowledged that epidemiological studies should be performed based on standard culture techniques for isolating pathogens in foods (EC 2073/2005).

Seafood (fish and shellfish), like other animals accommodate various types and number of pathogenic microorganisms, and the quantities differ in various parts of the body. In fish, gills and intestines are the resting place of pathogenic *Vibrios* (Cahill, 1990). Fish used gills for the movement of water in and out of their bodies, as a result; gills accommodate large quantities of foreign matters including bacteria. When the conditions are favorable for these bacteria, they grow and inhabit gills (Horsley, 1973).

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). When the fish die,

the bacteria that inhabit the gills and surface of the skin can penetrate into the inner parts such as intestine and contaminate them. All seafood contain certain doses of pathogenic bacteria and the prevalence of these pathogens is influenced by a number of extrinsic factors such as geographical zone, time of storage, and temperature fluctuations in the course of handling (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 *V. parahaemolyticus* to doses even higher than those obtained from the surrounding water (Yeung and Boor, 2004).

2.2 *Vibrio parahaemolyticus*

V. parahaemolyticus (Figure 2.2) is a human enteropathogenic, sucrose non-fermenting, facultative and halophilic bacterium that is widely distributed in both marine and estuarine habitats, and in seafood harvested from aquatic environments worldwide (Odeyemi, 2016). This marine-based enteropathogenic bacterium is responsible for the majority of seafood-borne bacterial illnesses leading to gastrointestinal problems (Su and Liu, 2007). The bacterium can be characterized by its high genetic diversity which, sometimes made the strain relatedness and epidemiological isolation complicated (Lüdeke et al., 2015). This is solely due to high rate of genetic transformation (Gonzalez-Escalona et al., 2008). Pertaining research and epidemiological studies, *V. parahaemolyticus* are the most widely observed among cholera and non-cholera *Vibrios* in the United States (Levine and Patricia, 1993), and isolates are often characterized for their unique virulence genes, ribotypes, serotypes and response to Pulsed-Field Gel Electrophoresis (Broberg et al., 2011; Jones et al., 2012; Banerjee et al., 2014 and Xu et al., 2015).

V. parahaemolyticus is generally less withstanding at higher temperatures, so also its numbers decline slowly at chill temperatures below its growth minimum and under frozen conditions a 2-log reduction has been observed after 8 days at – 18 °C (Adams and Moss, 2000).

V. parahaemolyticus is largely found in coastal inshore waters rather than the open sea. It is infrequently isolated from water with temperatures below 15°C (Adams and Moss, 2000 and ICMSF, 1996b).

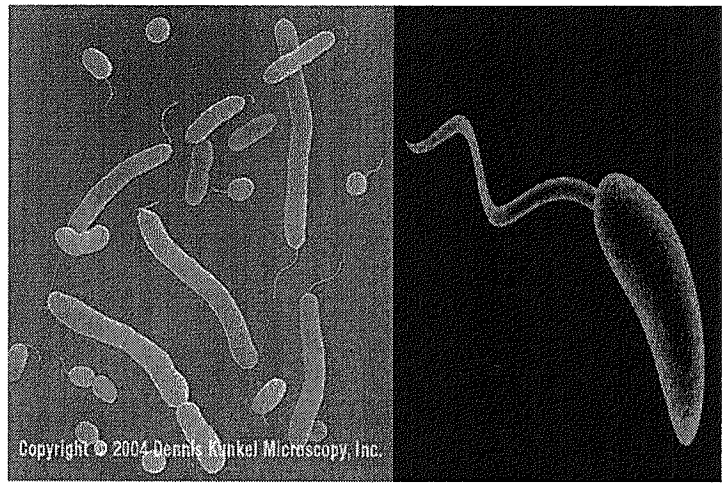

Figure 2.1: Images of *V. parahaemolyticus*
(https://kswfoodworld.wordpress.com)

Various studies revealed different D-values for *V. parahaemolyticus,* for example in a study with clam slurry, the D49 of *V. parahaemolyticus* is 0.7 min whilst it is 5 min in peptone water (3%NaCl) at 60°C with 4-5 log reductions. Pre-growth of *V. parahaemolyticus* in salt media enables the organism to increase heat resistance (Adams and Moss, 2000).

In terms of pH conditions, *V. parahaemolyticus* grows best at pH range slightly above neutral point (7.5-8.5). This unique property of V. *parahaemolyticus* is used as the basis for their isolation, although some growth has been detected at 4.5-5.0 (Adams and Moss, 2000). Table 2.1 contains the characteristics for the growth/survival of *Vibrio parahaemolyticus* (Appendix 2).

2.2.1 Classification of *V. parahaemolyticus* strains

Iniatially, *V. parahaemolyticus* starains has been classified based on antigens present in their cells (serotype) (Drake et al., 2007). Presently, more than 20 serovariants were available, these include; O3:K6, O4:K68, O1:K25 and O1:KUT (Nair et al., 2007). However, the present-day classifications focused on the presence of specific genes, and such particular genes determined the pathogenicity of *V. parahaemolyticus*.

Thus, for general species characterization, thermolabile hemolysin (*tlh*) can be applied. The presence of thermostable direct hemolysin (*tdh*) and/or TDH-related hemolysin (*trh*) genes in *V. parahaemolyticus* strains signifies that particular strain is pathogenic (Drake et

al., 2007). These genes (*tdh* and/or *trh*) and their relationship to pathogenicity are summarised in subsection below.

2.2.2 Pathogenicity of *V. parahaemolyticus*

Pathogenicity of *V. parahaemolyticus* depends on their hemolytic reaction on Wagatsuma agar, usually referred to as Kanagawa Phenomenon (KP). As a result, Kanagawa Phenomenon is used as a scientific frame for measuring the pathogenicity of *V. parahaemolyticus* (Honda and Iida, 1993). In fact majority of the virulence factors are seen to take part in the pathogenicity of *V. parahaemolyticus*. Among the virulence factors that are susceptible to cause disease include those associated with beta-hemolysis, various enzymes and the product of the *tdh*, *trh* and *ure* genes (Drake et al., 2007).

Nonetheless, some strains of *V. parahaemolyticus* are not pathogenic. Most often the clinical isolates are KP-positive (produce either TDH or TRH genes) meanwhile very little (1% to 2%) of the environmental isolates are KP-positive (Sakazaki et al., 1968; Miyamoto et al., 1969; Nashibuchi and Kaper, 1995).

Eventually, it was discovered that the thermostable direct hemolysin (TDH) protein is related to Kanagawa Phenomenon (KP) (Nashibuchi and Kaper, 1995), and it was named TDH because it withstand high temperature (100°C for 10 min) and because addition of lecithin does not affect its activity on erythrocytes (Sakurai et al., 1973; Nashibuchi and Kaper, 1995).

The first cloning of the TDH protein encoded gene from *V. parahaemolyticus* WP1, was conducted by Kaper and colleguages (1984) which was designated as *tdh*1. They subsequently applied the probes derived from this gene to detect tdh genes in other *V. parahaemolyticus* strains.

The following years Hida and Yamamota (1990) observed that *V. parahaemolyticus* strain WP1 contained another different *tdh* gene, so named *tdh*2. This was suppoted by a survey conducted by Nashibuchi and Kaper (1990) suggesting that all KP-positive (the clinical isolates) of *V. parahaemolyticus* possess 2 *tdh* genes while others (clinical and environmental isolates) that show weak response on wagatsuma agar (KP-intermediate) have only 1 *tdh* gene. By looking at the KP-negetive strains (mostly environmental isolates), it was discovered that only 16% contained 1 copy of the *tdh* gene, others are believed to have no *tdh* gene implying that TDH protein cannot be produce by KP-negetive strains (Nashibuchi et al 1985; Nashibuchi and Kaper, 1995).

Oftenly, some strains of other *Vibrios* including *V. cholerae non-O1*, *V. hollisae* and *V. mimicus* are said to contained the *tdh* gene (Nashibuchi and Kaper, 1995).

Irrespective of the role play by Kanagawa factor and TDH protein in *V. parahaemolyticus* infections, some outbreaks of gastroenteritis have been linked to KP-negetive strains of *V. parahaemolyticus*. For instance, Honda and colleagues (1987, 1988) showed that KP-negetive produced similar but somehow different type of TDH protein so-called TDH-related hemolysin (TRH) which was initially observed in O3:K6.

Additionally, TRH which is usually associated with environmental isolates was found to have adverse effects in the tested mouse (Sarkar et al., 1987). There is almost 69% similarity which shows that *trh* genes resemble the *tdh* genes in the nucleotide sequence indicating that they are from the same ancestor (Honda et al., 1987; Nashibuchi et al., 1989).

Furthermore, there is strong evidence indicating various forms of *trh* gene among some vibrios that vary in their nucleotide sequence and hemolytic activity and they equally share common ancestor (Kishishita et al., 1992).

It is well documented that both the *tdh* and *trh* genes are present in some clinical isolates, meanwhile most of the environmental isolates do not have the *tdh* and *trh* genes (Xu et al., 1994).

More recently, the CDC noted that many cases of *V. parahaemolyticus* infection are due to *V. parahaemolyticus* strains lacking any of the *tdh* and/or *trh* genes (Yu et al., 2006).

Studies indicated that adhesiveness plays a significant role in *V. parahaemolyticus* pathogenicity. For example, Hackney and colleagues (1980) revealed that all the tested clinical and environmental strains of *V. parahaemolyticus* were capable of adhering to HFI (human fetal intestinal) cells, although there is variability in the degree of adherence.

Regardless of their Kanagawa reaction, *V. parahaemolyticus* strains isolated from patients were found to have high adherence capacity compared to Kanagawa-negetive strains isolated from seafood which exhibited weak adherence. Accordingly, it was noted that the ability of *V. parahaemolyticus* clinical isolates to adhere to human intestinal mucosa is a function of hemagglutinin levels in human or erythrocytes in guinea pig (Yamamoto and Yakota, 1989).

Several enzymes were found to contribute to pathogenicity of *V. parahaemolyticus*. For instance, Baffone and colleagues (2001) tested various enzymatic (gelatinase, lipase and hemolysin), biological (cytotoxicity, enterotoxicity and adhesiveness) and

enteropathogenic activities of *V. parahaemolyticus* isolated from seawater. They concluded that all the strains had gelatinase and lipase activity. They also revealed that 80% and 90% had adhesive and cytotoxicity activities respectively.

For the previous few decades, urea hydrolysis has been used as a basis to measure the pathogenicity of *V. parahaemolyticus* strains. Findings from Abbot and others (1989) was the basis of this phenomenon. Briefly, it was found that urease-positive phenotype is linked to *V. parahaemolyticus* of O4:K12 serotype. Accordingly, Kaysner and others (1994) noted that *tdh*-positive isolates (clinical and environmental) were also urease-positive, correspondingly, Osawa and coworkers (1996) found that all clinical and environmental strains with *trh* gene were urease-positive.

Similarly, Iida and coworkers (1997) reported that urease production in *V. parahaemolyticus* was due to the presence of *ure* gene and as such *ure* and *trh* genes are related genetically as shown by restriction endonuclease digestion. Subsequent research by Lida and colleagues (1998) highlighted that there is close proximity among *tdh*, *trh* and *ure* genes on the chromosome of potentially pathogenic *V. parahaemolyticus*.

It was reported that consumption of raw or undercooked seafood that has been contaminated (at 10^7-10^8 CFU) of this organism may cause acute gastroenteritis with subsequent clinical manifestations such headaches, diarrhoea, vomiting, nausea, abdominal cramps and sometimes low fever (Yeung and Boor, 2004).

2.2.3 Maximum infective dose

V. parahaemolyticus is among most widely known non-cholera *Vibrios* implicated in food poisoning in the world. FAO recommended that organism of *V. parahaemolyticus* should be more 10^6 CFU/g to cause disease (FAO, 2002b). Hence, seafood containing 10^7-10^8 CFU/g can cause severe gastroenteritis with diarrhoea, abdominal cramps, nausea, vomiting, headaches and sometimes fever. Accordingly, the number of virulence factors and dose of *V. parahaemolyticus* determined the possibility of occurrence and intensity of gastroenteritis (Zhang and Austin, 2005).

Additionally, *V. parahaemolyticus* can cause wound infection to individuals exposed to polluted waters. Although, the number of this organism which can cause disease is high enough (10^7-10^8 CFU), its short generation time (less than 20min) enables it to increase rapidly at ambient temperatures thereby forming maximum infective dose within short intervals (FAO, 2002a).

2.3 Seafood Sampling and Sample Processing

Sampling is the cornerstone of any analysis. In microbiological investigations, the adequacy and condition of the sample are of paramount importance. Accordingly, the laboratory results will be valueless if samples are not systematically collected or could not represent the sampled lot.

Establishing sampling procedures must be uniformly applied to allow general interpretations on a large group of foods based on relatively small sample from the lot. Sampling procedures should be designed in a logical and coherent manner to provide the basis for valid results for the sample lot and/or the consignment (FDA/BAM, 2003). Samples should be taken independently and randomly. A number of factors should be considered in designing a good sampling plan; these include nature of the food, production processes, storage conditions, associated risks, targeted consumers and practical limitations (CFS, 2014). A comprehensive sampling plan should consider the following subjects:

1. The microbe or group of microbes in question.
2. Number of samples to be taken (n).
3. Method(s) of investigation.
4. Microbiological limit(s), c, m and M. Refer to Table 2.2 for more information (see Appendix 3).
    - Acceptable ($\leq$ m).
    - Marginally acceptable ($>$ m and $\leq$ M).
    - Unacceptable ($>$ M).
5. Number of samples which fall into each category of microbiological limit (i.e acceptable, marginally acceptable or unacceptable) (CFS, 2014).

To allow or ensure transparency and confidence in the sample collection, the food business operator should be involved. Information and rights of all the parties concern in ensuring food safety (food analysist, food business operator and food standards Authority) should be included in the final report (FSSI, 2012). Sampling can be done for many purposes; these include monitoring, surveying and checking the compliance with legislation (Reg. EC No 2073/2005). Several obligations were set down by regional and international regulatory authorities for food business operators to ensure that microbiological criteria are met. This will help establish efficient and effective traceability systems (EC 2073/2005; CAC/GL,

2008) and in ensuring the natural habitat and individuals involved in the food chain are protected (Denovan and van Netten, 1995).

Proper sampling, weighing and measurements of reagents and diluents should be correctly performed. Inefficient sample homogenization, unnecessary delay during analysis, and variations in media preparation and formulation, incubation temperature, atmosphere should be taken care of in order to minimize errors (Jarvis et al., 2007).

2.3.1 Sample size

Sample size is of paramount important as it determine the number of representative samples to be taken from the lot. ICMSF has recommended five (5) units per lot of fresh and frozen and cold-smoked finfish for *V. parahaemolyticus* investigations (ICMSF, 1986). A "lot" of seafood is a shipment or part of shipment of fresh fish produced and processed by the same producer in a period of one day. Representative sample is the one in which the units selected for analysis exhibit all the properties of the lot in an appropriate manner. Five (5) sample units of finfish (approximately 250g per unit) can be drawn in one lot size (CFIA, 2013). The procured sample should be carefully divided into three parts (representative portions), then labelled and sealed as quickly as possible to ensure clear and easy sample identification. However, if it is not possible to uniformly mix the samples from the three representative sample containers, then it is advisable to take one for analysis (FSSI, 2012).

2.3.2 Primary sample

Primary sample refers to the first portion of seafood generated from a lot in the initial stage of sampling. The primary sample should be drawn from the entire parts of the lot; any deviation should be taken care of. The samples should be sufficient enough to conduct laboratory analysis. Relevant procedures and precautions must be followed to keep the homogeneity and integrity of the samples such that laboratory samples fully represent the primary sample taken from the lot.

2.3.3 Composite sample preparation

This could be obtained by mixing the primary samples from the lot.

2.3.4 Laboratory sample preparation

All containers and equipment will be sterilized thoroughly before they can be used for sample preparation. The sample should be comminuted homogenously to obtain true representative analytical portion for liquids or semi-solid, if the sample is solid the analytical unit can be obtained from different parts within the representative unit (Kiiyukia, 2003). The sample should be measured separately in triplicate (25g each), dissolved, blended and homogenized in alkaline peptone water.

2.3.5 Final sample

The bulk or bulked sample should appropriately form the final sample ready for analysis. However, when it is not possible to analyze the bulk or bulked sample, the final sample may be extracted from it through appropriate 'Reduction Method' (FSSI, 2012).

Sample reduction can be done by dividing the sample into four equal parts (quartering) such that each part may represent the initial sample and can therefore be used for microbial analysis (FAO, 2012).

2.3.6 Sampling equipment

Equipment, materials and containers suitable for keeping the sample condition must be used when obtaining samples. Cleaning and sterilizing methods that may result in accumulation of residues on the equipment should be avoided, as it may affect the results. The sample meant for analysis must be taken in clean, portable and inert container capable of preventing subsequent damage, leakage or contamination during transportation. The container should be appropriately sealed, sampling document must be attached and the sample transported to the laboratory as quickly as possible. In addition, the container should have temper resistant closures and seals (FSSI, 2012). Some of the approved materials and apparatus for sampling include plastic bags, clean, hard-sided cooler and Ice packs, utility knife, hand towels, and hand coverings (CFIA, 2013).

2.3.7 Handling of the sample

Since all seafood samples must accurately meet the bacteriological conditions during sampling, it is imperative that analysis of samples is carried out in a short time following samples arrival; otherwise the samples must be stored in suitable temperatures that can

maintain the original flora without decreasing or increasing the number due to death or new population generation. This can be done in one of two ways:

i. Chilling: Samples intended for use in short periods of time may be stored at 0°C (32°F) by placing the sample containers in melting Ice.

ii. Freezing: This method can be employed for some reasons (example long distance from sampling area to laboratory) which may prevent the samples from being analysed within the possible time frame (say 8hrs). Care has to be taken because freezing can diminish the original bacterial flora or reduce the viability of the bacteria in the samples if the samples are stored under protracted conditions (Bonnell, 1994).

2.3.8 Sample storage

Clean, dry, leak-proof, wide-mouthed, sterile and portable containers can be used for sample storage. The containers must be clearly labelled with a marked strip of masking tape or etiquette to avoid confusion. Initial storage conditions of the samples should be maintained as appropriately as possible to nurture the microbial flora during the course of transportation. Rapid cooling destroys *Vibrios* and may results into false negative outcomes, but ambient temperatures favor the growth of *Vibrios* in seafood. *Vibrios* do not thrive or withstand extreme temperatures (heat and cold); storage of seafood under mild refrigeration is a best practice that enhances their survival. The procured samples should be aseptically collected, cooled (7-10°C) and analysed as quickly as possible and also storage under high temperatures is not encouraged, since *Vibrios* can grow significantly at ambient temperatures which may eventually change the initial content of microbial flora of the food (FDA/BAM, 2003).

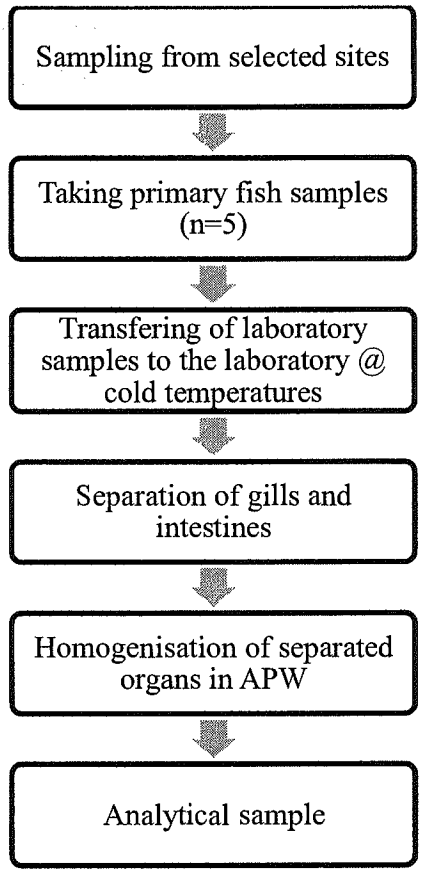

Figure 2.2: Sampling and preparation of analytical samples for the *Vibrio parahaemolyticus* investigation in fish

2.4 Conventional Culture Method

Microbiological methods that involved cultivation, isolation and serotyping of microbes gained more importance and are routinely used due to their effectiveness, sensitivity, reproducibility, ease of use and low cost (Gracias and McKillip, 2004). Culture method has been traditionally utilized for detection and enumeration of pathogenic bacteria for the past decades. The method is based on the growth and isolation of bacteria on selective culture media followed by standard biochemical tests for serological differentiation of the individual species (Kong et al., 2002; Amin and Salem, 2012).

Basically, culture method involved growing, isolating, and enumerating microorganisms of interest, while preventing the growth of unintended microorganisms by using appropriate culture media (López-Campos et al., 2012). Cultural detection methods are largely used for

qualitative analyses of pathogenic microbes, usually in 25g of food sample extracted out of small or large samples of food (Jasson et al., 2010).

Most of control laboratories are established under standardized methods, usually developed by the ISO protocols. These standardized methods are nothing but culture methods that involve growing, isolating and identifying target pathogens on appropriate culture media while inhibiting the growth of other indigenous microbial flora in the food. More interesting characteristics of these methods are the culture media and materials are abundantly found from a number of suppliers and the techniques are sufficiently described (Jasson et al., 2010).

Although molecular detection methods are faster, they are often hampered by limitations such as similarities in inter species 16Sr RNA sequence and occurrence of multiple copies of 16Sr RNA gene (Shikongo-Nambatorbi et al., 2012). Culture method is the oldest, standard and remained the most accurate and reliable technique for isolation, detection and identification of pathogenic microorganisms in foods including *Vibrios* (Lazcka et al., 2007; Velusamy et al., 2010).

Vibrios and many other Gram-negative bacteria can be grown under relatively high levels of bile salts condition. They are among facultative anaerobic microbes that grow best in high pH conditions. This phenomenon provides clue for their isolation in food samples, and the culture media used are prepared with compounds that have alkaline properties like NaCl. Various sort of enrichment media for the cultivation of *Vibrios* are available. Among these media, APW perform better and is widely accepted. Chemically, APW is made up of 10g peptone and 10g NaCl mixed in 1000ml distilled water. The pH level of approximately 8.5-9 and 3% NaCl (w/v) included in this medium favor the growth of Vibrio spp. while inhibiting other microbial flora present in the sample (Depaola and Kaysner, 2004; Letchumanan et al., 2014).

Alkaline peptone water is an enrichment medium chiefly used for the isolation of many species of *Vibrios* from food, water and clinical samples. Alkaline peptone water (APW) is a well nourished and enriched, cultured broth officially approved by the U.S. Food and Drug Administration (FDA), for the growth of all *Vibrios* including *V. parahaemolyticus* (Farmer et al., 2003; Depaola and Kaysner, 2004).

Thiosulphate citrate bile-salts sucrose (TCBS) agar has been strongly recommended for the enumeration of *Vibrio parahaemolyticus* (FDA/BAM 2001). It is a selective medium generally used for plating, propagating and isolating *Vibrios* from seafood (Elliot et al.,

1995 as cited in FDA/BAM, 2003; Blanco-Abad et al., 2009). Since its acceptance as a selective medium for the isolation of *Vibrios*, TCBS agar has been widely applied on food, water and clinical samples (Blanco-Abad et al., 2009). Typical composition of TCBS agar is presented in table 2.2 (FDA/BAM, 2003) (see Appendix 5). The medium acts by supporting the growth of *Vibrios* while inhibiting other non-vibrio species (Kobayashi, et al 1963 as cited in FDA/BAM 2003). It is known for its high selectivity for pathogenic *Vibrios* associated with seafood and environmental samples (Lotz et al., 1983). TCBS agar medium is superior to the newly developed culture media, thiosulphate chloride-iodide (TCI) for the isolation of *Vibrios* and it has, therefore been considered as the most effective selective medium for *Vibrio* spp. (Morris et al., 1979; Pfeffer and Oliver, 2003).

Although, conventional culture method is often associated with intensive labor and time consumption, scientists and researchers are continuously improving these methods for more accurate and reliable recoveries (Hara-kudo et al., 2001; Bisha, et al., 2012).

Additionally, a lot of improvements and automatization have been incorporated into conventional culture methods (Appendix 4) for the investigation of bacterial pathogens in foods. These include modifications and automation in sample preparation, plating techniques and test kits for enumeration and identification (De Boer and Beumer, 1999).

In the past decades, microbial colonies are counted manually without employing mechanical devices. Automatic colony-counters are now available and frequently used with the help of spiral platers, eliminating difficulty, slow and tedious nature of manual counting (Corry et al., 2007). There has been a progress in reducing inaccuracies from automatic counters as a result of coalescence of colonies and differences in colony size (Marotz et al., 2001), because of high percentage of counting errors in replicate manual counting (Fowler et al., 1978).

The time to detection could be reduced or at least the performance of the method could be improved by introducing little modifications to the classical methods. Nowadays, researchers developed interests towards reducing the time required for the preparation of enrichment broths by introducing one-step enrichment broths capable of preserving injured target cells and enabling rapid growth of isolatable numbers (Jasson et al, 2010). Various one-step enrichment broths are available in the market. Salt polymyxin broth (HyServe, Germany) is a typical example of one-step enrichment broths for isolation of *Vibrios* (Hara-kudo et al., 2001). In order to do away with cumbersome classical confirmation steps, numerous confirmation and identification kits were available. They are equipped

with miniaturized test tubes and dehydrated reagents which can be inoculated with presumptive cell colony. Various identification kits are available and can be in different formats, examples include BD Phoenix™, API® test kits, MicroLog™, and Vermicon Identification Technology kits (VIT®) (Jasson et al., 2010; Velusamy et al., 2010).

2.4.1 Confirmation

Automated microbiology system like BD Phoenix™ uses smart software to detect microbes without addition of reagents (Figure 2.3). BD Phoenix allows simultaneous identification, flexible data entry, reduced waste disposal, single or batch inoculation, and gives rapid and accurate results.

Thus, any modification or improvement in conventional culture method that may reduce labor and time of analysis can be regarded as rapid method (Mandal et al., 2011).

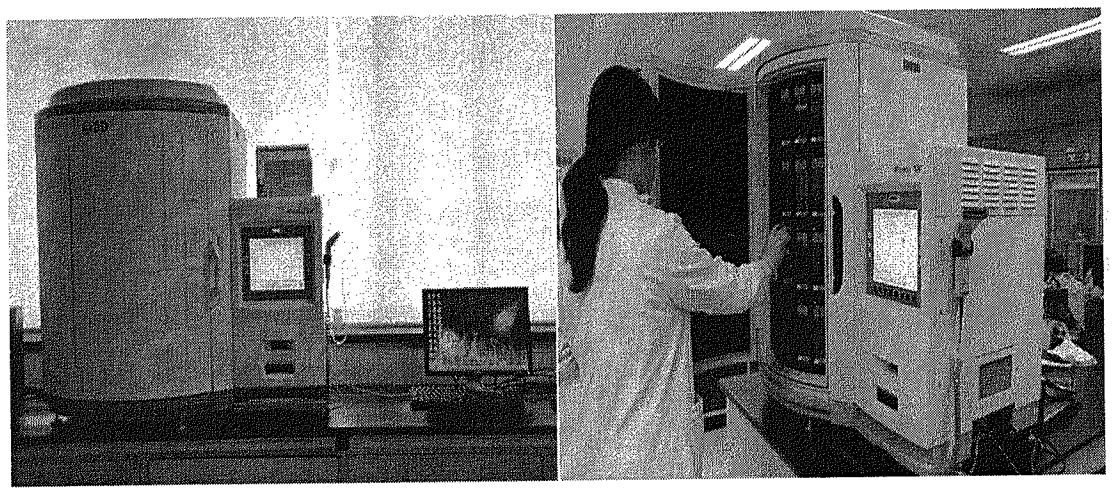

Figure 2.3: Automated BD Phoenix Instrument

CHAPTER 3

RELATED RESEARCH

This chapter provides information on similar studies carried out in different parts of the world by exploring the issues surrounding the prevalence and methods of detection and molecular characterization of pathogenic *Vibrios* particularly, *V. parahaemolyticus* in various seafood.

Various studies carried out on seafood in different parts of the world demonstrated the presence and contamination of these foods by pathogenic bacteria, predominantly *Vibrio species* (Colakoğlu et al., 2006; Adebayo-Tayo et al., 2011 and Francis et al., 2012).

V. parahaemolyticus is marine based bacterium and a leading seafoodborne pathogen causing severe clinical conditions mostly gastroenteritis coupled with mild to moderate diarrhea, nausea, vomiting, abdominal cramp, headache, chill and sometimes low fever (Chatterjee et al., 1970; Joseph et al., 1982). Although, few *V. parahaemolyticus* strains in seafood can cause disease (Drake et al., 2007). It is a leading cause of gastroenteritis associated with seafood consumption in the world (Letchumanan et al., 2014).

V. parahaemolyticus was originally discovered in 1950s by Tsunesaburo F. following shirasu (whitebit) outbreaks in Japan (Fujino et al., 1953). Presently, the illness occurs throughout the world (Daniels et al., 2000; Ansaruzzaman et al., 2005; Martinez-Urtaza et al., 2005; Fluenzalida et al., 2007; Iwamoto et al., 2010). Since then, *V. parahaemolyticus* is repeatedly isolated in seafood from different regions around the world (Martinez-Urtaza et al., 2005; Francis et al., 2012 and AbdElghany and Sallam, 2013).

With the emergence of highly virulent pandemic O3: K6 clone in 1996, the incidence of *V. parahaemolyticus*-associated infections has been increased (Okuda et al., 1997; Wong et al., 2000). This emerging serotype and its serovariants disseminated the aquatic environments and seafood in four different continents; Africa (Ansaruzzaman, et al 2005), Asia (Han et al 2016), America (DePaola et al., 2000) and Europe (Martinez-Urtaza et al., 2005) were resulting in several outbreaks. The outbreaks were mostly due to consumption of raw, undercooked or re-contaminated ready-to-eat seafood (Alam et al., 2003; DePaola et al., 2003; Tuyet et al., 2002; Wong et al., 2000; Martinez-Urtaza et al., 2005; Fluenzalida et al., 2007).

The continuing incidences of *V. parahaemolyticus* following the first outbreaks in 1950s is correlated with the frequent isolation of *V. parahaemolyticus* in foods resulting from 61 to

71% of the total outbreaks between 1996 and 1999 in Taiwan (Chiou et al., 2000). The incidence of V. *parahaemolyticus* in aquatic habitats is a function of many ecological factors. Seawater temperature and organic matter play a significant role for the survival of this organism in aquatic habitats. In japan, for example it accounts for about 20-30% of all food poisoning outbreaks (Alam et al., 2003).

V. parahaemolyticus strains were isolated following investigation of seafood-associated outbreaks in Chile during the summer of 2006. The outbreaks is the second of its kind that is connected with consumption of seafood carrying the pathogenic *V. parahaemolyticus* serovar O3:K6 pandemic clone. The outbreaks occurred unevenly between two geographical regions of the country. The outbreaks was reported in the summer of 1998 in Antofagasta (northern Chile), leaving some rare cases. The second and most frequent outbreaks was reported in 2004 at Puerto Montt (southern Chile). There was uneven occurrence of the outbreaks, large in Puerto Montt and rare in Antofagasta due to the differences in seawater temperatures between the two regions. For better understanding of the importance of seafood in the latter outbreaks, *V. parahaemolyticus* strains in clinical and shellfish samples from Puerto Montt were analysed following diarrheal outbreaks in 2006 and shellfish harvested from Antofagasta where there were no reported cases. *V. parahaemolyticus* was detected in 80% of the samples from Puerto Montt whereas no growth was detected in the samples from Antofagasta (Fuenzalida et al., 2007).

The increase in seafood-associated infections instigate the investigation of various seafood to ascertain the prevalence and levels of this organism and, when necessary, establish guidelines and control measures to protect seafood-consuming populace (Feldhusen, 2000; Adedeji et al., 2012).

The occurrence of *V. parahaemolyticus* in seafood has been documented in the southern part of Cyprus (Eleftheriadou et al., 2002). The investigated types of seafood carrying this medically important pathogen were frozen and raw shrimps and prawns. Standard culture method and ISO 891:1990 method were employed in this analysis, the presumptive isolates were subsequently taken for biochemical test and further confirmed by Analytical Profile Index (API) 20 E.

Jaksic et al (2002) used conventional culture technique in the investigation of pathogenic *Vibrios* associated with seafood. *V. parahaemolyticus* was the most prevalent among all human pathogenic *Vibrios* in the tested samples as revealed by the most probable number (MPN) quantitative method.

Microbiological quality of seafood has been assessed in the Adriatic Sea region of Croatia. Different varieties of seafood were investigated for the presence of pathogenic microbes including *Vibrios*. *V. parahaemolyticus* has been detected from the studied samples using standard culture method coupled with rapid detection test kit (API 20 NE) to facilitate the identification of the *Vibrio* isolates (Popovic et al., 2010).

Colakoğlu et al (2006) investigated the presence of some indigenous pathogenic bacteria of aquatic environs in various shellfish consumed in the Dardanelles coast of Turkey. Following traditional analysis by culture technique, a number of indigenous aquatic bacterial pathogens were isolated. Although, the occurrence of *V. parahaemolyticus* in this study was meager, other pathogenic *Vibrios* such as *V. alginolyticus* and *V. vulnificus* were significantly isolated.

Di pinto et al (2008) carried out research on the occurrence of pathogenic *V. parahaemolyticus* in shellfish harvested from the coastal regions of southern Italy. *V. parahaemolyticus* strains was isolated by conventional culture technique with confirmatory biochemical tests, followed by molecular (PCR) analysis indicating the presence of pathogenic TDH genes of *V. parahaemolyticus*. Eventhough the TRH gene was not detected in their study, the presence of *tdh* gene which is the major virulence factor, indicates safety concerns. Based on this study *V. parahaemolyticus* is regarded as dangerous organism seeking proper inspection to safeguard the health of shellfish consumers.

The distribution of *V. parahaemolyticus* in finfish and shellfish varieties has been reported. The bacterium occurs most commonly in shellfish than finfish. Effect of environmental condition on the distribution of this bacterium was also noted. Further, occurrences of *V. parahaemolyticus* in finfishes from different aquatic environs was analysed to provide insights on the unequal distribution of this bacterium from different sources (Das et al., 2009).

Additionally, incidences of pathogenic *Vibrios* were reported in freshly harvested seafood in Nigeria. Different species of finfish and shellfish were examined using standard microbiological techniques. The isolated *Vibrios* include *V. cholerae*, *V. mimicus*, *V. fluvialis* and *V .vulnificus*. The occurrence of these pathogens in seafood should not be neglected, raising the need for proper inspection at the very beginning of seafood harvest before allowance for human consumption (Adebayo-Tayo et al., 2011).

Salmonella and *Vibrios* species have been described as the most important microorganisms in terms of seafood-associated infections. Bakr and colleagues 2011 investigated the occurrence of *Salmonella* and some *Vibrios* in seafood marketed in the historic city of Alexandria, Egypt. TCBS was employed in the isolation of pathogenic *Vibrios*. *V. parahaemolyticus* was largely distributed and detected in the analysed seafood samples.

In another study, Francis et al (2012) investigated the prevalence and distribution of pathogenic *V. parahaemolyticus* in various finfish sold in Cochin, India. *V. parahaemolyticus* were identified from various organs of pelagic and demersal fish species by the standard conventional culture technique. The species specific thermolabile hemolysin (*tlh*) was confirmed by the polymerase chain reaction (PCR) method. This study revealed that there is high occurrence of *V. parahaemolyticus* on the skin and gills of pelagic fish, accordingly high strains of *V. parahaemolyticus* was isolated in the intestine of demersal fish. The authors mentioned that the occurrence of *V. parahaemolyticus* in finfish required control measures to avoid recontamination of seafood after harvest.

In their study, AbdElghany and Sallam (2013) examined the occurrence of potentially pathogenic *V. parahaemolyticus* in different shellfish sold in Mansoura, Egypt. The study is based on the detection of thermostable direct haemolysin (*tdh*) and *tdh* related (*trh*) virulent genes. Both conventional and molecular methods confirmed the presence of the target strains of *V. parahaemolyticus* although there was major variations between the methods. It was found that the tested samples of seafood (crab, cockle and shrimp) contain an adequate number of the target species with shrimp having the highest number of the verified pathogenic strains of *V. parahaemolyticus*. This study demonstrated that the analysed samples may be a threat to public health as they contained potentially pathogenic *V. parahaemolyticus* strains. The use of reliable molecular detection methods in the subsequent investigation of *V. parahaemolyticus* was also encouraged.

Alkaline peptone water (APW) broth was mostly used in the isolation of *V. parahaemolyticus* in seafood, but, advances in research showed that there are many other forms of enrichment broths that could be used to detect the pathogenic *V. parahaemolyticus* in seafood.

Sodium taurocholate (ST) broth, salt polymyxin broth (SPB), salt colistin broth (SCB), Alternative protein source (APS) broth and glucose salt teepol (GST) broth can replace APW broth (Bisha, et al 2012). In this regard, an alternative method for determining *V. parahaemolyticus* in seafood has been developed (Hara-kuda, et al 2000). The method is

based on enriching and plating of the homogenate in chromogenic agar. The study employed and tested two distinct media for *V. parahaemolyticus* isolation; the salt polymyxin broth (SPB) and salt tripticase soy (STS) broth, which are selective and non-selective media respectively. In essence, the method consists of two distinct steps; initial culturing of sample in STS broth followed by pouring the cultured STS onto SPB broth. The combine media yielded more growth of *V. parahaemolyticus* compared to SPB alone.

Raghunatath et al (2009) compared the modified enrichment broth containing bile salt and sodium taurocholate with traditional APW broth. The enrichment broth were compared following conventional isolation, colony hybridization and PCR. The authors revealed that the newly formed enrichment broth has higher efficiency over popularly used APW broth in terms of detection and isolation for the strains of pathogenic *V. parahaemolyticus* in seafood.

Hassan et al (2012) determined the frequency of occurrence of *V. parahaemolyticus* in various seafood marketed in some seafood outlets in The Netherlands by cultural and phenotypic detection techniques. The study is aimed at comparing the percent recoveries of two distinct selective culture media (TCBS agar and CHROMagr Vibrio (CV)) of the examined seafood samples. The study concluded that the two media showed no significant difference in terms of growth of *V. parahaemolyticus* in seafood samples.

Some studies emphasized on the importance of *V. parahaemolyticus* characterization following isolation and confirmation. Characterization is based on the presence or absence of pathogenic strains. The pathogenicity of *V. parahaemolyticus* is accessed by its hemolytic ability on wagatsuma agar (kanagawa phenomenon), urea hydrolysis, adherence factors (Drake et al 2007), and cytotoxicity on host body (Broberg et al., 2011; Zhen et al., 2014).

The thermostable direct hemolysin (*tdh*) and TDH related hemolysin (*trh*) genes were basically recognised as indicators of pathogenicity in *V. parahaemolyticus*, until recently two type III secretion systems designated as T3SS1 and T3SS2 were also discovered (Makino et al., 2003).

V. parahaemolyticus has been a major cause of seafood-borne diseases and understanding of its characteristic features could reduced an outbreak of seafood related diseases (Boonyawantang et al., 2012).

Vongxay et al (2008) investigated 216 *V. parahaemolyticus* isolates of seafood and clinical sources. The isolates were analysed for the presence of hemolytic and urea producing

phenotypes, and prominent virulence genes of *tdh* and *trh*. Pathogenetic strains of *V. parahaemolyticus* was tested for hemolytic activity, urease activity, adherence to Caco-2 cells and entheropathogenicity and cytotoxicity against test animals and cell lines.

Avşar and others (2016) investigated the frequency of *Vibrio* spp. of two fish varieties (anchovy and garfish) in the Sinop region of Turkey. Many *Vibrio* spp. including *V. parahaemolyticus* have been detected by conventional culture method and characterized by a series of morphological, physiological as well as biochemical examinations of the suspect isolates.

Several methods for detection of total and pathogenic strains of *V. parahaemolyticus* were available in the literature.

Raghunath et al (2008) used colony hybridization method to detect the total and *tdh*+ strains of *V. parahaemolyticus* in seafood. In this study, special set of seafood samples was used to evaluate the prevalence of *V. parahaemolyticus* in the selected seafood. The study was aimed at detecting the pandemic clones in different seafood samples.

Rosec et al (2009) compared the standard culture method with PCR protocols using pR72H and *tox*R and determined the total and pathogenic *V. parahaemolyticus* in various molluscan bivalve. The resultant primer pairs were compared based on *V. parahaemolyticus*-specific *toxR* gene, pR72H fragment as well as *tdh* and *trh* genes. Both methods were critically observed for their limits of detection (LOD). In *V. parahaemolyticus-toxR*, the LOD ranged from 7-24 pg of the purified DNA per reaction tube whereas pR72H depends on the *V. parahaemolyticus* strains used. The PCR protocols effectively identified thirty three (33) *V. parahaemolyticus* isolates in the samples , more than 3 *V. parahaemolyticus* isolates found with conventional culture method. PCR sequencing was applied to authenticate the presence of *V. parahaemolyticus* in the PCR prototocols (*toxR*) samples.

Khamesipour and others (2014) used culture method and PCR assay in the investigation of indigenous and non-indigenous bacterial pathogens in seafood. Samples of crayfish were analysed in this study. Vibrio species of concern in this research include: *V. vulnificus, V. alginolyticus*, *V. mimicus* and *V. harveyi*.

Malcolm and others (2015) investigated the quantity of potentially pathogenic *V. parahaemolyticus* in various shellfish using multiplex PCR and loop-mediated isothermal amplification assay (LAMP). Briefly, the multiplex PCR was used to detect the pathogenic genes (*tox R*+), (*tdh*+) *and* (*trh*+) in detected strains of *V. parahaemolyticus* while the

LAMP was used to detect pathogenic strains only. Significant number of the tested shellfish were positive for tdh+ whereas positive samples of trh+ were significantly low in terms of most probable number per gram (MPN/g). Although the key objective of this study was to compared the differences between these methods, detection of *tdh*+ across the sample shows no variation between the methods, however, in terms of *trh*+ singnificant difference was observed between the tested methods. In view of their findings, the authors suggested that *V. parahaemolyticus* present in shellfish could pose potential risk when preventive or safety measures are not properly applied.

In a study involving mussel, seawater and fish samples, Terzi Gulel and Martinez-Urtaza (2016) investigated the occurrence of pathogenic *V. parahaemolyticus* in the Black Sea region of Turkey using Pulsed-field gel electrophoresis (PFGE) and Real-time PCR following conventional culture method. The virulent strain markers (*tdh* and *trh* genes), serotype (O4:KUT, O2:KUT as well as O3:KUT serovars) and genetic profiles of *V. parahaemolyticus* were analysed.

CHAPTER 4
MATERIALS AND METHOD

4.1 Study Area

Cyprus, a third largest island in the historic Mediterranean Sea, is located at approximately 75 km to the South of Turkey, 200 km to the North West of Israel and Palestine territory, 800 km to the South East of Greece, and 380 km to the North of Egypt (The Early Prehistory of Cyprus: From Colonization to Exploitation, 2001). Demographically, Cyprus is broadly divided into two distinct ehtnic groups, one is Greek Cypriot and the other is Turkish Cypriot (2011 census, 2006 North Cyprus data, entire Island) (statistical service republic of Cyprus, 2011). The geographical location of Cyprus was found to be essential to the previous civilisations. Sequentially, the Island has been ruled by the Persians, Assyrians, Hellenistic, Arabs, Romans, Byzantine and Ottoman Empires.

In the TRNC, seafood are consumed in significant quantities and can be a source of vibriosis. Accordingly, ensuring the safety of seafood by investigating the pathogenic *V. parahaemolyticus* is crucial.

4.2 Sampling

Fish samples were taken from major seafood outlets of Nicosia (Lefkoşa), Famagusta (Mağusa), Kyrenia (Girne) and Morphou (Güzelyurt), and also directly from the coasts and/or bays of the Meditterranean Sea of the above named districts. A total of 60 seafood samples, (20 from seacoasts) and (40 from seafood outlets) of the four regions were asceptically taken during the summer period of 2016 as can be seen in Table 4.1. These representative samples were drawn in accordance with standardised procedures for fresh fish sampling (ICMFS, 1986; CFIA, 2013). See Appendix 3.

Seafood samples in this study include European Sea bass (*Dicentrarchus labrax*-Levrek), Gilt-head bream (*Sparus aurata* L.- Çipura), Blue whiting (*Micromesistius poutassou*-Voppa), Marbled spinefoot (*Siganus rivulatus*-Sokan) and Mackerel (*Scomber scombrus*-Palamut).

Table 4.1: Sampling regions in TRNC and number of primary samples taken

Region	Fish species	Number of primary samples
Famagusta (Mağusa)	Sea bass	5
	Sea bream	5
	Catch of the day: Mackerel	5
	Catch of the day: Marbled spinefoot	5
Kyrenia (Girne)	Sea bass	5
	Sea bream	5
	Catch of the day: Blue whiting	5
Nicosia (Lefkoşa)	Sea bass	5
	Sea bream	5
Morphou (Güzelyurt)	Sea bass	5
	Sea bream	5
	Catch of the day: Mackerel	5

These fish varieties were selected because they are widely consumed and are available in all seasons of the year.

Seafood samples were packed into a clean polyethylene bag then labelled 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 seafood 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 seafood samples.

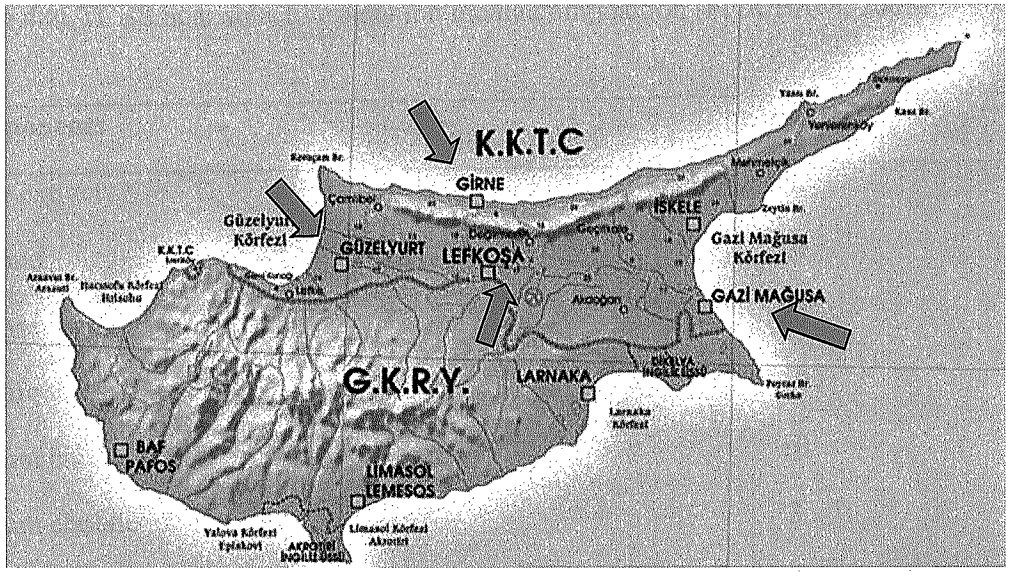

Figure 4.1: Map of Cyprus showing the study area in TRNC (KKTC)

4.3 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±2^{0}C), autoclave, pH meter, sterile forceps, pipettes, bottles, jars and glass wares.

4.3.1 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.

4.3.2 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 $5H_2O$	10
Sodium citrate $2H_2O$	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.

4.4 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 (FDA/BAM, 2001).

4.4.1 Analytical sample preparation

Seemingly, apathogenic and pathogenic bacteria live on the skin, the gills and in the intestines of fish (Feldhusen, 1999). Therefore the gills and intestines from each fish sample were separated and then homogenised in 225ml of alkaline peptone water (APW) with 3% NaCl for 1min. The homogenates were tranferred into sterile polythene stomacher bags and stored in an incubator (Thermo Scientific) at 37 ^{0}C for 18 – 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 ^{0}C for 18 – 24 hr. Figure 4.2 summarizes the distinctive steps involved in cultural detection of *Vibrio parahaemolyticus* from seafood samples.

Figure 4.2: Prepared APW enrichment media and homogenization of fish samples

4.4.2 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 hearth infusion broth and then inoculate onto TCBS agar at 37 °C for 24 hours. These control colonies were used for the detection of presumptive *Vibrio parahaemolyticus* colonies in the fish samples.

4.4.3 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 Confirmation

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

4.5.1 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 Phoneix AST Indicator was added to each AST-S broth tube. And then 25 µl of the Phonenix Inoculum broth suspension was transfered 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.

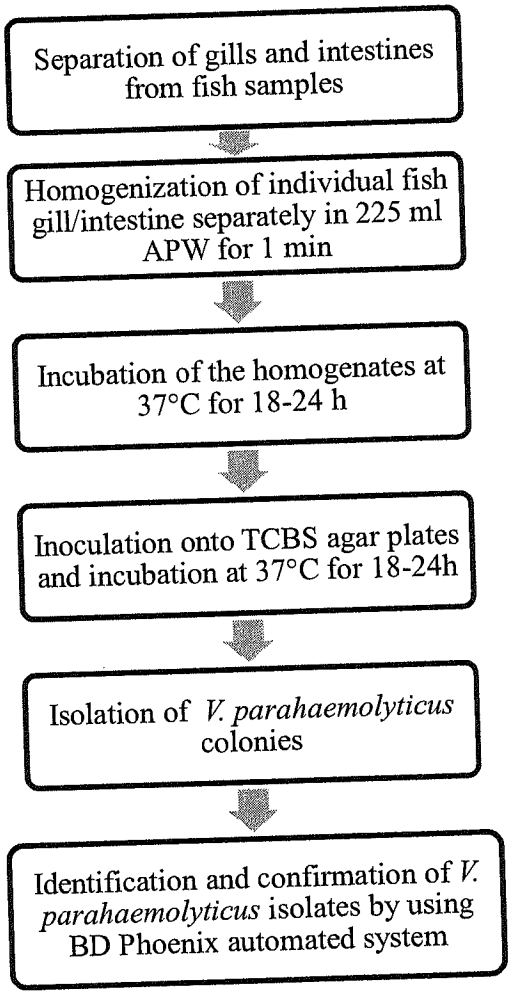

Figure 4.3: Steps for cultural detection of *Vibrio parahaemolyticus* in fish samples

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 finfish varieties.

5.1 Results

Apathogenic and pathogenic microorganisms are widely distributed in aquatic environment and of course in seafood harvested from such environment. Specifically, the present study investigated the occurrence of *Vibrio parahaemolyticus* in finfish marketed in four major cities and other species caught off from the Mediterranean coasts of the TRNC. Whereas *Vibrio parahaemolyticus* could not be found in any of the examined fish samples, other Gram- negative bacteria were detected in the intestines of sea bass from Kyrenia and sea bream from Morphou. Three bacterial species including *Photobacterium damselae* (formerly *Vibrio damsela*), *Providencia rettgeri* and *Pseudomonas fluorescens* were confirmed following biochemical test with BD Phoenix Identification Instrument. Two of these bacteria, namely *Photobacterium damsalae* (formerly *Vibrio damsela*) and *Providencia rettgeri* are pathogenic in humans and in animals. Results for fish species, locations and pathogens are presented in Table 5.1 and suspected bacterial colonies on TCBS agar in Figure 5.1.

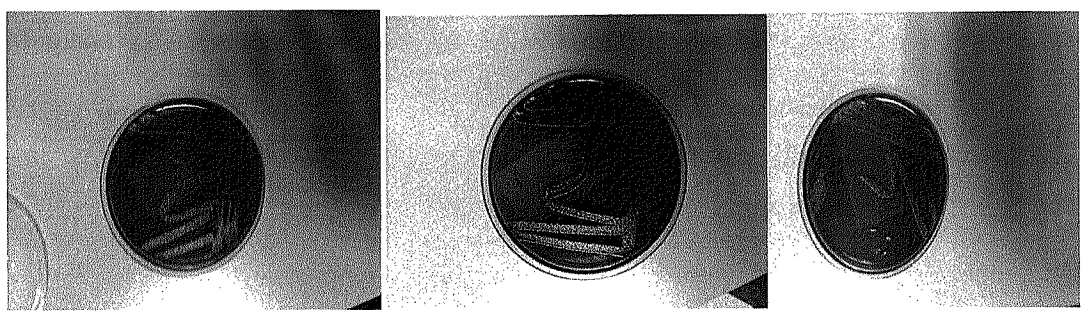

Figure 5.1: The suspected TCBS agar plates

Table 5.1: Occurrence of bacterial pathogens in various fish species in the TRNC

Region	Fish Species	Number of samples positive/number of samples examined	Biochemically identified pathogen	Concentration of pathogen (cfu/ml)
Famagusta	Sea Bass	0/5		
(Mağusa)	Sea Bream	0/5		
	Catch of the day (Mackerel)	0/5		
	Catch of the day (Marbled spinefoot)	0/5		
Kyrenia	Sea Bass	1/5	*Providencia rettgeri*	$> 10^5$
(Girne)	Sea Bream	0/5		
	Catch of the day (Blue whiting)	0/5		
Nicosia	Sea Bass	0/5		
(Lefkoşa)	Sea Bream	0/5		
Morphou	Sea Bass	0/5		
(Güzelyurt)	Sea Bream	1/5	*Photobacterium damselae* (formerly *Vibrio damsela)*	$> 10^5$
	Catch of the day (Mackerel)	0/5		

5.2 Discussion

Recent epidemiological studies identified the importance of minimizing or preventing pathogens contamination and control of temperature as a key to increasing shelf life and minimizing the occurrence of seafoodborne illnesses (Ronholm et al., 2016). 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 *Vibrios* (Ronholm et al., 2016). *V. cholerae, V. parahaemolyticus* and *V. vulnificus* are the most debilitating of all *Vibrios* in humans (DePaola et al., 2010).

Vibrio parahaemolyticus, a potential aquatic and seafood pathogen, happens to be the principal cause of seafoodborne diseases throughout the planet Earth. The debilitating effects of *V. 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; Mahoney et al., 2010; Letchumanan et al., 2014; Caburlotto et al., 2016).

To our knowledge, occurrence of *Vibrio parahaemolyticus* in the seafood consumed in TRNC has never been investigated. The present study examined the occurrence of the total *Vibrio parahaemolyticus* in fresh fish species marketed in four major cities of the TRNC.

Fortunately, *Vibrio parahaemolyticus* was not found in any fresh fish species sampled in our study, although some studies from the United States reported the elevations in the number of Vibrio infections associated with seafood (Iwamato et al., 2010), meanwhile in Europe, *Vibrio parahaemolyticus* has been considered as emerging foodborne pathogen responsible for most of the recent sporadic and epidemic seafoodborne infections (Powell et al., 2013).

However, our results are in agreement with previous study conducted in some European countries where fish samples sourced from France and Great Britain contain no *V. parahaemolyticus* (Davis et al., 2001).

Nonetheless, other aquatic bacterial pathogens like *Photobacterium damselae* (formerly *Vibrio damsela*) and *Providencia rettgeri* were found in our fish samples from Kyrenia and Morphou. *Photobacterium damselae* is a pathogen for several species of fish and shellfish. In humans, this bacterium can cause wide range of infections which may result into necrotizing fasciitis usually with severe clinical consequences.

Providencia rettgeri is one of the major causes of diarrhea in humans. It is also a major source of Tetrodotoxin, predominantly in some Asian countries and recently in Europe (Tu et al., 2014)

These bacteria could be isolated because they are also sugar-fermenting, Gram negative like *V. parahaemolyticus* and share similar growing conditions in sea.

CHAPTER 6
CONCLUSION AND RECOMMENDATIONS

Based on the results of our study, we could not find *Vibrio parahaemolyticus* in fish samples taken from different regions of the TRNC which is one of the most important seafoodborne pathogens. However seafood consumed in the TRNC might be a source of other bacterial pathogens like *Photobacterium damselae* (formerly *Vibrio damsela*) and *Providencia rettgeri* species, since the concentrations of these bacteria were found to be greater than 10^5 cfu/ml (minimum infective dose) in sea bass and sea bream fishes from Kyrenia and Morphou regions respectively.

It is highly recommended to investigate the occurrence of *Photobacterium damselae* (formerly *Vibrio damsela*) and *Providencia rettgeri* in various seafood products consumed in the TRNC.

REFERENCES

Adams, R.M., and Moss, O.M. (2000). Food Microbiology, Second Edition, The Royal Society of Chemistry, Thomas Graham House, Science Park, Milton Road, Cambridge CB4 OWF, UK.

Adams, R. M., and Moss, O. M. (2008). Food Microbiology, Third Edition, The Royal Society of Chemistry, Thomas Graham House, Science Park, Milton Road, Cambridge CB4 OWF, UK.

Adebayo-Tayo, B.C., Okonko, I.O., Esen, C.U., Odu, N.N., Onoh, C.C., and Igwiloh, N. J. P. (2011). Incidence of Potentially Pathogenic *Vibrio* Spp. In Fresh Seafood from Itu Creek in Uyo, Akwa Ibom State, Nigeria. *World Applied Sciences Journal, 15(7),* 985-991.

Adedeji O.B., Okerentugba P.O., Innocent-Adiele, H.C., and Okonko I.O. (2012). Benefits, Public Health Hazards and Risks Associated with Fish Consumption. *Journal of New York Science, 5(9),* 33-61.

Alam, M.J., Miyoshi, S.I., and Shinoda, S. (2003). Studies on pathogenic *Vibrio parahaemolyticus* during a warm weather season in the Seto Inland Sea, Japan. *Journal of Environmental Microbiology, 5(8),* 706-710.

Alasalvar, C., Shahidi, F., Miyashita, K., and Wanasundara, U. (2011). Handbook of Seafood Quality, Safety and Health Applications, Blackwell Publishing Ltd, Chichester, West Sussex, UK.

Amin, R.A., and Salem, A.M. (2012). Specific Detection of Pathogenic Vibrio species in Shellfish by Using Multiplex Polymerase Chain Reaction. *Journal of Global Veterinaria, 8(5),* 525-531.

Amusan, E., Oramadike C.E., Abraham-Olukayode A.O., and Adejonwo O.A. (2010). Bacteriological Quality of Street Vended Smoked Blue Whiting (*Micromesistus poutasou*). *Journal of Food Safety, 12,* 122-126.

Anonymous, (2011). Basin Bildirisi KKTC Nüfus ve Konut Sayimi. Retrieved March 28, 2016 from http://www.devplan.org/Nufus-2011/nufus%20son_.pdf.

Anonymous, (2012). Agriculture Sector Overview, Retrieved March 20, 2016 from http://idbgbf.org/assets/2012/5/2/pdf/ca41bf40-25a5-4df8-8bd7-748db6689401.pdf.

Ansaruzzaman, M., Lucas, M., Deen, J.L., Bhuiyan, N.A., Wang, X.Y., Safa, A., Sultana, M., Chowdhury, A., Nair, G.B., Sack, D.A., Von Seidlein, L., Puri, M.K., Ali, M., Chainagt, C., Clemens, J.D. and Baretto, A. (2005). Pandemic serovars (O3:K6 and O4:K68) of *Vibrio parahaemolyticus* associated with diarrhea in Mozambique: spread of the pandemic into the African continent. *Journal of Clinical Microbiology, 43,* 2559–2562.

Archer, D.L. (1984). Diarrhoeal episodes with diarrhoeal disease: acute disease with chronic implications. *Journal of Food Protection, 47,* 322-328.

Archer, D.L., and Kvenberg, V.E. (1985). Incidence and cost of foodborne diarrhoeal disease in the United States. *Journal of Food Protection, 48,* 887-894.

Abbott, S.L., Powers, C., Kaysner, C.A., Takeda, Y., Ishibashi, M., Joseph, S.W. and Janda, J. M. (1989). Emergence of a restricted bioserovar of *Vibrio parahaemolyticus* as the predominant cause of *Vibrio*-associated gastroenteritis on the west coast of the United States and Mexico. *Journal of Clinical Microbiology, 27,* 2891–3.

Avşar, C., Berber, I., and Yıldırım, A.K. (2016). Isolation and characterization of Vibrio spp. from anchovy and garfish in the Sinop province. *Turkish Bulletin of Hygiene and Experimental Biology, 73(2),* 121-130.

Baffone, W., Pianetti, A., Bruscolini, F., Barbieri, D. and Citterio, B. (2000). Occurrence and expression of virulence related properties of *Vibrio* species isolated from widely consumed seafood products. *International Journal of Food Microbiology, 54,* 9-18.

Baffone W., Citterio B., Vittoria, E., Casaroli, A., Pianetti A., Campana, R., Bruscolini, F., (2001). Determination of several potential virulence factors in *Vibrio* spp. isolated from sea water. *Journal of Food Microbiology, 18,* 479–88.

Baird-Parker, A.C. (1994). Foods and Microbiological Risks. *Journal of Microbiology, 140,* 687-695.

Baker-Austin, C., Stockley, L., Rangdale, R., and Martinez-Urtaza, J. (2010). Environmental occurrence and clinical impact of *Vibrio vulnificus* and *Vibrio*

parahaemolyticus: a European perspective, Mini review. *Environmental Microbiology Reports, 2(1),* 7–18.

Bakr, W.M.K., Hazzah, W.A. and Abaza, A.F. (2011). Detection of *Salmonella* and *Vibrio* species in some seafood in Alexandria. *Journal of American Sci*ence, *7(9),* 663-668.

Balebona, M.C., Zorrilla, I., Miguel A. Morinigo, M.A., Juan, J., and Borrego, J.J. (1998). Survey of bacterial pathologies affecting farmed gilt-head sea bream (*Sparus aurata* L.) in southwestern Spain from 1990 to 1996. *Journal of Aquaculture, 166 (1-2),* 19–35.

Bandekar, J.R., Chander, R., and Nerkar D.P. (1987). Radiation Control of *Vibrio parahaemolyticus* in Shrimp. *Journal of Food Safety, 8,* 83-88.

Banerjee, S.K., Kearney, A.K., Nadon, C.A., Peterson, C L., Tyler, K., Bakouche, L., Clark, C.G., Hoang, L., Gilmour, M.W., and Farber, J.M. (2014). Phenotypic and genotypic characterization of Canadian clinical isolates of *Vibrio parahaemolyticus* collected from 2000-2009. *Journal of Clinical Microbiology, 52(4),* 1081-1088.

Bisha, B., Simonson, J., Janes, M., Bauman, K., and Goodridge, L.D. (2012). A review of the current status of cultural and rapid detection of *Vibrio parahaemolyticus*. *International Journal of Food Science & Technology, 47,* 855–899.

Bonnell, A.D. (1994). Quality Assurance in Seafood Processing: A Practical Guide, Chapman & Hall, Inc., Dordrecht, P. 97. ISBN 978-1-4615-2642-1.

Boonyawantang, A., Mahakarnchanakul, W., Rachtanapun, C. and Boonsupthip, W. (2012). Behavior of pathogenic *Vibrio parahaemolyticus* in prawn in response to temperature in laboratory and factory. *Journal of Food Control, 26,* 479- 485.

Blanco-Abad, J.V., Ansede-Bermejo, Rodriguez-Castro, A., and Martinez-Urtaza, J. (2009). Evaluation of different procedures for the optimized detection of *Vibrio parahaemolyticus* in mussels and environmental samples. *International Journal of Food Microbiology, 129,* 229–236.

Broberg, C.A., Calder, T.J., and Orth, K. (2011). *Vibrio parahaemolyticus* cell biology and pathogenicity determinants. *Journal of Microbes Infections. 13(12-13),* 992–1001.

Butt, A.A., Aldridge, K E. and Sanders C.V. (2004). Infections related to the ingestion of seafood Part I: viral and bacterial infections; Reviews. *Journal of Lancet Infectious Diseases*, *4*, 201–212.

Canigral, I., Moreno, Y., Alonso, J.L., Gonzalez, A., and Ferrus, M.A. (2010). Detection of *Vibrio vulnificus* in seafood, seawater and wastewater samples from a Mediterranean coastal area. *Journal of Microbiology, 165,* 657- 664.

Canadian Food Inspection Agency (CFIA) (2013). *Fish Inspection Program Sampling Policy. Standards and Methods Manual sampling documents*, Canada. Retrieved May 20, 2016 from http://www.inspection.gc.ca/food/fish-and-seafood/manuals/standards-and-methods

Caburlotto, G., Suffredini, E., Toson, M., Fasolato, L., Antonetti, P., Michela Zambon, M. and Manfrin, A. (2016). Occurrence and molecular characterisation of Vibrio parahaemolyticus in crustaceans commercialised in Venice area, Italy. *International Journal of Food Microbiology*, *2,* 39-49.

Cahill, M.M. (1990). Bacterial flora of fishes: a review. *Journal of Microbial Ecology*, *19,* 21- 41.

Center for Disease Control and Prevention, CDC (2013). Increase in *Vibrio parahaemolyticus* Illnesses Associated with Consumption of Shellfish from Several Atlantic Coast Harvest Areas, United States. Retrieved 15th April, 2016. from http://www.cdc.gov /vibrio / investiga tions/index.html

Center for Disease Control and Prevention, CDC (2015). *Foodborne Germs and Illnesses*. Retrieved 27th May, 2016. From http://www.cdc.gov/foodsafety/foodborne-germs.html

Centre for Food Safety, CFS (2014). *Microbiological Guidelines for Food (For ready-to-eat food in general and specific food items), Food and Environmental Hygiene Department*, 66 Queensway, Hong Kong.

Chiou, C.S., Hsu, S.Y., Chiu, S.I., Wang, T.K., and Chao, C.S. (2000). *Vibrio parahaemolyticus* serovar O3:K6 as cause of unusually high incidence of food-borne

disease outbreaks in Taiwan from 1996 to 1999. *Journal of Clinical Microbiology 38,* 4621- 4625.

Codex Alimentarius Commission, CAC. (1997). *Principles and Guidelines for the Establishment and Application of Microbiological Criteria Related to Foods.* CAC/GL-21 (Pp. 1-6).

Codex Alimentarius Commission, CAC (2010). *Guidelines on the application of general principles of food hygiene to the control of pathogenic Vibrio species in seafood.* CAC/GL 69-2008 (Pp. 1-14).

Codex Alimentarius Commission, CAC (2008). *Guidelines for the validation of food safety control measures.* CAC/GL 73-2010 (Pp. 1-10).

Colakoğlu, F.A., Sarmasik, A. and Koseoğlu, B. (2006). Occurrence of *Vibrio* spp. and *Aeromonas* spp. in shellfish harvested off Dardanelles cost of Turkey. *Journal of Food Control, 17,* 648–652.

Commission Regulation (EC) No. 2073/2005 on microbiological criteria for foodstuffs. *Official Journal of the European Union, 22,* 338, 1-26.

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.

Corry, J.E.L., Jarvis, B., Passmore, S. and Hedges, A. (2007). A critical review of measurement uncertainty in the enumeration of food micro-organisms. *Journal of Food Microbiology 24,* 230–253.

Daniels, N.A., Mackinnon, L., Bishop, R., Altekruse, S., Ray, B., Hammond, R.M., Thompson, S., Wilson, S., Bean, N.H., Griffin, P M. and Slutsker, L. (2000). *Vibrio parahaemolyticus* infections in the United States, 1973–1998. *Journal of Infectious Diseases 181,* 1661–1666.

Davies, A.R., Capell, C., Jehanno, D., Nychas, G.J.E and Kirby, R.M. (2001). Incidence of foodborne pathogens on European fish. *Food Control, 12,* 67-71.

De Boer, E. and Beumer, R.R. (1999). Methodology for detection and typing of foodborne microorganisms. *International Journal of Food Microbiology, 50*, 119–130.

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.

DePaola, A., Kaysner, C.A., Bowers, J., and Cook, D.W. (2000). Environmental Investigations of *Vibrio parahaemolyticus* in Oysters after Outbreaks in Washington, Texas, and New York (1997 and 1998). *Journal of Applied and Environmental Microbiology, 66(11),* 4649–4654.

DePaola, A., Ulaszek, J., Kaysner, C.A., Tenge, B.J., Nordstrom, J.L., Wells, J., Puhr, N. and Gendel, S.M. (2003). Molecular, serological, and virulence characteristics of Vibrio parahaemolyticus isolated from environmental, food, and clinical sources in North America and Asia. *Journal of Applied and Environmental Microbiology, 69(7),* 3999-4005.

DePaola, A., and Kaysner, C.A. (2004). *Vibrio. Bacteriological Analytical Manual Online.* Washington, DC: U.S. Food and Drug Administration.

DePaola, A., Jones, J. L.,Woods, J., Burkhardt,W., Calci, K.R., Krantz, J.A., Bowers, J.C., Kaskuri, K., Byars, R.H. Jacobs, E., Williams-Hill, D. and Nabe, K. (2010). Bacterial and viral pathogens in live oysters: 2007 united states market survey. *Journal of Applied Environmental Microbiology,* 76, 2754–2768.

Di Pinto, A., Ciccarese, G., De Corato, R., Novello, L., and Terio, V. (2008). Detection of pathogenic *Vibrio parahaemolyticus* in southern Italian shellfish. *Journal of Food Control, 19,* 1037–1041.

Drake, S.L., Depaola, A. and Jaykus, L. (2007). Comprehensive Reviews in Food Science and Food Safety, "An Overview of *Vibrio vulnificus* and *Vibrio parahaemolyticus*". *Journal of Institute of Food Technologists, 6,* 120-144.

Donnenberg, M.S., Mandel, G.I., Bennet, J.E., John, R., and Mandel, D. (2005). *Enterobacteriaceae*, principles and practice of infectious diseases, Sixth Edition.

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

Eleftheriadou. M., Varnava-Tello, A., Metta-Loizidou, M., Nikolaou, A. and Akkelidou D. (2002). The microbiological profile of foods in the Republic of Cyprus. *Journal of Food Microbiology, 19,* 463-471.

Elliot, E.L., Kaysner, L., Jackson, and M.L. Tamplin. (1995). *Vibrio cholerae, V. parahaemolyticus, V. vulnificus* and other *Vibrio* spp, p. 9.019.27. *In* FDA Bacteriological Analytical Manual, Eighth Edition, AOAC International, Gaithersburg, MD, USA.

Farmer, J.J., Janda, J.M., and Birkhead, K. (2003). "*Vibrio*," in *Manual of Clinical Microbiology*, Editor, P.R. Murray, Washington, DC: ASM Press.

Feldhusen, F. (1999). Seafood related diseases. *Journal of Dtsch. Tierärztl. Wschr, 106,* 319–326.

Feldhusen, F. (2000). The role of seafood in bacterial foodborne diseases. *Journal of Microbes and Infection, 2,* 1651–1660.

Food and Agriculture Organization of the United Nations, FAO (1994*). Review of the State of the World Fishery Resources; Marine Fisheries*. FAO Fishery circular No 920. Rome. Retrieved on 16th April, 2016. from http://www.fao.org/3/a-y5852e.pdf

Food and Agriculture Organization, FAO (2002). *Discussion paper on Risk Management Strategies for Vibrio spp. in seafood.* FAO, Rome, CX/FH 03/5, Add.3, 1-23. Retrieved on 20th April 2015 from http://ftp.fao.org/codex/ccfh35/fh0305ce.pdf

Food and Agriculture Organization of the United Nations, FAO (2008). *Good Agricultural Practices benefits and challenges*, Rome. Retrieved on 28th April, 2016 from http://www.fao.org/prods/gap/

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 and Agriculture Organization of the United Nations, FAO (2011). *The State of World Fisheries and Aquaculture (SOFIA), 2010.* Rome. Retrieved on 17th May, 2016 from http://www.fao.org/docrep/013/i1820e/i1820e00.htm

Food and Agriculture Organization of the United Nations, FAO (2012*). Methods of sampling and analysis, Manual of Good Practices for the Feed Industry*. Rome.

Food and Agriculture Organization of the United Nations, FAO (2014). *The State of World Fisheries and Aquaculture (SOFIA).* Rome. Retrieved on 17th May, 2016 from http://www.fao.org/3/a-i3720epdf

Food and Agriculture Organization, FAO (2015). *Fishery utilization and trade*, Fisheries and Aquaculture Department, Rome, http://www.fao.org/fishery/utilization_trade/en. (Retrieved on 15th May, 2016).

Food and Agriculture Organization of the United Nations, FAO (2016). *Seafood Safety-Economics of Hazard Analysis Critical Control Point "Seafoodborne Disease and Illnesses", Fisheries and Aquaculture Department*, Rome. Retrieved on 27th May, 16 from http://www.fao.org/docrep/003/x0465e/x0465e04.htm

Food and Agriculture Organization of the United Nations, FAO (2016). *Fisheries and Aquaculture Department, National Aquaculture Legislation Overview, Turkey*. from http://www.fao.org/fishery/legalframework/nalo_turkey/en (Retrieved on May 2, 16)

Food and Agriculture Organization of the United Nations, FAO (2016). *Fisheries and Aquaculture Department*, Rome. Retrieved on February, 19th, 2016 from http://www.fao.org/fishe ry/culturedspecies/Dicentrarchus_labrax/en

Food and Drug Administration/Bacteriological Analysis Manual, FDA/BAM (2003). *"Food Sampling and Preparation of Sample Homogenate"*, Silver Spring, Md, USA, 2001. Retrieved on 25th May, 2016 from http://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm063335.htm

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

Fowler, J.L., Clark, W.S., Foster, J.F., Hopkins, A., (1978). Analyst variation in doing the standard plate count as described in Standard Methods for the Examination of Dairy Products. *Journal of Food Protection, 41*, 4–7.

Francis, B., Santha S., Puthenkandathil S.D., and Ammanamveetil A.M. Hatha. (2012) Prevalence and distribution of *Vibrio parahaemolyticus* in finfish from Cochin (south India). *Journal of Veterinaria Italiana, 48(3),* 269-281.

Freose, Rainer and Pauly, Daniel, eds. (2006). "*Dicentrarchus labrax*" in FishBase.

Fuenzalida, L., Armijo, L., Zabala, B., Hernández, C., Rioseco, M.L., Riquelme, C. and Espejo, R. T. (2007). *Vibrio parahaemolyticus* strains isolated during investigation of the summer 2006 seafood related diarrhea outbreaks in two regions of Chile. *International Journal of Food Microbiology, 117*, 270–275.

Fujino, T., Okuno, Y., Nakada, D., Aoyama, A., Mukai, T. and Ueho, T. (1953). On the bacteriological examination of shirasu-food poisoning. *Medical Journal of Osaka Univ*ersity, *4*, 299-304.

Gracias, K.S. and McKillip, J.L. (2004). A review of conventional detection and enumeration methods for pathogenic bacteria in food. *Canadian Journal of Microbiology, 50(11),* 883-890.

Gram, L. and Huss, H. H. (2000). Fresh and Processed Fish and Shellfish. In: Lund BM, Baird-Parker TC & Gould GW, editors. The Microbiological Safety and Quality of Food Volume I. Maryland, Aspen Publishers.

Gonzalez-Escalona, N., Martinez-Urtaza, J., Romero, J., Espejo, R.T., Jaykus, L.A., and DePaola, A. (2008). Determination of molecular phylogenetics of *Vibrio parahaemolyticus* strains by multi locus sequence typing. *Journal of Bacteriology, 190,* 2831–2840.

Gronder, R.M. and Hinton A.H (1986). Low dose gamma irradiation of *Vibrio cholerae* in crabmeat (*Callinectes sapidus*). Proceedings of the Eleventh Annual Tropical and Subtropical Fisheries Conference of the Americas, Texas Agricultural Extension Service.

Gronder, R.M. and Watson, M.A. (1990). Low dose gamma irradiation of *Vibrio vulnificus* in crabmeat (*Callinectes sapidus*). Proceedings of the Eleventh Annual Tropical and Subtropical Fisheries Conference of the Americas, Orlando, FL.

Hackney, C.R., Kleeman, E.G., Ray, B., and Speck, M.L. (1980). Adherence as a method for differentiating virulent and avirulent strains of *Vibrio parahaemolyticus*. *Journal of Applied and Environmental Microbiology, 40,* 652–8.

Han, F., and Ge, B. (2010). Quantitative detection of Vibrio vulnificus in raw oysters by real time loop-mediated isothermal amplification. *International Journal of Food Microbiology, 142,* 60-66.

Han, C., Tang, H., Ren, C., Zhu, X., and Han, D. (2016). Sero-Prevalence and Genetic Diversity of Pandemic *V.parahaemolyticus* Strains Occurring at a Global Scale. *Frontiers in Microbiology Journal, 7,* 567.

Hara-Kudo, Y., Nishina, T., Nakagawa, H., Konuma, H., Hasegawa, J. and Kuma-gai, S. (2001). Improved method for detection of *Vibrio parahaemolyticus* in seafood. *Journal of Applied Environmental Microbiology, 67,* 5819–5823

Hassan, Z.K., 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.

Helms, M., Simonsen, J., and Mølbak, K. (2006). Foodborne Bacterial Infection and Hospitalization: A Registry-Based Study. *Journal of Clinical Infectious Disease, 42(4),* 498-506.

Hida, T. and Yamamoto, K. (1990). Cloning and expression of two genes encoding highly homologous hemolysins from a Kanagawa-phenomenon-positive *Vibrio parahaemolyticus* T4750 strain. *Journal of Gene, 93,* 9–15.

Honda, S., Goto, I., Minematsu, I., Ikeda, N., Asano, N., Ishibashi, M., Kinoshita, Y., Nishibuchi, M., Honda, T., and Miwatani, T. (1987). Gastroenteritis due to Kanagawa negative *Vibrio parahaemolyticus*. *Journal of Lancet, 1,* 331–2.

Honda, T., Ni, Y., and Miwatani, T. (1988). Purification and characterization of a hemolysin produced by a clinical isolated of Kanagawa phenomenon-negative *Vibrio parahaemolyticus* and related to the thermostable direct hemolysin. *Journal of Infect Immun., 56,* 961–5.

Honda, T. and Iida, T. (1993). The pathogenicity of *Vibrio parahaemolyticus* and the role of thermostable direct hemolysin and related hemolysins, Rev. *Journal of Med. Microbiol*ogy, *43,* 106–113.

Hooper, W.L., Baurow, G.I., and Mcnab, D.J.N. (1974). *Vibrio parahaemolyticus* food poisoning in Britain. *Lancet Journal of Infectious Disease, 1(7866),* 1100-2.

Horsley, R.W. (1973). The bacterial flora of Atlantic salmon (*Salmon salar* L.) in relation to its environment. *Journal of Applied Bacteriology, 36,* 377-386.

Huss, H.H. (1997). Control of indigenous pathogenic bacteria in seafood. *Journal of Food Control, 8(2),* 91-98.

Huss, H.H., Reilly, A., and Ben Embarek, P.K. (2000). Prevention and control of hazards in seafood. *Journal of Food Control, 11,* 149-156.

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. Accessed on 13 February, 16 from http://www.fao.org/docrep/006/y4743e/y4743e00.htm

Hutt, P.B. and Hutt, P.B. (1984). A history of government regulation of adulteration and misbranding of food. *Food Drug Cosmetics Law Journal, 39,* 2-73.

Iida, T., Suthienkul, O., Park, K.S., Tang, G.Q., Yamamoto, R.K., Ishibashi, M., Yamamoto, K. and Honda, T. (1997). Evidence for genetic linkage between the *ure* and *trh* genes in *Vibrio parahaemolyticus*. *Journal of Medical Microbiology, 46,* 639–45.

International Atomic Energy Agency, IAEA (2001). *Irradiation to Control Vibrio Infection from Consumption of Raw Seafood and Fresh Produce.* Results of a coordinated research project organized by the Pan American Health Organization and the Joint

FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, Vienna, Austria. Vol. 12/13 Pp. 4-22.

International Commission on Microbiological Specifications for Foods, ICMSF (1986). *Microorganisms in Foods 2. Sampling for microbiological analysis: Principles and specific applications.* Second Edition, London: University of Toronto Press, Toronto; P. 192.

International Commission on Microbiological Specifications for Foods, ICMSF (1996). *Vibrio cholerae. In: Microorganisms in Foods 5. Characteristics of Microbial Pathogens.* London: Blackie Academic & Professional; P. 414-425.

International Commission on Microbiological Specifications for Foods, ICMFS (1996). *Vibrio parahaemolyticus. In: Microorganisms in Foods 5. Characteristics of Microbial Pathogens.* London: Blackie Academic & Professional; P. 426-435.

International Commission on Microbiological Specifications for Foods, ICMFS (1998). *Fish and Fish Products. In: Microorganisms in Foods 6. Microbial Ecology of Food Commodities.* London: Blackie Academic & Professional; P. 130-189.

Ito, H., Adulyatham, P., Sangthon, N., and Ishigaki I. (1989). Effects of gamma-radiation on frozen shrimps to reduce microbial contamination. *Journal of Radiation. Physics and Chemistry, 3,* 1009-1011.

Ito, H., Rashid, H., Sangthon, N., Adulyatham, P., Rattagool, P. and Ishigaki I. (1993). Effects of gamma-radiation on frozen shrimps for decontamination of pathogenic bacteria. *Journal of Radiation. Physics and Chemistry, 42(1-3),* 279-286.

Iwamoto, M., Ayers, T., Mahon, B. E. and Swerdlow, D. L. (2010). Epidemiology of seafood associated infections in the United States. *Journal of Clinical Microbiol. Rev*iew, *23,* 399-411.

Jaksic, S., Uhitil, S., Petrak T., Bazulic, D. and Karolyi L. G. (2002). Occurrence of Vibrio spp. in sea fish, shrimps and bivalve molluscs harvested from Adriatic Sea. *Journal of Food Control, 13,* 491–493.

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.

Jasson, V., Jacxsens L., Luning, P., Rajkovic, A. and Uyttendaele, M. (2010) Review. Alternative microbial methods: An overview and selection criteria. *Journal of Food Microbiol*ogy, *27*, 710–730.

Jay, J.M. (2000). Modern Food Microbiology, Sixth Edition, Aspen Food Science Series, Gaithersburg, (Maryland), USA.

Jawetz, Melnick and Adelberg (2013). Medical Microbiology, Twenty Sixth Editions, The McGraw Hill Companies, New York City, USA.

Jones, M.K. and Oliver, J.D. (2009). Vibrio vulnificus: disease and pathogenesis. *Journal of Infect. Immun., 77*, 1723-1733.

Kaferstein, F.K. and Sims, J. (1987) Food safety: a worldwide public health issue. *World Health Organiztion (3),* 308-328.

Kaysner, C.A., Abeyta, C.J., Trost, P.A., Wetherington, J.H., Jinneman, K.C., Hill, W.E. and Wekell, M.M. (1994). Urea hydrolysis can predict the potential pathogenicity of *Vibrio parahaemolyticus* strains isolated in the Pacific Northwest. *Journal of Applied Environmental Microbiology, 60*, 3020–2.

Kaysner, C.A., & De Paola, A. (2000). Outbreaks of *Vibrio parahaemolyticus* gastroenteritis from raw oyster consumption: Assessing the risk of consumption and genetic methods for detection of pathogenic strains. *Journal of Shellfish Research, 19,* 657–660.

Kishishita, M., Matsuoka, N., Kumagal, K., Yamasaki, S., Takeda, Y. and Nishibuchi, M. (1992). Sequence variation in the thermostable direct hemolysin related hemolysin (*trh*) gene of *Vibrio parahaemolyticus. Journal of Applied Environmental Microbiology, 58,* 2449–57.

Kiiyukia, C. (2003). *Laboratory Manual of Food Microbiology for Ethiopian Health and Nutrition Research Institute*. UNIDO Project (YA/ETH/03/436/11-52) (Vol. 1) P. 99.

Kobayashi, T., Enomoto, S. and R. Sakazaki. (1963). A new selective isolation medium for the vibrio group on modified Nakanishi's medium (TCBS agar). *Japanese Journal of Bacteriology, 18*, 387-392.

Kong, R.Y.C., Lee, S.K.Y., Law, T.W.F., Law, S.H.W. and Wu, R.S.S. (2002). Rapid detection of six types of bacterial pathogens in marine waters by multiplex PCR. *Journal of Water Resources, 36,* 2802-2812.

Kubuabola, I. & Vally, H. (2006). Isolation of *Vibrio cholerae* O1 strains similar to pre-seventh pandemic El Tor strains during an outbreak of gastrointestinal disease in an island resort in Fiji. *Journal of Medical Microbiology, 55*, 1559-1562.

Kusuda, R., Sake, H. & Kawai, K., (1979). Classification of vibrios isolated from diseased fishes. Part 1. On the morphological and biochemical properties. *Journal of Fish Pathology, 13*, 123-138.

Lazcka, O. Del Campo, F.J. and Mu˜noz, F.X. (2007). Pathogen detection: A perspective of traditional methods and biosensors, Review. *Journal of Biosensors and Bioelectronics, 22*: 1205–1217.

Letchumanan., Kok-GanChan, K. and Learn-HanLee, L. (2014). *Vibrioparahaemolyticus*: a review on the pathogenesis, prevalence, and advance molecular identification techniques. *Journal of Frontiers in Microbiology/Food Microbiology, 5*, 705-718.

Li, J., Yie, J., Foo, R.W.T., Ling, J.M.L., Xu, H. and Woo, N.Y.S. (1999). Antibiotic Resistance and Plasmid Profiles of Vibrio Isolates from Cultured Silver Sea Bream, Sparus sarba. *Marine Pollution Bulletin, 39*(1-12), 245-249.

Li, L., Lin, S.L., Deng, L. and Liu, Z.G. (2013). Potential use of chitosan nanoparticles for oral delivery of DNA vaccine in black seabream Acanthopagrus schlegelii Bleeker to protect from *Vibrio parahaemolyticus*. *Journal of Fish Diseases, 36*, 987–995.

Levine, W.C. and Patricia, M.G. (1993). *Vibrio* Infections on the Gulf Coast: Results of First Year of Regional Surveillance. *The Journal of Infectious Diseases, 16(2),* 479-483.

López-Campos, G., Martinez-Suarez, J.V., Aguado-Urda, M. and Victoria Lopez Alonso V.L. (2012). Microarray Detection and Characterization of Bacterial Foodborne Pathogens, Springer Briefs in Food, Health, and Nutrition, New York, USA.

Lotz, M.J., Tamplin, M.L. and Rodrick, G.E. (1983). Thiosulfate-citrate-bile salts-sucrose agar and its selectivity for clinical and marine vibrio organisms. *Journal of Ann Clin Lab Sci. 13(1),* 45-8.

Lüdeke, C.H.M., Gonzalez-Escalona, N., Fischer, M. and Jones, J.L. (2015). Examination of clinical and environmental *Vibrio parahaemolyticus* isolates by multi-locus sequence typing (MLST) and multiple-locus variable-number tandem-repeat analysis (MLVA),*frontiers in Microbiology Journal, 6,* 564.

Ma, C., Deng, X., Ke, C., He, D., Liang, Z., Li W., Ke, B., Li, B., Zhang, Y., Ng L., and Cui, Z. (2013). Epidemiology and Etiology Characteristics of Foodborne Outbreaks Caused by *Vibrio parahaemolyticus* during 2008–2010 in Guangdong Province, China. *Journal of Foodborne Pathogens and Disease, 11(1),* 21-29.

Mahoney, J.C., Gerding, M.J., Jones, S.H., and Whistler, C.A. (2010). Comparison of the pathogenic potentials of environmental and clinical *Vibrio parahaemolyticus* strains indicates a role for temperature regulation in virulence. *Journal of Applied Environmental Microbiology, 76,* 7459–7465.

Makino, K., Oshima, K.,Kurokawa, K., Yokoyama, K., Uda, T., and Tagomori, K. (2003). Genome sequence of *Vibrio parahaemolyticus*: a pathogenic mechanism distinct from that of *Vibrio cholerae. Lancet Journal, 361,* 743–749.

Mandal, P. K., Biswas, A. K., Choi, K. and Pal, U.K. (2011). Methods for Rapid Detection of Foodborne Pathogens: An Overview. *American Journal of Food Technology, 6(2),* 87-102.

Martinez-Urtaza, J., Simental, L., Velasco, D., DePaola, A., Ishibashi, M., Nakaguchi, Y., Nishibuchi, M., Carrera-Flores, D., Rey-Alvarez, C., and Pousa, A. (2005). Pandemic *Vibrio parahaemolyticus* O3:K6, Europe. *Journal of Emerging Infectious Diseases, 11*, 1319-1320.

Marotz, J., Lubbert, C., Eisenbeiss, W., (2001). Effective light recognition for automated counting of colonies in Petri dishes (automated colony counting). *Comput. Methods Prog. Biomed. Journal, 66,* 183–198.

Matches, J. and Liston J. (1971). Radiation destruction of *Vibrio parahaemolyticus. Journal of Food Science, 36(2)*, 339–340.

Mead, P.S., Slutsker L., Dietz V., McCaig L.F., Bresee J.S., Shapiro, C., Griffin P.M & Tauxe, R.V. (1999). Food-related illness and death in the United States. *Journal of Emerg Infect Dis*, 5, 607-625.

Mckane, L. and Kandel, J. (1996). Microbiology Essentials and Applications, Second Edition, York Graphic Services, New Aster, New York, USA.

Michael, T.M. and J., M.M. (2006). Block Biology of Microorganisms, Eleventh Editions, Pearson Education Inc., New Jersey, USA.

Miyamoto, Y., Kato, T., Obara, Y., Akiyama, S., Takizawa, K. and Yamai, S. (1969). In vitro hemolytic characteristic of *Vibrio parahaemolyticus*: its close correlation with human pathogenicity. *Journal of Bacteriology, 100,* 1147–9.

Molins, R.A., Motarjemi, Y. and Kaferstein, F.K. (2001). Irradiation: a critical control point in ensuring the microbiological safety of raw foods. *Journal of Food Control, 12,* 347-356.

Morris, G.K., Merson, M.H., Huq, I., Kibrya A.K. and Black, R. (1979). Comparison of four plating media for isolating *Vibrio cholerae. Journal of Clinical Microbiology, 9(1)*, 79-83.

Motarjemi, Y. (1993). Contaminated weaning food: a major risk factor for diarrhoea and associated malnutrition. *Bulletin of World Health. Organization, 71*, 79-92.

Mudoh, F.M., Parveen, S., Schwarz, J., Rippen, T. and Chaudhuri, A. (2014). The Effects of Storage Temperature on the Growth of *Vibrio parahaemolyticus* and Organoleptic Properties in Oysters. *Journal of Frontiers in Public Health, 45(2)*, 1-7.

Nafees, A., Lawrence, W.D. and James J.P. (2010). Medical Microbiology, Fifth Edition, Glyph International, New York, USA.

Nair, G.B., Safa, A., Bhuiyan, N.A., Nusrin, S., Murphy, D., Nicol, C., Valcanis, M., Iddings, S. and Nelson, J.S. (2006). Fisheries of the World. John Wiley & Sons, Inc., USA.

Nair, G.B., Ramamurthy, T., Bhattacharya, S.K., Dutta, B., Takeda, Y., and Sack, D.A. (2007). Global dissemination of *Vibrio parahaemolyticus* serotype O3:K6 and its serovariants, Review. *Journal of Clinical Microbiology, 20*, 39–48.

Nishibuchi, M. and Kaper, J.B. (1985). Nucleotide sequence of the thermostable direct hemolysin gene of *Vibrio parahaemolyticus. Journal of Bacteriology, 162,* 558–64.

Nishibuchi, M. and Kaper, J. B. (1990). Duplication and variation of the thermostable direct hemolysin (*tdh*) gene in *Vibrio parahaemolyticus. Journal of Molecular Microbiology, 4*, 87–99.

Nishibuchi, M. and Kaper, J.B. (1995). Thermostable direct hemolysin gene of *Vibrioparahaemolyticus*: a virulence gene acquired by a marine bacterium. Minireview. *Journal of Infections Immun., 63*, 2093–9.

Nishibuchi, M., Ishibashi, M., Takeda, Y. and Kaper, J.B. (1985). Detection of the thermostable direct hemolysin gene and related DNA sequences in DNA colony hybridization test. *Journal of Infections Immun., 49*, 481–6.

Okuda, J., Ishibashi, M., Hayakawa, E., Nishino, T., Takeda, Y., Mukhopadhyay, A. K., Garg, S., Bhattacharya, S. K., Nair, G. B. and 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.

Onwuka, G.I. (2014). Food Science and Technology, Naphtali Prints, Lagos, Nigeria.

Osawa, R., Okitsu T, Morozumi H, Yamai S. (1996). Occurrence of urease-positive *Vibrio parahaemolyticus* in Kanagawa, Japan, with specific reference to presence of thermostable direct hemolysin (TDH) and the TDH-related-hemolysin genes. *Journal of Applied Environmental Microbiology, 62*, 725–7.

Popovic, N.T., Skukan, A.B., Dzidara, P.D., Coz-Rakovac, R., Strunjak-Perovic, I.S., Kozacinski, L., Jadan, M. and Brlek-Gorski, D. (2010). Microbiological quality of marketed fresh and frozen seafood caught off the Adriatic coast of Croatia. *Journal of Veterinarni Medicina, 55(5),* 233–241.

Potter, N.N. and Hotchkiss, J.H. (2007). Food Science, Fifth Edition, CBS Publishers, New Delhi, India.

Powell, A., Baker-Austin, C., Wagley, S., Bayley, A. and Hatnell, R. (2013). Isolation of Pandemic *Vibrio parahaemolyticus* from UK Water and Shellfish Produce. *Journal of Microbial Ecology, 65*: 924- 927.

Pfeffer, C. and Oliver, J.D. (2003). A comparison of thiosulphate-citrate-bile salts-sucrose (TCBS) agar and thiosulphate-chloride-iodide (TCI) agar for the isolation of *Vibrio* species from estuarine environments. *Letters in Applied Microbiology Journal, 36(3)*, 150–151.

Quintoil M.N., Porteen, K. & Pramanik, A.K. (2007). Studies on occurrence of *Vibrio parahaemolyticus* in finfishes and shellfishes from different ecosystem of West Bengal. *Journal of Livestock Resources Rural Development, 19,* 215- 219.

Raghunath, P., Acharya, S., Bhanumathi, A., Karunasagar, I. and Karunasagar I. (2008). Detection and molecular characterization of *Vibrio parahaemolyticus* isolated from seafood harvested along the southwest coast of India. *Journal of Food Microbiology, 25*, 824– 830.

Raghunath, P., Karunasagar, I. and Karunasagar I. (2009). Improved isolation and detection of pathogenic *Vibrio parahaemolyticus* from seafood using a new enrichment broth. *International Journal of Food Microbiology, 129*, 200–203.

Rashid, H., Ito, H., and Ishigaki I. (1992). Distribution of pathogenic vibrios and other bacteria in imported frozen shrimps and their decontamination by gamma-irradiation. *World Journal of Microbiology and Biotechnology, 8,* 494-499.

Robert-Pillot, A., Guenole, A., Lesne, J., Delesmont, R., Fournier, J.M., & Quilici, M.L. (2004). Occurrence of the *tdh* and *trh* 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.

Ronholm, J., Lau, F. and Banerjee S.K. (2016). Emerging Seafood Preservation Techniques to Extend Freshness and Minimize Vibrio Contamination. *Frontiers in Microbiology Journal, 7,* 350.

Rosec, J., Simon, M., Causse, V. and 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.

Sakazaki, R., Tamura, K., Kato, T., Obara, Y., Yamai, S. and Hobo, K. (1968). Studies of the enteropathogenic, facultatively halophilic bacteria, *Vibrio parahaemolyticus.* III. Enteropathogenicity. *Japanese Journal of Medical Science Biology, 21,* 325–31.

Sakurai, J., Matsuzaki, and A, Miwatani, T. (1973). Purification and characterization of thermostable direct hemolysin of *Vibrio parahaemolyticus. Journal of Infection Immum., 8,* 775–80.

Sarkar, B.L., Kumar, R., De, S.P. and Pal, SC. (1987). Hemolytic activity of and lethal toxin production by environmental strains of *Vibrio parahaemolyticus. Journal of Applied Environmental Microbiology, 53,* 2696–8.

Shikongo-Nambabi, M.N.N.N., Petrus, N.P. and Schneider, M.B. (2012). The role, isolation and identification of Vibrio species on the quality and safety of seafood. *Journal of Biotechnology and Molecular Biology Review, 7(2),* 16-30.

Statistical Service Republic of Cyprus, 2011. Preliminary Results of the Census of Population. Retrieved May 25, 2016 from http://www.mof.gov.cy/mof/ cystat/statistics.nsf/ All/732265957BAC953AC2257983004069O3?

Su, Y. and Liu, C. (2007). *Vibrio parahaemolyticus*: A concern of seafood safety, Review. *Journal of Food Microbiology, 24(6)*, 549–558.

Terzi Gulel, G. and Martinez-Urtaza, J. (2016). Molecular characterizations of *Vibrio parahaemolyticus* in seafood from the Black Sea, Turkey. *Letters in Applied Microbiology Journal, 62(6)*, 494-500.

The Earliest Prehistory of Cyprus: From Colonization to Exploitation, (2001). In: Swinny, S. (Ed.) Cyprus American Archaeological Research Institute Monograph Series vol. 2. American Schools of Oriental Research Publications, Boston.

Twedt, R.M. (1989) *Vibrio parahaemeolyticus.* In *Foodborne Bacterial Pathogens,* ed. M. P. Doyle, pp. 543-568. Marcel Dekker Inc, New York, USA.

Tu, N., Tu, Q., Tung, H., Hieu, D. and Romeo-Jovel, S. (2014). Detection of tetrotoxin-producing bacteria *Providencia rettgeri* T892 in *Lagocephalus* Pufferfish. *World Journal of Microbiology and Biotechnology, 30(6)*, 1829-1835.

Tuyet, D.T., Thiem, V.D., Seidlein, L.V., Chowdhury, A., Park, E., Canh, D.G., et al. (2002). Clinical, epidemiological, and socioeconomic analysis of an outbreak of *Vibrio parahaemolyticus* in Khanh Hoa Province, Vietnam. *Journal of Infectious Diseases, 186*, 1615-1620.

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.

Venugopal, V. (2006). Seafood Processing "Adding Value through Quick Freezing, Retortable Packaging, and Cook-Chilling". CRC Press Taylor & Francis Group, United States.

Vongxay, K., Wang, S., Zhang, X., Wu, B., Hu, H., Pan, Z., Suyun Chen, S. and Fang, W. (2008). Pathogenetic characterization of *Vibrio parahaemolyticus* isolates from clinical and seafood sources. *International Journal of Food Microbiology, 126*: 71–75.

World Health Organization, WHO (2002). *Foodborne diseases, emerging. Fact sheet No. 124* Geneva. (http://www.who.int/mediacentre/factsheets/fs124/en/ (Retrieved on 3 May, 2016).

World Health Organization, WHO (2015): *Estimates of the Global Burden of Foodborne Diseases,* Geneva. http://www.who.int/mediacentre/news/releases/2015/foodborne-disease-estimates/en/ (Retrieved on 27 May, 2016).

Wong, H.C., Liu, S.H., Wang, T.K., Lee, C.L., Chiou, C.S., Liu, D.P., Nishibuchi, M. and Lee, B.K. (2000). Characteristics of *Vibrio parahaemolyticus* O3:K6 from Asia. *Journal of Applied Environmental Microbiology, 66,* 3981–3986.

Wong, H.C. and Wang, P. (2004). Induction of viable but nonculturable state in *Vibrio parahaemolyticus* and its susceptibility to environmental stresses. *Journal of Applied Microbiology, 96,* 359-366.

Wu, Y., Wen, J., Ma, Y., Ma, X. and Chen, Y. (2014). Epidemiology of foodborne Diseases Outbreaks by *Vibrio parahaemolyticus*, China, 2003-2008. *Journal of Food Control, 46,* 197-202.

Xu, M., Iida, T., Yamamoto, K., Takarada, Y., Miwatani T. and Honda T. (1994). Demonstration and characterization of simultaneous production of a thermostable direct hemolysin (TDH) and a TDH-related hemolysin (TRH) by a clinically isolated *Vibrio parahaemolyticus* strain, TH3766. *Journal of Infections Immun., 62*:166–71.

Xu, F., Ilyas, S., Hall, J.A., Jones, S.H., Cooper, V.S., and Whistler, C.A. (2015). Genetic characterization of clinical and environmental *Vibrio parahaemolyticus* from the Northeast USA reveals emerging resident and non-indigenous pathogen lineages. *Frontiers in Microbiology Journal, 6:* 272.

Yamamoto, T. and Yokota, T. (1989). Adherence targets of *Vibrio parahaemolyticus* in human small intestines. *Journal of Infections Immun., 57*: 24, 10–9.

Yeung, P.S. and Boor, K.J. (2004). Epidemiology, pathogenesis, and prevention of foodborne *Vibrio parahaemolyticus* infections. *Journal of Foodborne Pathogen Diseases, 1*, 74-88.

Yu, P., Puhr, N., Bopp, C., Gerner-Smidt, P. and Painter, J. (2006). *Vibrio parahaemolyticus* hemolysins associated with decreased hospitalization from shellfish-borne illness. Presented at the 2006 Intl. Conference on Emerging Infectious Diseases, Atlanta, Ga., 2006 March 19–22. Abstract 475.

Zhang, X.H. and Austin, B. (2005). Haemolysins in *Vibrio* species. *Journal of Applied Microbiology*, *98(5)*, 1011-1019.

Zhao, X., Lin, C., Wang, J. and Oh, D.H. (2014). Advances in Rapid Detection Methods for Foodborne Pathogens, Review. *Journal of Microbiology Biotechnology*, *24(3)*, 297–312.

APPENDICES

APPENDIX 1

VIBRIO SPECIES AND THEIR INFECTIONS

Table 1.1: *Vibrio* species and infections caused by each (Adams and Moss, 2008)

Species	Infections
V. cholerae , O1	Cholera, wound infection
V. cholerae, non-O1	Diarrhoea, gastroenteritis, wound infection, secondary septicaemia
V. mimicus	Diarrhoea, gastroenteritis, wound infection
V. parahaemolyticus	Gastroenteritis, wound infection, otitis media
V. fluvialis	Diarrhoea
V. furnissii	Diarrhoea
V. hollisae	Diarrhoea
V. vulnificus	Wound infection, primary septicaemia, secondary septicaemia
V. alginolyticus	Wound infection, otitis media
V. damsela	Wound infection

APPENDIX 2

SURVIVAL REQUIREMENTS OF *VIBRIO PARAHAEMOLYTICUS*

Table: 2.1: Characteristics for survival/growth of *Vibrio parahaemolyticus*

Parameters	Optimum	Extremes
Temperature (°C)	37	5-43
pH	7.8-8.6	4.8-11
Water activity(aw)	0.981	0.940-0.996
NaCl (%)	1.5-3	0.5-10

APPENDIX 3

MICROBIOLOGICAL LIMITS FOR *V. PARAHAEMOLYTICUS*

Table: 3.1 Recommended microbiological limits for *V. parahaemolyticus* in fish (ICMSF, 1986)

Product	n[1]	c[2]	Bacteria per gram or per cm^2	
			m[3]	M[4]
Fresh and frozen fish and cold smoked fish	5	2		
Frozen or raw crustaceans	5	1	10^2	10^3
Frozen cooked crustaceans	5	1	10^2	10^3
Cooked, chilled and frozen crabmeat	10	1	10^2	10^3
Fresh and frozen bivalve molluscs	10	1	10^2	10^3

[1] **n** is the number of representative samples

[2] **c** is the maximum number of acceptable samples with bacterial counts between m and M

[3] **m** is the maximum recommended bacterial counts for good quality seafood

[4] **M** is the maximum recommended bacterial counts for marginally acceptable seafood

APPENDIX 4

ADVANCEMENTS IN CULTURE METHODS

Table 4.1: Some improvements and automatization in conventional culture methods (De Boer and Beumer, 1999)

Method	Application
Sample preparation	
Gravimetric diluter	Diluent addition
Stomacher™ (Colworth)	Homogenization
Pulsifier™ (Kalyx)	Homogenization
Plating techniques	
Spiral plater	Enumeration
Dipslides	Enumeration
Chromogenic/fluorogenic culture media	Detection
Motility enrichment	Detection
Petrifilm™ (3M)	Enumeration and detection
HGMF	Enumeration and detection
Counting automation	Colony counting
Confirmation/identification test kits	Confirmation, characterization