STUDIES ON THE INCIDENCE OF *VIBRIO VULNIFICUS* IN AQUATIC FOODS AND ENVIRONMENTAL SAMPLES

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THESIS SUBMITTED TO THE
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CERTIFICATE

Dr.K.Y.SUBHASH has satisfactorily prosecuted the course of research and that the thesis entitled **"STUDIES ON THE INCIDENCE OF *VIBRIO VULNIFICUS* IN AQUATIC FOODS AND ENVIRONMENTAL SAMPLES"** submitted is the result of original research work and is of sufficiently high standard to warrant its presentation to the examination. I also certify that the thesis or part of has not been previously submitted by him for a degree of any university.

Date: (Dr. N. KRISHNAIAH)

Place: Hyderabad Major Advisor

CERTIFICATE

This is to certify that the thesis entitled **"Studies on the incidence of *Vibrio vulnificus* in aquatic foods and environmental samples"** submitted in partial fulfillment of the requirements for the degree of **MASTER OF VETERINARY SCIENCE** for **SRI P. V. NARASIMHA RAO TELANGANA STATE UNIVERSITY FOR VETERINARY, ANIMAL AND FISHERY SCIENCES, Hyderabad** is a record of the bonafied research work carried out by **Dr.K.Y.Subhash** under my guidance and supervision. The subject of the thesis has been approved by the student's Advisory committee.

No part of the thesis has been submitted for any other degree or diploma. All the assistance and help received during the course of investigation have been duly acknowledged by the author of the thesis.

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ABBREVIATIONS

APW	: Alkaine Peptone Water
bp	: base pairs
CFU	: Colony forming unit
cm	: Centimeter
CPC agar	: Cellobiose Polymyxin B Colistin agar
Da	: Dalton
DNA	: Deoxy Ribonucleic Acid
dNTP	: Deoxyribonucleoside triphosphate
DW	: Distilled Water
EDTA	: Ethylenediaminetetraacetic acid
ELISA	: Enzyme Linked Immuno Sorbent Assay
Fig.	: Figure
g	: Gram
h	: Hours
IU	: International Unit
kb	: Kilo base pairs
M	: Molar
m	: meters
mCPC	: Modified Cellobiose Polymyxin B Colistin
mg	: Milli gram
$MgCl_2$	: Magnesium Chloride
MH	: Muller Hinton
min	: Minutes
ml	: Milli litre
mM	: Millimolar

mol	:	Moles
mV	:	milli volts
MW	:	Molecular weight
NaCl	:	Sodium Chloride
ng	:	Nano gram
no.of	:	Number of
PCR	:	Polymerase chain reaction
PFGE	:	Pulsed Field Gel Electrophoresis
Pmol	:	Picomoles
ppt	:	parts per thousand
RNA	:	ribonucleic acid
rpm	:	Rotations per minute
Sl.No.	:	Serial number
TAE	:	Triacetate EDTA
TCBS	:	Thiosulphate Citrate Bile salt Sucrose agar
Viz.	:	Namely
µg	:	micro grams
µl	:	micro litre

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DECLARATION

I, **Dr.K.Y.Subhash** here by declare that the thesis entitled "**STUDIES ON THE INCIDENCE OF *VIBRIO VULNIFICUS* IN AQUATIC FOODS AND ENVIRONMENTAL SAMPLES**" submitted to Sri Venkateswara Veterinary University for the degree of **MASTER OF VETERINARY SCIENCE** is a result of original research work done by me. It is further declared that the thesis or any part there of has not been published earlier in any manner.

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ABSTRACT

The present study was undertaken to standardize PCR assay for detection of *Vibrio vulnificus* from aquatic foods and environmental samples and compare with conventional cultural methods. Primers derived from *vvhA* and *gyrB* genes gave specific amplification at 519 bp and 285 bp for *Vibrio vulnificus* respectively.

Four different template preparation methods viz. genomic DNA extraction, heat lysis, lysis buffers-1 and 2 were compared, of which heat lysis was found to be efficient and convenient. The specificity for *Vibrio vulnificus* was tested using primers from *vvhA* and *gyrB* genes with 3 *vibrio spp.* and 7 other organisms which gave a specific 519 bp and 285 bp products for *Vibrio vulnificus*. The minimum detection level with pure *Vibrio vulnificus* culture was found to be 0.25 CFU/ml.

APW and Luria Bertani enrichment broths were evaluated for PCR compatibility, it revealed that APW broth was superior. Spiking studies were carried out by inoculating with pure culture, it revealed that 18h incubation was better than 12h.

Out of 120 naturally contaminated food samples (30 each of fish, shrimps, crabs and oysters) screened for *Vibrio vulnificus,* PCR gave 28 positive results, out of which 6, 2, 14, and 16 were positive for the above given samples, respectively, where as

cultural methods gave 24 positive, of which the above given samples were positive for 5, 2, 3 and 14 samples respectively. Out of 150 environmental samples (30 each of aquatic farm water, esturine water, marine water, sediment and plankton) screened for *Vibrio vulnificus*, PCR gave 75 positive results, out of which 5, 21, 16, 16 and 17 were positive for the above given samples respectively, whereas cultural methods gave 67 positive, of which the above given samples were positive for 4, 19, 15, 14 and 15 samples, respectively.

The mean viable counts (CFU/g) are $2.9x10^2$ in fish, $5.1x10^2$ in shrimps, $5.0x10^2$ in crabs, $2.1x10^4$ in oysters , $5.3x10^3$ in shrimp pond water , $3.8x10^6$ in esturine water, $6.1x10^5$ in sediment and $6.2x10^3$ in plankton.

V.vulnificus was highly sensitive to Ciprofloxocin and Ceftazidime (96%), followed by Gentamicin(92%), Erythromycin and Tetracyclin (80%), Streptomycin (76%), Nalidixic acid (68%), Chloramphenicol (60%), Amikacin (52%), Kanamicin(44%). *V.vulnificus* was highly resistant to Ampicillin(96%) followed by Vancomycin(92%), Amikacin (20%), Kanamycin and Tetracyclin(12%), Nalidixic acid (8%), Erythromycin, Gentamycin and Streptomycin(4%).

CHAPTER I
INTRODUCTION

The genus Vibrio belongs to the family vibrionaceae, and are ubiquitous in the marine environment having thirty species.Thirteen species are pathogenic to humanbeings causing foodborne diseases,although *V.cholera, V.parahaemolyicus,V.vulnificus* are considered the most significant agents.

Vibrio vulnificus is a gram negative, non spore forming,halophilic,polarly flagellated, facultatively anaerobic short rod,capable of fermenting glucose to acid but not gas.It was first isolated by US centre for Disease Control (CDC) in 1964 (Strom and Paranjpye,2000).

It is part of natural flora of coastal marine environment worldwide and has been isolated from water,sediment, and a vaiety of seafoods including fish,shrimp,oysters and clams (Wright *et.al*., 1996; Baffone *et.al*., 2006). Low salinities (5-25 ppt) and warm temperatures (20-35^{0}C) have been reported to be favourable for this organism.

Vibrio vulnificus has been classified into three biotypes. Biotype 1 is found in esturine water and warm marine water, which is opportunistic pathogenic to humanbeings(Biosca *et. al.,* 1996). Biotype 2 is pathogenic to both eels and humanbeings and more virulent than biotype1(Biosca *et.al.,*1996). Biotype 3 is associated with either wound infection or septicaemia and is not associated with food consumption (Bisharat *et.al*., 1999). The infective dose is 10^3(Jackson *et. al.,* 1997).

Vibrio vulnificus produces endotoxins like Lipopolysacharide (LPS) and exotoxins like cytolysin-haemolysin toxin(*VvhA*), repeats in toxin (*RtxA*1) and protease (*VvpE).*Capsule and host serum iron are essential for multiplication of this bacterium.

In certain areas raw and undercooked seafoods are consumed in the form of wine preserved shrimps and crabs.It is a potential health hazard to the people handling and eating raw seafoods and /or exposure of open wounds to sea water (Lu *et. al.,* 2009).

In the United States contaminated seafood is responsible for 26.5% of all foodborne disease outbreaks with majority of these illnesses associated with consumption of shellfish.It is considered most serious and invasive of all human pathogenic vibrios in the United States,accounting for 95% of all seafood related deaths (Oliver,1989).

Liver damage or cirrhosis such as arising from alcoholism, chronic renal diseases, diabetis and immune compromising diseases are considered major factors in susceptibility (Oliver, 1989).

The clinical presentations produced by this organism are septicaemia, wound infection and gastroenteritis and rarely causes infection in meninges and lungs(West,1989). In the septicaemic form incubation period is 7 hours to several days with symptoms of fever,chills,nausea,and cardiovascular hypotention with 60% case fatality rate (Levine *et. al.*,1993; Oliver and Kaper, 1997). The wound infection is a cutaneous form, not foodborne but occurs due to punctured wounds after handling raw seafoods, with a fatality rate of 7-25% (Blake *et. al.*, 1980). Wound infections progress rapidly to cellulitis, ecchymoses and bullae which can progress to necrotizing fasciitis. In the gastrointestinal form symptoms are diarrhoea and abdominal spasms.

In order to increase the production of seafood and decrease the occurance of the disease in aquaculture some antibiotics are being misused and have resulted in the emergence of multidrug resistant *Vibrio vulnificus*. There is very little information on the abundance and ecology of *Vibrio vulnificus* in tropical waters in India.

Evaluating the risk of *Vibrio vulnificus*to public health requires determining the potential virulence and antibiotic susceptibility profile of the strains that are present in seafoods.After processing of seafoods,much of the *Vibrio vulnificus* organism will be under viable but non-culturable (VBNC) condition and the enumeration of this

organism by traditional conventional methods will underestimate the contamination levels.So the present study was undertaken with the following objectives.

1. To isolate and identify *Vibrio vulnificus* in aquatic foods and environmental samples.
2. To compare enrichment broths and media for isolation of *Vibrio vulnificus* from different samples.
3. To optimize PCR assay for detection of *Vibrio vulnificus*.
4. To study antibiotic sensitivity /resistance of *Vibrio vulnificus* against selected antibiotics.

CHAPTER II
REVIEW OF LITERATURE

2.1 *Vibrio vulnificus* in general

Vibrio vulnificus is a gram negative, motile, curved bacilli, halophilic, flourishes in warm temperatures and is a part of bacterial flora of the marine environment (Karunasagar *et. al*., 1987).

Vibrio vulnificus was first reported by the Centre of Disease Control in 1975 (Hollis *et. al*., 1976), described as "similar but not identical to *Vibrio parahaemolyticus* and *Vibrio alginolyticus*, it was initially named lactose positive (L+) *Vibrio* (Blake *et. al*., 1979), however this was later changed to *Beneckea vulnifica* (Reichelt *et. al*., 1976). It was later reported in 1979 that, due to the lack of use of the term *Beneckea vulnifica* by microbiologists, the organism should be referred to as *Vibrio vulnificus*, the naming nomenclature now commonly used today (Farmer, 1979).

Vibro vulnificus is an important seafood-borne pathogen widely distributed in esturine and coastal waters worldwide (Tamplin *et. al*., 1982; Oliver *et. al*., 1983; Karunasagar *et. al*., 1987).

The name *Vibrio vulnificus* (Latin, *vulnificus* = wound inflicting) was given in 1980 to a group of halophilic lactose fermenting vibrios isolated from blood cultures (Farmer, 1980).

Vibrio vulnificus is considered a highly pathogenic marine bacterium which implicated in a variety of infections in humans those consuming contaminated seafood or exposure of skin wounds for water contaminated with *Vibrio vulnificus* that cause primary septicaemia, in patients with chronic liver disease, immunodifficiency and iron storage disorders (Mead *et. al*., 1999).

Unlike other species of the *Vibrio* genus, the disease symptoms associated with *V. vulnificus* are often rapid and fulminating, this has led to *V. vulnificus* being considered as one of the most fatal human pathogens (Oliver 1995; Mahmud *et. al*., 2008; Li *et. al*., 2012; Ha *et. al*., 2014). In the United States *V. vulnificus* is the leading

cause of seafood related deaths (Thiaville *et. al*., 2011; Williams *et. al*., 2014) with a mortality rate that often exceeds 50%, and can rise to 100% if left untreated for longer than 72 hours (Bross *et. al*., 2007).

2.2 Taxonomy and classification

The genus *Vibrio* belongs to the family *Vibrionaceae*, which consists of Gram negative straight or curved rods, motile by means of single flagellum. According to the classification followed in Bergey's Manual of Systematic Bacteriology second edition(2005), *Vibrio vulnificus* belongs to Kingdom (*Bacteria*), Phylum (*Proteobacteria*), Class (*Gammaproteobacteria*), Order (*Vibrionales*), Family (*Vibrionaceae*), Genus (*Vibrio*) and Species (*vulnificus*). [Latin, *vulnificus* = inflicting wounds].

There are currently three main biotypes of *V. vulnificus*, based on characteristics that include; host range, serological characteristics and biochemical features (Amaro *et. al*., 1995; Arias *et. al*., 1997; Warner and Oliver, 1999; Vickery *et. al*., 2007; Broza *et. al*., 2012 ; Bier *et. al*., 2013) Biotype 1 is mainly associated with human infection (Baker-Austin *et.al*., 2012), whereas biotype 2 is associated with disease in eels and occasionally causes human disease (Biosca *et. al*., 1991). Biotype 3 is the most recently identified biotype and has been shown to be a hybrid of both biotypes 1 and 2 (Bisharat *et. al*., 1999; Horseman and Surani, 2011).

Several research groups have attempted to group *V. vulnificus* biotype 1 strains genotypically, with the aim of distinguishing hyper and lesser virulent strains. Currently, the most commonly referred to genotyping protocol for this approach is the "*vcg"* or "virulence correlated gene" typing method. The *vcg* typing system was developed as a way to distinguish between clinical and environmental isolates (Rosche *et. al*., 2005), as it was suggested that clinical and environmental isolates vary genetically, that produces 200 bp band by clinical strains (Warner and Oliver, 1999).

Analysis of this region at the DNA level identified that *V. vulnificus* strains could be divided into two groups, C-types and E-types. Therefore a PCR based method was established that could readily distinguish between the C-types and E-types (Warner and Oliver, 1999). Clinical isolates were grouped as C-type and reported to be "a strong indicator of potential virulence", whereas environmental isolates were grouped as E-type (Rosche *et. al*., 2005).

Analysis of the 16S ribosomal RNA led to the identification of two groups of *V. vulnificus* isolates i.e. type A (clusters of environmental isolates) and type B (clusters of clinical isolates) (Nilsson *et. al*., 2003; Vickery *et. al*., 2007). Bisharat *et. al*. (2007) used Multi-Locus Sequence Typing (MLST) and grouped into two clusters, cluster one which was mainly made up of environmental isolates and cluster two which was predominantly made up of clinical isolates.

Cohen *et. al*. (2007) published work on MLS having two groups which were termed lineages, lineage one was mainly made up of clinical isolates, whereas lineage two mostly consisted of environmental isolates.

A PCR method was developed to distinguish between *V.vulnificus* strains that are hazardous to human health, from those that are not, based on the difference in the DNA sequence of the *pilF* gene (Roig *et. al*., 2010). Pathogenic strains are reported to produce a PCR band that is 338bps in length, whereas non-pathogenic strains are PCR negative and do not produce a band (Roig *et. al*., 2010).

2.3 Distribution

Vibrio vulnificus is an important seafood-borne pathogen widely distributed in estuarine and coastal waters worldwide (Tamplin *et. al*., 1982; Oliver *et. al*., 1983; Karunasagar *et. al*., 1987). The abundance of *V. vulnificus* is influenced by seawater temperature and salinity. In temperate waters, moderate salinity (15–25 ppt) and warm temperatures (>18 °C) support the growth and proliferation of this organism (Kaspar and Tamplin, 1993; Tamplin, 1995; Motes *et. al*., 1998).

Physico-chemical parameters influencing growth of *vibrio vulnificus* in the environment, is influenced by various factors like temperature, pH, salinity, abundance of zooplankton, availability of nutrients and other unidentified factors (West and Colwell, 1984; De Lapena *et. al.,* 1993).

In tropical waters a different ecology has been reported recently with salinity as the key factor controlling the abundance of *V. vulnificus* in water (Parvathi *et. al*., 2004).

Salinities at or greater than 30 ppt will substantially reduce the burden of *V. vulnificus* regardless of the water temperature (Motes *et. al*., 1998). As a result, most cases of infection can be traced to tropical or subtropical sources. Even so, small outbreaks related to local seawater or seafood exposure have occurred in temperate climates as far north as Denmark during the summer months (Oliver *et. al*., 2005).

Quantitatively coastal water harbours more vibrio than the open sea (Jung and Shin, 1996), similar diminishing trend in dencity of *vibrios* towards open sea was reported from Indian coast also (Sreeja and Ravindran, 1999). Horizontal variation in the occurance was also noted in East China Sea by Shin and Jung (1996) and it contained $0.2x10^{1}$ to $9.0x10^{3}$ ml^{-1}.

Vibrio species have halophilic characterstics and occur most frequently in water with salinity ranging from 0.5 to 3 %, thus significantly limiting their presence to esturine and inshore coastal waters (West and Lee, 1982; Koh *et. al*., 1994a). *Vibrios* could survive in fresh water also and the interaction of high water temperature and elevated organic nutrient concentration might be the factors help to tide over the deleterious effect of low salinity (Singleton *et. al*., 1982 a and 1982 b; Miller *et. al*., 1984).

Vibrio population was positively correlated to the particulate matter of the water column (Monticelli and Crisafi, 1995). Shen *et. al.* (1996) studied the vertical

distribution of *Vibrio* and reported highest quantity in surface water (0m) followed by 100m and lowest in 25 m depth.

Kim and Kwon (1997) reported that at 40^0C, in bottom deposit solution of brackish water, survival time of *Vibrio vulnificus* was longer and rate of decline slower than that of brackish water.

Incidence of *Vibrio vulnificus* is higher during warmer months of the year, coinciding with zooplankton blooms in pre-monsoon (Motes *et. al*., 1998). *V.vulnificus* was correlated with indicator bacteria (Okpokwasi and Akajobi, 1996), where as negative correlation was reported by Tamplin *et. al*. (1982), O'Niell *et. al*. (1992) and Parvathi *et. al*. (2004).

In south India highest *V.vulnificus* levels were found during monsoon season when the salinities were less than 5ppt and at salinities higher than 25 ppt, these organisms were not detectable (Parvathi *et. al*., 2004).

2.3.1. Temperature

Temperature is one the most important factor determining the growth and survival of *vibrio vulnificus* in environment as well as sea food. *V. vulnificus* is usually found worldwide in coastal or estuarine environments with water temperatures from 9 to 31^0 C. The organisms preferred habitat, however, is considerably more selective and has been reported to be water temperatures in excess of 18 ^{0}C (Motes *et. al*., 1998; Strom and Paranjpye 2000; Oliver , 2005). *Vibrio vulnificus* shows poor survival below 8^0C. Growth was noted in sterilized sea water in the 6th day of incubation at 13 to 22°C and temperature outside this range reduced the time of survival up to 90% level after 6 days of incubation (Kasper and Tamplin, 1993).

Parker *et. al*. (1994) also reported a considerable reduction in the number of cells from 10^5 to 10^1 CFU/g of *V.vulnificus* in oysters stored at -20°C. For *V. vulnificus* low temperature induces the formation of viable but non-culturable state (VBNC)

(Oliver *et. al.*, 1991; Wolf and Oliver, 1992; Oliver, 1993; Oliver *et. al.,* 1995). In VBNC state, the bacteria survive for at least for 50 days at low temperature (Biosca *et. al.,* 1996).Therefore the hazard due to this organism exists even though the contaminated products are stored at low temperature.

2.3.2. pH

A pH range of 6-9 was reported for optimum growth of *Vibrio vulnificus* (De Lapena *et. al.*, 1993) Most of the species of *Vibrio* can tolerate moderately alkaline condition and can grow at pH 9. An optimum pH of 7.5 for *V. vulnificus* was described by Koh *et. al.* (1994b).

The minimum and maximum pH values for the growth of *V.vulnificus* have been reported to be 5 and 10, respectively (FDA, 1998). In a survey of cooked, ready –to- eat shellfish undertaken in the UK, no samples with a pH less than 5 (shellfish pickled in vinegar post processing) showed unsatisfactory levels of *vibrios*, including *V.vulnificus* (Little, 1997).

2.3.3 Salinity

Survival of *V.vulnificus* has been studied by using strains inoculated into sterilised seawater, the salinity of which had been adjusted with deionized water (Kaspar and Tamplin, 1993). At 14 °C there was a tendency for an increase in numbers over a 6 day incubation period at salinities of 5-25 ppt, while at higher salinities (30-38 ppt) there was a reduction over this period.

At 21°C, the numbers of *V.vulnificus* had dropped by 1-2 logs by 6 days at all salinities, although those between 5 and 15 ppt contained the greatest numbers.

Kelly (1982) reported that no growth of *V.vulnificus* took place at less than 0.1% or greater than 5% NaCl ,and optimal growth in 1-2 % NaCl. Oliver and Wanucha (1989) have observed an optimal NaCl concentration between 1% and 3% for *V.vulnificus* although 0.5% NaCl present in many routine laboratory media provides for

very good growth. The maximum tolerance for NaCl is 5- 6% for *V.vulnificus* (Mortimore and Wallace, 1994; FDA, 1998).

2.3.4 Other parameters

Many other parameters are also critical for the occurrence of *Vibrio vulnificus*. This included the availability of nutrients, products of metabolism and many other biological factors like presence of zooplankton and sediment etc. *V.vulnificus* could survive in sterilized sea water up to 14 days at 4^0C, but it could not survive in unsterilized sea water. This observation indicated the influence of biological factors in their survival in the environment (Kasper and Tamplin, 1993; McCarthy, 1996).

Kim and Kwon (1997) reported that at 4^0 C, in bottom deposit solution of brackish water, survival time of *V.vulnificus* was longer and rate of decline slower than that of brackish water.

Vibrio vulnificus density in the sediment was nearly three orders of magnitude higher than those in the overlying water (Wright *et. al*., 1996).

V.vulnificus has been isolated from small zooplankton (up to 200 micro m.) in nutrient rich waters in Italy, with a significant correlation being noted between plankton abundance and bacterial density (Montanari *et. al*., 1999). The effects of nutrients found in other studies (noted above) could therefore be due to the increase in plankton numbers rather than a direct effect on *V.vulnificus*. The association between chitinous zooplankton and *Vibrio vulnificus* have been established (Oliver *et. al*., 1983), due to production of chitinase by *V.vulnificus* (Strom and Paranjpyee, 2000).

2.4 The viable but non culturable state of *V. vulnificus*

It was shown that bacterial cells could enter a state whereby they were non-culturable on standard laboratory media, yet bacterial staining methods demonstrated that the cells were still alive (Xu *et. al*., 1982; Li *et. al*., 2014). As the cells were not dead, but had entered a non-culturable stage, this novel microbiological phenomenon

was termed viable but non culturable (VBNC) (Xu *et. al*., 1982). The VBNC state of bacterial cells is thought to be trigged by an extreme change in their environmental surroundings (Li *et. al*., 2014). When cells enter a VBNC state they undergo various changes which can include, changes to their cellular morphologies, an increase or decrease in metabolism and alterations to their cell wall and membrane compositions (Li *et. al*., 2014). Unlike dead cells however, VBNC cells are metabolically active, transcribe genes, utilise nutrients and carry out respiration (Lleo *et. al*., 1998; Lleo *et. al*., 2000).

V. vulnificus is known to enter a VBNC state (Oliver, 1995), which is reported to aid the bacterium in withstanding environmental stresses such as temperature shifts (Nowakowska and Oliver, 2013). For example, a decrease in temperature to 5 ^{0}C can induce *V. vulnificus* cells to become VBNC (Oliver *et. al*., 1991). During this period the cells undergo cellular membrane changes and reduced amino acid transport (Linder and Oliver, 1989; Oliver *et. al*., 1991). As previously mentioned, the ability to culture *V. vulnificus* cells from the environment during the winter months is greatly reduced. It is believed that this is due to the pathogen's ability to become VBNC. Accordingly the effect of temperature on the ability to culture *V. vulnificus* from the environment has been demonstrated experimentally (Smith and Oliver, 2006; Kong *et. al*., 2004). This was done by placing chambers filled with VBNC cells into estuarine water during August to November, when the water temperature was above 5 ^{0}C. When the cells were taken out again and plated onto laboratory media, the cells were culturable, demonstrating the resuscitation ability of temperature on the VBNC cells. However, when laboratory culturable cells of *V. vulnificus* were placed into estuarine water that was below 15 ^{0}C, when the cells were taken out again, the cells were no longer culturable on standard laboratory media, further demonstrating the effects of

environmental temperatures in inducing the VBNC state of *V. vulnificus* (Oliver *et. al*., 1995).

In addition to warm temperatures resuscitating VBNC cells, the quorum sensing auto-inducer molecule, AI-2, has also been shown to play a crucial role (Ayrapetyan *et. al*., 2014). This was demonstrated by adding the cell free supernatant from culturable *V.vulnificus* cells, to VBNC cells, causing the VBNC population to be resuscitated and become culturable. The molecule AI-2 was shown to be the important factor, as cell free 10 supernatants from *V. vulnificus* cells that were unable to secrete AI-2, failed to resuscitate VBNC cells (Ayrapetyan *et. al*., 2014).

To assess the virulence of VBNC *V. vulnificus* cells, iron over loaded mice were challenged with a VBNC population. The results showed that even after entering a VBNC state, *V. vulnificus* remains virulent, and able to cause disease (Oliver and Bockian, 1995).

2.5 History and epidemiology

In US and Korea *V.vulnificus* infection is a reportable disease, hence most epidemiological information is available and it is estimated that about hundred cases of primary septicemia due to this organism occur every year in United States (Drake *et. al*., 2007). All the cases have been associated with consumption of raw oysters from Gulf coast with 90% of cases occurring during April to November (FAO/WHO, 2005). A retrospective study of cases reported to the US Centre for Disease Control and prevention during 1988 to 1996 indicated that wound infections were almost as frequent as primary septicaemia (Strom and Paranjpye, 2000).

In 1996, major unexplained systemic *V.vulnificus* infection erupted among Isreal fish market workers (Bisharat and Raz, 1996). Out of 422 infections reported between 1988 and 1996, 45% were wound infections, 43% were primary septicaemia, 5% gastroenteritis and the remaining 7% from undetermined exposure.

The Korean CDC estimates 40-70 confirmed cases per year and this high rate was suspected to be due to consumption of raw seafood or higher prevalance of predisposing factors (Drake *et. al*., 2007).This apparent higher incidence of *V.vulnificus* infections in Korea may be the result of greater exposure due to high consumption of raw seafood or a higher prevalence of predisposing factors. It is well recognized that there are specific risk factors for the development of *V.vulnificus* sepsis (Hlady and Klontz, 1996). Not only is raw oyster consumption a risk factor, but underlying liver diseases, including cirrhosis, damage to the liver due to alcoholism and chronic hepatitis are strong predictors for fatal outcome of sepsis, with 80% of those who die from the infection falling into these risk groups (Shapiro *et. al*., 1998; Strom and Parnjpye, 2000). However, in Japan, Inoue *et. al*. (2008) estimated 12-24 cases per year and in Taiwan, there was a peak occurrence in 2000 with 26 cases per million population and these included both septicaemia and wound infections (Hsueh *et. al*., 2004).

A study of cases in Taiwan during 1995-2000 revealed that over 80% had underlying conditions like liver disease, diabetes or steroid use, and 23.8% cases had primary septicaemia. Interestingly in Japan oysters are not the primary source, since raw oysters are eaten only in winter and most infections occur during June-November with peak in July. A mud shrimp *Upogebia major* was the common agent associated with *V.vulnificus* infections (Inoue *et. al*., 2008). Most cases occurred in western Japan with about 50% of cases occurring in Kyushu. In Japan, 72.3% of infections had septicaemia and mortality rate was 75%. Most patients (86.5%) had liver function impairment with 56.9% having liver cirrhosis and 10.1% liver cancer (Inoue *et. al*., 2008).

In Europe, *V.vulnificus* infections are rare and mostly wound infections (Baker-Austin *et. al*., 2010). Rare cases of septicaemia have been reported from Thailand (Thamlikitkul, 1990) and India (Saraswathi *et. al*., 1989).

2.6 Transmission

Primary septicaemia seems most often to be a result of ingestion of bivalve shellfish. The route of infection in 10 of 11 cases in Denmark was a pre-existing skin lesion and none had consumed seafood (Dalsgaard *et. al*., 1996). In France, among 5 cases of *V.vulnificus* infections which occurred between 1995 and 1998, direct contact with the sea was established for 4 cases (Geneste *et. al*., 2000). In a recent review on the epidemiology and pathogenesis of *V.vulnificus*, it was mentioned that it is difficult to definitely state that *V.vulnificus* can cause seafood-related gastroenteritis (Strom and Paranjpye, 2000).

Since the consumption of seafood usually involves handling the products and often involves activities giving direct contact with estuarine water or sea water, the actual route of infection, whether ingestion or skin contact, is difficult to identify (Strom and Paranjpye, 2000). A marked seasonality correlated with high water temperature has also been reported for the incidence of human infections caused by this bacterium (Evans *et. al*., 1999; Geneste *et. al*., 2000).

2.7 Pathogenesis & virulence factors

The virulence of *V.vulnificus* is related to multiple factors such as evasion of host defences, iron uptake mechanism , motility and attachment, endotoxins, exotoxins etc.

2.7.1 Evasion of host defences by *V.vulnificus*

To overcome the host's immune system and establish infection *V.vulnificus* has certain mechanisms i.e. acid neutralization and capsule.

2.7.1.1 Acid neutralisation

As *V. vulnificus* is an acid-sensitive bacterium, it is imperative that the organism is able to neutralise gastric acids (Rhee *et. al*., 2002). Neutralisation of acidic conditions is achieved by the synthesis of cytoplasmic amino acid decarboxylases (Willey *et. al*., 2008). The *cadBA* operon of *V. vulnificus* encodes a lysine/cadaverine antiporter and a

lysine decarboxylase, which both aid in countering acidification by the synthesis and excretion of cadaverine (Rhee *et. al*., 2005).

Mutagenesis studies demonstrated that lysine decarboxylase encoded by the *cadA* gene plays an important role in acid tolerance, as a *cadA* mutant is more sensitive to acidic conditions than the wild-type strain. In order for the *cadBA* operon to be induced and provide cells with the ability to withstand acidic conditions, prior exposure of the cells to a low pH is required (Rhee *et. al*., 2002). Super oxide generation rather than acid stress may be initial triggering event for expression of enzymes (Jones and Oliver, 2009). *V.vulnificus* shown poor survival when exposed to low pH (Koo *et. al*., 2000) and dramatically improved survival if the organism was exposed to slightly acid pH prior to low pH (Rhee *et. al*., 2002).

2.7.1.2 Capsule

Capsule is involved in protecting bacteria from the host immune system and concealing pathogen associated molecular patterns (PAMPS), such as outer membrane proteins (Jeannin *et. al.,* 2002).The transformation of encapsulated isolates to the non encapsulated form is dependent on growth phase and temperature, which in turn affect bacterial cell morphology. Wright *et. al.* (1990) found an increase in the expression of capsular polysaccararide (CPS) during the logarithmic growth phase and a decrease during the stationary phase of growth for a clinical isolate of *V. vulnificus*.

V. vulnificus cells display two differing colony morphologies, designated opaque and translucent (Rosche *et. al.,* 2006), due to the surface expression of capsule (Joseph and Wright, 2004). Opaque morphologies represent encapsulated virulent strains expressing capsular polysaccharide (CPS), whereas translucent colonies represent non-encapsulated non virulent strains , or cells expressing decreased amounts of CPS (Rosche *et. al.,* 2006).

2.7.2 Iron uptake mechanisms by *V. vulnificus*

Iron in human hosts is generally unavailable to invading bacteria, as it is bound to transferrin (Willey *et. al.,* 2008).To overcome this *V. vulnificus* has been shown to produce two types of siderophores, vulnibactin and hydroxymate-type (Simpson and Oliver, 1983) .The vulnibactin (catechol siderophore) is the chief agent of acquiring iron for growth in iron limited media (Alice *et. al.,* 2008; Kim *et. al.,* 2006).

Several genes (*vvsA ,vvsB,venB* and *vis*) are involved in vulnibactin synthesis , where as *vuuA* under the control of *Fur* regulates uptake of iron (Alice *et. al.,* 2008; Kim *et. al.,* 2006; Kim *et. al.,* 2008a ; Webster and Litwin, 2000).

In addition to siderophores, *V. vulnificus* could also scavenge iron from the human host, through the use of the metalloprotease, VvpE. It was believed that the proteolytic ability of VvpE could cleave heme proteins from host transferrins and lactoferrins (Kim *et. al.,* 2006; Nishina *et. al.,* 1992). An increase in iron levels, caused a decrease in the amount of bacteria required to cause an infection from 10^6 to 1 bacterial cells and liver damage causes more susceptible to *V. vulnificus* infection (Wright *et. al.,* 1981).

2.7.3 Motility and attachment

In order to establish an infection, many bacteria will often move from the initial site of infection to another location within a host (Josenhans and Suerbaum, 2002). This motility is often achieved by the presence of flagella and as such flagella is often regarded as an important virulence factor for many pathogenic bacteria (Duan *et. al.,* 2013), including *V.vulnificus* (Lee *et. al.,* 2004a). The role of attachment in the pathogensis of *V.vulnificus* is well documented (Kim *et. al.,* 2008b).

2.7.3.1 Flagellum

The basic flagellum structure is made up of the basal body, hook and filament (Homma *et. al.,* 1984). In *V. vulnificus* the following genes encode for hook associated proteins (HAP), *flgK* (HAP1), *flgL* (HAP2) and *flgH*(HAP3), whereas *flgK* and *flgH*

form a junction between the hook and filament, with *flgL* forming a capping structure at the end of the flagellum.

Loss of two flagellar structural components (encoded by *flgC* and *flgE*) resulted in significant decrease in motility ,cellular adhesion and cytotoxicity (Kim and Rheem, 2003; Lee *et. al.,* 2004a).

2.7.3.2 Pili

Pili are used by many gram negative bacteria for adherence to host cells, mutations in *pilA*, which encodes a pilin structural protein, and *pilD*, which encodes a prepilin peptidase, were generated in *V. vulnificus* (Paranjpye and Strom 2005; Paranjpye *et. al.,* 1998). Both mutants demonstrated a loss of attachment to epithelial cells and a slight increase in LD50 (1 and 2 logs for *pilA* and *pilD*, respectively) compared to the parent strains. Furthermore, mutation in *pilD* reduced secretion of cytolysin, protease, and chitinase and was associated with an overall reduction in cytotoxicity (Paranjpye *et. al.,* 1998).

2.7.4 Endotoxins

Lipopolysaccharide (LPS), also known as endotoxin, is a molecule made up of a lipid and a polysaccharide, making up the outer leaflet of the outer membrane of Gram negative bacteria, is easily accessible to the host immune system(Bosshart and Heinzelmann, 2007).

Exposure of humans to *V. vulnificus* LPS results in a characterstic disease known as septic shock (Mayer *et. al.,* 2014). More number of male succumb to *V.vulnificus* sepsis when compared to females due to protective mechanis of estrogen (Jones and Oliver, 2009; Merkel *et. al.,* 2001).Low density lipoproteins (LDL), are also believed to help protect host organisms from *V. vulnificus* induced sepsis (Park *et. al.,* 2005).

2.7.5 Exotoxins

V. vulnificus produces and secretes many extracellular proteins and toxins. It is hypothesized that some of these secreted proteins contribute to the significant tissue damage observed during infection. The most well studied extracellular proteins have been the metalloprotease and haemolysin /cytolysine.

2.7.5.1 RtxA1 toxin

Although *V. vulnificus* is known to produce a range of secreted virulence factors, the most extensively studied toxin is the Rtx toxin or "repeats in toxin" is a class of toxin which is found extensively in Gram negative bacteria and can cause a range of biological effects (Linhartova *et. al.,* 2010). *V. vulnificus* RtxA has been shown to cause disruption to the cellular membrane of mammalian cells (Lee *et. al.,* 2007), as well as causing cytoskeleton rearrangement and blebbing of cells, ultimately leading to the necrotic cell death of mammalian cells (Kim *et. al.,* 2008b). This is in contrast to the *V. cholera* Rtx toxin, which has been shown not to disrupt the cellular membrane, but instead causes depolymerisation of actin leading to the rounding up of mammalian cells (Lee *et. al.,* 2007).

The RtxA toxin of *V.vulnificus*is secreted via the T1SS which contains an RtxE ATPase. The *rtxE* mutant was shown to be more attenuated than a *rtxA* due to the secretion of additional virulence factors (Lee *et. al.,* 2008). Following secretion of the RtxA toxin into host cells, the toxin has been shown to then be processed into two parts. The larger N-terminal region localises to the host cell membrane, whereas the smaller C-terminal region can be detected inside the host cell (Kim *et. al.,* 2013). The pore produced by the N-terminal fragment has been shown to cause an influx of calcium into host cells leading to an increase in intracellular calcium levels and which led to mitochondrial dysfunction and ultimately programmed necrotic cell death (Kim *et. al.,* 2013). .

The most abundant RtxA toxin present in clinical isolates has reduced virulence in comparison to the toxin from environmental isolates (Kwak *et. al.,* 2011)

2.7.5.2 Haemolysin

vvhA gene from *V. vulnificus* controlling hemolysin/cytolysin protein, is often used as a *V. vulnificus* species specific housekeeping gene that is commonly used to identify the provenance of isolates (Kim *et. al.,* 2010 ; Neogi *et. al.,* 2010).

The toxin is heat-labile, hydrophobic, able to cause lysis of red blood cells, toxic to CHO cells *in vitro* and lethal in an *in vivo* mouse model of infection (Gray and Kreger, 1985). As such *VvhA* is thought to be a potent *V. vulnificus* toxin due to the disease symptoms generated in mice at very low inoculation levels (Gray and Kreger, 1985; Lee *et. al.,* 2004b). The *vvhA* mutant was attenuated compared to the wild-type strain, and that VvhA in conjunction with RtxA, is required for gut pathogenesis (Jeong and Satchell, 2012).

V. vulnificus produces outer membrane vesicles (OMV) containing *VvhA* during *in vitro* infection that mediates apoptosis in epithelial cells (Kim *et. al.,* 2010). OMV will interact with cholesterol on host cells to deliver the toxin (Yu *et. al.,* 2007).

2.7.5.3 Proteases

An additional secreted protein by *V. vulnificus* is the extracellular metalloprotease (VvpE), requiring Zinc for its catalytic activity (Miyoshi *et. al.,* 1987), is produced maximally during the late exponential growth phase (Shao and Hor, 2001).

VvpE protein is 45 kDa, and upon secretion is cleaved into two fragments, a 34 kDa protein and a C-terminal pro-peptide that is 11 kDa in length (Park *et. al.,* 2008). 34 kDa protein is more efficient at degrading soluble proteins than insoluble proteins.VvpE is a virulence factor, manifesting with disease symptoms such as dermonecrosis and swelling, symptoms commonly seen in wound infected patients (Kothary and Kreger, 1987; Miyoshi and Shinoda, 1988).

It has also been further demonstrated that VvpE is able to degrade immunity proteins such as IgA and lactoferrins, suggesting that VvpE may play a role in establishment of infection by aiding *V. vulnificus* in adherence and colonisation, as well as carrying out removal of immunity proteins such as IgA (Kim *et. al.,* 2007).

2.7.5.4 Other factors

V.vulnificus enterotoxin was isolated by (Stelma *et. al.,* 1988), which is water soluble distinct from both the cytolysin (hemolysin) and the protease activity. Small regulatory protein *HlyU,* which is a regulator of virulence in *V.vulnificus* was identified (Kim *et. al.,* 2003).

2.8 Human infection

Vulnificus is known to be a fatal pathogen often causing fulminating systemic disease symptoms, yet the infectious dose for humans is currently undetermined (Strom and Paranjpye, 2000). Clinical presentation is often dependent on the route of infection, ingestion for example can lead to gastroenteritis, diarrhoea, vomiting and nausea. (Kumamoto and Vukich, 1998; Matsuoka *et. al.,* 2013).

The immune state of a patient has also been shown to play a significant role in the disease outcome (Daniels, 2011 and Matsuoka *et. al.,* 2013). For example, patients suffering from liver disease are at particular risk of dying from infection (Bross *et. al.,* 2007; Strom and paranjpye, 2000; Zaidenstein *et. al.,* 2008). On average, 80% of fatal *V.vulnificus* infections occur in individuals who have chronic liver disease (Horseman and Surani, 2011; Strom and Paranjpye, 2000; Haq and Dayal, 2005). Chronic liver diseases can include cirrhosis, hepatitis and alcoholic liver disease (Bross *et. al.,* 2007; Jones and Oliver, 2009). In addition to patients suffering with chronic liver problems, individuals who have high iron serum levels or weakened immune systems, such as AIDS and chemotherapy patients are also at risk of infection (Gulig *et. al.,* 2005; Daniels, 2011; Chen *et. al.,* 2002; Ward *et. al.,* 2011).

Males are most at risk of infection with the majority of cases occuring in males over the age of 40 (Jones and Oliver, 2009). One possible link to account for the small number of females contracting the infection is that oestrogen has been shown to be protective against the effects of the bacterium's lipopolysaccharide (LPS) (Merkel *et. al.,* 2001). However, the exact reason why more males contract *V. vulnificus* infection has not been fully elucidated.

The number of "considered at risk" individuals consuming raw oysters in Florida is around 70,000, yet there are only on average five to ten *V. vulnificus* infections a year in this region (Strom and Paranjpye, 2000; Jackson *et. al.,* 1997). This trend of low infection compared to the availability of the bacterium in the environment is typical in all areas, where *V. vulnificus* has been isolated (Horseman and Surani, 2011). Why there are so few fatal infections, despite the high mortality rate and natural prevalence of this pathogen, has been a question which has perplexed scientists for many years (Thiaville *et. al.,* 2011 and Warner and Oliver, 1999).

Infections with *V.vulnificus* as the etiological agent was for the first time described in 1970 when it occurred in a previously healthy man who developed a leg infection and diarrhoea after bathing and collecting shellfish in sea water (West, 1989).

Clinical presentation is often dependent on the route of infection, ingestion for example can lead to gastroenteritis, diarrhoea, vomiting and nausea, will develop into primary septicaemia a condition in which the mortality rate can exceed 50% (Jones and Oliver, 2009).

Infections caused by *V.vulnificus* show different clinical presentations, of which primary septicaemia, wound infection, and gastroenteritis are the most important. In rare cases, *V.vulnificus* has been reported to cause pneumonia, peritonitis, endometritis, meningitis, septic arthritis, osteomyelitis, endoptholmitis and keratitis (West, 1989).

(a) Gastroenteritis

Infections in the gastrointestinal tract presenting as diarrhoea seem to be relatively infrequent (Oliver and Kaper, 1997; West, 1989). Gastrointestinal infections with *V.vulnificus* were first described by Johnston *et. al.,* (1986) who reported abdominal spasms and diarrhoea in three males after consuming raw oysters. The reported case/fatality rate is low if the infection is limited to the gastrointestinal tract (West, 1989).

(b) Primary septicaemia

The major form of infection with *V.vulnificus* involves a rapidly progressing septicaemia with few gastrointestinal signs. Such infections have a reported incubation period from seven hours to several days (Levine *et. al.,* 1993). Severe cases of gastroenteritis will develop into primary septicaemia (Jones and Oliver, 2009). The most frequent symptoms are fever, chills, nausea and cardiovascular hypotension (Oliver and Kaper, 1997). This disease has a high fatality rate of up to 60% in reported cases, and is in the majority of cases affecting predisposed persons (Levine *et. al.,* 1993; Tacket *et. al.,* 1984; West, 1989). Several authors claim that primary septicaemia is predominantly associated with the consumption of raw bivalve shellfish (Chin *et. al.,* 1987; Johnston *et. al.,* 1985; Morris, 1988; West, 1989).

(c) Wound infections

Another form of infection with *V.vulnificus* is cutaneous infections. These are not foodborne in the strict sense as they usually occur in connection with puncture wounds after handling of raw seafood or trauma and exposure to saline environments (Blake *et. al.,* 1980; Johnston *et. al.,* 1985; Veenstra *et. al.,* 1993; West, 1989). Wound infections range from mild self-limiting lesions to rapidly progressing erythema, cellulitis and necrosis, occasionally developing to secondary septicaemia (Morris, 1995). This form is reported to affect both healthy and predisposed persons (Levine *et. al.,* 1993; West, 1989). Characteristic symptoms of wound infection include oedema,

necrosis and swelling. Secondary cutaneous lesions can occur and large blistered regions may present along with cellulitis and pain. The mortality rate associated with wound infection is ~25% (Baker-Austin *et.al.,* 2012; Kumamoto and Vukich 1998; Oliver, 2005; Vinoh *et. al.*, 2006; Morris, 2003). It is also not uncommon for wound infection patients to require amputation of infected sites (Horseman and Surani, 2011). As with ingestion of the bacterium, wound infection can also lead to life threatening septicaemia (Horseman and Surani, 2011; Lewis *et. al.*, 2005). Both wound infection and primary septicemia can present with fever and chills, and occasionally hypotension (Borenstein and Kerdel, 2003; Yamamoto *et. al.*, 2003).

Wound infections have a reported mean incubation period of 12 hours (Blake *et. al.,* 1980). Severe wound infections with this bacterium often require extensive surgery of affected tissue or even amputations, and have reported fatality rates ranging from 7 to 25 % (Levine *et. al.,* 1993; Oliver, 1981; Oliver and Kaper, 1997).

2.9 Samples

2.9.1 Fish

Schandevyl *et. al.* (1984) isolated *V.vulnificus* from marine fish in Senegal, Africa.

Thampuran and Surendran (1998) reported an incidence of 16.6% in freshly caught marine fish, which has increased to 17.8% after exposure to ambient temperature. They also reported that the incidence was more (80%) in intestinal contents compared to 20% in muscles.

Rajapandian *et. al.* (2009) reported that the incidence of *V. vulnificus* was 13% in fish collected from fish landing centers, Chennai.

Thampuran *et. al.* (1997) have reported the incidence of *V.vulnificus* in coastal waters of Cochin. In a survey conducted in Karnataka on the West coast of India, Karunasagar *et. al.* (1990) observed the incidence of *V.vulnificus* in fish samples collected from the markets. *V. vulnificus* was the dominant species isolated from fishes

along Kakinada in the eastern coast of India (Prasad and Rao, 1994). Sanjeev *et. al.* (2000) have reported the occurrence of this species in frozen fish and fish products collected from Kerala and Tamilnadu meant for export.

Finkelstein *et. al.* (2002) observed first fatal infection after eating and handling of *Tilapia* fish.

The distribution of *V.vulnificus* in the intestinal content of fish from US Gulf of Mexico was studied by De Paola *et. al.* (1994) and reported that the concentration of 10^6 cells per gram in particular in fish that feed on molluscs and crustacean.

The bacterium also causes a fish disease designated vibriosis, causing considerable economical losses in European eel culture (Amaro *et. al.,* 1992).

V. vulnificus was isolated in 1996 from 2 disease outbreaks on a Danish eel farm which used brackish water (Dalsgaard, 1999).

Tison *et. al.* (1982) isolated strains of *V. vulnificus* from lesions on eels and proposed that these strains should be grouped under biotype 2.

2.9.2 Shrimps and prawns

A mud shrimp *Upogedia major* was the common agent associated with *v. vulnificus* infection (Inoue *et. al.,* 2008).

V.vulnificus has been isolated from larval shrimps (Karunasagar *et. al.,* 1990). Thampuran and Surendran (1998) reported zero percent prevalence of *V.vulnificus* in prawns from Cochin.

Chan *et. al.* (1989) studied the prevalence of vibrios in seafood from markets in Honkong during the summer season and reported 3 % prevalence of *V.vulnificus* in prawns.

Berry *et. al.* (1994) studied the microbial quality of raw shrimp imported into United States from China, Ecuador and Mexico which has been frozen during transportation and reported that 17% of the products were positive for *V.vulnificus*.

V. vulnificus and five other vibrio organisms (*V. harveyi*, *V. parahaemolyticus*, *V. alginolyticus*,*V. anguillarum* and *V. splendidus*) were found to be associated with five

types of diseases, i.e., tail necrosis, shell disease, red disease, loose shell syndrome (LSS), and white gut disease (WGD) in cultured shrimps from Andhra Pradesh (Jayasree *et. al.*, 2006).

Vaseeharan and Ramaswamy (2003) isolated *V.vulnificus* from shrimp eggs, post larvae, rearing tank water, seawater and feed.

Yano *et. al.* (2004) isolated *V.vulnificus* from giant tiger prawns and mantis shrimp samples collected from the market in Coastal cities of China.

Gopal *et. al.* (2005) studied the occurance of *Vibrio sps* in shrimps from East and West coast of India and reported that the incidence of *V. vulnificus* was 1-4 %.

Rao and Surendran (2013) studied the prevalence of *Vibrio sps* in post larvae from hatcheries and shrimp from farms and reported that the incidence of *V. vulnificus* (2.4%) in post larvae from hatcheries where as no incidence in whole shrimp.

2.9.3 Crabs

Davis and Sizemore (1982) examined 140 blue crabs (*Callinectessapidus*) collected from Galveston Bay, Texas, between November 1979 and November 1980 and reported that the incidence of *V.vulnificus* in Crabs was 7 % and the incidence was more in summer.

Morris (1988) reported that the prevalence of *V.vulnificus* in crabs was 11% from the Atlantic coast of the United States during the summer months.

Chan *et. al.* (1989) studied the prevalence of *vibrios* in seafood from markets in Honkong during the summer season and reported 3 % prevalnce of *V.vulnificus* in crabs

Rodgers *et. al.* (2014) examined the prevalence of *Vibrio vulnificus* in blue crabs (*Callinectessapidus*) and sediments from Maryland Coastal Bays, USA and reported that an incidence of 5.43 log MPN g^{-1}, which was higher than the incidence observed in bivalves.

2.9.4 Oysters

Since the soft body of the oyster is consumed whole, either raw or lightly cooked, it is generally classified as a high-risk food (Desenclos *et. al.,* 1991). They can accumulate large numbers of bacteria due to their filter-feeding habit, (Pruzzo *et. al.,* 2005; Pujalte *et. al.,* 2010) and *Vibrio* species are commonly isolated with 100-fold higher concentration in filter feeding organisms than in the surrounding water (Wright *et. al.,* 1996).

It is estimated that about hundred cases of primary septicemia due to *V.vulnificus* occur every year in United States, which are associated with consumption of raw oysters (Drake *et. al.,* 2007).

The post-harvest multiplication of *V.vulnificus* was examined by Cook (1994; 1997) and it was found that *V.vulnificus* was capable of multiplying in oysters at temperatures above 13^0C underlining the importance of short post-harvest storage times and low storage temperatures.

Oliver (1981) reported a rapid reduction in the original numbers on the survival of *V.vulnificus* in experimentally contaminated raw oysters stored on ice. Freezing alone does not eliminate *V.vulnificus* from oysters, as the organism can still be detected in oysters kept at – 20ºC for 12 weeks (Cook and Ruple, 1992; Cook, 1997; Parker *et. al.,* 1994). When contaminated oysters were transferred from a temperature of 35°C directly to a temperature of 6°C, the present *V.vulnificus* cells entered a viable but not culturable (VBNC) state (Bryan *et. al.,* 1999).

Morris (1988) reported that the prevalence of *V.vulnificus* in oysters was 50% from the Atlantic coast of the United States during the summer months.

Chan *et. al*. (1989) studied the prevalence of vibrios in seafood from markets in Honkong during the summer season and reported 6 % prevalnce of *V.vulnificus* in raw oysters.

Tamplin and Capers (1992) reported that higher densities of *V.vulnificus* organisms were found in oyster digestive tissue as compared to muscle tissue.

Little *et. al.* (1997) examined 2500 samples of cooked ready to eat oyster products from UK and reported lower prevalence 0.4 % *V. vulnificus.*

Oliver *et. al.* (1983) reported the average no of *V.vulnificus* in oysters to be 6x10 ^{4} CFU/ g in samples taken from East coat of USA.

Parvathi *et. al.* (2004) reported a prevalence of 56.6% in oyster samples collected from two estuaries along the Southwest coast of India using Colony hybridisation method and after 18 h of enrichment in alkaline peptone water the prevalence was 85 % by nested PCR.

2.9.5 Other sea foods

Various levels of incidence of *V.vulnificus* in mussels were reported i.e. 32.6% by Pinto *et. al.* (2008) in Italy, 11.2% in Spain by Blanco Abad *et. al.* (2009).

Adebayo Tayo *et. al.* (2011b) studied the occurance of vibios in Oron Creek and repoted that the incidence of *V.vulnificus* in aquatic snails was 100%.

Canigral *et. al.* (2010) studied the occurance of vibrios in mussel from Mediterranian sea and reported that 10% of samples are positive.

2.9.6 Environmental samples

V.vulnificus is wide spread in the environment and has been isolated from esturine waters of most US coastal states (Tilton and Ryan, 1987; Pfeffer *et. al.,* 2003).

Higher levels of *V.vulnificus* has been isolated from U.S waters as far North as the great bay of marine, Long island New York, Chesapeake Bay and Gulf of Mexico waters ,where the water temperatures were warmer year round and lower densities from Pacific, Canadian and North Atlantic waters, where the water temperatures were generally cooler year round(O'Neil *et. al.,* 1992; Motes *et. al.,* 1998).

Canigral *et. al.* (2010) studied the incidence of *V.vulnificus* in environmental samples from Mediterranian coastal area and reported that 32% of sea water samples and 13% of waste water samples were positive by real time PCR asaay.

Adebayo Tyo *et. al.* (2011b) studied the occurance of *vibrios* in Mediterranian sea and reported that all the 20 water sampes were negative for *V. vulnificus*.

2.10 Isolation & Identification

2.10.1 Conventional isolation methods

Currently PBS is recommended as diluent for the enumeration of *V.vulnificus* in Bacteriological Analytical Mannual of the USFDA (US Food and Drug Administration, 1995).

Azanza *et. al.* (1996) reported that 0.1% peptone solution containing 3% sodium chloride gave higher recovery than peptone buffer saline in both broth culture and oyster homogenate.

Parvathi *et. al.* (2004) compared the efficiency of enrichment broth and plating media for isolation of *V. vulnificus* and reported that enrichment in alkaline peptone water with polymixine B was found to be better than plain alkaline peptone water for 18 h.

Massad and Oliver (1987) were the first to use Cellobiose-polymixinB Colistin (CPC) Agar for isolation and identification of *V.vulnificus*. They also reported that the Colistin and Polymixin B helps in resistance of *V.vulnificus* and fermentation of Cellobiose for differentiation and high temperature incubation (40^0C) eliminates many marine bacteria.

Sun and Oliver (1995) and Oliver *et. al.* (1992) reported that CPC Agar was clearly superior to TCBS, Sodium do decylsulphate –polymixin B –sucrose (SPS) Agar and *V.vulnificus* enumeration (VVE) Agar for isolation of *V.vulnificus* from environmental samples.

In Spain, only 8% of the presumptive colonies from CPC Agar were identified as *V.vulnificus* by PCR which might be due to the difference in background flora in samples from Mediterranian and difference in brands of reagents used (Arias *et. al.,* 1998).

A selective modification of the CPC Agar termed *m*CPC with reduced concentration of Colistin is effective in isolating *V.vulnificus* from environmental sources (Tamplin *et. al.,* 1991; Tamplin and Capers, 1992; Dalsgard and Hoi, 1997; Hoi *et. al.,* 1998b).

In Denmark more than 95% of the presumptive colonies on *m*CPC Agar were identified as *V.vulnificus* taking into consideration the typical colony morphology of *V.vulnificus* i.e. flat yellow colonies of 2 mm in diameter (Hoi *et. al.,* 1998a; Hoi *et. al.,* 1998b).

Colistin and Polymixin B are both fatty acyl deca peptide antibiotics with bactericidal activity against most gram negative bacteria and these two differ only in single aminoacid, mode of action and microbiological activity are identical (Sogaard, 1982).

Parvathi *et. al.,* (2004) reported that the rate of *V .vulnificus* isolation was 56.6% on Cellobiose-Colistin agar followed by Modified Cellobiose-PolymixinB-Colistin agar (50%), Cellobiose-Polymixine-Colistin (33.3%) and Thiosulphate Citrate Bilesalt Sucrose agar (1.3%).

Hoi *et. al.* (1998a) reported that Cellobiose Colistin (CC Agar) was better for recovery of *V.vulnificus* than TCBS, CPC and *m*CPC Agar with pure culture, Danish water and sediment samples. They also isolated *V.vulnificus* from 40% of the samples using CC Agar compared to 35% using *m*CPC Agar from the same water and sediment samples. They also reported that TCBS Agar gave very low plating efficiency (1%) of both clinical and environmental *V.vulnificus* strains.

Brayton *et. al.* (1983) and Beazley and Palmer (1992) also reported low recovery of *V.vulnificus* on TCBS. Although, *V.vulnificus* has been described to be resistant to colistin and polymixin B, plating efficiency experiments showed that the CC agar, which has the lowest concentration of colistin compared to the other media gave best recovery of *V.vulnificus* (Massad and Oliver, 1987).

SPS Agar lacks selectivity for *V.vulnificus* as the sulphatase positive isolates will also be identified as *V.vulnificus* whereas TCBS agar had better specificity in this regard (Oliver *et. al.,* 1992).

Warner and Oliver (2007) developed a new medium CPC + for direct isolation of *V.vulnificus* from water and oyster samples using isolation A and B .

Micelli *et. al.* (1993) developed direct plating agar i.e. *V.vulnificus* Enumeration (VVE) medium containing oxgall,sodiumcholate, sodium taurocholate and potassium tellurate and reported that it has reduced 61.99% marine associated background microflora without adversely affecting the recovery of *V.vulnificus*. They also reported that the detection limit was 10 culturable cells in 100 grams of shellfish.

2.10.2 PCR methods

A number of PCR detection methods have been described for *V. vulnificus* which are based on the cytolysin–haemolysin gene (*vvhA*) (Brauns *et. al.,* 1991; Hill *et. al.,* 1991; Coleman and Oliver,1996; Lee *et. al.,* 1999).

Hill *et. al.* (1991) were the first to develop a PCR procedure for the detection of *V.vulnificus* by seeding into oyster homogenates, targeting cytotoxin-hemolysin gene, using vvp1

and vvp2 primers which has yielded 519 bp product. They also reported that the limit of detection was 10^2CFU/g following 24h enrichment.

Aono *et. al.* (1997) used the same primers developed by Hill *et. al.,* (1991) for identifying *V.vulnificus* from marine environments and reported that 713 samples out of 13,325 samples produced 519 bp products, which were confirmed by cultural methods.

Lee *et. al.* (1997) used primers Choi-1 and Choi-2 for PCR amplification of 704 bp sequence of the *vvhA* gene and reported that the sensitivity of detection was 10CFU/ml of homogenate and also reported that modified brain heart infusion broth containing 2% Nacl was superior to APW and the sensitivity was 10CFU/ml.

Hervio-Heath *et. al.* (2002) used VV-1 and VV-2 are primers targeting 704bp sequence *vvhA* gene and Vvoligi 1 and Vvoligo 3 targeting 386 bp fragment and reported that 20 samples were positive out of 242 samples, of which 19 belongs to Biotype 1 .

Kumar *et. al.* (2006) developed a PCR method based on *gyrB* gene sequence for the detection of *V.vulnificus* in seafoods and reported that 75% of the natural oyster samples were positive after 18h enrichment in APW producing 285bp sequence. They also reported that the sensitivity was 300 CFU/g without enrichment where as the sensitivity was 30 CFU/g with 18h enrichment in APW.

Arias *et. al.* (1995) developed nested PCR using 23S r RNA gene universal external primers and *V.vulnificus* specific internal primers and reported a sensitivity of 120 cells without enrichment. These primers missed some strains that are positive by *vvhA* PCR (Parvathi *et. al.*, 2005). Nested PCR amplifying *vvhA* gene detected as low as 1CFU/ g when coupled with direct extraction of template DNA (Lee *et. al.*, 1999).

Lee *et. al.* (1998) developed a nested PCR for direct identification of *V.vulnificus* in blood serum using P1 and P2 primers amplifying 704 bp sequence and P3 and P4 amplifying 222 bp sequence and reported that 17 out of 18 culture positive specimens and 8 from 19 culture negative specimens were positive for *V .vulnificus.*

Wang and Levin (2005) developed a quantitative detection of *V.vulnificus* in shellfish via competitive PCR and reported that the sensitivity was 220 CFU/g without enrichment, whereas the sensitivity was reduced to 7 CFU/g after a10 h enrichment.

2.10.3 Real-time PCR

Panicker *et. al.* (2004) developed SYBR Green I–based real-time PCR for detection of *V.vulnificus* in oyster tissue targeting *vvhA* gene amplifying 205 bp sequence and reported a detection limit of 10^2 CFU/g which has been increased to 1 CFU/g with 5h enrichment.

Wang and Levin (2006) developed TaqMan Real-time PCR assay for discrimination between viable and non viable cells of *V.vulnifics* using DNA inter chelating agent Ethidium Bromide.

Canigral *et. al.* (2010) developed a Taqman probe based real-time PCR assay targeting *vvhA* gene for determining the occurrence of *V.vulnificus* in water and seafood samples from coastal area near the Mediterranian and reported that 32% of sea water samples, 13 % waste water samples and 10% of seafood samples were positive by producing 205 bp band. They also reported that the detection level of the method was 10 CFU g^{-1}.

Campbel and Wright (2003) developed a Real time PCR for quantification of *V.vulnificus* targeting *vvhA* gene and reported the sensitivity of 100 cells/g in oyster homogenate or sea water without enrichment and enrichment for five hours increased the sensitivity of detection to one cell.

Han and Ge (2008) developed loop mediated isothermal amplification (LAMP) using primers binding to *vvhA* of *V.vulnificus* which had a detection limit of 10^7 CFU/g of oyster and the detection limit has been reduced to 7 CFU/g after 5h enrichment. Surasilp *et. al.*, (2011) combined LAMP targeting *rpoS* gene with amplicon detection by chromatographic lateral flow dipstick assay (LFD) with a detection limit of $1.2X10^4$ CFU/g. LAMP assay using virulence-coregulated gene (*vcg*) with a detection limit of $2.5x10^3$ CFU/g which has been increased to 1CFU/g using 6h enrichment.

2.10.4 Multiplex PCR

Wang *et. al.,* (1997) developed multiplex PCR method to detect 13 different foodborne pathogens including *V.vulnificus* (targeting *vvhA* gene) and reported that the detection limit was 100 cells per reaction.

Brasher *et. al.,* (1998) developed a multiplex PCR using L-CTH and R-CTH primers targeting 205 sequence *cth* gene and reported that the sensitivity of detection was 10^1-10^2 CFU.

Bauer and Rorvik (2007) developed a novel multiplex PCR for simultaneous identification of *Vibrio* organisms using different primers and reported that UtoxF/vvtox R primers amplified 640 bp sequence of the toxR gene for *V.vulnificus*. The sensitivity and specificity was 100%.

2.11 Antibiotic sensitivity

Antibiotics and other chemotherapeutic agents are commonly used in fish farms either as feed additive s or immersion baths to achieve either prophylaxis or therapy (Li *et. al.,* 1999).

However, extensive use of these drugs has resulted in an increase in drug resistant bacteria as well as R-plasmids (Son *et. al.,* 1997; Saitanu *et. al.,* 1994).

In EU member states, only four or five antimicrobial agents are licensed for use in finfish cultutre. In USA, Canada and Norway regulatory control is equally vigorous. But in many countries there is either no or no effective control on the use of antibiotics in food fish or shellfish species (Alderman and Hastings, 1998).

Traditionally, *Vibrio vulnificus* is considered to be susceptible to all antibiotics, except ampicillin (Joseph *et. al.,* 1978; Zanetti *et. al.,* 2001). However, some studies indicated that antimicrobial resistance has been surfaced into *V.vulnificus* in Austria (Akinbowale *et. al.,* 2006), the Philippines and Thailand (Maluping *et. al.,* 2005) by characterizing the antimicrobial susceptibility profile.

More than 80% isolates of *V.vulnificus* strains isolated from the coastal waters of Italy were resistant to ampicillin (Zanetti *et. al.,* 2001).

Hollis *et. al.* (1976) and Sanjeev and Mukundan (2003) have reported sensitivity of *V.vulnificus* strains towards ampicillin, cholramphenicol, tetracycline and gentamicin.

Li *et. al.* (1999) reported that *V.vulnificus* was sensitive to streptomycin, nalidixic acid, rifampicin and ceftriaxone and allmost all were sensitive to chloramphenicol (98%), sulphamethoxazole (98%) and ceftazidime (96%). French *et. al.,* (1989) reported a similar antibiotic susceptibility profiles for *V.vulnificus* in a clinical and environmental setting.

Nascimento *et. al.* (2001) reported that *V.vulnificus* strains isolated from shrimps purchased from Brazil were sensitive to nitrofurantoin, ciprofloxacin, gentamicin and chloramphenicol and resistant to clindamycin, pencillin and ampicillin.

In clinical therapy, tetracycline has been recommended as the antimicrobial of choice to treat severe *V.vulnificus* infections (Morris and Tenney, 1985), and alternative treatments are a combination of a third-generation cephalosporin (e.g., ceftazidime) and doxycycline, or a fluoroquinolone alone (Tang *et. al.,* 2002). Trimethoprim-sulfamethoxazole plus an aminoglycoside are used to treat children in whom doxycycline and fluoroquinolones are contraindicated (Centers for Disease Control and Prevention, 2005).

Chiang and Chuang (2003) observed that tetracycline was the drug of choice for *V.vulnificus* infection and also reported that combination of cefotoxime and minocycline was distinctly more advantageous than a single antibiotic treatment and newer fluoroquinolones as single agent was equally efficient as combination therapy.

Thakur *et. al.* (2003) studied the antibiotic susceptibility of *V.vulnificus* isolated from moribund shrimps in Maharashtra and reported that resistance was observed against ampicillin, sensitivity against oxytetracycline, erythromycin, chloramphenical and streptomycin whereas intermediate for polymixin B.

Han *et. al.* (2007) studied the antimicrobial susceptibility of 151 *V.vulnificus* isolates recovered from oysters and reported that susceptibility was observed for ampicillin, ceftazidime, chloramphenicol, ciprofloxacin, gentamicin, imipenem and tetracycline.

A study on sensitivity of *V.vulnificus* of 151 coastal isolates and 10 primary septicemia isolates against 26 antimicrobial agents and reported that 45% of the environmental sourced *V. vulnificus* strains were resistant to doxycycline tetracycline, aminoglycosides and cephalosporins (Baker-Austin *et. al.,* 2009).

Sharshar and Azab (2008) reported that *V.vulnificus* isolated from diseased fresh water prawns were highly sensitive to rifadin, virbamycin, ofloxacin, garamycin, flummox, and trimethoprim/sulfamethoxzole and resistant to nalidixic acid, unasyn, velosef, claforan, negram and amoxicillin.

Okoh and Igbinosa (2010) studied the antibiotic susceptibility of *V.vulnificus* stains isolated from waste water final effluents in South Africa and reported that *v.vulnificus* isolates were resistant to ampicillin, sulfamethoxazole, and sensitive to imepenem, meropenem and norfloxacin.

Elhadi (2012) studied the antibiotic resistance of *V.vulnificus* isolated from Saudi Arabia coastal water and reported that some isolates showed high resistance to gentamicin , ticarcillin, cefaclor, amikacin, and ampicillin and all isolates were susceptible to tetracycline and ampicillin.

Raissy *et. al.* (2012) studied the antimicrobial resistance of *V.vulnificus* isolated from seafoods and reported that the resistance was ampicillin (97.2%), gentamicin (83.3%) and pencillin (77.7). They also reported that 27.7 isolates contained one to three of the antibiotic resistance genes *StrB, tetS* and *ermB* coding for streptomycin, tetracycline and erythromycin.

Pan *et. al.* (2013) studied antibiotic susceptibility of *V. vulnificus* strains to 21 antimicrobial agents and reported that the resistance was cefepime (3.03%), tetracycline (6.06%), aztreonam (24.24%), streptomycin (45.45%), gentamicin (93.94%), tobramycin (100%), and cefazolin (100%).

Shaw *et. al.* (2014) evaluated antimicrobial susceptibility of *V.vulnificus* from recreational and commercial areas of Chesapeake Bay and Maryland Coastal Bays and reported that *V.vulnificus* isolates were susceptible to 14 antibiotics i.e. tetracycline, quinolone and folate pathway inhibitor, intermediate against ceftazidime, apramycin, streptomycin, amikacin and sensitive to gentamicin.

CHAPTER- III

MATERIAL AND METHODS

3.1 BIOLOGICALS, CHEMICALS AND EQUIPMENT

The stock and working solutions used in this study have been listed in the Annexure – 1 and 2.

3.1.1 Media, Chemicals, Molecular Biologicals and Buffers

Media and chemicals used in this study were purchased from SRL, Qualigens, Himedia, Merck and other reputed firms. All the media used in the present study were prepared according to standard procedures. Media were sterilized by autoclaving at 121^{o}C at 15 psi pressure for 15 min, unless otherwise specified. Sterility of the media was checked by incubating at 37^{0}C for 24 h. The composition of media and any specific procedure followed in its preparation are appended in Annexure 1. Molecular biologicals were obtained from Merck. The recipe for various buffers and other reagents used in this study are appended in Annexure 2.

The various cultures used in this study are *Vibrio parahaemolyticus ., Vibrio cholera ,Vibrio vulnificus ,Escherichia coli, Bacillus cereus, Pseudomonas spp., Staphylococcus aureus, Klebsiella spp., Yersinia enterocolitica* and *Clostridium perfringens*

3.1.2 Equipment

Equipment from national / international firms used in this study are given in the Table.1.

Table 1: Equipment used in this study

S.NO	NAME OF EQUIPMENT	COMPANY	MODEL
1.	Ultra centrifuge	Beckman	Optima TLX 120
2.	Thermal cycler	Eppendorf	MC Gradient
3.	U.V transilluminator	B.Genei	MD-20,312nm
4	Dry bath	B.Genei	DB 900
5	Water bath	Biotechnics-India	---
6	Homogenizer	Remi motor Ltd.	RQ-127A
7	Micro centrifuge	B.Genei	SLM-CFT-10K
8	Biomedical freezer	Sanyo	MDF-U333
9	Gel documentation unit	Syngene	GBOX HR
10	Gel electrophoresis	B.Genei	---

3.2 EVALUATION OF ENRICHMENT BROTHS

Two enrichment broths i.e. Alkaline Peptone Water (APW) and Luria Bertani broth were evaluated to find out their suitability for PCR assay. The broths were inoculated with a standard culture of *Vibrio vulnificus* and incubated (37^0C, 18h).

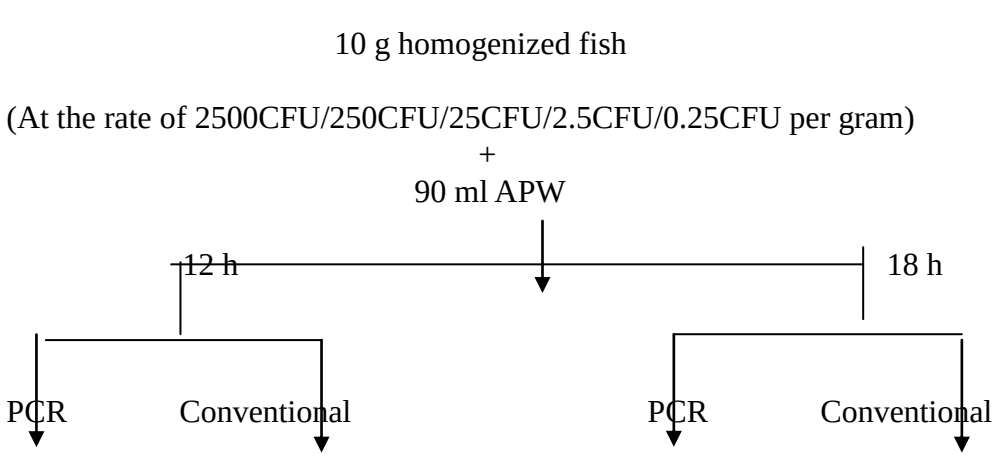
The enrichment broth cultures were alliquoted in microcentrifuge tubes and centrifuged at 8000 rpm for 10 min, the supernatant was discarded and pellet was resuspended in 50 µl of sterile distilled water. This was subjected to heat treatment and snap chilling, the bacterial lysate was subjected to centrifugation at 13,000 rpm for 5 min and 2 µl of the supernatant was used as template for PCR assay.

3.3 ARTIFICIAL INOCULATION (SPIKING) STUDIES

Fish samples intended for spiking studies were initially screened for the presence of *Vibrio vulnificus*, by inoculating 10 g of the sample in 90 ml of Alkaline Peptone Water . The rest of the isolation steps were as described in section 3.8. Remaining portion of the sample was stored at -20°C for spiking studies. Samples confirmed to be *Vibrio vulnificus* free were used for spiking, as out lined in Fig.1.

Homogenized fish samples were artificially inoculated with *Vibrio vulnificus* at the rate of 250 CFU, 25 CFU, 2.5 CFU, and 0.25 CFU per 10 g of homogenized fish with a negative control also included in the study. 90 ml of Alkaline Peptone Water was added to each fish portion and incubated at 37^0C. Aliquots were collected from the enrichment broths at 18 h post-inoculation for PCR-assay for all the primers. CPC, mCPC and TCBS plates were streaked with a loopful of culture from the broth. After an incubation period of 18 h, yellow (CPC, mCPC), green (TCBS) colonies were observed on agar plates.

Fig.1: Spiking studies in fish



3.4 ANALYSIS OF RESULTS OF SPIKING STUDIES

The results obtained in the PCR and cultural methods in experimental inoculation studies were analyzed and the sensitivity and specificity of these detection methods were calculated as per the formulae described below:

1)Sensitivity (the ability to detect positive samples) = a/a+c

2)Specificity (the ability to detect negative samples) = d/b+d

a = True positive, b = False positive, c = False negative, d = True negative

3.5 IDENTIFICATION OF *Vibrio vulnificus*

Identification of *Vibrio vulnificus* as and where necessary was done using the following procedures. The presumptive colonies were subjected to the following biochemical identification tests (Table 2).

Table 2: Biochemical tests

S.No	Tests	**Typical reactions of** *V. vulnificus*
1.	ONPG	Positive
3.	Indole	Positive
4.	Methyl Red	Positive
5.	Voges Proskauer	Negative
6.	Citrate (Simmons)	Positive
7.	Motility	Positive
8.	Oxidase test	Positive
9.	Lysine decorboxylase	Positive
10.	Lactose to acid	Positive
11.	Sucrose to acid	Negative
12.	Arabinose to acid	Negative
13.	Galactose	Positive
14.	Urease	Negative
15.	Arginine dihydrolase	Negative
16.	Growth in 0%Nacl	Negative
17.	Growth in 3%Nacl	Positive
18.	Growth in 8%Nacl	Negative

3.6 COMPARISON OF VARIOUS TEMPLATE PREPARATION PROCEDURES TO ASSESS THEIR SUITABILITY FOR PCR ASSAY.

Different methods for preparation of template were tried to determine the most suitable one for use in PCR assay. Genomic DNA extraction / bacterial lysis procedures were done using standard cultures of *Vibrio vulnificus.*

3.6.1 Genomic DNA Extraction

The sequence of unit operations shown as flow diagram as shown below *Vibrio vulnificus* inoculated into APW and incubated at 37^0C for 18

1 ml of 1M NaCl is taken in eppendorf tube

↓

A loopful of *Vibrio vulnificus* inoculated into 1 ml of 1M NaCl in eppendorf tube, in order to kill (or) inactivate bacteria.

↓

Centrifuged it in micro centrifuge at 5000 rpm for 5 min, pellet was formed. Supernatant discarded; again 1 ml of 1M NaCl was added. Mixed properly, then centrifuged at 5000 rpm for 5 min.

↓

Supernatant discarded, 700 µl of 50X TAE buffer was added. Centrifuged at 5000 rpm for 5 min.

↓

Supernatant discarded, 600 µl of 50X TAE buffer was added and mixed.

↓

20 µl lysozyme and 10µl RNAase were added and mixed. Incubated at 37^oC for 30 min in waterbath. (50X TAE contain EDTA which lyses the outer wall of bacteria. The remaining layers of bacteria and Peptidoglycon are lysed by lysozyme, so that Nucleic acid is released).

↓

30 µl of 20% SDS was added to it and incubated at 65^0C for 10 min in a waterbath.

↓

30 µl proteinase – K was added and incubated at 37^0C for 1h in waterbath.

↓

Phenol: Chloroform: isoamyl was added as 500:480:20 µl in a ratio of 25:24:1 (700 µl), then centrifuged at 13,000 rpm for 15min.

↓

Supernatant wascollected with broad open mouth tip into the eppendorf tube(250µl vol.)

↓

25 µl of cold 3M Ammonium acetate was added to supernatant and mixed. Kept it at -20^{0}C for overnight.

↓

Sample was centrifuged at 13000 rpm for 20 min and sedimented the precipitated DNA.

↓

700 µl of cold 70% ethanol was added and centrifuged at 13,000 rpm for 20 min.

↓

The pellet was air dried i.e. kept open with lid open. 100 µl of 1X TAE buffer is added and stored at -20^{0}C for future use.

3.6.2 Bacterial lysis methods

3.6.2.1 Bacterial lysis by heat application (boiling and snap chilling)

About 1.5 ml of alkaline peptone water broth culture of *Vibrio vulnificus* was taken in a micro centrifuge tube. The tube was then centrifuged at 8000 rpm for 10 min and the supernatant was discarded. 50 µl of sterile distilled water was added to the tubes and boiled in a water bath at 100^{0}C for 10 min and immediately transferred onto ice. Then centrifuged at 13,000 rpm for 5 min. For PCR assay, 2 µl of the bacterial lysate was taken as template.

3.6.2.2 Bacterial lysis using lysis buffer-1

A modification of the method suggested by Fluit *et. al.,* (1993) was used in which 1.5 ml of the broth culture was heated at 95^{0}C for 5 min to which 60 µl of 0.05% SDS and 7.5 µl of 20% Triton X-100 was added and then kept in a boiling water bath for 5 min. This was then centrifuged briefly and 2 µl of the supernatant was used as template for the PCR assay.

3.6.2.3 Bacterial lysis using lysis buffer-2

Another method suggested by Wang *et. al.,* (1997) was followed in which the cell pellet from 1.5 ml of broth culture was suspended in 50 µl of 1% TritonX-100 and heated at 100^{0}C for 5 min and immediately cooled on ice. 2 µl of the bacterial lysate was used for the PCR assay.

3.7 POLYMERASE CHAIN REACTION (PCR)

3.7.1 Oligonucleotide Primers

The primers targeting *vvhA* gene (Hill *et. al.*, 1991) and *gyrB* (Kumar *et. al.*, 2006) used for detection of *Vibrio vulnificus.*These primers were custom synthesized by Integrated DNA technologies (IDT) which are given in Table.3.

3.7.2. Standardization of the PCR protocol

PCR amplification of the *vvhA* and *gyrB* gene fragments was set up to 20 µl reactions. The PCR protocol was initially standardized by optimizing the concentration of the components of the reaction mixture in the PCR assay and by varying the annealing temperature and cycling conditions.

Reaction mixture:

The components of the reaction mix were finally optimized as given in Table 4. The master mix was made upto 20 µl using molecular grade water. Routinely, master mix was set up and 18 µl each was distributed to the PCR tubes, to which 2 µl of the template was added. In this study, the template preparation was done throughout the experiment by heat lysis application. PCR assay was performed in Eppendorf gradient Thermal Cycler with a heated lid. The cycling conditions used are given in Table 5. PCR products were stored at -20^{o}C until further use.

2 µl of the bacterial lysate or 8 ng of purified DNA, 2 µl of 10x assay buffer for Taq polymerase containing 1.5 mM $MgCl_2$, 0.8 µl of 10 mM dNTP mix, 2 µl each of forward and reverse primer (10 pmol/µl) and 1U/µl of Taq DNA polymerase, which was made upto 20 µl using molecular grade water. Routinely, master mix was set up and 18 µl, each was distributed to the PCR tubes, to which 2 µl of the template was added.

Table 3: Details of primers used in this study

Primer	Target Gene	Length	Primer sequence	Amplification product (bp)	Reference
*gyr-vv*1	*gyrB*	20	Foward 5'-	285	Kumar *et. al.* (2006)
*gyr-vv*2	*gyrB*	20	GTCCGCAGTGGAATCCTTCA -3' Reverse 5'- TGGTTCTTACGGTTACGGCC-3'	285	Kumar *et. al.* (2006)
*VVp*1	*vvhA*	20	Forward 5'-CCGGCGGTACAGGT	519	Hill *et. al.* (1991)
*VVp*2	*vvhA*	19	TGGCGC-3' Reverse 5'-CGCCACCCACTTTCGGGCC-3'	519	Hill *et. al.* (1991)

Table 4: Components of reaction mixture

S.No.	Name of the Reagent	Quantity (µl)
1.	10X Taq polymerase buffer	2.0
2.	dNTP mix	0.8
3.	Primer-F	2.0
4.	Primer-R	2.0
5.	Taq DNA polymerase	0.5
6.	Purified DNA /Bacterial lysate	2.0

Table 5: Cycling conditions used for the primers

S.No.	Step	*gyrB*	*vvhA*
1.	Initial denaturation	94^{o}C/5min	94^{o}C/10min
2.	Final denaturation	94^{o}C/30sec	94^{o}C/1.75min
3.	Annealing	64 ^{o}C /30sec	66^{o}C/2min
4.	Initial extension	72^{o}C/30 sec	72^{o}C/2min
5.	Final extension	72^{o}C/5min	72^{o}C/10min
6.	Hold	4^{o}C	4^{0}c

3.7.3 Agarose Gel Electrophoresis

Agarose gel (1.5%) was prepared by boiling agarose in an appropriate volume of 1X TAE buffer. After cooling for about 3 min, ethidium bromide (Biogenei, USA) was added to the agarose solution to a final concentration of 0.5 µg/ml. The molten agarose was then poured into the tray and the comb was fitted into the slots on the tray. The tray was kept undisturbed till the gel had solidified. The comb was then taken out carefully and the tray containing the gel was then placed in a submarine horizontal electrophoresis unit filled with 1X TAE buffer upto a level of 1 mm above the gel surface.

About 5 µl of each PCR product was mixed with 2 µl of bromophenol blue (6x) loading dye and loaded into each well. Electrophoresis was performed at 5 V/cm and the

motility was monitored by the migration of the dye. After sufficient migration, the gels were observed under UV transilluminator to visualize the bands. The PCR product size was determined by comparing with a standard molecular weight marker and was photographed by the gel documentation system.

3.8 SPECIFICITY OF THE PCR ASSAY

The specificity of the PCR assay was validated by subjecting various Gram-negative and Gram-positive cultures to PCR assay. The bacterial isolates were inoculated in APW, incubated overnight and subjected to heat lysis treatment (section 3.6.2.1) and 2 µl of each bacterial lysate was subjected to PCR for the two primers sets as outlined in section 3.7.2 and agarose gel electrophoresis as in section 3.7.3

3.9 EVALUATION OF THRESHOLD SENSITIVITY OF THE PCR ASSAY

Trials were conducted to evaluate theoretically lowest quantum of bacterial cells that would produce a visible signal by the PCR assay.

3.10 SCREENING OF NATURAL SAMPLES

3.10.1 Sample Collection

3.10.1.1 . Fish

Thirty fish samples each of 50g were aseptically collected from local markets and outlets of Fisheries Dept.

3.10.1.2 Crabs

Thirty crab samples each of 50g were aseptically collected from local markets and outlets of Fisheries Dept.

3.10.1.3 Shrimps

Thirty shrimp samples each of 50g were aseptically collected from local markets and outlets of Fisheries Dept.

3.10.1.4 Oysters

Thirty oyster samples of 50 g each were collected from different regions of coastal Andhrapradesh.

3.10.1.5 Pond Water samples

Thirty pond water samples each of 100 ml from aquatic farms and esturine water sources were collected from different places of coastal Andhrapradesh.

3.10.1.6 Esturine Water samples

Thirty esturine water samples each of 100ml from different sources in coastal region of Andhrapradesh were collected.

3.10.1.7 Marine Water samples

Thirty marine water samples each of 100ml from different places of coastal region of Andhrapradesh were collected.

3.10.1.8 Sediment samples

Thirty sediment samples each of 100g from different places of coastal region of Andhrapradesh were collected

3.10.1.9 Plankton samples

Thirty plankton samples each of 100g from different places of coastal region of Andhrapradesh were collected.

3.10.2 Enrichment

About 10 g of each fish, crab, shrimp and oyster samples were inoculated into 90 ml APW and Luria Bertani broths in individual sterile polythene bags homogenized

thoroughly in a stomacher for 3 to 5 min and incubated at 37^{o}C for 18 h. 10 ml of each water samples were inoculated into 90 ml in APW and Luria Bertani broths in individual sterile polythene bags and incubated (37^{o}C, 18 h).

3.10.3 Conventional isolation method

The enriched inoculum from the broths was streaked onto different selective media agar plates like CPC, mCPC, TCBS agars and incubated at 37^{0}C for 18 h. The presumptive colonies of *V.vulnificus* were picked up and subjected to biochemical tests as listed in Table 2.

3.10.4 Enumeration of *V.vulnificus* in the samples

The samples after enrichment were plated on *m*CPC agar for counting of number of organisms present (per gram or per ml) in the samples (Tamplin *et. al.,* 1991).

3.11 ANTIBIOTIC SENSITIVITY TEST

3.11.1 Materials

1. Broth cultures of *V.vulnificus.* isolates
2. Antibiotic discs
    - Ampicillin - 10 µg
    - Chloramphenicol - 30 µg
    - Ciprofloxacin - 5 µg
    - Erythromycin - 15µg
    - Gentamicin - 10 µg
    - Kanamycin -30 µg
    - Streptomycin - 10 µg
    - Tetracycline - 30 µg
    - Ceftazidime - 30 µg
    - Vancomycin -30 µg
    - Amikacin -30 µg

- Nalidixic acid -30 µg

3. Sterile flexi inoculation loops
4. Sterile forceps
5. Sterile cotton swabs
6. Muller Hinton Broth
7. Muller Hinton Agar plates

3.11.2 Method

Antimicrobial susceptibility of the isolates was established by the disc diffusion assay with MH (Muller-Hinton) agar as described by Bauer *et. al.*, (1966).

MH broth was inoculated with five colonies from the sample and tubes were incubated at 37^0C for 2-8 h until achieving a turbidity equivalent to 0.5 on the Mac Farland scale.

- After turbidity adjustment, a sterile swab was introduced, pressed against the tube well in order to remove any excess liquid, and then seeded on the surface of a petridish containing MH agar, rotating atleast twice.
- After the lid was placed, the dish was left at rest for five minutes to absorb any excessive humidity.
- Using sterile forceps seven discs (Himedia) impregnated with antimicrobials were placed at equal distances from each other on the surface of each dish.
- Subsequently the dish was inverted and incubated at 37^0C in anaerobiosis.
- Dish readings were performed 18 h after incubation and the diameter of inhibition halos was measured with the aid of a ruler.
- The interpretation was made as per the zone size interpretation chart provided by manufacturer of discs.

CHAPTER IV

RESULTS

4.1 STANDARDIZATION OF PCR ASSAY

The PCR assay for the detection of *V.vulnificus* and cytotoxin-haemolysin from aquatic foods and environmental samples were standardized by using the primers targeting *vvhA* and *gyrB* genes. Genomic DNA prepared from *V.vulnificus* culture and samples were used as templates. The details of the primers used are shown in Table 3.

Initial experiments to optimize PCR reaction conditions for *vvhA* and *gyrB genes* were empirical variation of annealing temperatures (61 - 67^{0}C for *gyrB* and 64 -69^{0}C for *vvhA genes*), concentration of primer (5-15 pmol), $MgCl_2$ (1.5 mM - 3 mM), template volume (2 µl - 8 µl) and the cycling conditions.

Optimal results were obtained using 2 µl of bacterial lysate or 8 ng of diluted DNA as template in a reaction mixture consisting of 2 µl of 10X assay buffer for Taq polymerase containing 1.5 mM $MgCl_2$, 0.8 µl of dNTP mix, 2 µl of each primer and 1U/µl of Taq DNA polymerase in a final reaction volume made upto 20µl with molecular grade water.

Initial denaturation at 94^{0}C for 10 min, followed by 30 cycles each of denaturation at 94^{0}C for 1.75min, annealing at 66^{0}C for 2 min and extension at 72^{0}C for 2 min with a final extension period of 10 min at 72^{0}C was found to be optimum for obtaining the desired PCR amplicon of 519 bp from *vvhA gene* of *V.vulnificus.* Electrophoretic analysis of the PCR product revealed the specific amplification of 519 bp fragment, without the presence of any spurious product (Fig. 2).

Initial denaturation at 94^{0}C for 5 min, followed by 35 cycles each of denaturation at 94^{0}C for 30 sec, annealing at 64^{0}C for 30 sec and extension at 72^{0}C for 30 sec with a final extension period of 5 min at 72^{0}C was found to be optimum for

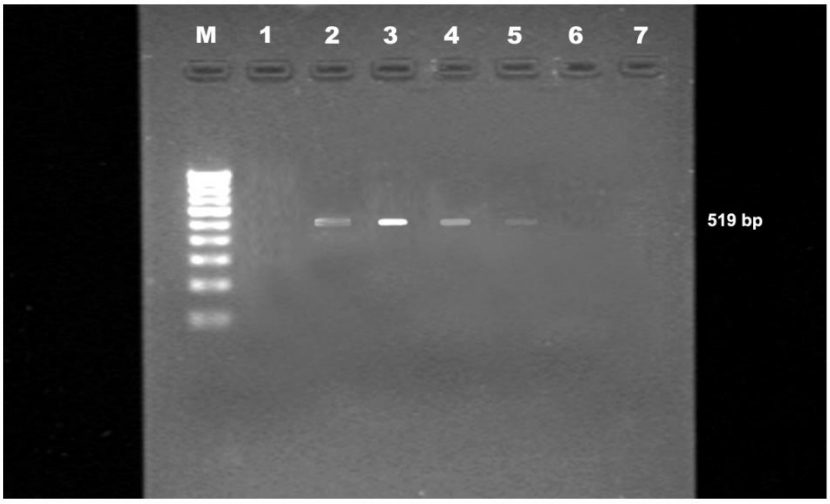


Fig.2: Standardization of PCR assay for detection of *Vibrio vulnificus* targeting *vvhA* gene

Lane M: 100 bp DNA ladder
Lane 2: PCR product at an annealing temperature 65°C
Lane 3: PCR product at an annealing temperature 66°C
Lane 4: PCR product at an annealing temperature 67°C
Lane 5: PCR product at an annealing temperature 68°C

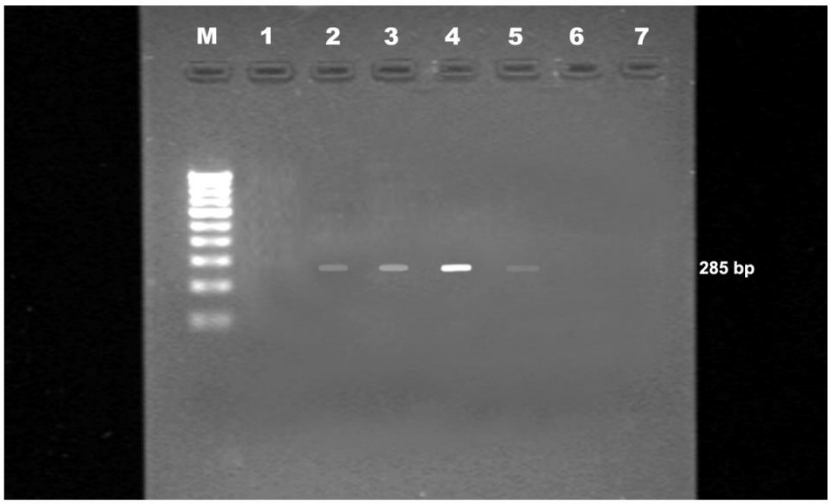


Fig.3: Standardization of PCR assay for detection of *Vibrio vulnificus* targeting *gyrB* gene

Lane M: 100 bp DNA ladder
Lane 2: PCR product at an annealing temperature 62°C
Lane 3: PCR product at an annealing temperature 63°C
Lane 4: PCR product at an annealing temperature 64°C
Lane 5: PCR product at an annealing temperature 65°C

obtaining the desired PCR amplicon of 285 bp from *gyrB* gene of *V.vulnificus*. Electrophoretic analysis of the PCR product revealed the specific amplification of 285 bp fragment, without the presence of any spurious product (Fig.3).

4.2 COMPARISION OF TEMPLATE PREPARATION METHODS

Four different template preparation methods viz: genomic DNA extraction, heat lysis, lysis buffer-1 and lysis buffer-2 were compared to determine the most sensitive, rapid and simple method suitable for PCR assay (Fig.4 and 5). Template prepared by isolation of genomic DNA gave a clear cut amplicon of 519 bp and 285 bp length on PCR assay for *vvhA* and *gyrB* respectively. Heat lysis method was used for all the further experiments. Heat lysis method was preferred as it saves time, less cumbersome and results almost comparable clarity of genomic DNA extraction. Templates prepared by lysis buffers 1 and 2 also gave positive amplicon but it was lesser in intensity than other methods.

4.3 SPECIFICITY OF THE PCR ASSAY

The specificity of the standardized PCR assay for primers from *vvhA* gene to detect cytotoxic haemolysin gene in *V.vulnificus* was tested by subjecting three *Vibrio spp*. and seven other organisms. Only *V.vulnificus* carrying *vvhA* gene yielded specific PCR products of desired length (519bp). No specific PCR product was obtained from other organisms tested. (Fig. 6).

The specificity of the standardized PCR assay for primers from *gyrB* gene to detect *V.vulnificus* was tested by subjecting three *Vibrio spp*. and seven other organisms. Only *V.vulnificus* yielded specific PCR products of desired length (285bp). No specific PCR product was obtained from any other organisms tested (Fig.7).

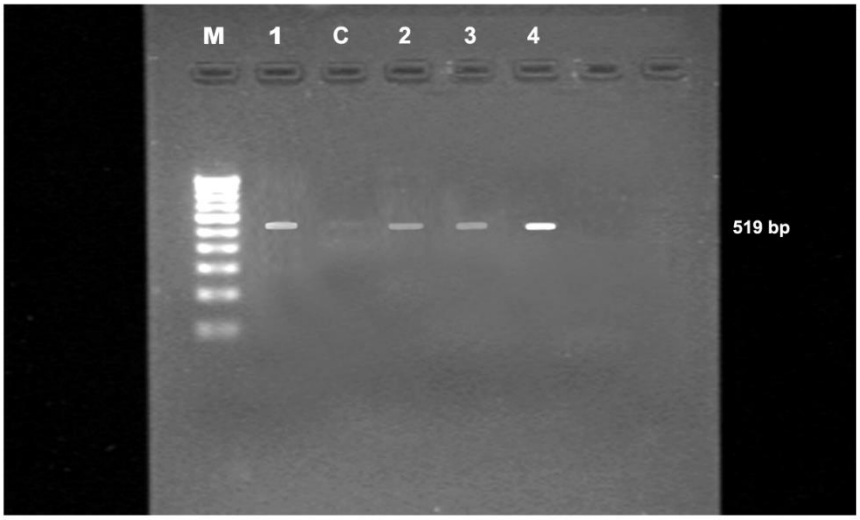


Fig.4: Comparison of template DNA preparation methods (targeting *vvhA* gene)

Lane M: 100 bp DNA ladder
Lane C: Negative control
Lane 1: Boiling and snap chilling
Lane 2: Lysis buffer-1 (SDS and TritonX-100)
Lane 3: Lysis buffer-2 (TritonX-100)
Lane 4: Genomic DNA extraction

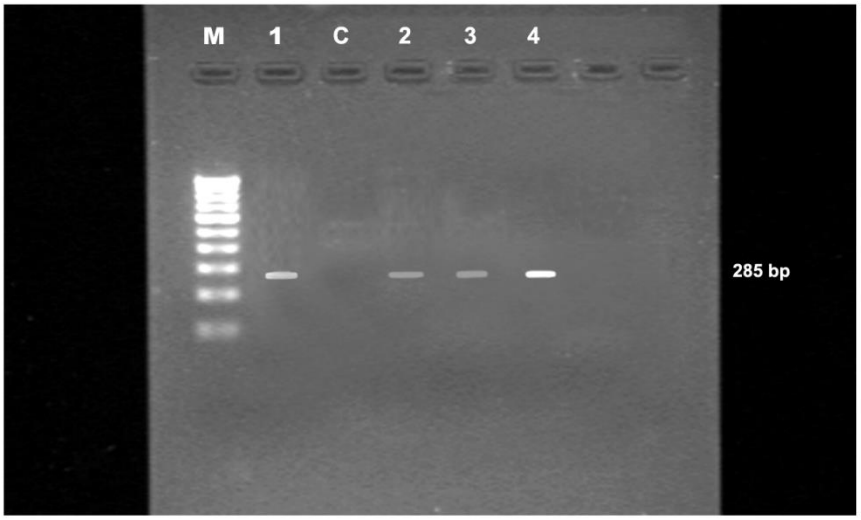


Fig.5: Comparison of template DNA preparation methods (targeting *gyrB* gene)

Lane M: 100 bp DNA ladder
Lane C: Negative control
Lane 1: Boiling and snap chilling
Lane 2: Lysis buffer-1 (SDS and TritonX-100)
Lane 3: Lysis buffer-2 (TritonX-100)
Lane 4: Genomic DNA extraction

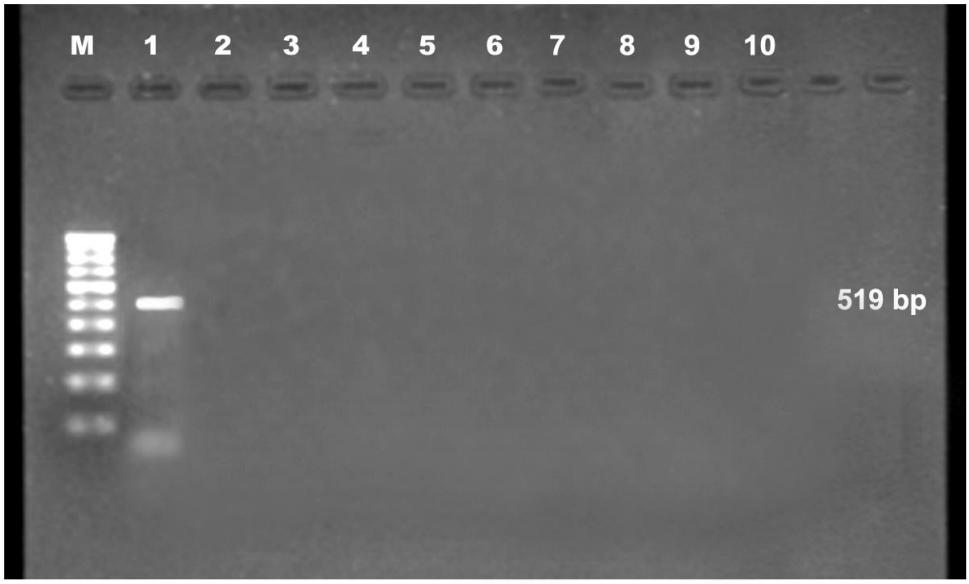


Fig.6: Specificity of the PCR assay for detection of *Vibrio vulnificus* targeting *vvhA* gene

Lane M: 100 bp DNA ladder
Lane 1: *Vibrio vulnificus*
Lane 2: *Vibrio parahaemolyticus*
Lane 3: *Vibrio cholera*
Lane 4: *Escherichia coli*
Lane 5: *Bacillus cereus*
Lane 6: *Pseudomonas spp.*
Lane 7: *Staphylococcus aureus*
Lane 8: *Klebsiella spp.*
Lane 9: *Yersinia enterocolitica*
Lane 10: *Clostridium perfringens*

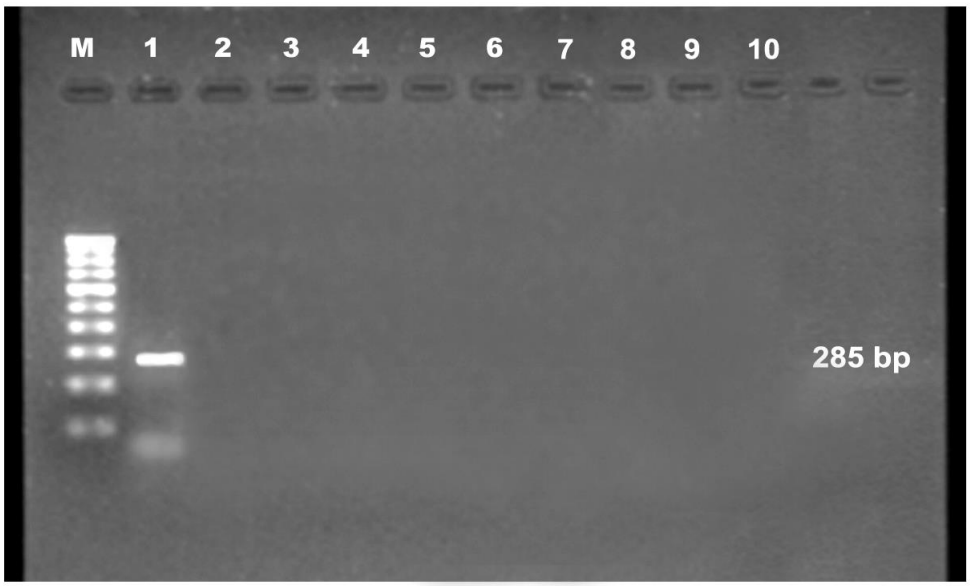


Fig.7: Specificity of the PCR assay for detection of *Vibrio vulnificus* targeting *gyrB* gene

Lane M: 100 bp DNA ladder
Lane 1: *Vibrio vulnificus*
Lane 2: *Vibrio parahaemolyticus*
Lane 3: *Vibrio cholera*
Lane 4: *Escherichia coli*
Lane 5: *Bacillus cereus*
Lane 6: *Pseudomonas spp.*
Lane 7: *Staphylococcus aureus*
Lane 8: *Klebsiella spp.*
Lane 9: *Yersinia enterocolitica*
Lane 10: *Clostridium perfringens*

4.4 EVALUATION OF THRESHOLD SENSITIVITY OF THE PCR ASSAY

The results of evaluation of threshold sensitivity of the PCR assay were presented in Fig.8 and 9.

The sensitivity of the PCR assay was evaluated by subjecting serial 10 fold dilutions of a pure culture of *V.vulnificus*, ranging from $2.5x10^3$ CFU/ml to 0.25 CFU/ml to PCR for the primer of *vvhA*. The minimum detection level was found to be 0.25 CFU/ml for the primer. The intensity of band at the lowest detection level i.e. 0.25 CFU/ml was clearly discernible. The samples of higher bacterial concentration were found to produce signal of increasing intensity.

4.5 EVALUATION OF ENRICHMENT BROTHS

The results of evaluation of enrichment broths to assess their PCR compatibility were presented in Fig. 10 and 11.

Two enrichment broths i.e. APW and Luria Bertani broths were inoculated with *V.vulnificus* culture incubated at 37^0C for 18h and subjected to boiling and snap chilling for DNA extraction.APW broth produced brighter bands compared to Luria Bertani broth for both the primers.

4.6 ARTIFICIAL INOCULATION (SPIKING) STUDIES

Homogenized fish was inoculated with *V.vulnificus* at the rate of 2500 CFU, 250 CFU, 25 CFU, 2.5 CFU and 0.25 CFU per 1g and transferred to two enrichment broths i.e. APW and Luria Bertani broths and incubated at 37^0C for 18h. Later they were subjected to PCR assay and conventional method of testing for *V.vulnificus.*

The results obtained for *V.vulnificus* for 12h and 18h by cultural and PCR methods are presented in Table 6.

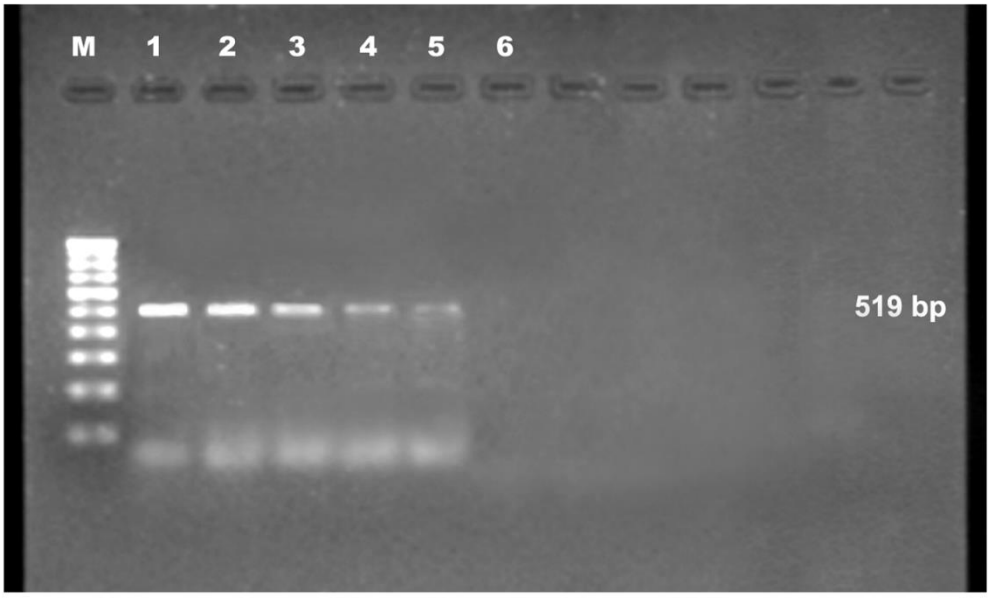


Fig. 8 : Evaluation of threshold sensitivity of PCR assay targeting vvhA gene

Lane M: 100 bp DNA ladder
Lane 2: 2.5x10^2 cfu/ml
Lane 4: 2.5 cfu/ml
Lane 6: Negative control
Lane 1: 2.5x10^3 cfu/ml
Lane 3: 2.5x10 cfu/ml
Lane 5: 0.25 cfu/ml

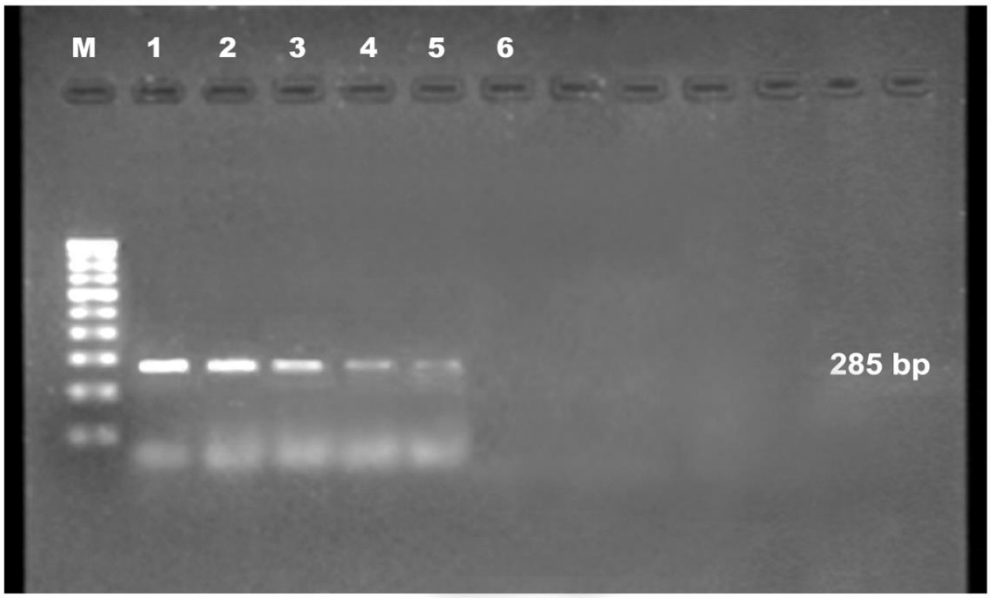


Fig. 9 : Evaluation of threshold sensitivity of PCR assay targeting *gyrB* gene

Lane M: 100 bp DNA ladder
Lane 2: 2.5x10^2 cfu/ml
Lane 4: 2.5 cfu/ml
Lane 6: Negative control
Lane 1: 2.5x10^3 cfu/ml
Lane 3: 2.5x10 cfu/ml
Lane 5: 0.25 cfu/ml

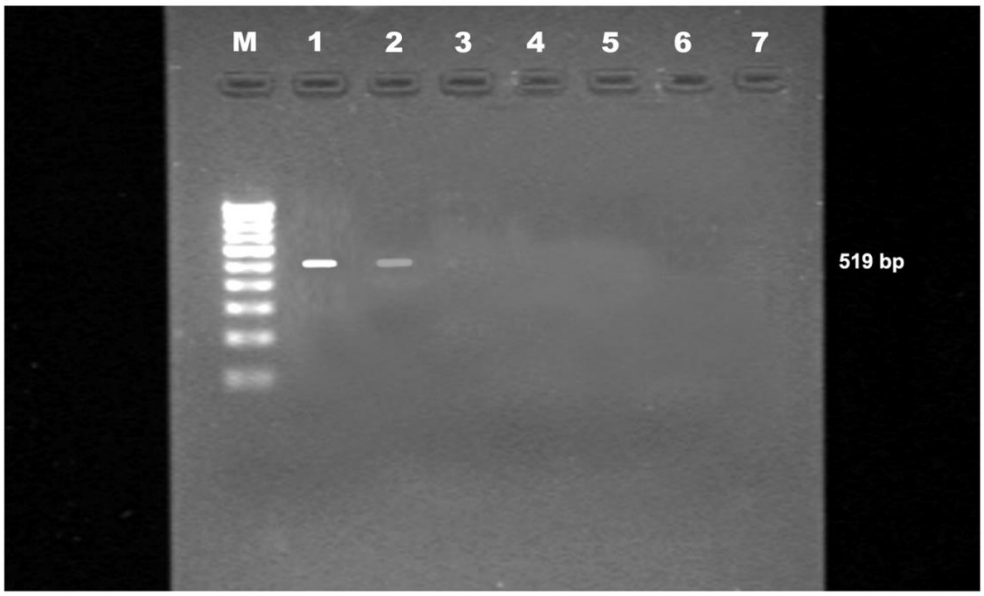


Fig.10 Evaluation of PCR compatibility of enrichment broths for the detection of *Vibrio vulnificus* targeting *vvhA* gene

Lane M: 100 bp DNA ladder
Lane 1: Alkaline peptone water broth
Lane 2: Luria Bertani broth

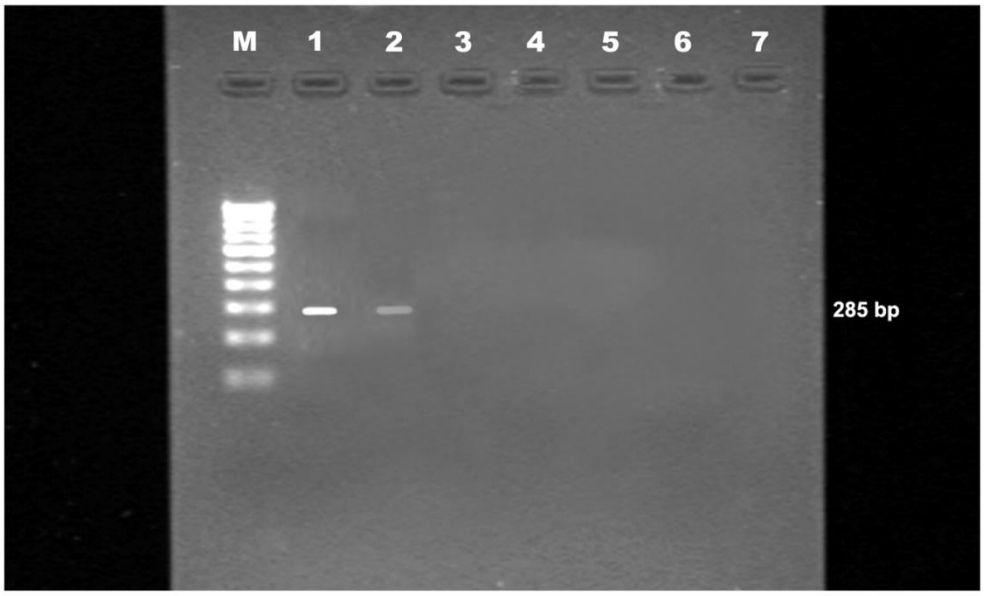


Fig. 11 Evaluation of PCR compatibility of enrichment broths for the detection of *Vibrio vulnificus* targeting *gyrB* gene

Lane M: 100 bp DNA ladder
Lane 1: Alkaline peptone water broth
Lane 2: Luria Bertani broth

4.6.1 RESULTS AT 12 h ENRICHMENT

APW and Luria Bertani broths yielded positive results for *V.vulnificus* at lowest inoculation level of 2.5 CFU by PCR method, whereas by cultural method it gave positive result upto 25 CFU only.

4.6.2 RESULTS AT 18 h ENRICHMENT

APW and Luria Bertani broths yielded positive result for *V.vulnificus* at lowest inoculation of 0.25 CFU by PCR method, whereas by cultural methods both the broths gave positive result upto 2.5 CFU.

4.7 SCREENING OF NATURAL SAMPLES

The results of natural and environmental samples by cultural and PCR methods for *V.vulnificus* are shown in Table 7 and 8 and Fig. 12 (fish), 13 (shrimp), 14 (crab), 15 (oyster), 16 (pond water), 17 (esturine water), 18 (marine water), 19 (sediment), 20 (plankton).

Out of 30 fish samples, 5 (16.66%) were positive by cultural method and PCR assay targeting *gyrB* gene, whereas 6 (20%) were positive for *V.vulnificus* by PCR method targeting *vvhA* gene. The efficiency of cultural method compared to PCR assay targeting *gyrB* gene was 100%, whereas it was 83.3% compared to PCR assay targeting *vvhA* gene.

Out of 30 shrimp samples 2 (6.66%) were positive for *V.vulnificus* by cultural and both PCR methods targeting *vvhA* and *gyrB* genes. The efficiency of cultural method compared to PCR assay was 100%.

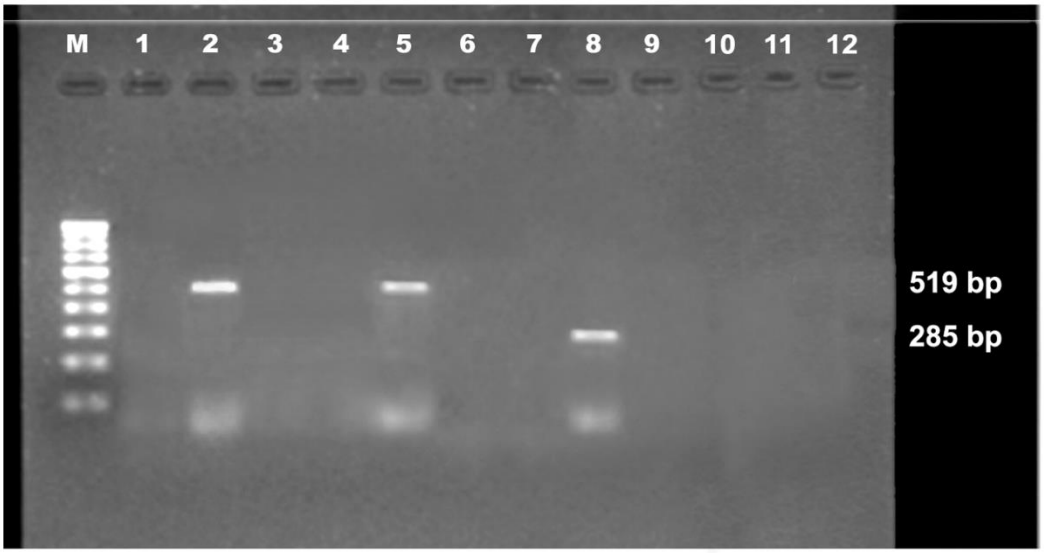


Fig.12 Results of some fish samples for *Vibrio vulnificus* (targeting *vvhA* and *gyrB* genes)

Lane M: 100 bp DNA ladder
Lane 1: Fish samples showing negative result
Lane 2: Fish samples showing positive result
Lane 3: Fish samples showing negative result
Lane 4: Fish samples showing negative result
Lane 5: Fish samples showing positive result
Lane 6: **Negative control**
Lane 7: Fish samples showing negative result
Lane 8: Fish samples showing positive result
Lane 9: Fish samples showing negative result
Lane 10 : Fish samples showing negative result
Lane 11 : Fish samples showing negative result
Lane 12 : **Negative control**

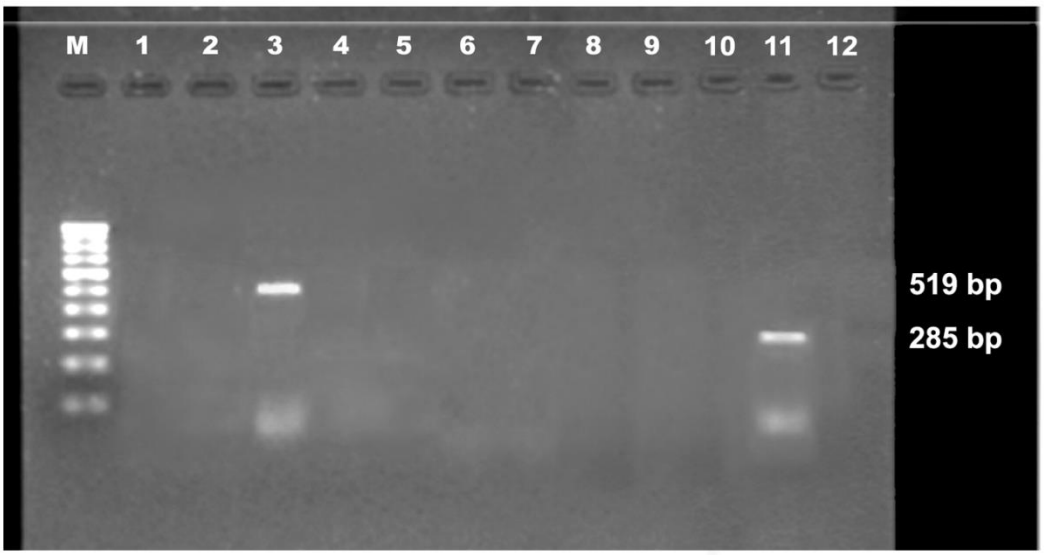


Fig.13 Results of some shrimp samples for *Vibrio vulnificus* (targeting *vvhA* and *gyrB* genes)

Lane M: 100 bp DNA ladder
Lane 1: Shrimp samples showing negative result
Lane 2: Shrimp samples showing negative result
Lane 3: Shrimp samples showing positive result
Lane 4: Shrimp samples showing negative result
Lane 5: Shrimp samples showing negative result
Lane 6: **Negative control**
Lane 7: Shrimp samples showing negative result
Lane 8: Shrimp samples showing negative result
Lane 9: Shrimp samples showing negative result
Lane 10 : Shrimp samples showing negative result
Lane 11 : Shrimp samples showing positive result
Lane 12 : **Negative control**

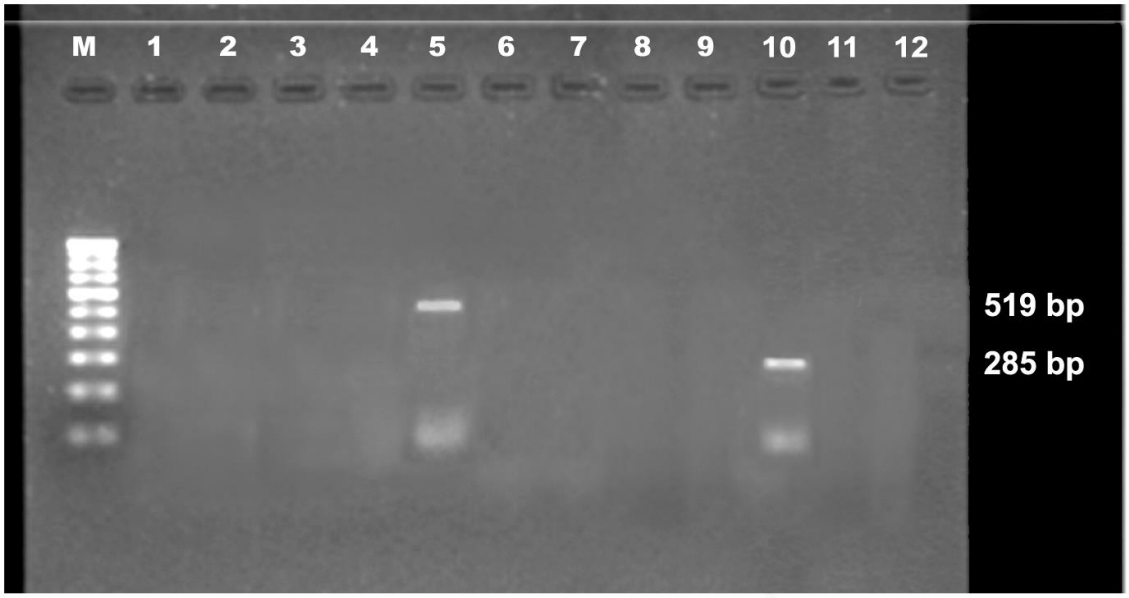


Fig.14 Results of some crab samples for *Vibrio vulnificus* (targeting *vvhA* and *gyrB* genes)

Lane M: 100 bp DNA ladder
Lane 1: Crab samples showing negative result
Lane 2: Crab samples showing negative result
Lane 3: Crab samples showing negative result
Lane 4: Crab samples showing negative result
Lane 5: Crab samples showing positive result
Lane 6: **Negative control**
Lane 7: Crab samples showing negative result
Lane 8: Crab samples showing negative result
Lane 9: Crab samples showing negative result
Lane 10 : Crab samples showing positive result
Lane 11 : Crab samples showing negative result
Lane 12 : **Negative control**

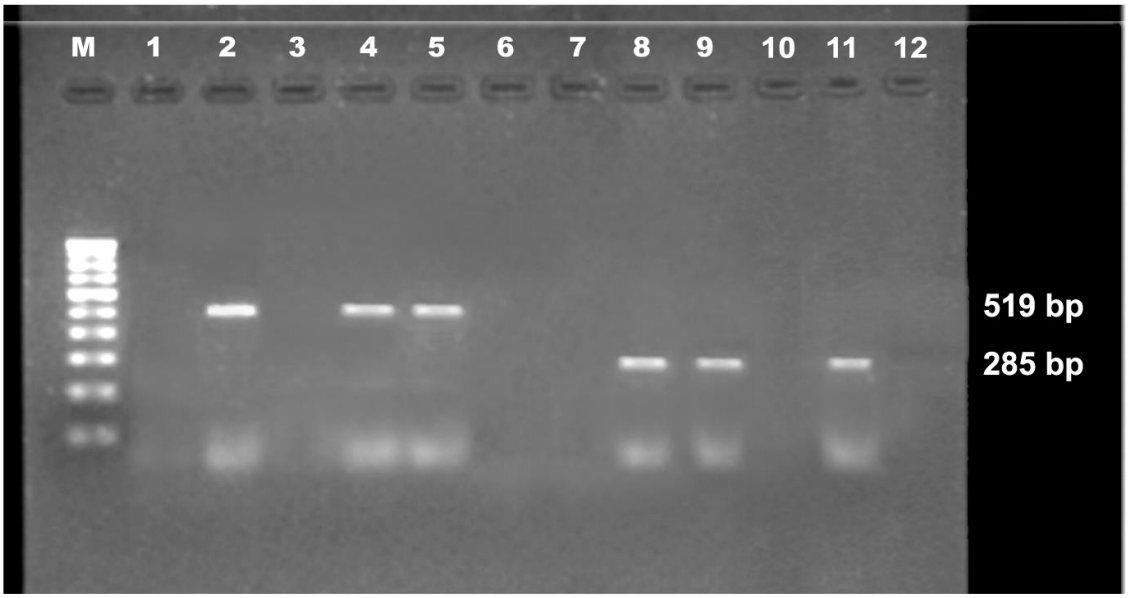


Fig.15 Results of some oyster samples for *Vibrio vulnificus* (targeting *vvhA* and *gyrB* genes)

Lane M: 100 bp DNA ladder
Lane 1: oyster samples showing negative result
Lane 2: oyster samples showing positive result
Lane 3: oyster samples showing negative result
Lane 4: oyster samples showing positive result
Lane 5: oyster samples showing positive result
Lane 6: **Negative control**
Lane 7: oyster samples showing negative result
Lane 8: oyster samples showing positive result
Lane 9: oyster samples showing positive result
Lane 10 : oyster samples showing negative result
Lane 11 : oyster samples showing positive result
Lane 12 : **Negative control**

Out of 30 crab samples 3 (10%) were positive by cultural method whereas 4 (13.33%) were positive for *V.vulnificus* by both PCR methods targeting *vvhA* and *gyrB* genes . The efficiency of cultural method compared to PCR assay was 75%.

Out of 30 oyster samples 14 (46.6%) were positive by cultural method and 16 (53.33%) were positive for *V.vulnificus* by both PCR methods targeting *vvhA* and *gyrB* genes . The efficiency of cultural method compared to PCR assay was 87.5 %.

Out of total 120 aquatic food (30 each of fish, shrimp, crab and oyster) samples 24 (20%) and 28 (23.33%) were positive for *V.vulnificus* by cultural and PCR methods respectively. The efficiency of cultural method compared to PCR assay for detection of *V.vulnificus* in aquatic foods was 85.71%.

Out of 30 pond water samples 4 (13.33%) and 5 (16.66%) were positive for *V.vulnificus* by cultural and PCR methods (targeting both *vvhA* and *gyrB* genes) respectively. The efficiency of cultural method compared to PCR assay was 80 %.

Out of 30 esturine water samples19 (63.33%) were positive by cultural method whereas 21 (70.00%) were positive for *V.vulnificus* by both PCR methods targeting *vvhA* and *gyrB* genes. The efficiency of cultural method compared to PCR assay was 90.48%.

Out of 30 marine water samples 15(50%) were positive by cultural method and 16(53.33%) were positive for *V.vulnificus* by both PCR methods targeting *vvhA* and *gyrB* genes. The efficiency of cultural method compared to PCR assay was 93.75%.

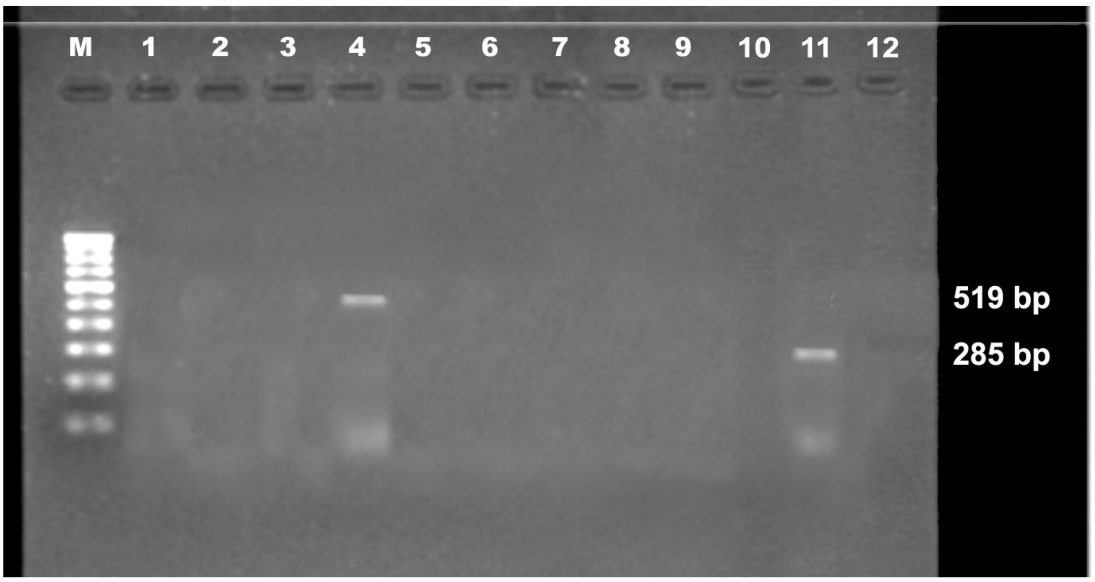


Fig.16 Results of some pond water samples for *Vibrio vulnificus* (targeting *vvhA* and *gyrB* genes)

Lane M: 100 bp DNA ladder
Lane 1: Pond water samples showing negative result
Lane 2: Pond water samples showing negative result
Lane 3: Pond water samples showing negative result
Lane 4: Pond water samples showing positive result
Lane 5: Pond water samples showing negative result
Lane 6: **Negative control**
Lane 7: Pond water samples showing negative result
Lane 8: Pond water samples showing negative result
Lane 9: Pond water samples showing negative result
Lane 10 : Pond water samples showing negative result
Lane 11 : Pond water samples showing positive result
Lane 12 : **Negative control**

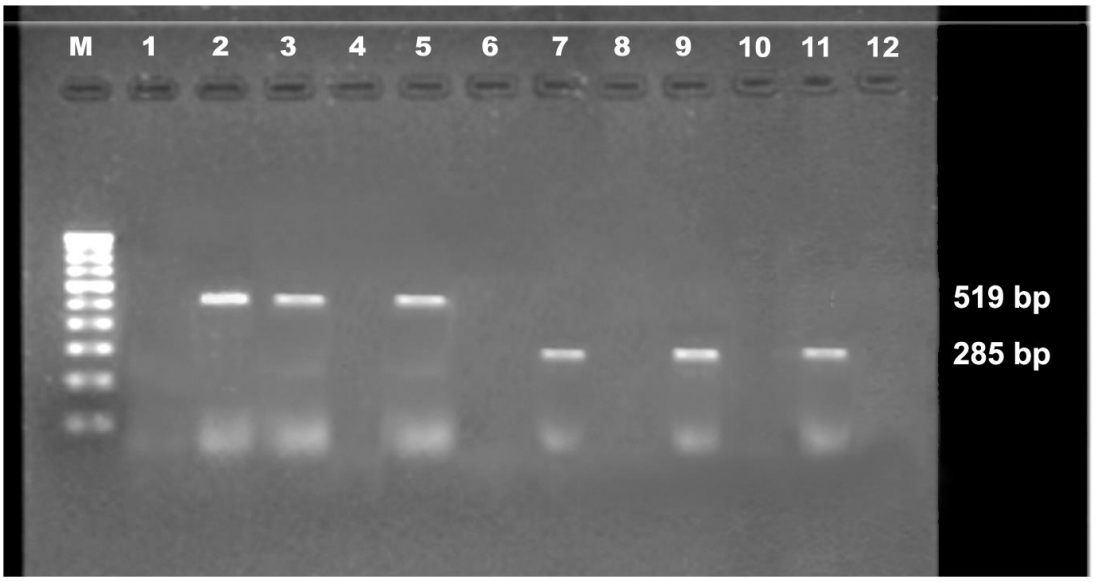


Fig.17 Results of some esturine water samples for *Vibrio vulnificus* (targeting *vvhA* and *gyrB* genes)

Lane M: 100 bp DNA ladder
Lane 1: Esturine water samples showing negative result
Lane 2: Esturine water samples showing positive result
Lane 3: Esturine water samples showing positive result
Lane 4: Esturine water samples showing negative result
Lane 5: Esturine water samples showing positive result
Lane 6: **Negative control**
Lane 7: Esturine water samples showing positive result
Lane 8: Esturine water samples showing negative result
Lane 9: Esturine water samples showing positive result
Lane 10 : Esturine water samples showing negative result
Lane 11 : Esturine water samples showing positive result
Lane 12 : **Negative control**

Out of 30 sediment samples 14 (46.66%) were positive by cultural method and 16 (53.33%) were positive for *V.vulnificus* by both PCR methods targeting *vvhA* and *gyrB* genes. The efficiency of cultural method compared to PCR assay was 78.5%.

Out of 30 plankton samples 15 (50%) were positive by cultural method and 17 (56.66%) were positive for *V.vulnificus* by both PCR methods targeting *vvhA* and *gyrB* genes. The efficiency of cultural method compared to PCR assay was 88.23%.

Out of total 150 environmental (30 each of pond water, esturine water, marine water, sediment and plankton) samples 67 (44.66%) were positive by cultural method and 75 (50%) were positive for *V.vulnificus* by both PCR methods targeting *vvhA* and *gyrB* genes. The efficiency of cultural method compared to PCR assay was 89.33%.

The mean viable counts (CFU/g) of *V.vulnificus* in various samples are presented in Table 9. The counts in different aquatic foods were $2.9x10^2$ in fish, $5.1x10^2$ in shrimps, $5.0x10^2$ in crabs and $2.1x10^4$ in oysters.

The mean counts of *V.vulnificus* in different environmental samples were $5.3x10^3$ CFU/ml in pond water, $3.8x10^6$ CFU/ml in esturine water, $9.4x10^4$ CFU/ml in marine water, $6.1x10^5$ CFU/g in sediment and $6.2x10^3$ CFU/g in plankton.

4.8 ANTIBIOTIC SENSITIVITY.

To know about the sensitivity and/or resistance of *V.vulnificus* for different antibiotics, antibiotic sensitivity test was conducted. The results of antibiotic sensitivity of *V.vulnificus* are presented in Table 10.

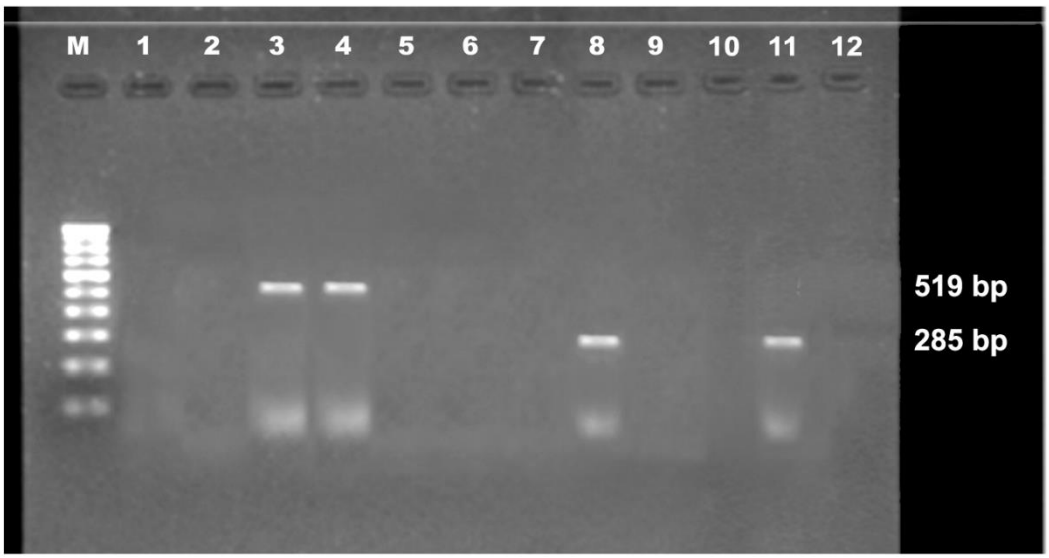


Fig. 18 Results of some marine water samples for *Vibrio vulnificus* (targeting *vvhA* and *gyrB* genes)

Lane M: 100 bp DNA ladder
Lane 1: Marine water samples showing negative result
Lane 2: Marine water samples showing negative result
Lane 3: Marine water samples showing positive result
Lane 4: Marine water samples showing positive result
Lane 5: Marine water samples showing negative result
Lane 6: **Negative control**
Lane 7: Marine water samples showing negative result
Lane 8: Marine water samples showing positive result
Lane 9: Marine water samples showing negative result
Lane 10 : Marine water samples showing negative result
Lane 11 : Marine water samples showing positive result
Lane 12 : **Negative control**

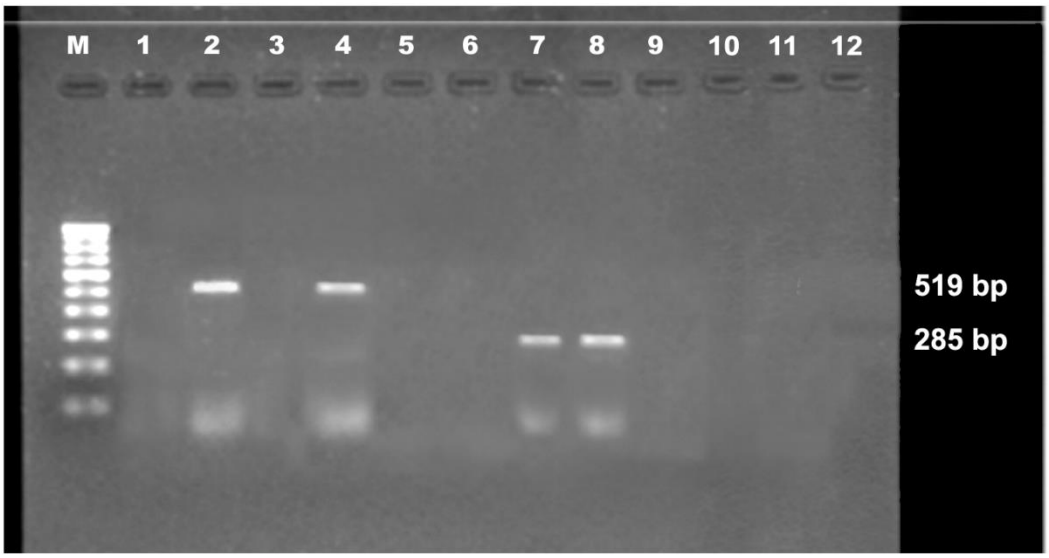


Fig. 19 Results of some sediment samples for *Vibrio vulnificus* (targeting *vvhA* and *gyrB* genes)

Lane M: 100 bp DNA ladder
Lane 1: Sediment samples showing negative result
Lane 2: Sediment samples showing positive result
Lane 3: Sediment samples showing negative result
Lane 4: Sediment samples showing positive result
Lane 5: Sediment samples showing negative result
Lane 6: **Negative control**
Lane 7: Sediment samples showing positive result
Lane 8: Sediment samples showing positive result
Lane 9: Sediment samples showing negative result
Lane 10 : Sediment samples showing negative result
Lane 11 : Sediment samples showing negative result
Lane 12 : **Negative control**

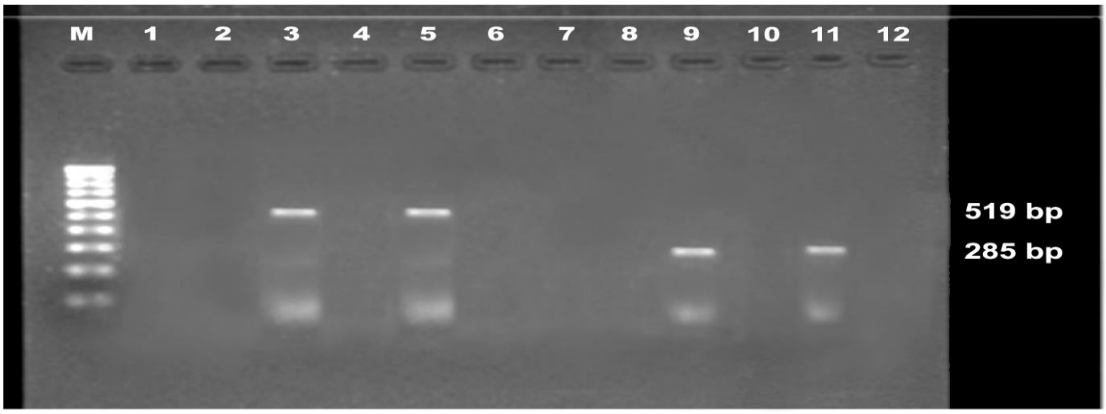


Fig. 20 Results of some plankton samples for *Vibrio vulnificus* (targeting *vvhA* and *gyrB* genes)

Lane M: 100 bp DNA ladder
Lane 1: Plankton samples showing negative result
Lane 2: Plankton samples showing negative result
Lane 3: Plankton samples showing positive result
Lane 4: Plankton samples showing negative result
Lane 5: Plankton samples showing positive result
Lane 6: **Negative control**
Lane 7: Plankton samples showing negative result
Lane 8: Plankton samples showing negative result
Lane 9: Plankton samples showing positive result
Lane 10 : Plankton samples showing negative result
Lane 11 : Plankton samples showing positive result
Lane 12 : **Negative control**

25 isolates of *V.vulnificus* obtained from different samples, were subjected to antibiotic sensitivity test by disc diffusion method.

The antibiotic sensitivity of *V.vulnificus* was tested for antibiotics like Ampicillin (10µg), Chloramphenicol (30µg), Ciprofloxacin (5µg), Erythromycin (15µg), Gentamicin (10µg), Kanamycin (30µg), Streptomycin (10µg), Tetracycline (30µg), Ceftazidime (30 µg), Vancomycin (30µg), Amikacin (30µg) and Nalidixic acid (30 µg).

V.vulnificus was highly sensitive to Ciprofloxocin and Ceftazidime (96%), followed by Gentamicin (92%), Erythromycin and Tetracyclin (80%), Streptomycin (76%), Nalidixic acid (68%), Chloramphenicol (60%), Amikacin (52%), Kanamicin (44%). The sensitivity was zero to Ampicillin and Vancomycin.

V.vulnificus was highly resistant to Ampicillin (96%) followed by Vancomycin (92%), Amikacin (20%), Kanamycin and Tetracyclin (12%), Nalidixic acid (8%), Erythromycin, Gentamicin and Streptomycin (4%).

This organism was intermediately resistant to Kanamicin (44%) followed by Chloramphenicol (40%), Amikacin (28%), Nalidixic acid (24%), Streptomycin (20%), Erythromycin (16%), Tetracycline and Vancomycin (8%), Ampicillin, Ciprofloxacin, Gentamicin and Ceftazidime (4%).

Table 6: Detection of *V.vulnificus* by cultural and PCR methods from spiked fish.

Selective broth	Time of aliquot collected	Inoculation level (CFU /g of fish)									
		2500		250		25		2.5		0.25	
		PCR	Cultural	PCR	Cultural	PCR	Cultural	PCR	Cultural	PCR	Cultural
APW	12h	+	+	+	+	+	+	+	-	-	-
	18h	+	+	+	+	+	+	+	+	+	-
Luria Bertani broth	12h	+	+	+	+	+	+	+	-	-	-
	18h	+	+	+	+	+	+	+	+	+	-

Table 7: Cultural and PCR results of different natural samples for *V.vulnificus* *vvhA* and *gyrB* genes.

Type of sample	No. of samples	Positive results & per cent incidence for *V.vulnificus*							
		Cultural method		*vvhA* PCR assay		% of cultural method compared to *vvhA* PCR	*gyrB* PCR assay		% of cultural method compared to *gyrB* PCR
		No	%	No	%		No	%	
Fish	30	5	16.66	6	20	83.33	5	16.66	100
Shrimps	30	2	6.66	2	6.66	100.0	2	6.66	100
Crabs	30	3	10.00	4	13.33	75.0	4	13.33	75
Oysters	30	14	46.66	16	53.33	87.5	16	53.33	87.5
Total	120	24	20	28	23.33	85.71	27	22.5	88.88

Table 8: Cultural and PCR results of different environmental samples for *V.vulnificus* *vvhA* and *gyrB* genes.

Type of sample	No. of samples	Positive results & per cent incidence for *V.vulnificus*							
		Cultural method		*vvhA* PCR assay		% of cultural method compared to *vvhA* PCR	*gyrB* PCR assay		% of cultural method compared to *gyrB* PCR
		No	%	No	%		No	%	
Farm Water	30	4	13.33	5	16.66	80.00	05	16.66	80.00
Esturine water	30	19	63.33	21	70.00	90.48	21	70.00	90.48
Marine water	30	15	50.00	16	53.33	93.75	16	53.33	93.75
sediment	30	14	46.66	16	53.33	87.50	16	53.33	87.50
plankton	30	15	50.00	17	56.66	88.23	17	56.66	88.23
Total	150	67	44.66	75	50.0	89.33	75	89.33	89.33

Table 9: Mean counts (CFU/g) and Range (CFU/g) of *V.vulnificus* in various samples.

S.no	Sample (n=30)	Mean counts (CFU/g) of *V.vulnificus*	Range(CFU/g)
1.	Fish	$2.9x10^2$	$4.1x10^1 – 6.2x10^2$
2.	Shrimps	$5.1x10^2$	$0.8x10^1 – 1.2x10^3$
3.	Crabs	$5.0x10^2$	$1.1x10^1 – 1.4x10^3$
4.	Oysters	$2.1 x10^4$	$1.8x10^1 – 2.1x10^5$
5.	Shrimp Pond water	$5.3x10^3$	$6.2x10^1 – 1.2x10^4$
6.	Estuarine water	$3.8x10^6$	$3.8x10^3 – 1.1x10^7$
7.	Marine water	$9.4x10^4$	$1.2x10^2$ - $2.8x10^5$
8.	Sediment	$6.1x10^5$	$3.2x10^2 – 1.8x10^6$
9.	Plankton	$6.2x10^3$	$4.6x10^2 – 1.6x10^4$

Table 10: Antibiotic sensitivity of *V. vulnificus.*

Sl.no	Antibiotic	Antimicrobial resistance and No.positive(%)		
		Sensitive	Intermediate	Resistant
1.	Ampicillin (10 µg)	-	1(4%)	24(96%)
2.	Chloramphenicol (30 µg)	15(60%)	10(40%)	-
3.	Ciprofloxacin (5 µg)	24(96%)	1(4%)	-
4.	Erythromycin (15µg)	20(80%)	4(16%)	1(4%)
5.	Gentamicin (10 µg)	23(92%)	1(4%)	1(4%)
6.	Kanamycin (30µg)	11(44%)	11(44%)	3(12%)
7.	Streptomycin (10 µg)	19(76%)	5(20%)	1(4%)
8.	Tetracycline (30 µg)	20(80%)	2(8%)	3(12)
9.	Ceftazidime (30 µg)	24(96%)	1(4%)	-
10.	Vancomycin (30 µg)	-	2(8%)	23(92%)
11.	Amikacin(30 µg)	13(52%)	7(28%)	5(20%)
12.	Nalidixic acid (30 µg)	17(68%)	6(24%)	2(8%)

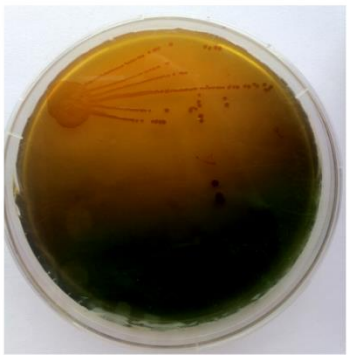


plate:-1 *Vibrio vulnificus* on mCPC agar

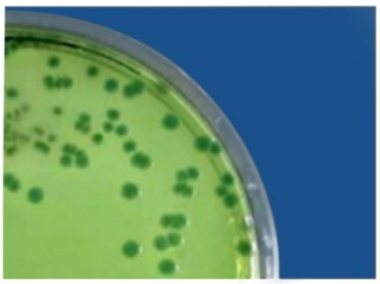


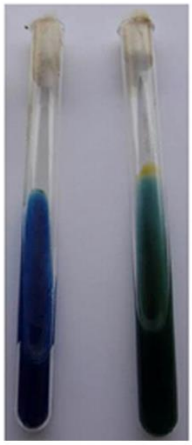
plate:-2 *Vibrio vulnificus* on TCBS agar

+ve control

plate:-3 ONPG test

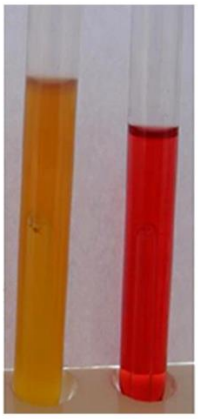
+ve control

plate:-4 Oxidase test



+ve control

plate:-5 Simmons citrate test



+ve control

plate:-6 Lactose test (Lactose to acid)

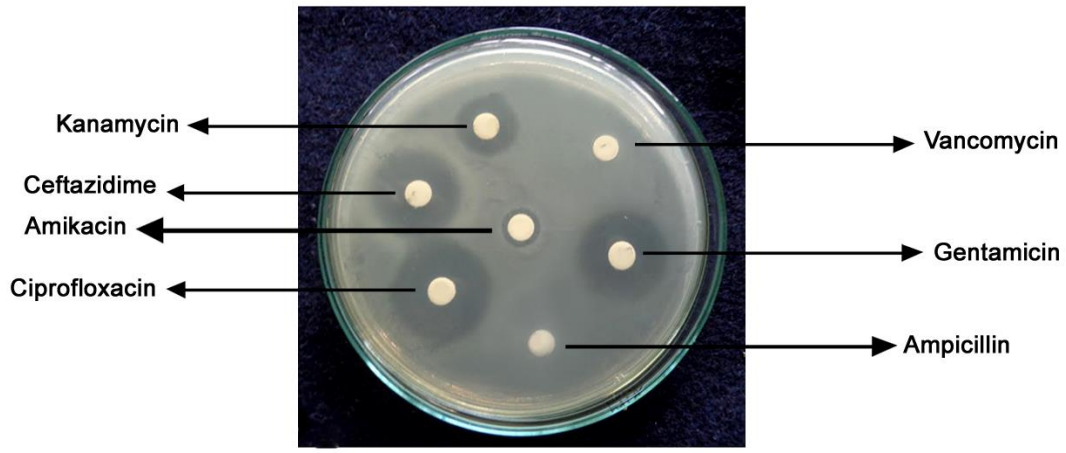


plate:- 7 Antibiotic sensitivity test for *Vibrio vulnificus*

CHAPTER V

DISCUSSION

V.vulnificus is a major economic problem for the seafood industry especially in developing countries and it is responsible for food borne illness in many countries leading to hospitalization and sometimes fatal also (Mead *et. al.*, 1999). As per Rapid Alert System for Food and Feed (RASF) of the European Union, *V.vulnificus* infected samples must be quickly identified so that the spread of contamination can be controlled (Rao and Surendran, 2013). *V.vulnificus* is a main cause of seafoodborne infection (Oliver, 1989) and it has been a major causative agent of food borne septicaemia,wound infection and gatroenteritis in humans (Desenclos *et. al.*, 1991).

In India, *V.vulnificus* has been isolated from variety of aqua foods including fish, shrimps,crabs,oysters and canned fish products and also from environmental samples collected from different sources (Thampuran and Surendran, 1998; Rajapandyan *et. al.,* 2009). There is wide difference in isolation of *V.vulnificus* by various scientists from various sources ranging from 0% (Adebayo–Tayo *et. al.*, 2011b) to 81% (Parvathi *et. al*., 2005). These differences might be due to geographic, seasonal, salinity, temperature variations and procedures adopted for isolation (Kelly, 1982; Kasper and Tamplin, 1993).

Current standard culture procedures for detection of *V.vulnificus* takes approximately 3-4days (DePaola *et. al*., 1994) and are laborious as well as time consuming. The detection of *V.vulnificus* from foods is difficult due to presence of high number of contaminating and indigenous bacteria and a low number of *V.vulnificus.* Tamplin *et. al*. (1982) reported a contamination level of less than 10

V.vulnificus in naturally contaminated foods. Jackson *et. al*. (1997) reported a low level of infective dose (10^3organisms) caused outbreak of *V.vulnificus.* Therefore, there is a significant need for more rapid and/or sensitive methods for identification of *V.vulnificus*. Several techniques have been developed to address this need, including DNA hybridization (Parvathi *et. al.,* 2004; Kaysner and DePaola, 2004), ELISA (Parker and Lewis, 1995), DNA probe (Nishibushi *et. al.*, 1985); RFLP-PFGE (Tamplin *et. al.*, 1996), RAPD (Ryang *et. al*., 1999), AFLP(Arias *et. al.*, 1997; Vos *et .al.*, 1995), supplementing Dot blot Hybridization (Cuellar *et. al.*, 2000) and Covalink Hybridisation Method (Lee *et. al*., 2003) with PCR assay. However sensitivity and specificity problems have limited the application of such methods, and there is still a need for a rapid, sensitive and user-friendly method. One possible approach involves PCR based assay, which has become a powerful and increasingly popular tool in other areas of microbial detection and identification. As PCR relies on the detection of specific gene fragments, it can be applied in mixed microbial cultures, avoiding problems which may arise by using other biochemical and morphological tests (Barry *et. al.*, 1991).

5.1 OLIGONUCLEOTIDE PRIMERS

Several workers have used PCR with varied success for detection of *V.vulnificus* from foods targeting specific gene sequences (Brauns *et. al.*, 1991; Hill *et. al.*,1991; Coleman *et. al.*,1996; Lee *et. al.*,1999). Of these *vvhA* gene has been most frequently targeted for PCR based detection of *V.vulnificus* (Hill *et. al.*, 1991). The mostly targeted gene for detection of cytolysin haemolysin toxin is *vvhA* (Brauns *et. al.*, 1991). Therefore, in this study primers derived from *vvhA* gene (Hill *et. al.*, 1991), and *gyrB* gene (Kumar *et. al.*, 2006) were used for detection of *V.vulnificus.*

5.1.1 STANDARDIZATION OF PCR ASSAY

The PCR procedure using *vvhA* and *gyrB* derived primers were standardized by optimizing the annealing temperature, primer concentration, $MgCl_2$ concentration, template volume and cycling conditions. The specific PCR products of 519bp for *vvhA* gene and 285bp for gyrB gene of *V.vulnificus* have to be stored at -20^0C, as it was observed that storage at a temperature of 4^0C for a longer period resulted in the degradation of the product. This degradation might be due to action of thermostable endogenous nucleases as reported by Gibson and McKee (1993).

5.2 COMPARISON OF TEMPLATE PREPARATION METHODS TO ASSESS THEIR SUITABILITY FOR PCR ASSAY

Various efficient template preparation methods should be tried to fully exploit the potential of PCR technique for the detection of *V.vulnificus* in foods (Kaysner and De Paola FDA ,BAM ,2004). Direct application of PCR technique to complex food substrate results in no amplification products (Li *et. al.,* 1988) or poor sensitivity (Fluit *et. al.,* 1993).This might be due to compository effect caused by complex composition of food (Yoo *et. al.,* 1997), but these methods are not reliable due to their poor repeatability, inapplicability to a variety of food samples and poor sensitivity (Olsen, 2000). Four template DNA preparation methods were evaluated to determine the most efficient one, in recovery of DNA from broth culture for PCR assay. Heat lysis method (boiling and snap chilling method) gave comparable results to that obtained, when pure genomic DNA was used in PCR assay. The other two methods i.e. lysis buffer-1 and lysis buffer-2 gave acceptable results, but they are not reliable because of the components of lytic reagents like SDS and Triton X-100. If

these are present in the reaction mixture above a certain level, may reduce the detection limit or have an inhibitory effect on PCR (Rossen *et. al.*, 1992).

Genomic DNA extraction method is ideal for the culture inoculated and incubated in broths like APW/Luria Bertani Broth, In addition, genomic DNA extraction method is time consuming and laborious and therefore not suitable for testing of more number of samples. Boiling and snap chilling was the template preparation method employed in all the experiments in this study, as it is simple, rapid, reliable and this method was followed by many workers (Kumar *et. al.*, 2006).

5.3 SPECIFICITY OF THE PCR ASSAY

The specificity of PCR assay was done for *V.vulnificus* (Primers derived from *vvhA and gyrB* genes) by testing three species of Vibrios i.e. *V.vulnificus, V.cholera, V.parahaemolyticus* and other 7 organisms. Only *V.vulnificus* gave specific products of 519 bp and 285 bp respectively, whereas other species did not yield any amplification product. These findings are in agreement with the results of Hill *et. al.* (1991), Coleman *et. al.* (1996) and Lee *et. al.* (1998).

5.4 EVALUATION OF THRESHOLD SENSITIVITY OF THE PCR ASSAY.

The minimum detection level of *V.vulnificus* by PCR assay in this study was 0.25 CFU/ml in pure culture using primers for both *vvhA* and *gyrB* genes. Almost similar threshold sensitivity for *V.vulnificus* was reported by Kaysner and DePaola (2004). A threshold sensitivity of 1 cell/gm (Lee *et. al.*, 1999; Campbell and Wright, 2003; Parvathi *et. al.*, 2004), 2 cells/g (Coleman *et. al.*, 1996), 7 cells/g(Wang and Levin, 2005), 10 cells/ml (Lee *et. al.*, 1997), 100cells/gm (Campbell and Wright, 2003; Lee *et. al.*, 2003), 220-270 cells/g (Wang and

Levin, 2005), 10^3 cells (Kim *et. al.*, 2008) and 10^4cells/gm (Morris *et. al.*, 1987) were reported.

The level of sensitivity obtained in the present study was different from studies involving modification of PCR like Chromatographic lateral flow dipstick assay (Surasilp *et. al.*, 2011), SYBR Green Real time PCR (Panicker *et. al.*, 2004), Duplex PCR (Hossain *et.al.*, 2013), Microtitre plate sandwich hybridization(Lee *et. al.*, 2003), Amplified fragment length polymorphism(Arias *et. al.*, 1997), Multiplex PCR (Lee *et. al.*, 2003; Bauer and Rervik, 2007), TaqMan Real time PCR (Wang and Levin, 2006), Nested PCR (Lee *et. al.*, 1998), Loop mediated isothermal amplification (Han and Ge, 2008) and Random Amplification of Polymorphic DNA (Radu *et. al.*, 1998).

The threshold sensitivity for *V.vulnificus* was 0.25 CFU/ml in the present study was almost similar to the findings of Kaysner and DePaola (2004). Thus the levels of sensitivity obtained from pure cultures in this study employing boiling and snap chilling for template DNA preparation is quite excellent.

5.5 EVALUATION OF ENRICHMENT BROTHS

Certain components of the selective media may cause inhibition of PCR assay either totally or partially (Rossen *et. al.*, 1992; Olsen, 2000). Therefore, methods have to be developed to extract and concentrate the target bacteria into a suitable small test volume and also remove the inhibitory substances from the target bacteria (Olsen, 2000). In this present investigation, the PCR compatibility of two enrichment (APW and Luria Bertani) broths were assessed for compatibility of the PCR assay for the primers of *V.vulnificus.*

APW broth produced brighter bands compared to Luria Bertani broth for the primers targeting *vvhA* and *gyrB* genes . Lee *et. al.* (1997), Lee *et. al.* (1999) and Parvathi *et. al.* (2004) used APW medium for enrichment of *V.vulnificus* to detect *vvhA* gene and reported that brighter bands were obtained with this medium. Dalsgaard *et. al.* (1996) used APW with Polymixin B with higher recovery rate than APW. Luria Bertani broth was used for the primers targeting *gyrB* gene by Kumar *et. al.* (2006) and Cai *et. al.* (2006) and reported that it produced brighter bands. Phosphate Buffer Saline was used by Cuellar *et. al.* (2000) for enrichment and obtained satisfactory isolation.

5.6 SPIKING STUDIES

Spiking studies in homogenized fish were done to determine the ideal enrichment protocol, which can detect the least concentration of inoculum, at the earliest by PCR assay and also to compare the results with cultural method. Low counts of *V.vulnificus,* if ingested produced cytolysin haemolysin toxin (Wright *et. al.,* 1991). Therefore, detection method should have high level of sensitivity i.e. able to detect low counts. Though sensitivity of PCR technique is quite high, the direct application of PCR to food samples often results in an absence of amplification product or poor sensitivity (Dickinson *et. al.,* 1995) due to the inhibitory effect of food components on PCR (Wang *et. al.,* 1997).

Some workers extracted DNA directly from clinical specimens (Lee *et. al.,* 1998) but it is time consuming, laborious (Brauns *et. al.,* 1991), lack of repeatability, inapplicable to variety food products and poor sensitivity (Hill *et. al.,* 1991; Arias *et. al.,* 1995). The ability of PCR to detect even a single organism is offset by the lack of methods that can release the organism from the matrix into the test volume of 5µl. To overcome this problem an enrichment step should be included (Dalsgaard *et. al.,* 1996).

The enrichment step will reduce the negative influence of food matrix by dilution and also enhances the sensitivity of the assay by increasing DNA from dead and non-culturable cells. The single step or multi-step enrichment procedure and PCR combination is by far the most used setup in food safety (Olsen, 2000).

Even though the inclusion of sophisticated technique like IMS, DNA hybridization and ELISA along with PCR assay helped to increase threshold sensitivity of PCR assay slightly, the inclusion of enrichment step enhanced the minimum detection level to much higher levels (Hoi *et. al*., 1998a). Increase in the threshold sensitivity with an increase in enrichment time was observed by many scientists (Kumar *et. al*., 2006, Parvathi *et. al*., 2004 and Tamplin *et. al*., 1991), whereas Hoi *et. al*., (1998a) reported that 6-8 h enrichment in APW was more favourable than overnight enrichment.

After enrichment in broths, a loopful of inoculum should be transferred onto selective media for obtaining good counts (Azanza *et. al*., 1996).

In naturally contaminated seafood samples less recovery of *V.vulnificus* by PCR was observed without enrichment (Arias *et. al*., 1995; Kumar *et. al*., 2006; Drake *et. al*., 2007).The enrichment step prior to PCR technique has been followed by many workers (Hsu *et. al*., 1998; Tamplin and Capers, 1992; Hagen *et. al*., 1994). Extraction of DNA directly from naturally contaminated samples without enrichment is not advisable, as low number of stressed or injured specific organisms present in natural samples may not be able to compete competitive microflora, which are dominant and will not allow specific organisms to multiply to detectable levels leading to false negative results (Colwell , 1984; Gauthier, 2000). The enrichment is recommended by all regulatory agencies for the conventional cultural isolation method and this protocol was used successfully by many workers (De Paola *et. al*., 1997; Biosca *et. al*., 1997 and Hoi *et. al*., 1998a).

5.6.1 DETECTION OF *V.vulnificus* BY PCR FROM SPIKED FISH

The results showed that in APW and Luria Bertani broths, the *V.vulnificus* were detected at an inoculation level of 2.5 CFU after 12h of incubation and 0.25 CFU after 18h of incubation by PCR technique.

Panicker *et. al*. (2004) reported a detection level of 100cells/gm without enrichment and the sensitivity has been increased to 1 cell/g with 5 h enrichment in Gulf Water Peptone (GWP) broth. Lee *et. al*. (2003) detected a level of 100 cells/g after 3h enrichment in APW broth. Kaysner and DePaola (2004) obtained a sensitivity of 0.1cell/g after 12-18h enrichment. The present study results which has sensitivity of 2.5 cells/g and 0.25 cells/g with enrichment after 12h and 18h respectively with both APW and Luria Bertani broth, which is almost similar to the results observed in above studies.

A detection level of 120 cells of *V.vulnificus* without enrichment (Arias *et. al*., 1995), 100cells/g(Lee *et. al*., 2003; Campbell and Wright, 2003) were reported. Enrichment has improved the detection levels in many studies i.e. 7 CFU/g after 5h (Han and Ge, 2008) and 1 cell/g with 6h (Han *et. al*., 2011; Lee *et. al*., 1999). As the period of incubation increased, the sensitivity of both PCR and cultural methods has increased in both the broths in the present study. Kumar *et. al*. (2006) detected *V.vulnificus* in 4, 36 and 59 samples out of 79 samples after 0,6 and 18 h enrichment, which clearly indicates that only 5 % of the samples were positive for *V.vulnificus* without enrichment and which has increased to 45.5% and 75% after 6h and 18h enrichment in APW broth.

The variation in threshold sensitivity in various studies can occur due to various factors like differences in DNA extraction method, duration of enrichment, temperature of incubation and type of food used for spiking studies (Arias, 1995; Harwood *et. al*., 2004; Kumar *et. al*., 2006).

Though the artificial inoculation studies gave a high sensitivity for PCR assay, we should keep in mind the fact that *V.vulnificus* present in food samples are not in the same physiological conditions, as in pure laboratory cultures and also the microflora present in frozen meat used for spiking studies may be suppressed. So it can be assumed that the sensitivity of the method followed might have given over estimation results.

5.7 SCREENING OF NATURAL SAMPLES

For screening of naturally contaminated aquatic food samples (fish, shrimp, crab and oyster) and environmental samples(pond water, esturine water, marine water, sediment and plankton), 2 enrichment broths were used namely APW and Luria Bertani broth, as regulatory agencies recommended the use of at least 2 selective media to increase the sensitivity (Waltman, 2000). Incubation temperature of 37^0C was followed for both the broths (Kaysner and DePaola, 2004).

CPC, mCPC and TCBS plating media are used for traditional cultural methods, based on the results obtained in spiking studies as well as based on high sensitivity (99%) reported by Tamplin *et. al*. (1991) and Dalsgaard and Hoi (1997).The excision method of sampling for raw aquatic food samples was followed based on reports of consistent recovering of bacteria (Canigral *et. al*., 2010).

Out of 30 fish samples, 5 (16.66%) were positive by cultural method and PCR assay targeting *gyrB* gene ,whereas 6 (20%) were positive for *V.vulnificus* by PCR method targeting *vvhA* gene. Tison and Kelly (1986), Stelma *et. al*. (1992) and Kumar *et. al. (*2006) reported that all *v.vulnificus* strains irrespective of the source

possess *vvhA* gene. one fish sample positive for *vvhA* was negative for *gyrB*. Higher incidence of *V.vulnificus* of 25% and 26% in fish by cultural method than the present study (16.66%) was reported by Thampuran and Surendran (1998) and Hoi *et. al*. (1998b) respectively. Low incidence (1.9% and 2.5%) by cultural method was reported by Adebayo Tayo (2011b) and Adebayo Tayo (2011a) respectively. The incidence by PCR in the present study (20%) was more than the incidence of 8.4% (Sudha, 2001) and 15% (Wang *et. al*., 1997) reported.

Out of 30 shrimp samples 2 (6.66%) were positive for *V.vulnificus* by cultural and both PCR methods targeting *vvhA* and *gyrB* genes. Low incidence of 0.4 %, 2.4%, 4%, 4.2% and 5% than the present study (6.6%) was reported by Little *et. al*., (1997), Koralage *et. al*. (2012), Chan *et. al*. (1989), Landgraf *et. al*. (1996) and Otta *et. al*. (2001) respectively. An incidence of 6.6% in the present study by PCR was similar to the results reported by Merwad *et. al*. (2011). Higher incidence of 13.3%, 16.6%, 17%, 18-35%, 42.3%, 43.7 %, 83.3% and 90% in shrimps by PCR was reported by Gopal (2005), Colakogu *et. al*. (2006), Berry *et. al*. (1994), Hoi *et. al*. (1998b), Pan *et. al*. (2013), Fukushima (2006), Arias *et. al*. (1999) and Hlady and Klontz (1996) respectively. 100% incidence by PCR was reported by Yano *et. al*. (2004), whereas low incidence of 2.2% by PCR was reported by Gopal *et. al*. (2005).

Out of 30 crab samples 3 (10%) were positive by cultural method whereas 4 (13.33%) were positive for *V.vulnificus* by both PCR methods targeting *vvhA* and *gyrB* genes. The incidence by cultural method in the present study (10%) was almost similar to the incidence (11%) reported by Morris (1988). A lower incidence of 1.9% and 4.0% by cultural method was reported by Adebayo Tayo *et. al*. (2011b) and Chan *et. al*. (1989) respectively. Higher incidence of 25% by cultural method was reported by Adebayo Tayo *et. al*. (2011a). Wang *et. al*. (1997) reported an incidence of 15%

by PCR assay, which was almost similar to the incidence (13.3%) in the present study, whereas low incidence of 2% was reported by Merwad *et. al*. (2011).

Out of 30 oyster samples 14 (46.6%) were positive by cultural method and 16 (53.33%) were positive for *V.vulnificus* by both PCR methods targeting *vvhA* and *gyrB* genes. Kumar *et. al*. (2006) reported an incidence of 45.5% in oysters by cultural method, which was almost similar to the incidence in the present study. Higher incidence of 50% by cultural method was reported by Morris (1988) than in the present study. Low incidence of 43%, 40%, 29% and 6% by cultural method than the present study was reported by Parvathi *et. al.* (2005), Tamplin (1990), Landgraf *et. al*. (1996) and Chen *et. al*. (2010) respectively. Higher incidence of 100%, 86.3%, 85% and 60% by PCR was reported by Panicker *et. al*. (2004), Johnson *et. al*. (2012), Parvathi *et. al*. (2004) and Han *et. al*. (2007) respectively. Low incidence of 17.2%, 10% and 2% was reported by Kirs *et. al*. (2010), Canigral *et. al*. (2010) and Merwad *et. al*. (2011) respectively. Parvathi *et. al.* (2005) reported varied incidence depending upon the incubation of enrichment in APW i.e. 11.4%, 60.8% and 81% after 0, 6, 18h enrichment respectively.

Out of total 120 aquatic food samples 24 (20%) were positive for *V.vulnificus* by cultural method. The PCR assay targeting *vvhA* gene has given 28 (23.33%) positive whereas the PCR assay targeting *gyrB* gene has given 27(22.5%) positive for *V.vulnificus*.

Out of 30 pond water samples 4 (13.33%) and 5 (16.66%) were positive for *V.vulnificus* by cultural and PCR methods (targeting both *vvhA* and *gyrB* genes) respectively. The incidence of 13.3% by cultural method in the present study was higher than the incidence of 4% reported by Gopal *et. al*. (2005). Very low incidence of 2% and 3.7% in pond water by PCR assay was reported by Masini *et. al*. (2007)

and Gugliandolo *et. al*. (2005) respectively than the incidence (16.66%) in the present study.

Out of 30 esturine water samples 19 (63.33%) were positive by cultural method whereas 21 (70.00%) were positive for *V.vulnificus* by both PCR methods targeting *vvhA* and *gyrB* genes. Low incidence (25%) by cultural method in esturine water was reported by Adebayo Tayo *et. al*. (2011b) than the incidence (63.33%) in the present study. The incidence by PCR in the present study was 70%, which was higher than the incidence of 5% reported by Merwad *et. al*. (2011) and lower than the incidence of 79.2% reported by Johnson *et. al*. (2012).

Out of 30 marine water samples 15 (50%) were positive by cultural method and 16 (53.33%) were positive for *V.vulnificus* by both PCR methods targeting *vvhA* and *gyrB* genes. The incidence of 50% by cultural method in the present study in marine water was less than the incidence of 68.4% and 53% reported by Hoi *et. al*. (1998b) and Cuellar *et. al.* (2000) respectively and higher than the incidence of 33% reported by Oliver *et. al*. (1983).Very low incidence of 0.6% by cultural method was reported by Sudha (2001). The incidence (53.3%) by PCR in the present study was higher than the incidence of 32% reported by Canigral *et. al*. (2010) and lower than the incidence of 80% reported by Wright *et. al*. (1996).

Out of 30 sediment samples 14 (46.66%) were positive by cultural method and 16 (53.33%) were positive for *V.vulnificus* by both PCR methods targeting *vvhA* and *gyrB* genes. Higher incidence of 76% (Hoi *et. al*., 1998b), 62.5% (Oliver *et. al*., 1983) by cultural method than the incidence (46%) in the present study was reported. Low incidence of 3.3% and 10.4% by cultural method were reported by Gopal *et. al*. (2005) and Sudha (2001) respectively. Higher incidence of 61% than the incidence of 53.33% in the present study by PCR assay was reported by Johnson *et. al*. (2012).

Out of 30 plankton samples 15 (50%) were positive by cultural method and 17 (56.66%) were positive for *V.vulnificus* by both PCR methods targeting *vvhA* and *gyrB* genes. The incidence (50%) of *V .vulnificus* in sediment by cultural method in the present study was higher than the incidence of 42.5%, 38.5%, 20% and 3.3% reported by Oliver *et. al*. (1983), Tamplin (1990), Baffone *et. al*. (2006) and Sudha (2001) respectively. Wright *et. al*. (1996) reported 100% incidence in zooplankton.

Out of total 150 environmental samples 67 (44.66%) were positive by cultural method and 75 (50%) were positive for *V.vulnificus* by both PCR methods targeting *vvhA* and *gyrB* genes.

The mean viable count of *V.vulnificus* (CFU/g) in fish in the present study was $2.9x10^2$. Higher counts than the present study were reported i.e. $1.8x10^3$(Oliver *et. al*., 1983), $1.2x\ 10^3$ in fish fed on plankton and $2.1x10^6$ in bottom fed fish (DePaola *et. al*., 1994).

The mean viable count of *V.vulnificus* (CFU/g) in shrimp was $5.1x10^2$ in the present study, which is almost similar to the counts reported by Fukushima (2006). Higher counts of $3.8x10^4$ and $4.36x10^4$ in shrimp were reported by Gopal *et. al*. (2005) and Sudha (2001) respectively. Very low counts of 15 CFU/g was reported by Cantet *et. al*. (2013).

The mean viable count of *V.vulnificus* (CFU/g) in crabs was $5.0x10^2$ in the present study, which was less than the counts of $1.2X\ 10^3$ and $1.8x10^3$ reported by DePaola *et. al*. (1994) and Oliver *et. al*. (1983) respectively.

The mean viable count of *V.vulnificus* (CFU/g) in oysters was $2.1x10^4$ in the present study, which was higher than the counts of $1.1x10^3$, $1.8x10^3$ and $3x10^3$ reported by DePaola *et. al*. (1994), Motes *et. al*. (1998) and Tamplin *et. al*. (1982) respectively and lower than the counts of $4.7x10^4$, $5.7X10^4$, $6x10^4$ and $2.1x10^5$ reported by Wright *et. al*. (1996), Drake *et. al*. (2007), Oliver and Kaper (1997), and

Tamplin and Capers (1992) respectively. Parvathi *et. al*. (2004) reported counts almost similar to the present study during summer and no counts were found during winter.

The mean viable count of *V.vulnificus* (CFU/ml) in pond water was $5.3x10^{3}$ in the present study which was less than the counts of $3x10^{4}$– $2x10^{5}$ reported by (Wright *et. al.*, 1996).

The mean viable count of *V.vulnificus* (CFU/ml) in esturine water was $3.8x10^{6}$ in the present study which was higher than the counts of $5.8x10^{2}$, $7.2X10^{2}$, $4.2x10^{3}$, $4.6 X10^{4}$ and $9.3x10^{4}$ reported by Lipp *et. al*., (2001), Kasper and Tamplin (1993), Pfeffer *et. al*. (2003), Tamplin *et. al*. (1982) and Fukushima and Seki (2004) respectively.

The mean viable count of *V.vulnificus* (CFU/ml) in marine water was $9.4x10^{4}$ in the present study. Low counts of $1.9X10^{1}$, $2.1x10^{2}$, $4.7x10^{3}$, $1.2x10^{4}$, $4.69x10^{4}$ and $5x10^{4}$ in marine water was reported by Hoi *et. al*. (1998b), Wright *et. al*. (1996), Oliver *et. al*. (1983), Pfeffer *et. al*. (2003), Gopal *et. al*. (2005) and Arias *et. al*. (1999) respectively.

The mean viable count of *V.vulnificus* (CFU/g) in sediment was $6.1x10^{5}$ in the present study which was almost similar to the counts reported by Wright *et. al*. (1996).Lower counts of $1.1x10^{2}$, $2.02x10^{2}$, $5.5x10^{2}$ and $4.2x\ 10^{3}$ than the present study were reported by Cantet *et. al*. (2013), Gopal *et. al*. (2005), Oliver *et. al*. (1983) and DePaola *et. al*. (1994) respectively.

The mean viable count of *V.vulnificus* (CFU/g) in plankton was $6.1x10^{3}$ in the present study which was higher than the counts of $4.7x10^{1}$ and $1.2x10^{3}$ reported by Oliver *et. al*. (1983) and Wright *et. al*. (1996) respectively and lower than the counts of $2.8x10^{5}$ reported by Sudha (2001).

- **5.8 ANTIBIOTIC SENSITIVTY**

- In order to increase the production of seafood and decrease the occurance of the disease in aquaculture some antibiotics are being used and have resulted in the emergence of multidrug resistant *Vibrio vulnificus*. So there is a need to evaluate the antibiotic sensitivity/resistance of the isolates obtained from different samples so as to safeguard the public health by opting effective antibiotics for treatment.

Vibrio vulnificus isolates tested in the present study were highly resistant (96%) to Ampicillin, with zero sensitivity. Many workers reported high resistance of Ampicillin i.e. Nascimento *et. al*. (2001), Ottaviani *et. al*. (2001), Zanetti *et. al*. (2001) and Elhadi *et. al*. (2012).Vijaya Joseph (2013) reported 61% resistance to Ampicillin, whereas Sunith Shine *et. al*. (2014) reported high levels intermediate resistance. On contrary to the present finding Shaw *et. al*. (2014) reported a sensitivity of 97% to Ampicillin, whereas Biosca *et. al*. (1996), Dalsgaard and Hoi (1997) and Pan *et. al*. (2013) observed moderately higher sensitivity.

The sensitivity to Chloramphenicol in the present study was 60%, zero resistance and 40% intermediate. Higher sensitivity than the present study was observed by Radu *et. al*. (2000), Nascimento *et. al*. (2001), Thakur *et. al*. (2003), Pan *et. al*. (2013) and Sunith Shine *et. al*. (2014). Low sensitivity of 22% and 78% intermediate than the present study was observed by Shaw *et. al*. (2014). Pan *et .al*. (2013) and Sunith Shine *et. al*. (2014) also observed low sensitivity against cholaramphenicol.

Vibrio vulnificus was highly sensitive (96%) to ciprofloxacin and zero resistance was observed. 100% sensitivity to ciprofloxacin was reported by Shaw *et. al*. (2014) and high level of sensitivity was reported by Nascimento *et. al*. (2001), Pan *et. al*. (2013), and Sunith Shine *et. al*. (2014).

A sensitivity of 80% and 16% intermediate was observed against Erythromycin in the present study. High sensitivity was also reported by Biosca *et. al*. (1996), Dalsgaard and Hoi (1997), Thakur *et. al*. (2003) and Sunith Shine *et. al*. (2014).

The sensitivity to rifampicin was 60% and resistance was 4% in the present study. A resistance of 6% reported by Vijaya Joseph (2013) was almost similar to the resistance in the present study.

The sensitivity to gentamicin was 92% and 4% resistance was observed in the present study. 100 percent sensitivity was reported by Shaw *et. al*. (2014). Higher sensitivity than the present study was reported by Biosca *et. al*. (1996), Dalsgaard and Hoi (1997), Nascimento *et. al*. (2001), Sunith Shine *et. al*. (2014), whereas moderate sensitivity was reported by Sharshar and Azab (2008). A resitance of 6% reported by Vijaya Joseph (2013) was almost similar to the resistance in the present study Very high resistance to gentamicin was reported by Baker - Austin *et. al*. (2009), whereas moderately high resistance was reported by Elhadi (2012). Pan *et. al*. (2013) reported 93.94 % intermediate to gentamicin.

A sensitivity of 44% to Kanamycin and 44% intermediate was observed in the present study. Biosca *et. al*. (1996) and Dalsgaard and Hoi (1997) reported susceptibility of *V.vulnificus* to Kanamycin.

Vibrio vulnificus had a sensitivity of 76% and 20% intermediate to streptomycin. Thakur *et. al*. (2003) and Shaw *et. al*. (2014) reported higher sensitivity of *V.vulnificus* to streptomycin. Baker –Austin *et. al*. (2009) reported highly resistance to streptomycin whereas, Pan *et. al*. (2013) and Sunith Shine *et. al*. (2014) reported higher intermediate and /or resistance.

80% sensitivity, 12% resistance and 8% intermediate was observed against tetracycline in the present study. 100% sensitivity to tetracycline was reported by Shaw *et. al*. (2014), where as higher sensitivity was reported by Radu *et. al*. (2000)

and Sunith Shine *et. al*. (2014). A resistance of 6.06% was reported Pan *et. al*. (2013), which was almost similar to the resistance (8%) observed in the present study, whereas Vijaya Joseph (2013) reported a resistance of 39%.

V.vulnificus was highly sensitive (96%) to ceftazidime. The sensitivity in the present study was almost similar to the sensitivity (98%) reported by Shaw *et. al*. (2014). Pan *et. al*. (2013) also reported higher sensitivity of *V.vulnificus* to ceftazidime.

Vibrio vulnificus was highly resistant (92%) to vancomycin in the present study, which was almost similar to the resistance (94%) reported by Vijaya Joseph (2013).

The sensitivity of *V.vulnificus* to Amikacin was 52%, whereas 20% resistance and 28% intermediate was observed in the present study. Shaw *et. al*. (2014) reported 98% sensitivity to Amikacin, whereas, Sharshar and Azab (2008) and Sunith Shine *et. al*. (2014) reported higher sensitivity. Elhadi (2012) reported higher resistance to Amikacin.

A sensitivity of 68% to Nalidixic acid was observed in the present study. Higher sensitivity was reported by Biosca *et. al*. (1996), Dalsgaard and Hoi (1997), Radu *et. al*. (2000) and Pan *et. al*. (2013).The resistance to Nalidixic acid in the present study was 24%, which was higher than the resistance (6%) reported by Vijaya Joseph (2013). Higher resistance was reported by Sharshar and Azab (2008).

CHAPTER VI

SUMMARY

Vibrio vulnificus is one of the most important pathogenic organisms implicated in food borne bacterial outbreaks, both in developed and developing countries and constitutes an important public health problem. It has been isolated from wide variety of aqua foods such as shrimps, oysters, crabs and fish. Detection of *Vibrio vulnificus* from food stuffs is problematic, since they are present at low level together with competing aerobic microflora and also they may be injured by different food processing methods. Traditional methods for detecting and identifying *Vibrio vulnificus* by cultural methods are labour intensive and time consuming, requiring four to six days for getting a confirmed positive result. Hence, there is a need to develop reliable and rapid methods for detection of *Vibrio vulnificus* from foods. The present study was undertaken to standardize PCR assay for detection of *Vibrio vulnificus* from foods and compare its efficacy with conventional cultural methods.

Two sets of primers derived from *vvhA* and *gyrB* genes were used for *Vibrio vulnificus* detection by PCR assay. The PCR assay was initially standardized by optimizing the concentration of the components of the reaction mixture, annealing temperature and cycling conditions for two sets of primers. Optimal results were obtained for the two primers by using 2 µl of bacterial lysate or 8 ng of diluted DNA as template in a reaction mixture consisting of 2.0 µl, 10X assay buffer for Taq polymerase containing 1.5 mM of $MgCl_2$, 0.8 µl of 10 mM dNTP mix, 10 Pmoles of each primer and 1U/µl of Taq DNA polymerase in a final reaction volume made upto 20 µl with molecular grade water.

For the primers derived from *vvhA* gene the initial denaturation was at 94^0C for 10 min followed by 30 cycles of each of denaturation at 94^0C for 1.75 min, annealing

at 66^0C for 2 min and extension at 72^0C for 2 min with a final extension period of 10min at 72^0C was found to be optimum with specific amplification products at 519bp fragment without any spurious products.

For the primers derived from *gyrB* gene the initial denaturation was at 94^0C for 5 min followed by 35 cycles of each of denaturation at 94^0C for 30sec, annealing at 64^0C for 30 sec and extension at 72^0C for 30 sec with a final extension period of 5 min at 72^0C was found to be optimum with specific amplification products at 285 bp fragment without any spurious products.

Four different template preparation methods viz. genomic DNA extraction, heat lysis and two lysis buffers were compared to determine the most sensitive, rapid and simple method suitable for PCR technique. Desired amplification products were obtained with all four template preparation methods, but genomic DNA extraction and heat lysis methods gave specific products with good intensity, whereas two lysis buffers resulted in lesser intensity. In this study heat lysis method was employed, as it is simple, rapid and reliable and the efficiency was comparable to genomic DNA extraction method.

The specificity of the standardized PCR assay for the two primers was tested by subjecting 10 isolates including 3 species of *vibrio* (*Vibrio cholera, Vibrio parahaemolyticus, Vibrio vulnificus*) and 7 other bacteria. *Vibrio vulnificus* yielded a specific product of 519 bp for *vvhA* gene and 285 bp for *gyrB* gene primer.

The sensitivity of the PCR assay was evaluated by subjecting serial 10 fold dilution of pure culture of *V. vulnificus* from $2.5x10^3$ CFU/ml to 0.25 CFU/ml to PCR assay with primers for *vvhA* .The minimum detection level was found to be 0.25 CFU/ml in APW and Luria Bertani broth but APW produced brighter bands compared to Luria Bertani broth.

Spiking studies were conducted to determine an ideal enrichment protocol suitable for detecting the minimum inoculum at earliest by both PCR and cultural methods. Homogenized fish was inoculated with *V.vulnificus* at levels of 2500, 250, 25, 2.5 and 0.25 CFU per 1 g and enrichment was done in APW and Luria Bertani incubated at 37^0C for 12 h and 18h. The minimum detection levels in both APW and Luria Bertani by PCR assay were 2.5 CFU and 0.25 CFU after 12 h and 18 h of incubation, respectively. By cultural methods the minimum detection levels were 25 CFU and 2.5 CFU after 12 h and 18 h of incubation, respectively.

Out of 120 naturally contaminated food samples (30 each of fish, shrimps, crabs and oysters) screened for *Vibrio vulnificus,* PCR gave 28 positive results, out of which 6, 2, 14, and 16 were positive for the above given samples, respectively, where as cultural methods gave 24 positive, of which the above given samples were positive for 5, 2, 3 and 14 samples respectively.

Out of 150 environmental samples (30 each of farm pond water, esturine water, marine water, sediment and plankton) screened for *Vibrio vulnificus,* PCR gave 75 positive results, out of which 5, 21, 16, 16 and 17 were positive for the above given samples respectively, whereas cultural methods gave 67 positive, of which the above given samples were positive for 4, 19, 15, 14 and 15 samples, respectively.

The mean viable counts of *Vibrio vulnificus* (CFU/g) are $2.9x10^2$ in fish, $5.1x10^2$ in shrimps, $5.0x10^2$ in crabs, $2.1x10^4$ in oysters , $5.3x10^3$ in shrimp pond water, $3.8x10^6$ in esturine water, $9.4x10^4$ in marine water, $6.1x10^5$ in sediment and $6.2x10^3$ in plankton.

V.vulnificus was highly sensitive to Ciprofloxocin and ceftazidime (96%), followed by Gentamicin (92%), Erythromycin and Tetracyclin (80%), Streptomycin (76%), Nalidixic acid (68%), Chloramphenicol (60%), Amikacin (52%), Kanamicin(44%). The sensitivity was zero to Ampicillin and Vancomycin.

V.vulnificus was highly resistant to Ampicillin (96%) followed by Vancomycin (92%), Amikacin (20%), Kanamycin and Tetracyclin(12%), Nalidixic acid (8%), Erythromycin, Gentamycin and Streptomycin (4%).

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ANEXURE -1

Medium	Units
1. Alkaline peptone water	
Peptone	10g
Sodium chloride	5g
P^H	8.6±0.2
2. Luria Bertani broth	
Tryptone	10g
Yeast extract	5g
NaCl	5 g
P^H	7.0±0.2
3. Thiosulphate Citrate Bile salt Sucrose agar	
Proteose peptone	10g
Yeast extract	5g
Sodium thiosulphate	10g
Sodium citrate	10g
Oxgall	8g
Sodium chloride	10g
Ferric citrate	1g
Bromthymol blue	0.04g
Thymol blue	0.04g
Agar	5g
P^H	8.6±0.2

4. CPC Agar Base w/ 1% Cellobiose

Ingredients	Gms / Litre
Peptic digest of animal tissue	10.000
Beef extract	5.000
Cellobiose	10.000
Sodium chloride	20.000
Bromothymol blue	0.040
Cresol red	0.040
Agar	15.000
Final pH (at 25°C)	7.6±0.2

5. CPC Agar

Ingredients	Gms / Litre
Peptic digest of animal tissue	10.000

Beef extract	5.000
Cellobiose	15.000
Sodium chloride	20.000
Bromothymol blue	0.040
Cresol red	0.040
Agar	15.000
Colistin	13,60,000Units
Polymyxin B sulphate	1,00,000Units
Final pH (at 25°C)	7.6±0.2

6. Modified CPC Supplement

Colistin	4,00,000 units
Polymyxin B sulphate	1,00,000 units

7. mCPC Agar (modified CPC Agar = CPC Agar Base w/ 1% Cellobiose + Modified CPC Supplement)

Ingredients	Gms / Litre
Peptic digest of animal tissue	10.000
Beef extract	5.000
Cellobiose	10.000
Sodium chloride	20.000
Bromothymol blue	0.040
Cresol red	0.040
Agar	15.000
Colistin	4,00,000Units
Polymyxin B sulphate	1,00,000Units
Final pH (at 25°C)	7.6±0.2

8. Mueller Hinton agar

Ingredients	Gms / Litre

Beef infusion from	300.000
Casein acid hydrolysate	17.500
Starch	1.500
Agar	17.000
Final p^H (at 25^0 C)	7.3±0.2

8. Phenol Red broth base

Ingredients	Gms / Litre
Proteose peptone	10.000
Beef extract	1.000
Sodium chloride	5.000
Phenol Red	0.018
Final p^H(at 25^0 C)	7.4±0.2

9. Simmons citrate agar

Ingredients	Gms / Litre
Magnesium sulphate	0.200
Ammonium dihydrogen phosphate	1.000
Dipotassium phosphate	1.000
Sodium citrate	2.000
Sodium chloride	5.000
Bromothymol blue	0.080
Agar	15.000
Final pH (at 25°C)	6.8±0.2

ANNEXURE-2

A. Reagents for genomic DNA extraction

1. Sodium dodecyl sulphate (10%)

SDS	10g
Deionized water	100 ml

Warmed to dissolve completely and adjusted PCR assay^{H} to 7.0

2. Proteinase-K (20 mg/ml)

Proteinase-K	20mg
Distilled water	1 ml

Stored at -20^{0}C

3. RNase (10mg/ml)

RNA	10 mg
Distilled water	1 ml

4. Lysozyme (10mg/ml)

Lysozyme	10 mg
Distilled water	1 ml

5. Tris-EDTA (TE) buffer (P^{H} 8.0)

Tris base	121.1g

Dissolved in 800 ml water, adjusted the P^{H} to 8.0 by adding concentrated HCL and made the volume to 1000 ml. Autoclaved and stored at 4^{0}C.

B. Reagents for agarose gel electrophoresis

1. Tris-acetate EDTA (TAE) buffer (50x)

Tris base	54g
Acetic acid	27.5g
EDTA (0.5M; PCR assay^{H} 8.0)	20 ml
Distilled to make upto	1000 ml