ISOLATION AND MOLECULAR CHARACTERIZATION OF PATHOGENIC *Vibrio parahaemolyticus* FROM CRUSTACEAN AND MOLLUSCS AND ANTAGONISTIC EFFECT OF A PROBIONT

Thesis

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Dr. S. Parthasarathy
Roll No. 5341

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF

Master of Veterinary Science
(Veterinary Public Health)

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Dedicated to....

My Parents, Wife

&

Daughter

भ.कृ.अनु.प.–भारतीय पशु चिकित्सा अनुसंधान संस्थान
(सम विश्वविद्यालय)
इज्जतनगर –243122, (उ.प्र.), भारत

DIVISION OF VETERINARY PUBLIC HEALTH
ICAR-INDIAN VETERINARY RESEARCH INSTITUTE
(Deemed University)
IZATNAGAR - 243 122, U.P., INDIA

Dr. Suresh Chandra Das
M.V.Sc., Ph.D.
Principal Scientist

Dated: June 29th, **2015**

Certificate

This is to be certified that the research work embodied in this thesis entitled **"Isolation and molecular characterization of pathogenic *Vibrio parahaemolyticus* from crustacean and molluscs and antagonistic effect of a probiont"** *submitted by* **Dr. S. Parthasarathy, Roll No. M-5341,** *for the award of* **Master of Veterinary Science** *Degree in* **Veterinary Public Health** *at Indian Veterinary Research Institute, Izatnagar, is the original work carried out by the candidate himself under my supervision and guidance.*

It is further certified that **Dr. S. Parthasarathy, Roll No. M-5341,** *has worked for more than 21 months in the Institute and has put in more than 150 days attendance under me from the date of registration for the* **Master of Veterinary Science** *Degree in this Deemed University, as required under the relevant ordinance.*

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We the undersigned members of Advisory Committee of **Dr. S. Parthasarathy , Roll No. M-5341,** *a candidate for the degree of* **Master of Veterinary Science** *with the major discipline in* **Veterinary Public Health,** *agree that the thesis entitled* **"Isolation and molecular characterization of pathogenic *Vibrio parahaemolyticus* from crustacean and molluscs and antagonistic effect of a probiont"** *may be submitted in partial fulfillment of the requirement for the degree.*

We have gone through the contents of the thesis and are fully satisfied with the work carried out by the candidate, which is being presented for the award of **Master of Veterinary Science** *Degree of this Institute.*

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Signature
Name DR. S.K. KOTWAL
External Examiner

Date : 28/7/15

(Suresh Chandra Das)
Chairman
Advisory Committee

Date : 24-6-2015

MEMBERS OF STUDENT'S ADVISORY COMMITTEE

Dr. Ashok Kumar, Principal Scientist & Head
Division of Veterinary Public Health, ICAR-IVRI, Izatnagar

Dr. D.K. Sinha, Principal Scientist
Division of Epidemiology, ICAR-IVRI, Izatnagar

Dr. Z.B. Dubal, Senior Scientist
Division of Veterinary Public Health, ICAR-IVRI, Izatnagar

Dr. P. Dandapat, Senior Scientist
Division of Bacteriology and Mycology, ICAR-IVRI, ERS, Kolkata

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S Parathasarathy

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(S. Parthasarathy)

ABBREVIATIONS

APW	:	Alkaline peptone water
AWDA	:	Agar well diffusion assay
bp	:	Base pair
CFS	:	Cell free supernatant
conc.	:	Concentration
DNA	:	Deoxy-ribonucleic acid
dNTPs	:	Deoxy-nucleotide triphosphates
DW	:	Distilled water
EDTA	:	Ethylene diamine tetra-acetic acid
et al	:	et alibi
g	:	Gram
GS	:	Group-specific
h	:	Hour
i.e.	:	That is
K/A	:	Kaper's alkaline reaction
Kb	:	Kilo base
kDa	:	Kilo Dalton
KP	:	Kanagawa phenomenon
LB	:	Luria-Bertani
M	:	Molar
mA	:	Milli ampere
mg	:	Milligram
$MgCl_2$	:	Magnesium chloride
MHA	:	Muller Hinton Agar
min	:	Minute
ml	:	Milliliter
MR	:	Methyl red
mM	:	Millimole
MRS	:	deMan, Rogosa and Sharpe
MW	:	Molecular weight
NaCl	:	Sodium chloride
NB	:	Nutrient broth
ng	:	Nano gram
NSS	:	Normal Saline Solution
pmol	:	Pico mole

PBS	:	Phosphate buffered saline
PCR	:	Polymerase chain reaction
PFGE	:	Pulsed Field Gel Electrophoresis
PGS	:	Pandemic group specific
pH	:	- Log hydrogen ion concentration
RAPD	:	Randomly Amplified Polimorphic DNAs
RBC	:	Red blood corpuscles
rpm	:	Revolutions per minute
TAE	:	Tris Acetate EDTA
TCBS agar	:	Thiosulfate citrate bile salt sucrose Agar
TDH	:	Thermostable direct haemolysin
TE	:	Tris-EDTA
TRH	:	TDH-related haemolysin
Tris	:	Tris-hydroxy methyl amino ethane
UV	:	Ultraviolet
V	:	Volts
VP	:	Voges Proskauer
viz.	:	Namely
w/v	:	Weight by volume
%	:	Percentage
µg	:	Micro gram
µl	:	Micro liter

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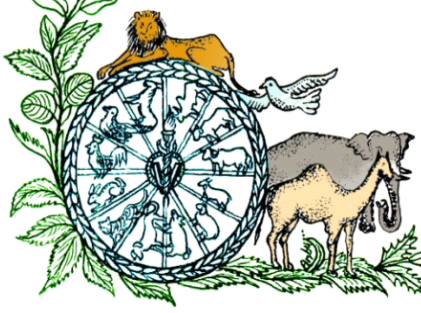


1 INTRODUCTION

Vibrio parahaemolyticus, a halophilic member of the genus *Vibrio*, has emerged globally as one of the leading enteric pathogen associated in food poisoning (toxi-infection), diarrhoea and gastroenteritis resulting from the consumption of raw or under cooked seafoods (Venktinarayanan and Doyle, 2001; Faruque and Nair, 2006; Drake *et al.,* 2007; Newton *et al.,* 2012) especially bivalve molluscs (Joseph *et al.,* 1982; Daniels *et al.,* 2000). It is ubiquitous in estuarine environments and frequently isolated from various seafood including oysters, mussels, scallops, octopus, shrimp, clam, crab, mackerel, flounder, crawfish, sardines, codfish etc. all over the world (Wong *et al.,* 2000a; Pereira *et al.,* 2007; Su and Liu, 2007).

V. parahaemolyticus is a member of the family *Vibrionaceae* and genus *Vibrio* that comprises 30 species including 13 human pathogens (McLaughlin, 2005). The members of this genus are Gram-negative, straight or curved, non-spore forming rods (0.5–0.8 µm wide by 1.4–2.6 µm long), oxidase positive, facultative aerobe and motile with polar flagellum (Drake *et al.,* 2007).

It was first identified as a cause of food borne illness in Osaka, Japan in late 1950 leading to 20 fatalities among 272 patients (Fujino *et al.,* 1953). It is one of the 13 *Vibrio* species known pathogenic causing enteric disease in human and a leading cause of foodborne infection worldwide (Joseph *et al.,* 1982; Wong *et al.,* 2000a) and causes about 25% of total foodborne diseases in comparison to other *Vibrio* species (Feldhusen *et al.,* 2000). It is a common cause of food borne illnesses in many Asian countries including China (31.1%), Japan (20–30%) and Taiwan (69%) (Mauramatsu, 1999; Wong *et al.,* 2000a; Alam *et al.,*

2002; Liu *et al.,* 2004; Anon, 2005; Nair *et al.,* 2007). In Europe, the risk of *V. parahaemolyticus* infections is considered to be very low (Anonymous, 2001; Yi-Cheng and Chengchu, 2007); however, sporadic outbreaks have been reported in countries such as Spain (important outbreaks reported in 1989, 1999 and 2004) (Lozano-Leon *et al.,* 2003) and France (a serious outbreak reported in 1997) (Robert-Pillot *et al.,* 2004). Moreover, it is acknowledged as the leading cause of human gastroenteritis associated with seafood consumption in the United States (Kaysner *et al*., 1990; Mead *et al.,* 1999; Su and Liu, 2007).

This organism accounted for about 10% of gastroenteritis cases admitted to the Infectious Diseases Hospital in Kolkata, India (Deb *et al.,* 1975) and recent studies say that *V. parahaemolyticus* infections have increased globally (Park *et al*., 2004; Nair *et al.*, 2007). Gastroenteritis is mainly characterized by diarrhoea, abdominal cramps (burning sensation in the stomach), vomiting, headache, nausea and low grade fever (Honda and Iida, 1993). Less commonly, it can cause skin infections when an open wound is exposed to warm seawater (>15°C). Individuals who are immune-compromised or burdened with pre-existing health conditions (liver disease) are at high risk for severe complications that can result in death (Morris and Black, 1985).

In Vibrios, the *toxR* gene is recognized as a regulatory gene controlling the expression of different genes encoding important extracellular virulence factors and other virulence-associated genes (Miller and Mekalanos, 1984). This *toxR* gene stimulates expression of the *tdh* gene encoding thermostable direct haemolysin, the major virulence factor of this microorganism (Lin *et al.,* 1993). The *toxR* gene together with *toxS* gene encode transmembrane proteins involved in the regulation of virulence-associated genes, which appears to be well conserved among the bacteria within *Vibrionaceae* (Osorio and Klose, 2000) but sufficiently divergent to distinguish between different *Vibrio* spp. (Lin *et al.,* 1993). The *toxR* gene fragment (~368 bp) which is specific for *V. parahaemolyticus* presents in all strains and therefore, recognized as a species specific marker for all strains of *V. parahaemolyticus* (Kim *et al.,* 1999; Dileep *et al.,* 2003).

Thermostable direct haemolysin (TDH) encoded by *tdh* gene and TDH-related haemolysin (TRH) encoded by *trh* gene are considered as two major virulence factors of this pathogen and most of the clinical isolates carry either *tdh* or *trh* and in some cases both of the genes. In contrast, the *tdh* and *trh* genes are detected in a very low frequency in the environmental strains of *V. parahaemolyticus* (Nishibuchi and Kaper, 1995; Jones *et al.,* 2012). Only 1% of environmental isolates have been reported to produce TDH (Thompson and Vanderzant, 1976) and this could relate to the limited report in occurrence of pathogenic strains of *V. parahaemolyticus* in the estuarine environment compared to its non-pathogenic strains. Alternatively, on the basis it may be explained that the pathogenic stains are more sensitive to dystrophic conditions in the aquatic environment that may rapidly become non-culturable.

Since early 1996, association of *V. parahaemolyticus* in diarrhoeal disease has witnessed a considerable higher frequency primarily due to the emergence and dominance of unique serovar, O3:K6, in India and other parts of the world (Okuda *et al.,* 1997; Bag *et al.,* 1999; Chowdhury *et al.,* 2000a; Daniels *et al.,* 2000; Matsumoto *et al.,* 2000). Surveillance for *V. parahaemolyticus* infection was initiated in January 1994 in Kolkata, India where infections caused by this organism were found to be associated with diverse serovars. However, a group of strains belonging to serovar O3:K6 and possessing the *tdh* gene but not *trh* gene appeared suddenly in February 1996 and was shown to be responsible for the high incidence (50 to 80%) of *V. parahaemolyticus* infection in Kolkata from February to August 1996 (Okuda *et al.,* 1997 and Bag *et al.,* 1999) and was subsequently found responsible for gastroenteritis in other parts of the world like Laos, Taiwan, Japan and also in US (DiRita, 1992; Nishibuchi *et al.,* 1992; Matsumoto *et al.,* 2000). Besides, the O3:K6 strains isolated in Kolkata were shown to exhibit unique profiles in arbitrarily primed PCR (AP-PCR) analysis (Matsumoto *et al*., 2000). Strains of same serovar, O3:K6, with similar genotypic traits and AP-PCR profile were also detected in 8 different countries including the United States (Matsumoto *et al.,* 2000) suggesting that the Kolkata O3:K6 strains and those isolated from different countries belonged to a single clone (Bag *et al.,* 1999; Chowdhury *et al.,* 2000b; Matsumoto *et al.,* 2000). The O3:K6 strains isolated before 1995 (referred to as Old O3:K6) and those isolated since 1995 (referred to as New O3:K6) were analysed for variation in nucleotide sequence of the *toxRS* region which encodes the transmembrane proteins involved

in the regulation of virulence-associated genes in *V. parahaemolyticus*. The difference in the sequence between the new and the old O3:K6 strains ranged from 11 to 14-bp within the 1364-bp region covering 95·4% of the *toxRS* coding regions and the sequences of these two groups (Old O3:K6 and New O3:K6 clone) differed invariably at 7 base positions (Matsumoto *et al.,* 2000). On detail nucleotide sequence study of *toxRS* region of O3:K6 (old and new clone) the sense and anti-sense primers were designed to accomplish a PCR [group specific PCR (GS-PCR)] to amplify the *toxRS* region of the O3:K6 new clone. Identification of pandemic strains by GS-PCR and various DNA fingerprinting methods revealed that in addition to the pandemic serovar, O3:K6, other serovars such as O4:K68; O1:K25; O1:KUT; O4:K12; O1:K41; O1:K56; O3:K75; O4:K8; O4:KUT; O5:KUT; O5:K17; O5:K25; O1:K33; O2:K3; OUT:KUT; O3:KUT; O3:K5; O4:K4; O4:K10 and O6:K18 were also identified for pandemic potentials (Ansaruzzaman *et al.,* 2005; Nair *et al.,* 2007). Another PCR assay developed by Okura *et al.* (2004) to identify the pandemic property of *V. parahaemolyticus* using a marker pair PGS-1 and PGS-2 derived from the group-specific sequence of an arbitrarily primed-PCR (AP-PCR) fragment (930bp) that encodes for a "hypothetical protein". This PCR assay was found to be specific to the pandemic sequence and was recommended for use in identification of pandemic *V. parahaemolyticus* from clinical source. With detail study, it was concluded that PGS-PCR assay may be a useful molecular tool not only for identification of pandemic *V. parahaemolyticus* strains but also for direct detection of the organism contaminating food and environmental samples (Okura *et al*., 2004).

Pathogenic Vibrios are involved in significant mortalities in the larviculture and grow out phases of crustacean aquaculture (Gomez-Gil *et al.,* 2000). Species such as *Vibrio harveyi, V. anguillarum, V. alginolyticus, V. parahaemolyticus* and *V. vulnificus* have frequently been associated with mortalities both in hatcheries and grow out ponds (Singh, 1986; Mohney *et al.,* 1994). Prophylactic and therapeutic use of antibiotics has been practised in commercial hatcheries to control the proliferation of pathogenic Vibrios creating the more serious event of antibiotic resistance among the microflora in the environment (Karunasagar *et al.,* 1994; Amabile-Cuevas *et al.,* 1995; Hameed *et al.,* 2003) that directed to the exploration of several alternate approaches for disease management in aquaculture systems. Of these, the most popular

and practical approach is the use of probiotics as prophylactics. Probiotics are live microbial feed supplements that can beneficially affect the host animal by improving intestinal balance (Fuller, 1989). The range of probiotics examined for use in aquaculture encompasses both Gram-negative and Gram-positive bacteria, bacteriophages, yeasts and unicellular algae. Lactic acid bacteria and bifidobacteria have been intensively employed as probiotic strains due to their recognition as members of the indigenous microflora of the animals, safety and the evidence supporting their positive role (Gaggia *et al.,* 2011). This concept is now increasingly being considered as an eco-friendly approach to mitigate public health consequences (Irianto and Austin, 2002; Nayak, 2010). The mechanism responsible for most of the effects of probiotic intervention have been extensively investigated and can be roughly classified as modification of host immune reactivity (Rook and Brunet, 2005) and interference with colonic bacterial metabolism (Ndagijimana *et al.,* 2009; Vitali *et al.,* 2012). Disease preventing abilities of probiotics are achieved through their adhesion abilities to pathogen, production of antimicrobial peptides, immune-stimulation and exclusion of pathogens (Barbosa *et al*., 2005; Balcazar *et al*., 2006). Among the probiotics, different *Lactobacillus* spp. including the *L. plantarum* have been gaining increasing attention as biological control agent against pathogenic fungi and bacteria including Vibrios in aquaculture (Nuphet *et al*., 2003; Vieira *et al*., 2013). *L. plantarum* produces plantaricin, a bacteriocin which is also effective against both the Gram-positive and Gram-negative bacteria (Enan *et al.,* 1996). Competition between *L. plantarum* and pathogenic bacteria in binding with receptors reduces the degree of adherence of the latter to the human cells and may protect the host from infection including the modulation of host immunity (Khalid, 2011).

With the enormous increase in human population and growing demand of more food vis-à-vis depletion of traditional terrestrial resources, the aquatic domain has been considered as an alternate source of dietetic protein to meet up the burgeoning need of the community. Fish and other seafood are among the most important protein rich foods for human beings especially in a country like India. Today's world is witnessing the resurgence in the consumption of fish particularly sea fishes due to growing awareness about its content of quality protein with high biological value, content of good cholesterol (HDL) as well as low in bad cholesterol

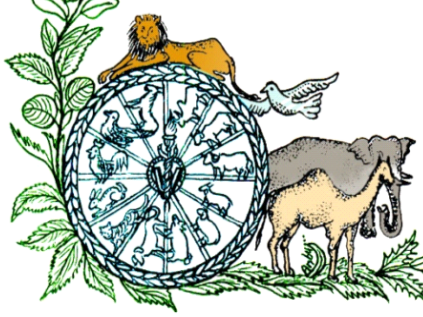
(LDL) that having salutary effect. In Odisha especially the people in coastal districts like Puri, Ganjam, Balasore, Bhadrak, Kendrapara and Jagatsinghpur prefer marine fishes and shellfishes including crab, snail, oyster, clam and small shrimp in their daily dishes that supplement the commonly available dietary protein at affordable cost. Since long, consumption of salt water fishes is well accepted among people of Kolkata and occurrence of *V. parahaemolyticus* detected from 3.5–23.9% of acute human diarrhoea cases (Pal *et al*., 1985). Since 1996, the incidence of *V. parahaemolyticus* associated infections has increased remarkably with emergence of highly virulent pandemic O3:K6 clone (Okuda *et al*., 1997; Wong *et al*., 2000b).

Over the time period, *Vibrio* of seafood origin have gained increasing attention as it is found to be one of the important causes of food poisoning in human (Nair *et al.,* 2007). Globally and regionally, most of the reported data are generated on the potentials of seafoods causing gastroenteritis, especially molluscan shellfishes (Daniels *et al.,* 2000; DePaola *et al.,* 2003; Dileep *et al.,* 2003; Deepanjali *et al.,* 2005). Although *V. parahaemolyticus* is distributed in freshwater environs in low frequencies than in seawater; however, its occurrence in various marine water and freshwater shellfish has received little attention (Sarkar *et al.,* 1985; Charles-Hernandez *et al.,* 2006). Further, magnitude of occurrence of pathogenic *V. parahaemolyticus* in different crustacean and molluscs of marine origin commonly marketed in the coastal areas of West Bengal and Odisha has not been explored, so far. It is a growing issue in respect of increasing incidence of *V. parahaemolyticus* in clinical diarrhoea and gastroenteritis in global context (Nair *et al.,* 2007). This draws attention to determine the real occurrence of pathogenic *V. parahaemolyticus* in common niche foods of crustacean and molluscs origin e.g. oysters, crab and shrimp as well as the benefit in use of probiotic(s) as an antagonist to *V. parahaemolyticus*. Keeping this in view, the present investigation was designed with the following objectives:

I. To know the incidence of pathogenic V*ibrio parahaemolyticus* in crustacean and molluscs common in coastal West Bengal and Odisha.

II. To evaluate the antagonistic effect of *Lactobacillus plantarum* on the representative isolates of *V. parahaemolyticus.*

Review of Literature



2 REVIEW OF LITERATURE

2.1 History

Association of *V. parahaemolyticus* in food borne infection was first discovered by Fujino *et al*. (1953) in Japan which was related with consumption of semi dried juvenile sardines (shirasu). For the next 18 years of its discovery, it was conceived that *V. parahaemolyticus* mediated gastroenteritis was a problem native to Japan and Far East and restricted to these countries only. However, the ubiquitousness of this organism around the world became a reality and has drawn international attention with the passage of time and advancement of studies. Since its discovery, *V. parahaemolyticus* has been recognized as a leading cause of seafood derived food poisoning throughout the world. Transmission of pathogenic strains of *V. parahaemolyticus* is mediated via ingestion of raw or improperly cooked seafoods causing acute gastroenteritis in human (Newton *et al.,* 2012). The unique combination of pathogenicity and halophilism manifested by *V. parahaemolyticus* has created much interest and consequently it has become the cynosure of food safety, marine microbiology, food hygiene, food sanitation, fisheries and public health. The organism causes three major syndromes of clinical illness in human such as gastroenteritis, septicaemia and wound infections. The gastroenteritis is characterized by mild bloody diarrhoea (meat washed) with abdominal cramps, nausea, vomiting, headache and low-grade fever (Honda and Iida, 1993). The condition become worse in immunocompromised and older people (Morris and Black, 1985).

The detailed ecological studies of *V. parahaemolyticus* have been carried out in temperate and sub-tropical waters. An ecological study conducted in tropical environs like

Kolkata, India detected *V. parahaemolyticus* in 3.5–23.9% of acute human diarrhoeal cases (Pal *et al.,* 1985) and their frequent isolation from freshwater locales have compounded its halophilic status (De *et al.,* 1977; Sarkar *et al*., 1985).

Fyfe *et al*. (1998) reported seafood associated outbreak of *V. parahaemolyticus* infection in North America and British Columbia. *V. parahaemolyticus* food poisoning cases doubled in 1998 compared to 1997 and exceeded the number of *Salmonella* cases in Japan. This has influenced to pay more attention on *V. parahaemolyticus* along with other gastroenteric pathogens (WHO, 1999).

Till 1995, *V. parahaemolyticus* was found to occur in local and sporadic clinical diarrhoea and was never associated with a pandemic occurrence. However, the past few years have witnessed an unusual change in its occurrence pattern, primarily due to the emergence and dominance of unique O3:K6 serovar, causing diarrhoea in India and other parts of the world (Okuda *et al.,* 1997; Bag *et al.,* 1999; Matsumoto *et al.,* 2000). This serovar reported in 50-80% of cases infected with *V. parahaemolyticus* in Kolkata from February to August 1996 (Okuda *et al.,* 1997; Bag *et al.,* 1999). Subsequently, gastroenteritis cases were reported in other parts of Southeast Asia and also in the United States (Matsumoto *et al.,* 2000). A sudden increase in *V. parahaemolyticus* serotype O3:K6 infections were detected in a hospital based survey in Kolkata, India (Chowdhury *et al.,* 2000a; Nair *et al.,* 2007). This highly virulent strain accounted for about 63% of all *V. parahaemolyticus* strains isolated from patients in Kolkata between September 1996 and April 1997. Subsequently, obtained in high rates from patients in other Southeast Asian countries and from travellers arriving in Japan from this region (Chiou *et al.,* 2000; Vuddhakul *et al.,* 2000) intending its pandemic occurrence.

2.2 Ecology and Epidemiology

Environmental factors influence the survival and proliferation of this bacterial pathogens and water temperature, salinity, zooplankton blooms, tidal flushing and dissolved oxygen play an important role in dictating its spatial and temporal distribution (Kaneko and Colwell, 1975). Being a halophilic (require salt for growth) bacterium, it is widely disseminated in marine and coastal environments throughout the world. However, it is vulnerable to environmental stresses

and generally rapidly inactivated at 48ºC to 55ºC with salinity below 0.5% or pH 4.0 (Beuchat, 1975). *V. parahaemolyticus* strains with high resistance to environmental stresses may survive and spread better in environmental substrates. This pathogen is typically not recovered from seawater during winter months when the water temperature is below 13ºC-15ºC (Kaneko and Colwell, 1975) and probably exists in a viable but non-culturable state (Jiang and Chai, 1996). Water temperatures have been shown to influence the growth of *V. parahaemolyticus* (Kaneko and Colwell, 1975; Thompson and Vanderzant, 1976; Kelly and Stroh, 1981) and the importance of water temperature in the epidemiology of infections is reflected by the fact that most outbreaks occur during the warmer months. It is postulated that warmer sea temperatures (the *El Nino* effect) has resulted in the emergence of more virulent serotypes (Daniels *et al.*, 2000) and both marine and freshwater fishes provide ideal substrates for the survival and proliferation of *V. parahaemolyticus* (Sarkar *et al.*,1985).

2.3 Important Outbreaks

First reported as cause of food poisoning in Japan by Fujino *et al.* (1953) and since been encountered in gastroenteritis cases from various parts of the world including Thailand, Philippines, Australia (Battey *et al.,* 1970), Malaysia and Calcutta (Chatterjee *et al.,* 1970). Sakazaki *et al*. (1971) detected large number of *V. parahaemolyticus* strains from diarrhoeal patients in Calcutta and three outbreaks of food borne gastroenteritis in Maryland (United States).

It was isolated from ill travellers who ate crab meat on a flight from Bangkok to London in 1972. An outbreak of acute gastroenteritis with an attack rate of 51% occurred in late August 1972 involving 600 persons in Covington, Louisiana following a "shrimp boil" (Barker, 1974). Contaminated seafood associated *V. parahaemolyticus* outbreaks on two Caribbean cruise ships affecting 27 passengers in 1974 and 1975 reported by Lawrence in 1979.

The largest reported outbreak (1997) was in North America affecting 209 persons linked with eating of raw oysters harvested from California, Washington and Oregon in the United States and from British Columbia in Canada which claimed one death. The total number

of food borne outbreaks due to *V. parahaemolyticus* in Tokyo between 1989 and 2000 were 710 (Obata *et al.,* 2001). This bacteria is a common cause of food borne illnesses in many Asian Countries, including China (31.1% outbreaks reported between 1991 and 2001), Japan (20–30% of food borne infection cases from 1981 to 1993) and Taiwan (1,495 cases reported between 1981 and 2003), representing 69% of all bacterial food borne outbreaks during this period) (Mauramatsu, 1999; Liu *et al.,* 2004; Nair *et al*., 2007).

Hong Kong Department of Health reported in 1999, a total of 90 confirmed outbreaks of *V. parahaemolyticus* food poisoning which affected 519 persons and was also responsible for 36% of all outbreaks in 2000 (Mok, 2001). Countries such as Spain (important outbreaks reported in 1989, 1999 and 2004) (Lozano-Leon *et al.,* 2003) and France (a serious outbreak reported in 1997) (Robert-Pillot *et al*., 2004) witnessed sporadic outbreaks.

A retrospective prevalence study on *Vibrio* spp. by Srifuengfung *et al*. (2004) from January 1994 to December 2001 at Siriraj Hospital, Bangkok, Thailand reported 35.5% prevalence of *V. parahaemolyticus* among diarrhoeal patients. In Antofagasta, Chile in 1998 about 300 human cases of infection with *V. parahaemolyticus* caused by consumption of contaminated seafood were reported (Cordova *et al.,* 2002). Since then outbreaks have not been observed in this region but during 2004–2007, about 7,000 cases were reported in Puerto Montt (Gonzalez-Escalana *et al*., 2005; Fuenzalida *et al.,* 2006; Cabello *et al.,* 2007). Subsequently, the prevalence of V. *parahaemolyticus* infection was reported from Italy (24.3%) (Ottaviani *et al.,* 2005), India (55%) (Chakraborty *et al.,* 2008) and China (47.2%) (Chao *et al*., 2009).

2.4 Species Specific Identification

The *toxR* gene was first identified as the positive transcriptional regulator of the *ctx* gene encoding the cholera toxin in *Vibrio cholerae* (Miller and Mekalanos, 1984), but later shown to be involved in the regulation of many other genes in *V. cholerae* (Miller *et al.,* 1987; DiRita, 1992). The *toxR* gene is a regulatory gene controlling the expression of the genes encoding important extracellular virulence factors and other virulence-associated genes in Vibrios. Further, the *toxR* gene was also detected in *V. parahaemolyticus* and was shown to

stimulate expression of the *tdh* gene encoding Thermostable Direct Haemolysin (TDH); a major virulence factor of this organism (Lin *et al.,* 1993). The *toxR* gene together with *tox*S gene encode transmembrane proteins involved in the regulation of virulence-associated genes, which appears to be well conserved among the bacteria within *Vibrionaceae* (Osorio and Klose, 2000), but sufficiently divergent to distinguish between different *Vibrio* spp. (Lin *et al.,* 1993). Cloning and analysis of the *toxR* gene sequences from *V. fisheri* and two other species of *Vibrio* done by Reich and Schoolnik in 1994 and they stated that the *toxR* gene appeared to be well conserved among *Vibrio* species. The degree of homology of the *toxR* gene between *V. parahaemolyticus* and *V. cholerae* is 52%. The *toxR* gene fragment (~368 bp) which is specific for *V. parahaemolyticus* presents in all strains and therefore, recognized as a species specific marker for all strains of *V. parahaemolyticus* (Kim *et al.,* 1999; Dileep *et al.,* 2003).

Karunasagar *et al*. (1997) reported that PCR targeting *toxR* gene found to be useful for confirmation of this species. It has been demonstrated that this PCR technique can detect a low number of specific bacteria against a large background of other prokaryotic and eukaryotic cells and organic materials which may present in the samples (Tsai and Olson, 1992; Thiem *et al*., 1994; Laser *et al*., 1995). These properties make PCR a suitable method for analysing environmental samples. Kim *et al*. (1999) established a *toxR* targeted PCR protocol for the specific detection of *V. parahaemolyticus* by inferring with 373 identified strains of *V. parahaemolyticus.*

Martinez-Urtaza *et al.* (2005) investigated an outbreak in A Coruna, Spain with 80 patients where *V. parahaemolyticus* recovered from stool samples of 3 patients and all isolates yielded the *toxR* gene. Elamparithi and Ramanathan (2011) demonstrated positive result for species specific-PCR targeting *toxR* gene in fish sample (Sangara–Red snapper) collected from costal belt of Cuddalore district in Tamilnadu, India. Hassan *et al*. (2012) analysed 200 retail seafood samples such as marine fish, oyster, shrimp and mussel in The Netherland and reported 19% of samples were *V. parahaemolyticus* positive (38/200) and mostly belonged to mussels (93%) and oysters (70%).

2.5 Virulence Factors

V. parahaemolyticus is frequently present in seafood; however, a subset of this organism bearing *tdh* and *trh* are known to be pathogenic and able to cause gastro-enteric problems in humans (Nishibuchi and Kaper, 1995). These two genes encode thermostable direct haemolysin (TDH) and the TDH-related haemolysin (TRH), respectively (Nishibuchi and Kaper, 1995), which are considered as the major virulence factors of *V. parahaemolyticus* (Kaysner and DePaola, 2001; Nelapati and Krishnaiah, 2010). Wagatsuma, (1968) reported that TDH is responsible for the induction of β-haemolysis on Wagatsuma blood agar containing human erythrocytes and this phenomenon is popularly known as Kanagawa phenomenon (KP). The isolates of *V. parahaemolyticus* which produce TDH are referred as Kanagawa phenomenon positive (KP^+) (Wagatsuma, 1974). TDH also have been reported to produce enterotoxic, cardiotoxic and cytotoxic activities along with haemolytic activity (Wagatsuma, 1974; Shirai *et al*., 1990; Nishibuchi *et al*., 1992; Honda and Iida, 1993). There are five sequence variants of *tdh* (named *tdh*1 to *tdh*5), but only *tdh*2 appears to have a high level of transcription (Nishibuchi and Kaper, 1990; Nakaguchi and Nishibuchi, 2005). TDH damages the eukaryotic cells by acting as a pore forming toxin that alters the ion balance of cells (Honda *et al*., 1992). Another virulence factor, named TDH-related haemolysin (TRH) has been discovered in clinical stains of *V. parahaemolyticus* lacking *tdh*, is encoded by the *trh* gene (Honda *et al.,* 1987; Honda *et al.,* 1988). The *trh* gene sequence can vary from strain to strain to some extent, but *trh* gene sequences can be clustered into two subgroups, represented by *trh*1 and *trh*2 (Kishishita *et al*., 1992). Since 1990s, resent, pathogenic *V. parahaemolyticus* isolates are identified using PCR based methods that amplify *tdh* and *trh* gene sequences (Shirai *et al.,* 1990).

More than 90% of clinical *V. parahaemolyticus* isolates possess *tdh* (DePaola *et al.,* 1990; Kaysner *et al.,* 1990; DePaola *et al.,* 2000; Wong *et al.,* 2000a). In contrast, the *tdh* and *trh* genes were rarely detected in the environmental strains of *V. parahaemolyticus* (Shirai *et al*., 1990; Kishishita *et al*., 1992). European Commission stated that the practice of judging seafood exclusively based on total *V. parahaemolyticus* counts without consideration of the virulent factors such as *tdh* and *trh* is not appropriate (Anonymous, 2001).

Hara–Kudo *et al.* (2003) analyzed 329 seafood samples (mostly molluscan shellfish) by using PCR method for detection of the *tdh* gene and found 10.0% (33 samples) are *tdh*$^+$. Similarly, DePaola *et al*. (2003) reported *tdh*$^+$ *V. parahaemolyticus* in 12.8% of Alabama oysters. Mohammad *et al*. (2005) also reported the prevalence of *tdh* gene in 8%, 11% and 17% of frozen shrimp, raw shrimp and pond water, respectively in Malaysia.

McLaughlin *et al*. (2005) reported the high prevalence of *tdh* gene in environmental *V. parahaemolyticus* (74%) and were frequent during the warmer month. However, other researchers reported low frequency (0 to 6%) of *tdh* in environmental samples and seafoods (Kiiyukia *et al*., 1989; Ogawa *et al*., 1989; Cook *et al*., 2002). Further, pathogenic strains with *tdh* and/ or *trh* have been detected in 0.3 to 3% of total *V. parahaemolyticus* environmental population (Nordstorm *et al*., 2007).

In a case-control study among expatriates and adults from 2001 to 2002 in Thailand, 95 isolates of *V. parahaemolyticus* were detected positive for the species-specific gene *toxR,* 91 were positive for *tdh*, 5 for *trh* and 4 for both *tdh* and *trh*. All five *trh* gene positive isolates were urease positive. Interestingly, three isolates (two from diarrhoea cases and one asymptomatic control) have been found to be negative for both *tdh* and *trh* (Serichantalergs *et al.,* 2007). Sujeewa *et al.* (2009) analyzed 251 samples collected from frozen shrimps and aquatic environment attempting the *toxR, tdh* and *trh* genes by PCR assay and reported 51 %, 15% and 7% isolates, respectively were positive for concerned genes.

Analysis of 120 seafood samples collected from Samsun region at the middle Black Sea coast of Turkey by biochemical reactions and *tl*-targeted gene amplification by conventional PCR done by Terzi *et al*. (2009). They reported , out of 24 mussel isolates 13 (54.2%) were *tdh* positive and *trh* negative, 11 isolates (45.8%) were both *tdh* and *trh* positive while out of 8 fish isolates six (75%) were *trh* positive and *tdh* negative and two isolates (25%) were both *tdh* and *trh* positive. Nelapati and Krishnaiah (2010) examined 105 samples (35 each of retail fresh water fish, sea fish and mutton) in Hyderabad, India and recovered 28.6% and 37.1% positivity by cultural and PCR methods, respectively and of these, 5.7%, 26.6% and 8.6% were positive for *tdh, trh* and both *tdh* and *trh*.

Chang *et al.* (2011) examined 94 virulent strains of *V. parahaemolyticus* containing *tdh* and/or *trh* genes isolated from seawater, sediment and oysters collected in Penghu, Taiwan, and Changhua Lukang coastal water and characterized by analysis of O-group antigens, PFGE after digestion with *SfiI* enzyme and RAPD. Analysis of virulence markers revealed that 60.6% of the isolates possessed only *tdh*, 13.8% of them possessed only *trh* and 25.5% of them possessed both *tdh* and *trh*.

2.6 Serotyping and Pandemic Properties

Serotyping based on 'O' and 'K' antigens can differentiate isolates of *V. parahaemolyticus* and accordingly 13 'O' groups and 71 'K' types were identified by using the commercial antisera (Iguchi *et al*., 1995). Till 1995, *V. parahaemolyticus* associated gastroenteritis was caused by many different serogroups, although in some geographic regions specific serogroups predominated like predominance of the O4 serogroup among clinical isolates in the United States (Okuda *et al*., 1997; Depaola *et al*., 2000; Kaufman *et al*., 2002).

In 1995, an outbreak of *V. parahaemolyticus* infection occurred in Kolkata, India, which caused rapid hospitalization and found to be caused by a single serotype, the new O3:K6 highly virulent strain (Okuda *et al*., 1997). Since then *V. parahaemolyticus* O3:K6 and its clonal derivatives O4:K68, O1:K25 and O1:KUT has received increasing attention as it is the first documented *V. parahaemolyticus* serotype to cause pandemic disease (Okuda *et al*., 1997; Matsumoto *et al*., 2000; Chowdhury *et al*., 2004). After that, incidence of *V. parahaemolyticus* O3:K6 gastroenteritis has been reported in many Asian countries, the United States (Matsumoto *et al*., 2000), Europe (Martinez- Urtaza *et al*., 2005), South America (Gonzalez- Escalona *et al*., 2005) and Africa (Ansaruzzaman *et al*., 2005).

The O3:K6 strains isolated before 1995 referred as old O3:K6 and those isolated since 1995 referred as new O3:K6. The difference in the sequence between the old and new O3:K6 strain ranged from 11 to 14-bp within the 1,364-bp region covering 95.4% of the *toxRS* coding region and the sequences of these two groups (old O3:K6 and new O3:K6 clone) differed invariably at 7 base position (Matsumoto *et al*., 2000).

The pandemic strains exhibit a unique sequence within the *toxRS* operon, which encodes transmembrane proteins in the regulation of virulence-associated genes conserved in the genus *Vibrio* and development of GS-PCR was a milestone in simplifying the identification of the O3:K6 isolate and also for detecting other serotypes that share identical molecular traits (Matsumoto *et al*., 2000). Bhuiyan *et al*. (2002) isolated sixty-six (66) strains of *V. parahaemolyticus* belonging to 14 serotypes from hospitalized patients in Dhaka, Bangladesh. Among these, 48 strains belonging to four serotypes had the pandemic genotype and possessed the *tdh* gene and 14 different serotypes were isolated with O3:K6 (42.4%) being the dominant serotype, followed by O4:K68 (19.7%), O1: KUT (9.1%) and O5: KUT (6.1%).

Okura *et al.* (2003) reported that neither toxRS/new nor orf8 is a reliable genetic marker for PCR-based identification of the pandemic strains; detection of tox*RS*/new is necessary but not always sufficient for the identification of the pandemic strains, while detection of orf-8 is sufficient but not always necessary for the identification of pandemic strains. However, they concluded that a strain possessing both tdh and tox*RS*/new can be considered a pandemic strain.

Okura *et al*. (2004) described that, PGS-PCR assay can be a useful molecular tool for identification of pandemic *V. parahaemolyticus* strains as well as for direct detection of this organism contaminating food and environmental samples by analysing 38 pandemic and 44 non pandemic group strains. In this study all 38 pandemic group strains yielded a 235 bp amplicon while all 44 non pandemic group of various serotype did not.

Cabanillas-Beltran *et al*. (2006) reported more than 1230 cases of gastroenteritis attributed to the consumption of raw or undercooked shrimp in the south of Sinaloa State, north-western Mexico during 2003 and 2004, which was the first outbreak of gastroenteritis caused by the pandemic strains of O3:K6 *V. parahaemolyticus* in Mexico.

Bhoopong *et al*. (2007) studied 629 isolates from 63 patients and reported that 87.4%, 0.5% and 7% possessed only *tdh* gene, only *trh* gene and both genes, respectively. Remaining 5.1% (32 isolates) lack both the genes and as whole, 70% of the *V. parahaemolyticus* isolates (440 of 629) were GS-PCR positive which belonged to serotypes O3:K6 (43.7%), O1:K25

(16.4%), O1: KUT (5.0%), O4:K68 (4.6%), and O4:K8 (0.3%). A total of 62% of the GS-PCR-positive isolates carried *orf-8*. Serichantalergs *et al.* (2007) reported the prevalence of pandemic isolates of serotype O3: K6 in expatriates 71% (36/51) and Thai adults 34% (15/44).

Han *et al*. (2008) studied the genome plasticity of *V. parahaemolyticus* and concluded that the depiction of an evolutionary history of the pandemic group (clone), strains of new-O3:K6 and its serovariants (post-1996 O4:K68, O1:K25, O1: KUT and O6:K18) constituted the pandemic group. New-O3:K6 (*trh*$^-$, *tdh*$^+$ and GS-PCR$^+$) was emerged from the old-O3:K6 clone (*trh*$^+$, *tdh*$^-$ and GS-PCR$^-$) by the stepwise acquisition of genomic islands. A small group of O3:K6 strains (*trh*$^-$, *tdh*$^-$ and GS-PCR$^+$) (named as the intermediate-O3:K6 clade) served as the phylogenetic intermediate between new-O3:K6 and old-O3:K6. They performed PCR to screen the presence of various DNA markers, including *toxRS*/new sequence (GS-PCR), ORF-8, Hu-α/insertion, PGS-PCR sequence, *V. parahaemolyticus*-specific sequences of VPM, *gyrB* , *toxR*, *tdh, trh, tl* genes and defined the pandemic group based on the previous genotypic definition (GS-PCR$^+$, *tdh*$^+$ and *trh*$^-$) (Okura *et al*., 2003; Wong *et al*., 2007) and concluded that none of *toxRS*/new sequence, orf-8 and Hu-α/insertion was reliable for PCR-based identification of the pandemic strains. However the DNA marker (namely the PGS-PCR sequence) was specific to the pandemic group. Ottaviani *et al*. (2010) analysed 559 samples of Italian mussels and recorded *V. parahaemolyticus* in 65 (11.6%) samples; however, none of the isolate was positive for *tdh* and *toxRS*; while, five (7.7%) were positive for *trh* and belonged to O1: KUT, O3: KUT, O1:K37 and OUT: KUT (both 'O' and 'K' untypable) serotype.

Serotyping has been widely used for identifying isolates in epidemiological studies. In fact, serotypes have been used extensively to distinguish between clinical isolates of *V. parahaemolyticus* (Jones *et al.,* 2012). Furthermore, some serotypes have been reported to be highly virulent and have been identified as the causative agents of foodborne illnesses (Nair *et al.,* 2007; Jones *et al*., 2012). For serotyping of *V. parahaemolyticus* isolates, traditional serological techniques that use antisera for antigen detection are commonly employed. However, traditional serotyping methods are time consuming and expensive. Therefore, for rapid and

accurate identification of a wide array of *V. parahaemolyticus* isolates, a combinatorial analytical approach involving PCR-based detection and serotyping is required.

Pazhani *et al*. (2014) examined a total of 178 strains of *V. parahaemolyticus* isolated from 13,607 acute diarrheal patients admitted in the Infectious Diseases Hospital, Kolkata between January 2001 and December in 2012 for serovar prevalence, antimicrobial susceptibility and genetic traits with reference to virulence and clonal lineages. The prevalence of pandemic strains was 68% as confirmed by group-specific PCR (GS-PCR) in contrast to non-pandemic strains i.e. 32%. Serovars such as O3:K6 (19.7%), O1:K25 (18.5%), O1: KUT (11.2%) were more commonly found and other serovars like O3: KUT (6.7%), O4:K8 (6.7%), and O2:K3 (4.5%) were newly detected. GS-PCR positive strains frequently associated with virulence gene *tdh*.

2.7 Environmental Investigation: Indian Context

Environmental studies in India have shown widespread distribution of the bacteria in coastal, estuarine and freshwater environments, especially during summer months (Nair *et al*., 1980; Sarkar *et al*., 1983; Sarkar *et al.,* 1985; Dileep *et al*., 2003; Deepanjali *et al*., 2005). As salinity strongly influences the distribution of this halophilic bacteria in open waters and in seafoods (Kaper *et al*., 1981; Charles-Hernandez *et al*., 2006) its occurrence in freshwater systems remains inexplicable. Some studies indicate that the survival of the bacteria in such alien environments is greatly aided by its association with a biological host, particularly fish (Sarkar *et al*., 1985). Survival of the organism in freshwater environments increases in organically rich water associated with planktons and fish (Kaper *et al*., 1981; Sarkar *et al*., 1985).

In a multicentric project conducted by the Indian Council of Medical Research (ICMR), it was found that the incidence of *V. parahaemolyticus* diarrhoea in coastal areas was <1%. However, in Kolkata this organism constitutes one of the major health hazards, with a detection rate ranging from 3.5 to 23.9% among the acute hospitalized diarrhoeal cases during different months (Pal *et al*., 1985).

Sarkar *et al*. (1985) studied the seasonal distribution of *V. parahaemolyticus* in freshwater environments in association with freshwater fishes in 1982 and 1983 and reported its occurrence in water and sediments was very infrequent which was restricted to the summer months and progressively declined with the onset of monsoons, remaining below detectable levels during the post monsoon and winter months. The incidence and counts of *V. parahaemolyticus* were consistently higher in association with plankton than with water and sediment samples. Most of the strains isolated were untypable and all isolates were Kanagawa negative and finally concluded that the survival of *V. parahaemolyticus* in freshwater ecosystems is transient and dependent on a biological host. Subsequently, Sarkar *et al*. (2003) investigated the incidence of *V. parahaemolyticus* in sewage water in Kolkata and reported 8 (26.7%) strains of this bacterium from 30 samples. Among these 5 (62.5%) were positive for *tdh* gene, out of which 2 strains belonged to O5:K3 serovar and remaining 3 to O5: KUT. However, no strain found positive for GS-PCR and *orf*-8.

Dileep *et al*. (2003) analysed 86 samples including molluscan shellfish, water and sediment by conventional method and *toxR* targeted PCR performed directly on enrichment broth lysate. In conventional method *V. parahaemolyticus* was identified in 28 samples; however, it was 53 by direct *tox*R PCR assay. One isolate found positive for *tdh* gene and three others for *trh*.

Deepanjali *et al*. (2005) studied the occurrence, seasonal abundance and density of pathogenic *Vibrio parahaemolyticus* in 49 oysters samples from the southwest coast of India by colony hybridization using non-radioactive labelled oligonucleotide probes of the corresponding genes (*tox*R/ *tlh* and *tdh*) and by PCR-assay for *toxR*, *tdh* and *trh*. They reported *V. parahaemolyticus* in 93.87% of the samples with the densities ranged from <10 to 10^4 organisms per g. For detection of pathogenic (*tdh*$^+$) isolates, colony hybridization was superior (10.2%) to *tdh* PCR (6.1%). Moreover, 59.3% samples were identified to be positive in *trh* PCR and one sample was positive for pandemic serotype O3:K6. Authors concluded that *trh* positive *V. parahaemolyticus* were common in oysters available in southern part of India.

Quintoil *et al*. (2007) investigated the occurrence and pathogenicity of *V. parahaemolyticus* in 1620 finfishes and shellfishes in West Bengal, India by various biochemical tests and Kanagawa reaction and reported *V. parahaemolyticus* in 38 (23.46%) samples and 5 of these were positive for Kanagawa reaction. Marine samples produced the highest isolation (28.81%) followed by brackish water (24.6%) and freshwater samples (14.3%). Khuntia *et al*. (2008) performed the characterization and pathogenicity study of *V. parahaemolyticus* isolated from diseased freshwater prawn (*Macrobrachium rosenbergii)* in Bhubaneswar, India.

Raghunath *et al.* (2008) examined 83 seafood samples from southwest coast of India by colony hybridization, conventional isolation technique and PCR. Out of 83 samples tested, *V. parahaemolyticus* were detected in 74 (89.2%) samples and *tdh*$^{+}$ *V. parahaemolyticus* in 5 (6.0%) samples by colony hybridization. The virulence genes i.e. *tdh* and *trh* were detected in 8.4% and 25.3% samples respectively, in the sample enrichment broths by PCR. Das *et al*. (2009) examined 293 samples of finfish and shellfish from wholesale fish markets in Kolkata, India and reported the isolation from shellfish (45.83%) and finfish (16.73%) with comparatively high occurrence in sea fishes (21.74%) as compared to estuarine (16.04%) and freshwater fishes (13.83%) and in demersal fish species (19.35%). Anjay *et al.* (2014) reported high prevalence (75.9%) of *V. parahaemolyticus* in marine fishes (Lote, Parse, Bhetki, Pabda, Pomfret) and shellfish (Tiger prawn) from retail shops in Kolkata, India by analysing 224 samples.

2.8 *Lactobacillus* as Probiotic against *Vibrio* spp.

There is an ever-increasing demand for aquaculture products and a similar increase in the search for alternatives to antibiotics. Use of probiotics in aquaculture is now increasingly being considered as an eco-friendly approach to mitigate health related problems (Irianto and Austin, 2002; Nayak, 2010). Disease preventing abilities of probiotics are achieved through enhancement of immunity and exclusion of pathogens (Barbosa *et al*., 2005; Balcazar *et al*., 2006). The field of probiotics intended for aquacultured live components is now attracting considerable attention and a number of commercial products are available, particularly directed at shrimp larval culture (Watson *et al.,* 2008).

Many types of microorganisms have been used as probiotics; however, the lactic acid bacteria (LAB) are predominating in probiotic preparations particularly the *Lactobacillus* species including *L. acidophilus, L. lactis, L. delbrucekii, L. salivarius, L. plantarum, L. paracasei, St. thermophilus, E. faecalis* and *Bifidobacterium* spp. (Fuller, 1989; Saavedra, 2001). In recent years, LAB have attained major attention for probiotic activity because they are generally regarded as safe (GRAS) (Tannock, 1998; Belicova *et al.,* 2013).

Nuphet *et al*. (2003) isolated *L. plantarum* (designated as PSU-LA71) from fermented pork and examined its antagonistic effect on ten pandemic strains of *V. parahaemolyticus* O3:K6 both in agar spot technique and co-culture technique. The agar spot method revealed inhibition zone between 11.1 and 15.0 mm and 52.1 to 92% inhibition of pandemic strains of *V. parahaemolyticus* was seen in co-culture method.

Ogunbanwo *et al.* (2003) isolated bacteriocin producing *L. plantarum* F1 and *L. brevis* OG1 from Nigerian fermented food products that had broad spectrum of inhibition against both pathogenic, food spoilage organisms and various lactic acid bacteria with inhibition zone between 6 mm to 12 mm in well diffusion method and concluded that the ability of bacteriocins produced by these isolates are of potential interest for food safety and may have future applications as food preservative.

Lash *et al.* (2005) demonstrated that the cell-free supernatant of *L. plantarum* (ATCC 8014) which is a 122 KDa protein has the growth inhibitory effect against a wide range of Gram-positive and Gram-negative bacteria including *Staphylococcus aureus*, *Escherichia coli*, *Listeria innocua* and *Pseudomonas aeruginosa*. The inhibitory compound lost activity when heated to temperatures greater than 30^0C, subjected to pH changes (< 4 or > 5) and various proteases enzymes.

Chowdhury *et al.* (2012) identified four *L. plantarum* isolates from homemade yoghurt samples which were resistant to NaCl (1- 9%) and bile-salt (0.05-0.3%) and showed good growth in the acidic condition being maximum growth at pH around 6.0. Further the isolates were examined for their antibacterial activity against nine different test pathogens. Growth of all pathogens were inhibited to some extent but maximum zone of inhibition was observed

against *Bacillus cereus* (53.20 mm) and minimum was against *Staphylococcus aureus* (19 mm) after 72 hour incubation. Out of these, 3 isolates showed inhibition zone of about 25 mm to 45 mm against *V. parahaemolyticus* ATCC 17802.

Sivakumar *et al.* (2012) evaluated the probiotic potential of *L. acidophilus* 04 (home made curd isolate) on pathogenic *Vibrio* in shrimp juveniles by feeding and immersion challenge method. They found that, *L. acidophilus* has antibacterial activity against *Vibrio parahaemolyticus, Vibrio cholerae, Vibrio harvey*i and *Vibrio alginolyticus* and thus can be used as a probiotic to control pathogenic *V. alginolyticus* in shrimp aquaculture. Ravishankar *et al*. (2012) isolated bacteriocin producing *L. plantarum* strain from raw cow milk that showed broad range of antibacterial activity against important food borne pathogens such as *S. aureus*, *E. fecalis*, *E. coli* and *L. monocytogenes* with corresponding inhibition zone of 21 mm, 17 mm, 18 mm and 11 mm respectively in well diffusion assay.

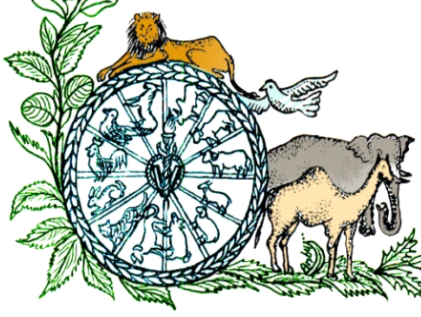
Iyapparaj *et al.* (2013) advocated the use of a candidate bacterial strain *Lactobacillus* sp. MSU3IR isolated from goat milk and having 16s RNA similarity with *L. casei* as a potential bacteriocin producing probiotic in shrimp culture systems to replace the usage of conventional chemotherapeutics. Yang *et al.* (2013) evaluated the probiotic effects of some *Lactobacillus* strains against *V. parahaemolyticus* TGqx01 (serotype O3:K6) by feeding and challenge method in mouse model. The results showed that severe intestinal fluid accumulation (FA) and villi damage in control mice which were pretreated with PBS in contrast to significant alleviation of FA in mice pretreated with a high dose of *Lactobacillus* strains ($P < 0.05$, $n = 6$) but not in mice that received low-dose pretreatments. They concluded that *Lactobacillus* strains can alleviate pathogenic *V. parahaemolyticus* induced intestinal FA and villi damage in the mouse model and suggested the use of some strains of *Lactobacillus* as non-chemotherapeutic agents for controlling *V. parahaemolyticus* causing gastroenteritis.

Francois *et al*. (2013) isolated and characterized the bacteriocin produced by *L. plantarum* 29V from raw cow milk in Cameroon with fairly broad bacteriocinogenic activity that inhibited the growth of several *Lactobacillus* spp., pathogenic and food spoilage microorganisms. The antimicrobial compound was sensitive to some proteolytic enzymes,

stable at high temperatures and in presence of organic solvents, detergents and surfactants, active in pH range 2-10 and NaCl range of 1-7%.

Use of *L. fructivorans* and *L. plantarum* significantly decreases larval and fry sea bream mortality. A mixture of *L. plantarum, L. salivarius* and *L. rhamnosus* applied to water reported to improve the survival of fish larvae. Use of *Lactobacillus* sp. enabled improved survival and growth of pearl oyster (*Pinctada mazatlanica* (Newaj fyzul *et al*., 2014).

Materials and Methods



3 MATERIALS AND METHODS

3.1 Materials

3.1.1 Design of study

The study was designed for isolation and identification of *Vibrio parahaemolyticus* from saline water origin shellfishes such as crab, shrimp and oyster by cultural isolation and biochemical characterization followed by determination of pathogenic isolates by molecular characterization and to evaluate the *in-vitro* growth inhibitory effect of *Lactobacillus plantarum* (MTCC No.1407), a probiont, on the pathogenic *V. parahaemolyticus* isolates.

3.1.2 Sample source

All the samples were collected from different retail fish markets and fishing harbors in and around Kolkata, West Bengal and Bhubaneswar, Odisha.

3.1.3 Experimental samples

A total of two hundred and twenty seven (227) saline water shellfishes were collected from different established fish markets and fishing harbours in and around Kolkata and Bhubaneswar. The details of shellfish species and number of samples used in the present study have been shown in Table 1.

Table 1: Sample from different types of shellfish

Sl. No.	Type of shellfish (species)	Area	Sample no.
1.	Crab (*Scylla serrata*)	In and around Kolkata	40
	(*Scylla tranqueberia*)	In and around Bhubaneswar	42
2.	Shrimp (*Penaeus monodon*)	In and around Kolkata	40
	(*Metapenaeus spp.*)	In and around Bhubaneswar	45
3.	Oyster (*Crassostrea gigantica*)	Paradeep and Chilika area, Odisha	60
		Total	**227**

3.1.4 Collection and transportation of samples

One piece of live crab and 4 to 5 small shrimps from one retail stall vendor in the market was collected and considered as single sample in respect of crab and shrimp and placed aseptically in sterile sample container and properly caped with identification label to avoid contamination and for right identity. The collected crab and shrimp samples were transported to the laboratory with ice packs at the earliest and processed immediately. In the same manner, live oysters were collected directly from the fishing harbor near bank of Chilika Lake and Paradeep sea coast situated in Odisha. Total number of samples collected from different area and sources were mentioned in the Table 1.

3.1.5 Reference bacterial strains

Reference *V. parahaemolyticus* strains (Vp-Kx-V_{138} and Vp-230) obtained by kind courtesy of Dr. T. Ramamurthy, Senior Deputy Director, National Institute of Cholera and Enteric diseases, Kolkata were used in this study. The culture Vp-Kx-V_{138} used as positive control for *toxR, tdh,* GS-PCR and PGS-PCR assay and likewise, VP-230 for *trh* gene in *trh* PCR assay.

Reference *Lactobacillus plantarum* strain (MTCC No. 1407) was obtained by kind courtesy of Dr. SVS Malik, Principal Scientist, Division of Veterinary Public Health, ICAR-Indian Veterinary Research Institute, Izatnagar, Bareilly.

3.1.6 Media, buffers and reagents

Bacteriological media used in the study were Thiosulfate Citrate Bile salt Sucrose (TCBS) agar, Muller Hinton Agar (MHA), Luria Bertani (LB) broth, Alkaline peptone water (APW), Nutrient Broth (NB) etc. The details of these media, buffers and reagents along with their composition and preparations (as per the instructions given by the manufacturers) that were used in the present study are given in the appendix I to III.

3.1.7 Chemicals used for molecular studies

All the chemicals used in the study were of molecular biology grade except the requirements for cultural isolation. Chemicals were procured from SIGMA (USA), BD (Germany), Qiagen (Germany), Bangalore Genei (India), SRL (India), Hi-media (India), Tiatn (India) and other reputed firms. PCR reagents used in this study such as *Taq* DNA polymerase, 10X PCR amplification buffer with magnesium chloride and dNTP mixture were procured from Thermo Fisher Scientific, USA. Other reagents like 6X gel loading dye, DNA 100 bp ladder plus ruler, 1.0 Kb ladder plus ruler were from (MBI Fermentas), 0.5 M ethylene diamine tetra acetic acid (0.5 M EDTA), ethidium bromide (SIGMA, USA), Tris base, Sea Kem LE Agarose from (Cambrex Bioscience, USA), Nuclease free water from (Ambion, India) and 0.22 µM syringe filter from (Millex® GV Millipore, Carrigtwohill, Co. Cork, Ireland).

3.1.8 Other routine chemicals

The other chemicals included in the study were Tris hydrogen chloride, glysate peptone, tryptone, Glucose, Methyl red, Conc. HCl, sodium thiosulphate, sodium acetate, potassium chloride, bromo cresol purple, yeast extract procured from (Titan, India), chloroform, acetic acid glacial (100%), sodium hydroxide pellets, hydrochloric acid, sulphuric acid, sucrose pure, lactose monohydrate pure, mannitol, bromocresol purple indicator, L-Arginine mono hydrochloride (MERCK, India), ammonium ferric citrate, sodium chloride (Hi-media, India) and dehydrated alcohol (BCPL, India).

3.1.9 Plasticwares and glasswares

Plasticwares used in this study were obtained from Tarsons (India), Nunc (Denmark), Greiner (Germany) and Axygen (USA). Glasswares were obtained from Borosil (India) and Schott Duran (Germany). Glasswares and plasticwares were thoroughly washed and sterilized before use following the recommended procedures.

3.1.10 Equipments

The important equipments used in the study were thermal cycler (Agilent Sure cycler 8800, Agilent Technologies, USA; Gene Amp PCR 9700, Applied Biosystems, USA), horizontal gel electrophoresis apparatus (Bangalore Genei, India), gel documentation system (Axygen, USA), deep freeze (-20ºC and -70^{0}C) (Blue Star, India), micro centrifuge (Biofuge Fresco, USA), circulatory water bath (Grant, UK), Spinwin micro centrifuge (Tarson, India), UV spectrophotometer (Scientronic, India), electronic balance (Afcoset, Germany), high speed refrigerated centrifuge (Sorvall RC, Dupont, USA), autoclave and hot air oven (Instrumentation India, India), Laminar air flow (SD Instruments & Equipments, Kolkata, India), incubator (REMI, India), pH meter (Corning, USA), wave reflector system (Kenstar, India) and water distillation apparatus (Millipore, Ireland).

3.1.11 Oligonucleotide primers

Oligonucleotide primers used in different PCR assays in the study were procured from Xcelris genomics, India. The oligonucleotide sequences of the primers are given in Table 2.

Table 2. Primers used in different PCR assays

Sl. No.	Primer Sequences (5 '- 3 ')	Target gene	Amplicon size	References
1.	F: GTC TTC TGA CGC AAT CGT TG R: ATA CGA GTG GTT GCT GTC ATG	*toxR*	368bp	Kim *et al.,* 1999
2.	F: CCA AAT ACA TTT TAC TTG G R: GGT ACT AAA TGG CTG ACA TC	*tdh*	199bp	Tada *et al.,* 1992
3.	F: GGC TCA AAA TGG TTA AGC G R: CAT TTC CGC TCT CAT ATG C	*trh*	250bp	Tada *et al.,* 1992
4.	F: TAA TGA GGT AGA AAC A R: ACG TAA CGG GCC TAC A	*toxRS* new/ GS PCR	651bp	Matsumoto *et al.,* 2000
5.	F: TTC GTT TCG CGC CAC AAC T R: TGC GGT GAT TAT TCG CGT CT	PGS-PCR	235bp	Okura *et al.,* 2004

F: Forward primer, R: Reverse primer

3.2 Methods

3.2.1 Enrichment of sample

Alkaline peptone water (1% peptone, 3% NaCl) [APW] was prepared in measured conical flask and the pH was adjusted to 8.5 by adding 1N NaOH. Then 30 ml volume of the prepared APW was dispensed in 50 ml capacity test tubes and autoclaved (121°C, 15psi, 15 minutes). The necessary broths, agar petri plates were prepared and stored properly before processing the samples.

Gastro-intestinal parts of crab and oyster and whole body of shrimp were considered as sample of respective sources. With the help of a pair of sterile forceps and scissors the gastrointestinal mass were eviscerated from crab and oysters and grinded aseptically before adding to APW for pre-enrichment. Similarly for shrimp, 4 to 5 pieces were grinded in sterile pestle and mortar and make ready to add in APW. About 10-15 g of each grinded sample was taken and inoculated in 50 ml capacity test tubes containing 30 ml of APW (pre-enrichment broth) and was incubated aerobically for 24 hour at 37ºC.

3.2.2 Detection of *V. parahaemolyticus* by conventional method

3.2.2.1 Isolation on selective media

Thiosulfate Citrate Bile salt Sucrose (TCBS) agar was used as selective media for isolation of *V. parahaemolyticus* from the collected shellfish samples. TCBS agar plates were prepared and stored in refrigerator as per the instruction of the manufacturer (BD, Difco, Germany). Before inoculation, the stored TCBS plates were removed from refrigerator and allowed to attain the ambient temperature. A loop full of APW grown overnight culture was streaked on the TCBS agar and plates were incubated at 37ºC for 24 hour. Characteristic colonies of the *V. parahaemolyticus* on TCBS agar i.e. typical, round, 2-3 mm in diameter, with green or blue centers were selected from each plates for further biochemical characterization.

3.2.2.2 Biochemical characterization of *V. parahaemolyticus* isolates

The characteristic colony of the *V. parahaemolyticus* on TCBS agar was confirmed by biochemical test with Kaper's multi test medium (Kaper *et al*., 1980). Five (5) typical colonies from each plate (each sample) were selected for biochemical characterization. Each colony was picked up separately with the help of sterile toothpick and they were distinctly inoculated on to the nutrient agar plates supplemented with 3.0 % NaCl. Thus each colony was labeled as a representative of colony selected from TCBS plate and incubated for 24 hour at 37ºC to have a master copy of original culture colony.

In the Kaper's multitest medium, the *V. parahaemolyticus* produce acidic (yellow) butt and alkali (purple) slant (K/A) reaction. The colony exhibited positive result in Kaper's multitest medium was considered as presumptive *V. parahaemolyticus* and were maintained in LB agar plate with 3% Nacl on sub-culture. Such presumptive isolates were subjected to species specific *Vp-toxR* PCR assay for confirmation of *V. parahaemolyticus* as described in section 3.2.2.5.

3.2.2.3 Genomic DNA extraction (template DNA) by snap chill method

1. About 1.5 ml of 24 h broth culture of APW inoculated with samples were taken in sterile 2 ml eppendorf tube and centrifuged at 10,000 rpm for 10 min to pellet the bacteria and supernatant was discarded.

2. One ml of normal saline solution (NSS) was then added to the bacterial pellet and tube was vortexed for complete mixing of the pellet with NSS.

3. The tube again centrifuged at 10,000 rpm for 10 min to obtain the pellet and supernatant was discarded.

4. Then 100 µl distilled water was added to the bacterial pellet and vortexed for mixing.

5. Then the tube was subjected to boiling for 10 min.

6. After boiling, the micro centrifuge tube was immediately transferred to 4ºC and kept for 5 min. Thereafter stored at -20ºC till preparation of reaction mixture for PCR assay.

7. Tube was taken out of freezer and kept in ice box at room temperature for thawing and subsequently, centrifuged at 8,000 rpm for 6 min before running PCR.

8. Supernatant was taken as genomic DNA template (bacterial lysate) and diluted with sterile nuclease free water as required for different PCR method.

3.2.2.4 Dilution of genomic DNA

Genomic DNA extracted by snap and chill method (section 3.2.2.3) was diluted as 1:10 with sterile deionized water for Vp-*toxR* PCR assay.

3.2.2.5 Identification of *V. parahaemolyticus* by species specific Vp-*toxR* PCR assay

A PCR assay was standardized for the detection of *V. parahaemolyticus* by identifying species specific *toxR* gene as per the method described by Kim *et al*. (1999) with some modification. The PCR was performed using the bacterial lysate as template DNA prepared directly from APW broth by snap chill method (section 3.2.2.3). Amplification reaction was performed in 25 µl PCR reaction mixture containing 2.5 µl 10X PCR amplification buffer (500 mM KCl, 100 mM Tris-HCl, pH-8.3; 15 mM $MgCl_2$), 0.5 µl of dNTP mix (containing 10 mM each of dATP, dTTP, dGTP and dCTP), 1 µl (10 pmol/ µl) each of forward and reverse primers of *toxR* gene, 0.2 µl (1 unit) of Taq DNA polymerase, 5.3 µl of bacterial lysate (1:10dilution) and 14.5 µl of nuclease free water. The

cycling condition for the PCR programme included initial denaturation at 95ºC for 5 min followed by 20 cycles consisting of denaturation (94ºC for 1 min), annealing (63ºC for 1.30 min) and elongation (72ºC for 1.30 min). Final extension was carried out at 72ºC for 7 min. The PCR products for amplicon size (368 bp) were identified comparing with Vp-Kx-V_{138} as positive control by submarine gel electrophoresis on 1.5% agarose gel and visualized under UV light after staining with ethidium bromide (0.5 µg/ ml). DNA ladder 100bp (Thermo Fischer Scientific, USA) was used as molecular weight (MW) marker.

3.2.3 Preservation of the isolates

V. parahaemolyticus isolates that found positive in both biochemical (Kaper's reaction) as well as in Vp-*toxR* PCR assay (*toxR* gene, single colony PCR) were cultured in nutrient agar stab containing 3% NaCl with overnight incubation at 37ºC. Next day nutrient agar stab was capped with sterile rubber cork and finally sealed with hot paraffin to store at room temperature.

3.2.4 Re-use of the preserved isolates

Preserved isolates in nutrient agar stabs were revived on the LB agar plates containing 3% of NaCl with 24 hour incubation at 37ºC. The DNA templates (lysate) were prepared from these cultures by snap chill method and diluted as 1:10 (3.2.2.3) for screening of *tdh*, *trh* and PGS-PCR assay in order to do the molecular characterization. The DNA templates were diluted as 1:50 and used in GS-PCR assay.

3.2.5 Molecular characterization of virulent genes

3.2.5.1 Detection of thermostable direct haemolysin (*tdh*) gene by PCR

The PCR method for detection of haemolysin producing gene (*tdh*) was standardized and used in this study employing the method as described by Tada *et al.* (1992) with minor modification. The PCR reaction mixture was prepared by taking 2.5 µl of 10X PCR amplification buffer (500 mM KCl, 100 mM Tris-HCl, pH-8.3; 15 mM $MgCl_2$), 0.5 µl dNTP mix (10 mM each of dATP, dTTP, dGTP and dCTP), 1µl (10 pmol/µl) each of forward and reverse primers (Table 2), 0.2 µl (1 unit) of Taq DNA polymerase, 5.3 µl of bacterial lysate (1:10 dilution)

prepared by boiling and snap chilling method and sterile deionized water to make the final volume upto 25 µl. The reaction mixture was cycled in thermal cycler at 94ºC for 5 min for initial denaturation, then 30 cycles of denaturation at 94ºC for 1.30 min, annealing at 50ºC for 1.30 min and elongation at 72ºC for 1.30 min followed by final extension for 7 min at 72ºC. At the end of reaction the amplified product (199bp) was electrophoresed on 1.5% agarose gel, visualized under UV light after staining with ethidium bromide (0.5 µg/ml) using 100bp DNA ladder [molecular weight (MW) marker] and result was recorded.

3.2.5.2 Detection of *tdh* related haemolysin (*trh*) gene by PCR

PCR method for *trh* gene assay as adapted by Tada *et al.* (1992), was standardized in laboratory and isolates were screened for the presence of *trh* gene. The reaction mixture consisted 2.5 µl of 10X PCR amplification buffer (500 mM KCl, 100 mM Tris-HCl, pH-8.3; 15 mM $MgCl_2$), 0.5 µl of dNTP mix (10 mM each of dATP, dTTP, dGTP and dCTP), 1 µl (10 pmol/µl) each of forward and reverse primers, 0.2 µl (1 unit) of Taq DNA polymerase, 5.3 µl of bacterial lysate (1:10 dilution) prepared by boiling and snap chilling method and sterile deionized water to make final volume 25 µl. Cycling condition involved initial denaturation at 95ºC for 5 min followed by 30 cycles with denaturation at 94ºC for 1 min, annealing at 55ºC for 1.30 min, extension at 72ºC for 1.30 min and final extension step at 72ºC for 7 min. Amplified products (250bp) were electrophoresed through 1.5% (w/v) agarose gel stained with ethidium bromide (0.5 µg/ml) and viewed under UV light.

3.2.6 Molecular characterization of pandemic gene

3.2.6.1 Group specific PCR (GS-PCR)

The PCR-assay for determining pandemic character of *V. parahaemolyticus* isolates i.e. group specific gene (*toxRS*-new region) was performed by adopting the method described by Matsumoto *et al.* (2000) with certain modifications. The reaction mixture was optimized to contain 2.5 µl of 10X PCR buffer (500 mM KCl, 100 mM Tris-HCl, pH-8.3; 15 mM $MgCl_2$), 0.5 µl of dNTP mix (containing 10 mM each of dATP, dTTP, dGTP and dCTP), 1µl (10 pmol/µl) each of forward and reverse primers, 0.2 µl (1 unit) of Taq DNA polymerase, 5.3 µl of bacterial lysate (1:50 dilution) prepared by boiling and snap chilling method and sterile deionized

water to make final volume upto 25 µl. The reaction was performed in a thermal cycler with preheated lid (lid temp 105ºC). The cycling condition comprised was an initial denaturation at 96ºC for 5 min, followed by 25 cycles each of denaturation at 96ºC for 1 min, annealing at 45ºC for 2 min, elongation at 72ºC for 3 min and final extension step at 72ºC for 7 min. On completion of the reaction the amplified products (651bp) were held briefly at 4ºC and then analyzed by agarose (1.2%) gel electrophoresis using 100 bp DNA ladder as molecular weight (MW) marker to detect the desired amplicon size.

3.2.6.2 Pandemic group specific (PGS) PCR

Another gene recognized for pandemic character i.e. pandemic group specific gene was detected by employing the method followed by Okura et *al.* (2004) with certain modifications. The reaction mixture was prepared as done in GS-PCR using the concerned primers with exception of bacterial lysate used as 1:10 dilution in this study. The reaction was performed in a thermal cycler with preheated lid (lid temp 105ºC). The cycling condition comprised an initial denaturation at 94ºC for 5 min, followed by 25 cycles each of denaturation at 94ºC for 1 min, annealing at 59ºC for 1 min, elongation at 72ºC for 1 min and final extension step at 72ºC for 7 min. On completion of the reaction the amplified products (235bp) were held briefly at 4ºC and then analyzed by agarose (1.5%) gel electrophoresis.

3.3 Antagonistic Activity of *Lactobacillus plantarum* on Pathogenic *V. parahaemolyticus*

The *in-vitro* antagonistic activity of *Lactobacillus plantarum* on pathogenic *V. parahaemolyticus* (*tdh*$^+$) isolates was initially determined using both disc diffusion method and agar well diffusion assay following the method described by (Schillinger and Lucke, 1989; Ogunbanwo *et al*., 2003; Lash *et al*., 2005) with minor modifications. However, there was lack of consistency and distinction in the zone of inhibition in the disc diffusion method in comparison to the agar well diffusion assay where the zone of inhibition was well defined and consistent in standardizing the assay. Considering this, agar well diffusion method was used for screening the antagonistic activity as described below.

3.3.1 Preparation of broth and media

The media and broth used for agar well diffusion assay (AWDA**)** were MHA, de-Man, Rogosa and sharpe (MRS) broth, MRS agar, 1% agar, LB broth with 3% NaCl, 1N NaOH etc. These were prepared following the instructions by the manufacturers.

3.3.2 Selection of *V. parahaemolyticus* isolates

All the twenty six (26) *tdh*$^+$ *V. parahaemolyticus* isolates along with three (3) clinical isolates *viz.* Vp O3:K6, Vp O4:K8 and Vp MS-79 (obtained by the courtesy of Dr. T. Ramamurthy, Senior Deputy Director, NICED, Kolkata) were selected for studying the antagonistic effect of *L. plantarum* in agar well diffusion assay.

3.3.3 Revival of *V. parahaemolyticus* isolates

The preserved *V. parahaemolyticus* isolates in nutrient agar stab were inoculated to Luria-Bertani (LB) Broth with 3% NaCl and incubated at 37^{o}C for 24 hour for revival and use.

3.3.4 Revival of *Lactobacillus plantarum* strain (MTCC No. 1407)

Single colony from the supplied *L. plantarum* pure culture was picked up from the deMan Rogosa and Sharpe (MRS) agar plate with sterile loop and inoculated to 5 ml freshly prepared MRS broth (Himedia, India) in 20 ml capacity sterile test tubes. Then the test tubes were incubated in a desiccating jar with candle lit (to make partial anaerobic) at 37^{0}C for 48 hour. A loop full of 48 hour MRS broth culture was streaked on MRS agar and incubated anaerobically (as above) at 37^{o}C for 48 hour for morphological and biochemical identification. Confirmation of the strain was done based on growth characteristics on MRS (HiMedia, India) agar, morphology (Gram staining) and major biochemical test results *viz.* Indole test, Methyl Red (MR), Voges Proskauer (VP), Oxidase test and Catalase test. The results suggestive of the organism as *L. plantarum* are mentioned in the following table (Holt, 1984).

Growth on MRS agar	Round creamy white colony
Gram staining	Gm positive, non-spore forming rod
Catalase and oxidase test	Negative
Indole, MR, VP and citrate (IMViC)	All negative

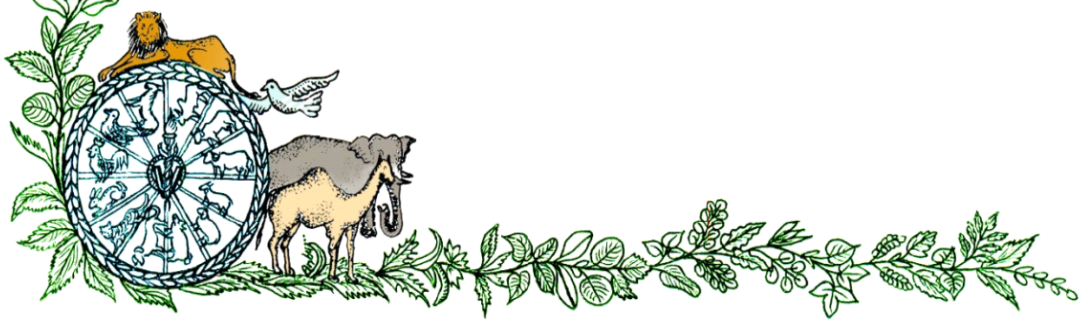
3.3.5 Preparation of cell free supernatant (CFS) of *L. plantarum*

Two (2) ml of 48 hour MRS broth culture of *L. plantarum* was taken in 2 ml eppendorf tubes and centrifuged at 10000 rpm for 10 minutes at 4°C. The pH of supernatant was adjusted to 6 - 6.5 with 1 N NaOH and known as crude supernatant. The crude supernatant was filter sterilized by filtering through 0.22 µM syringe filter (Millipore, Ireland) to another sterile eppendorf tubes and were stored at 4°C for further use.

3.3.6 Antagonism assay of *L. plantarum* by agar well diffusion method

The agar well diffusion assay was carried out following the procedure described by Schillinger and Lucke, (1989) with certain modifications. Briefly, 100 µl 24 hour LB broth culture of *V. parahaemolyticus* was first swabbed on Muller Hinton Agar plates with sterile cotton swab and plates were left for some time at room temperature for air drying. Wells (8 mm diameter) were made on the MHA plates by using sterile cork borer (8mm diameter). Five equidistant wells were made on one plate and numbered as 1 to 5 as shown in Fig. 12. The base of the wells were sealed with sterile 1% molten agar and allowed to solidify. The wells were then filled with previously prepared filtered cell free supernatant of *L. plantarum* using different volumes *viz.* 100 µl in well No.1 & 2, 120 µl in well No. 3 & 4, 140 µl in well No. 5 to know the variations in antagonistic effect with different volume of CFS. A well filled with sterile distilled water served as negative control. Plates were then incubated aerobically at 37°C in topside up position. The diameter of clear inhibitory zones around each well were measured by using Hi-Antibiotic Zone Scale (Himedia, India) at 12 h, 24 h and 36 h of post incubation and results were recorded (Table 7).

Results



4 RESULTS

The results of the present study for isolation and molecular characterization of pathogenic *V. parahaemolyticus* from saline water origin crustacean and molluscs and antagonistic effect of the probiont, *Lactobacillus plantarum* on the pathogenic *V. parahaemolyticus* isolates are presented in this section.

4.1 Detection of *V. parahaemolyticus* by Conventional Method

A total of 227 saline water origin shellfish samples [crab (n=82), shrimp (n=85), oyster (n=60)] were first pre-enriched in Alkaline Peptone Water (APW) with 24 hour aerobic incubation at 37^{0}C followed by streaking of loop full of cultured broth on the selective Thiosulfate Citrate Bile salt Sucrose (TCBS) agar. Of the 227 shellfish samples, 194 (85.46%) produced typical characteristic (round, 2-3 mm in diameter with green or blue center) colonies on TCBS agar after 24 hour incubation at 37^o C (Table 3; Fig. 1). Such typical colonies on TCBS agar were recovered in samples of crab 71 (86.58%) followed by shrimp 70 (82.35 %) and oyster 53 (88.33%) (Table 3).

4.1.1 Biochemical characterization

For each sample, 5 characteristic colonies on TCBS agar were selected for biochemical (acid/ alkali) reaction in Kaper's multitest medium and only one K/A positive colony (Kaper's reaction positive) from each sample was considered as representative isolate of that sample. Accordingly, out of 194 shellfish isolates, 159 (81.95%) produced positive K/A reaction (Fig. 2) and belonged to crab 46 (56.09%), shrimp 66 (77.64 %) and oyster 47 (78.33%) (Table 3).

Out of the total 227 samples, 165 yielded the characteristic colony of *V. parahaemolyticus* on TCBS agar. Except 6 samples, all other 159 samples showed positive reaction in Kaper's multitest medium with the 5 colonies randomly selected from each sample.

4.1.2 Confirmation of *V. parahaemolyticus* by species specific Vp-*toxR* PCR assay

All the Kaper's reaction positive isolates (n=159) were tested for the presence of species specific *toxR* gene by PCR assay. All the K/A^{+} isolates were found positive and generated specific amplicon (368 bp) in Vp-*toxR* PCR assay as compared with the positive control strain Vp-Kx-V_{138} (Fig. 3). Accordingly, out of 227 shellfish samples, *V. parahaemolyticus* was detected in 159 samples that belonged to crab 46 (56.09%), shrimp 66 (77.64 %) and oyster 47 (78.33%) (Table 3; Fig. 8).

Table 3: Occurrence of *Vibrio parahaemolyticus* in different shellfishes of saline water origin.

Type of Shell fish	Sample (No.)	Isolation on TCBS agar	Kaper's reaction	Species specific gene	Virulence genes		Pandemic marker (gene)	
				toxR	*tdh*	*trh*	*toxRS*/new PGS-PCR (GS-PCR)	
Crab	82	71 (86.58)	46 (56.09)	46 (56.09)	10 (12.2)	0	2 (2.4)	6 (7.31)
Shrimp	85	70 (82.35)	66 (77.64)	66 (77.64)	10 (11.76)	1 (1.17)	0	5 (5.88)
Oyster	60	53 (88.33)	47 (78.33)	47 (78.33)	6 (10)	0	0	3 (5)
Total	227	194 (85.46)	159 (70.04)	159 (70.04)	26 (11.45)	1 (0.44)	2 (0.88)	14 (6.16)

***** The value in parenthesis indicates the percentage and calculated on the basis of total number of samples in each category of shellfish.

4.2 Molecular Characterization of Virulence Determinants

All the *V. parahaemolyticus* isolates that yielded the positive amplicon in Vp-*toxR* PCR assay were further screened for virulence genes i.e. *tdh* gene and *trh* gene that are

responsible for production of thermostable direct haemolysin (TDH) and TDH related haemolysin (TRH) associated in mediating gastroenteritis and diarrhoea.

4.2.1 PCR assay for detection of thermostable direct haemolysin (*tdh*) gene

Out of 159 Vp-*toxR* positive *V. parahaemolyticus* isolates, 26 (16.35%) were found positive for *tdh* gene and were recovered from crabs 10 (21.73%) followed by shrimps 10 (15.15%) and oysters 6 (12.76%) (Table 4; Fig. 10).

Overall the occurrence of *tdh*$^+$ *V. parahaemolyticus* in shellfish samples was 11.45% (26/227) and detected highest in crab 10 (12.2%) and shrimp 10 (11.76%) followed by oyster 6 (10%) (Table 3; Fig. 9). All the *tdh*$^+$ isolates of *V. parahaemolyticus* gave a specific 199 bp amplicon in the *tdh* PCR assay that was comparable with the amplicon of the reference strain Vp-Kx-V_{138} (Fig. 4).

4.2.2 PCR for TDH-related haemolysin (*trh*) gene

Like *tdh* PCR, all the 159 identified isolates were tested for *trh* gene by *trh*-PCR assay and only 1 (0.62%) was found positive (Table 4). Overall the occurrence of *trh*$^+$ isolate was 0.44% (1/227) and recorded in shrimp (1.17%) only (Table 3; Fig. 9). The *trh*$^+$ isolate gave a specific 250 bp amplicon in *trh* PCR that was comparable with the amplicon of the reference strain Vp-230 (Fig. 5).

4.3 Molecular Characterization for Pandemic genes

All 27 isolates (26 *tdh*$^+$ and 1 *trh*$^+$) of *V. parahaemolyticus* from shellfish samples were selected and screened for two common pandemic clone markers by adopting GS-PCR and PGS-PCR assays.

4.3.1 Group specific PCR (GS-PCR)

Of the 27 [*tdh*$^+$ (n=26); *trh*$^+$ (n=1)] pathogenic isolates, 2 (7.69%) isolates gave the positive amplicon (651bp) corresponding to *toxRS*-new region gene in GS-PCR assay and belonged to crabs (20%) (Table 5; Fig. 11). The amplicon produced by the isolates were compared with the amplicon produced by the reference strain VP-Kx-V_{138} (Fig. 6). The *trh* positive only isolate (1) did not give amplicon in this assay. Out of total 159 Vp-*toxR* PCR positive isolates, 2 (1.25%) were found to be GS-PCR positive (Table 4). These 2 isolates

were recovered from crab (4.34%) samples only whereas none of the shrimp and oyster samples revealed GS-PCR positive isolates (Table 4; Fig. 10). However, overall occurrence of GS-PCR positive isolates among 227 saline water origin shellfish samples was 0.88% (2/227) i.e. in crab 2 (2.4%) (Table 3; Fig. 9).

4.3.2 Pandemic group specific PCR (PGS-PCR)

Like GS-PCR, all the *tdh*$^+$ (n=26) and *trh*$^+$ (n=1) isolates were subjected to PGS-PCR and 14 (53.8%) isolates were found to produce the corresponding amplicon (235bp). Such PGS-PCR positive isolates were from crab 6 (60%) followed by shrimp 5 (50%) and oyster 3 (50%) (Table 5; Fig. 11). The *trh* positive only isolate (1) did not give amplicon in this assay. Considering the total number of Vp-*toxR* PCR positive isolates (159), positivity reflected as 8.8% (14/159) and belonged to crab 6 (13.04%), shrimp 5 (7.57%) and oyster 3 (6.38%) (Table 4; Fig. 10). Overall occurrence of PGS-PCR positive isolates among 227 shellfish samples was 6.16% (14/227) i.e. in crab 6 (7.31%), shrimp 5 (5.88%) and oyster 3(5%) (Table 3; Fig. 9). All PGS-PCR positive isolates produced 235bp amplicon in PCR assay which was comparable to reference strain Vp-Kx-V_{138} (Fig. 7).

Table 4: Distribution of virulence and pandemic genes in *Vibrio parahaemolyticus* isolates.

Type of Shellfish	Vp-*toxR* positive isolates	Virulence genes		Pandemic clone marker	
		tdh	*trh*	*toxRS*/new (GS-PCR)	PGS-PCR
Crab	46	10 (21.73)	0	2 (4.34)	6 (13.04)
Shrimp	66	10 (15.15)	1 (1.5)	0 0	5 (7.57)
Oyster	47	6 (12.76)	0	0	3 (6.38)
Total	159	26 (16.35)	1 (0.62)	2 (1.25)	14 (8.8)

***** The value in parenthesis indicates the percentage and calculated on the basis of number of Vp-*toxR* positive isolates in each category of shellfish.

Table 5: Distribution of pandemic genes in virulent (*tdh*$^+$) *Vibrio parahaemolyticus* isolates.

Type of Shellfish	Virulent isolates (*tdh*$^+$)	Pandemic clone marker	
		toxRS/new (GS-PCR)	PGS-PCR
Crab	10	2 (20)	6 (60)
Shrimp	10	0	5 (50)
Oyster	6	0	3 (50)
Total	26	2 (7.69)	14 (53.8)

***** The value in parenthesis indicates the percentage and calculated based on number of virulent (*tdh*$^+$) *Vibrio parahaemolyticus* isolates in each category of shellfish.

4.4 Area wise Occurrence of *V. parahaemolyticus* in Shellfishes

The data for occurrence of this pathogen were extrapolated considering the area for collection of samples. The findings showed that 10% of crab sample from Kolkata, West Bengal and 14.28% from Bhubaneswar, Odisha were harboring the pathogenic strains of this organism. Similarly for the shrimp samples, 17.5% from Kolkata, West Bengal and 6.66% from Bhubaneswar, Odisha were carrying the pathogenic *V. parahaemolyticus* (Table 6). The findings indicated that in comparison to Odisha, the shrimp from West Bengal are more potential source of this pathogen but the frequency of occurrence of this pathogen in crabs from both the areas were almost same. The only *trh*$^+$(2.5%) *V. parahaemolyticus* isolate was detected in shrimp sample of Kolkata, West Bengal. The results also suggested that 5% of crab samples from West Bengal are GS-PCR positive. The PGS-PCR assay revealed 10% positivity in shrimp from Kolkata, West Bengal; however, 14.28% of crab and 2.2% of shrimp collected from Bhubaneswar, Odisha were positive in this assay (Table 6). As oysters were collected only from Odisha, the value represented in Table 3 is the data for Odisha state only.

Table 6: Area wise occurrence of *V. parahaemolyticus* in crab and shrimp.

Area	Type of Shell fish	No. of sample	Isolation on TCBS	Species specific gene (*toxR)*	Virulence genes		Pandemic marker (gene)	
					tdh	*trh*	GS-PCR	PGS-PCR
West Bengal (Kolkata)	Crab	40	34 (85)	22 (55)	4 (10)	0	2 (5)	0
	Shrimp	40	31 (77.5)	28 (70)	7 (17.5)	1 (2.5)	0	4 (10)
Odisha (Bhubaneswar)	Crab	42	37 (88.09)	24 (57.14)	6 (14.28)	0	0	6 (14.28)
	Shrimp	45	39 (86.66)	38 (84.44)	3 (6.66)	0	0	1 (2.2)

*Value in the parenthesis denotes percentage and calculated on the basis of on total number of samples examined in each category of shellfishes.

4.5 Antagonistic Activity of *L. plantarum* on Pathogenic *V. parahaemolyticus* Isolates

Antibacterial activity *in-vitro* assays are often used as an important indicator to evaluate the potential of probiotic candidates (Spanggaard *et al*., 2001; Jensen *et al*., 2012). Antagonistic activity of *L. plantarum* (MTCC No. 1407) was studied against all 26 pathogenic *V. parahaemolyticus* isolates (*tdh*$^+$) including the characterized isolates Vp-O_3:K_6, Vp-O_4:K_8 and Vp-MS79 adopting the agar well diffusion (AWD) assay as described by Schillenger and Lucke, (1989) with minor modifications. In the AWD assay, growth of all the *V. parahaemolyticus* isolates were inhibited by the cell free supernatant (CFS) of *L. plantarum* with a filtrate volume ranging from 100 µl to 140 µl (Table 7). The diameters of inhibition zone ranges between 11 and 24 mm and the test volume 140 µl of CFS exhibited highest zone of inhibition against Vp-Oys23 isolate (Fig. 12). Further, the CFS also exhibited same degree of antagonistic activity against the pandemic strain of *V. parahaemolyticus* i.e. Vp-O3:K6. The diameter of the inhibition zone was initially recorded after 12 hour of incubation at 37^{0}C and thereafter 24 h, 36 h and 72 h; however, the magnitude of inhibition in 24 h, 36 h and 72 h of incubation remain same as with 12 hour. The data for all the isolates of *V. parahaemolyticus* in AWD assay have been shown in Table 7 and Fig 13.

Table 7: Growth inhibitory effect of *L. plantatum* on pathogenic *V. parahaemolyticus*

Sl No.	Pathogenic *V. parahaemolyticus* isolates	Diameter of inhibition zone (mm) Volume of *L. plantarum* CFS		
		100µl	120µl	140µl
1	O3:K6 *	14	15	18
2	O4:K8 *	11	12	14
3	MS 79 *#	18	19	20
4	CRB 1 #	18	19	21
5	CRB 2	17	18	20
6	CRB 4 #	18	19	21
7	CRB 7 #	14.5	16	19
8	CRB 25 #	12	14	16
9	CRB 40 #	13	15	18
10	CRB 41 #	13	18	20
11	CRB 57*	15.5	16.5	18.5
12	CRB 58*	13	15	17
13	CRB 71	12	14	17.5
14	SHM 4 #	13	15	16
15	SHM 7	13	14.75	16
16	SHM 8	13	13.75	15.5
17	SHM 27 #	13	14.5	18.5
18	SHM 34	13	14	16
19	SHM 38 #	14	16	17
20	SHM 39 #	12	13	15
21	SHMO 9	13.5	14.5	16
22	SHMO 11	13.75	14	15.5
23	SHMO 40 #	13	14	16
24	OYS 6	13	13.5	16
25	OYS 10	14	15	18
26	OYS 23 #	20	21	24
27	OYS 35 #	16	17	19
28	OYS 43 #	14	15	16
29	OYS 60	13	14	15.5

*** GS PCR Positive isolates** **#PGS-PCR positive isolates**
CRB-Crab, SHM and SHMO-Shrimp, OYS-Oyster

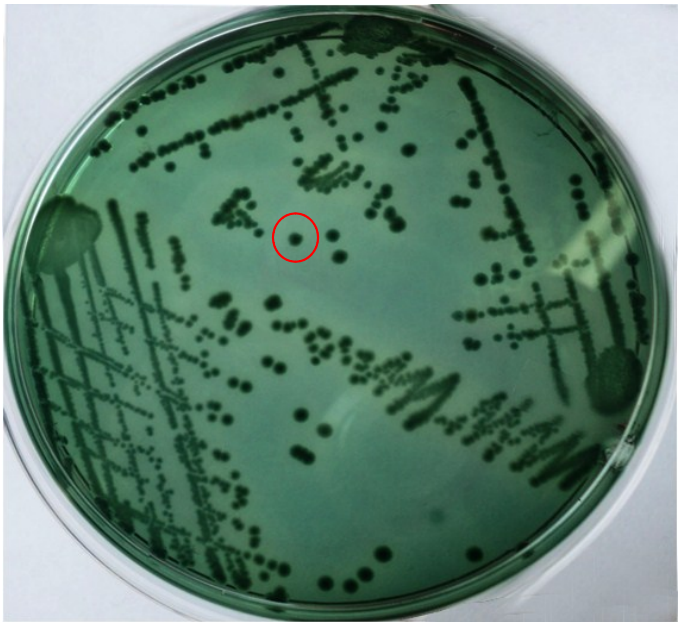


Fig. 1: Typical round (2-3 mm in diameter) green colonies of *Vibrio parahaemolyticus* on TCBS agar

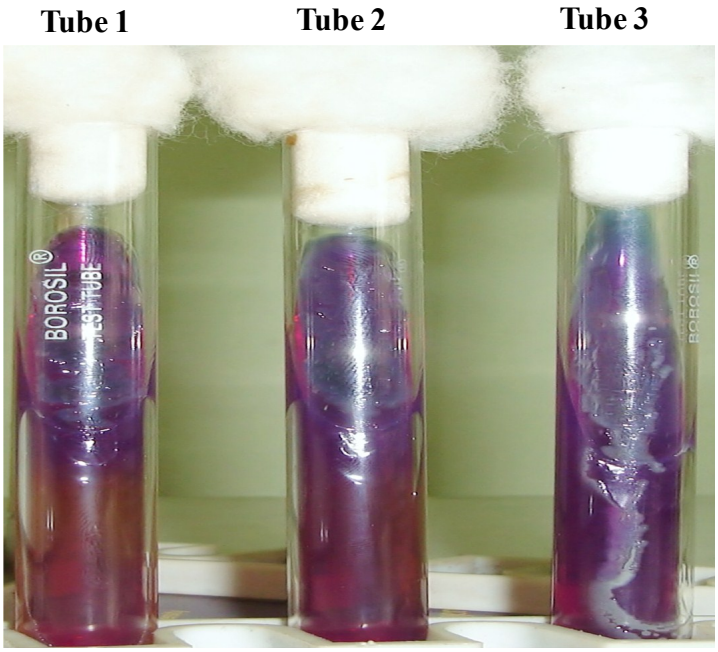


Fig. 2: Biochemical reaction of *Vibrio parahaemolyticus* in Kaper's medium
Tube 1-2 : Yellow acidic butt and purple colour alkali slant (K/A^+) reaction
Tube 3 : Negative Kaper's reaction

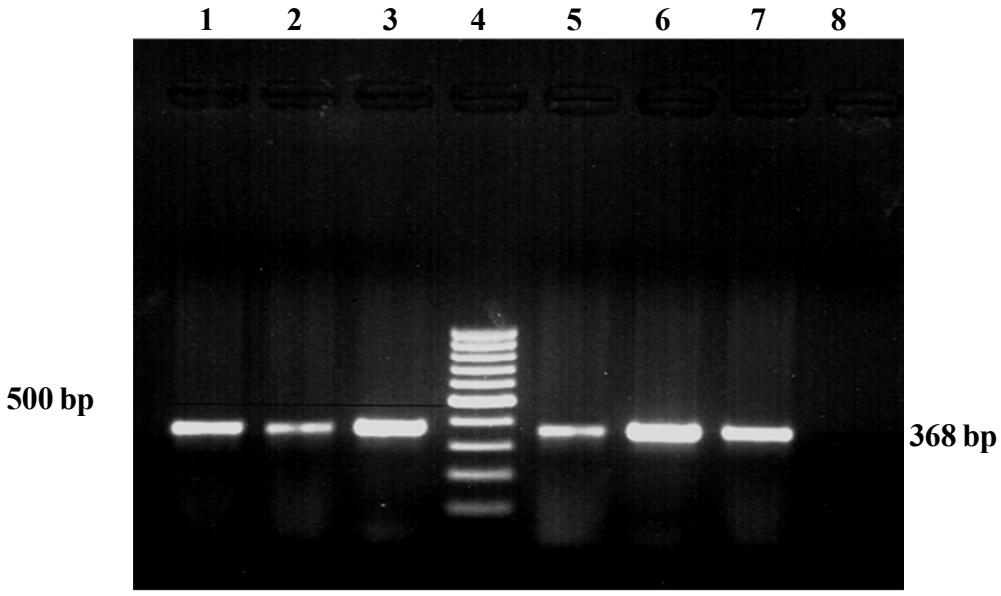


Fig. 3: PCR amplification of *toxR* gene of *Vibrio parahaemolyticus*
Lane 1, 2, 3, 5 & 7 : Sample isolates with (+) ve amplicon (368bp)
Lane 4 : DNA ladder of MW 100bp (Thermoscientific)
Lane 6 : Positive control of *V. parahaemolyticus* (Vp-Kx-V_{138})
Lane 8 : Negative control

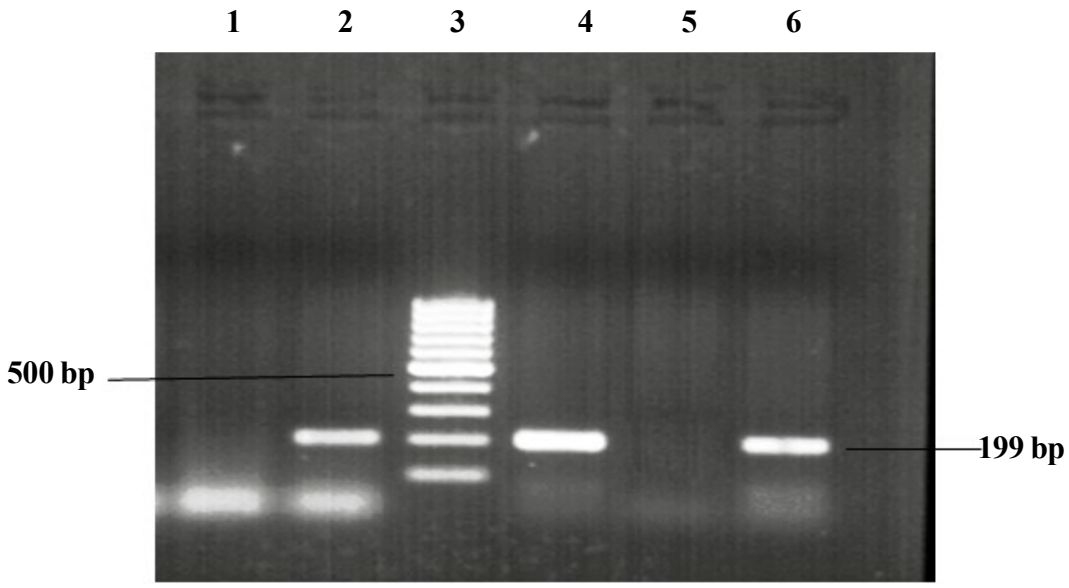


Fig. 4: PCR amplification of *tdh* gene of *Vibrio parahaemolyticus*
Lane 1: Negative control
Lane 2, 5, 6: Sample isolates with (+) ve amplicon (199bp)
Lane 3 : DNA ladder of MW 100 bp (Thermoscientific)
Lane 4: Positive control of *V. parahaemolyticus* (Vp-Kx-V_{138})

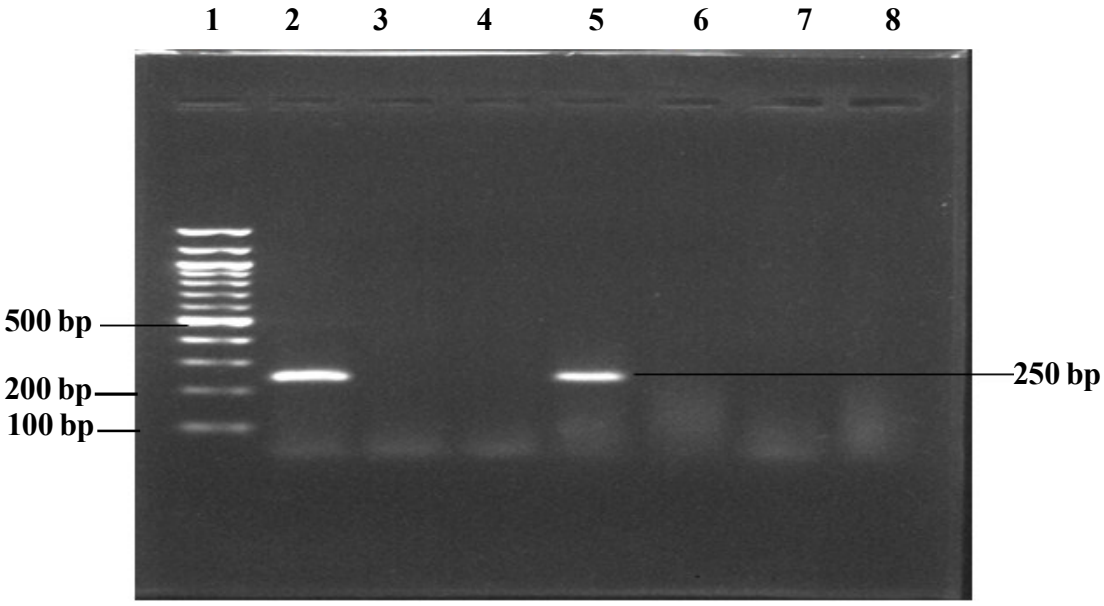


Fig. 5: PCR amplification of *trh* gene of *Vibrio parahaemolyticus*
Lane 1 : DNA ladder of MW 100 bp (Thermoscientific)
Lane 2 : Positive control of *V. parahaemolyticus* (Vp-230)
Lane 5 : Sample isolates with *trh* positive amplicon (250bp)
Lane 3, 4, 6 & 7 : Sample isolates with no amplicon
Lane 8: Negative control

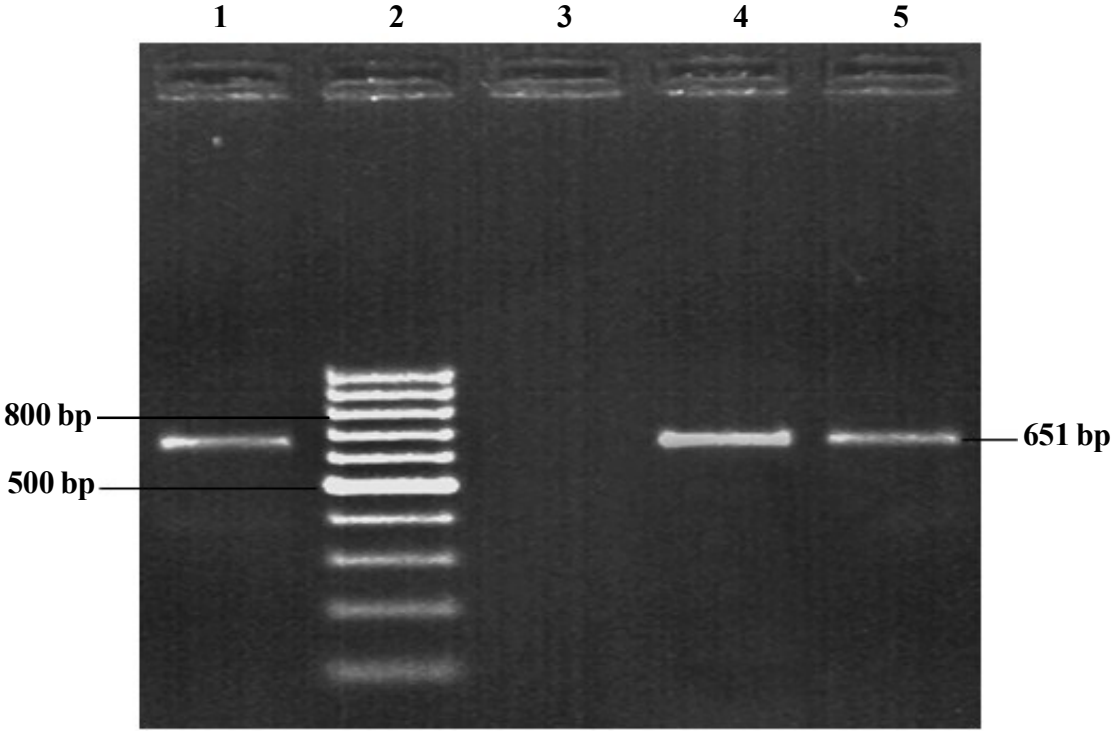


Fig. 6: PCR amplification of *toxRS* / new region (GS-PCR)
Lane 1 and 5: Sample isolates with positive amplicon (651 bp)
Lane 2: DNA ladder of MW 100 bp (Thermoscientific)
Lane 3: Negative control
Lane 4: Positive control of *V. parahaemolyticus* (Vp-Kx-V_{138})

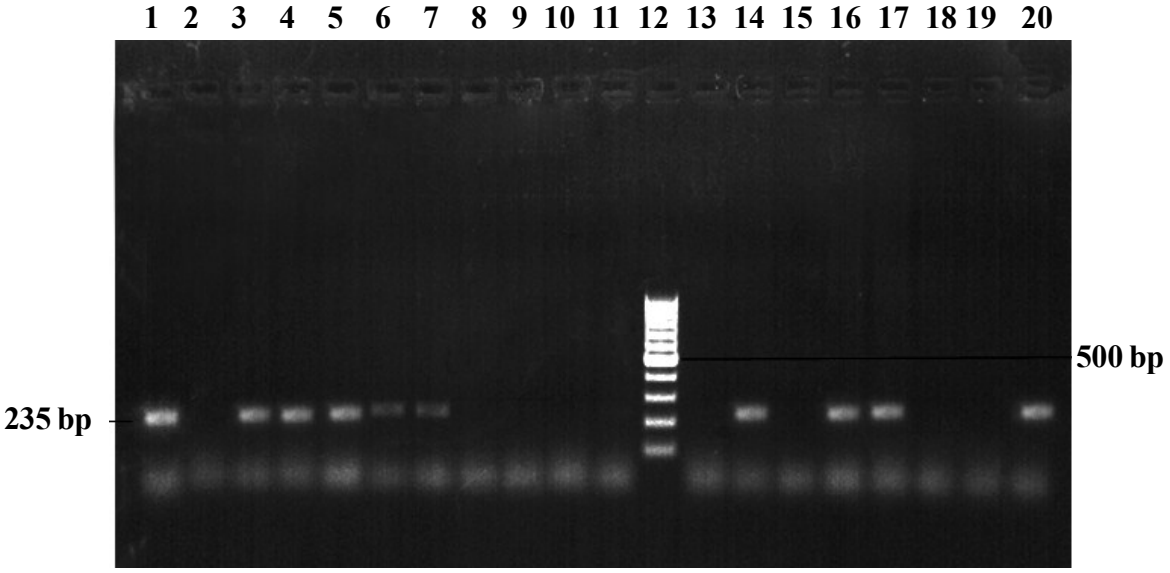
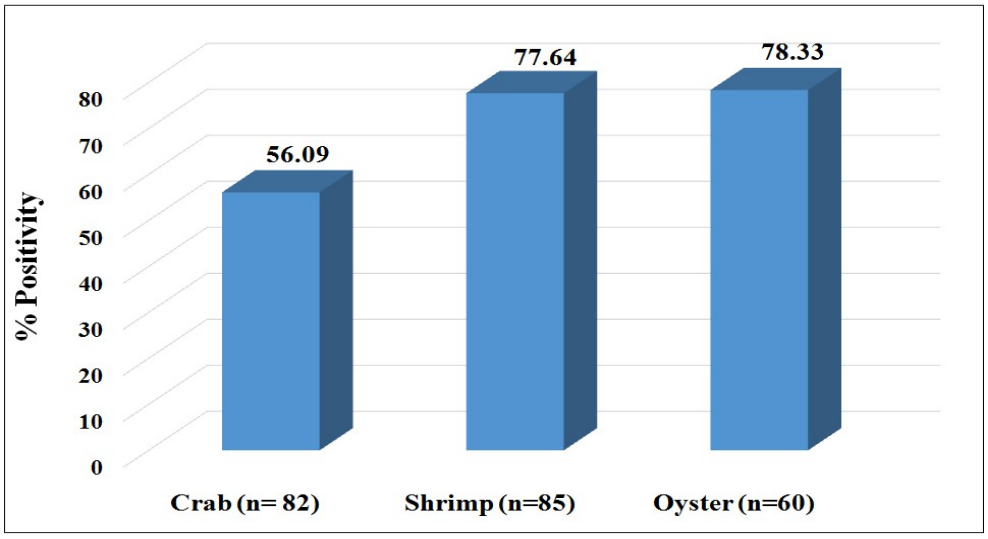
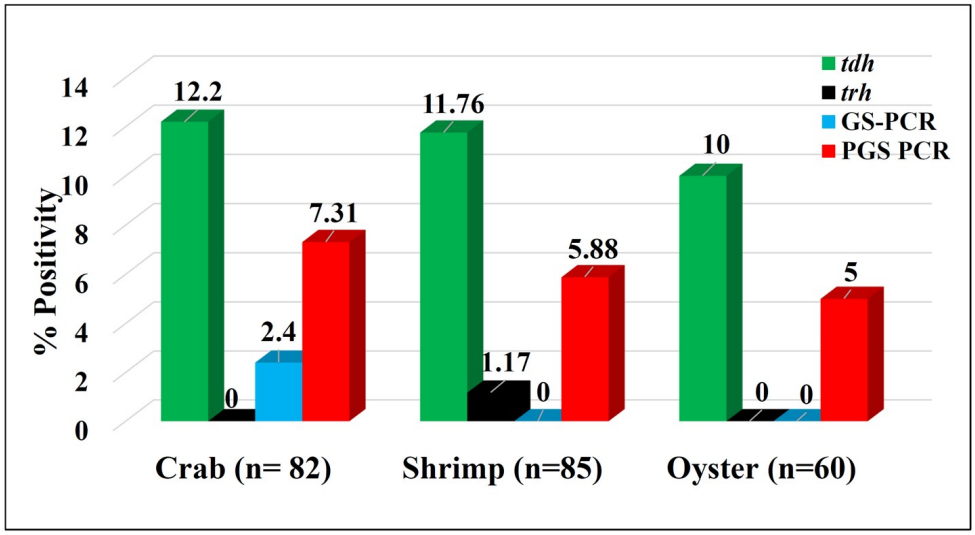


Fig. 7: PGS-PCR of *Vibrio parahaemolyticus*
Lane 1 : Positive control of *V. parahaemolyticus* (Vp-Kx-V_{138})
Lane 3, 4, 5, 6, 7, 14, 16, 17 and 20: Sample isolates with positive amplicon (235 bp)
Lane 12 : DNA ladder of MW 100 bp (Thermoscientific)
Lane 19 : Negative control



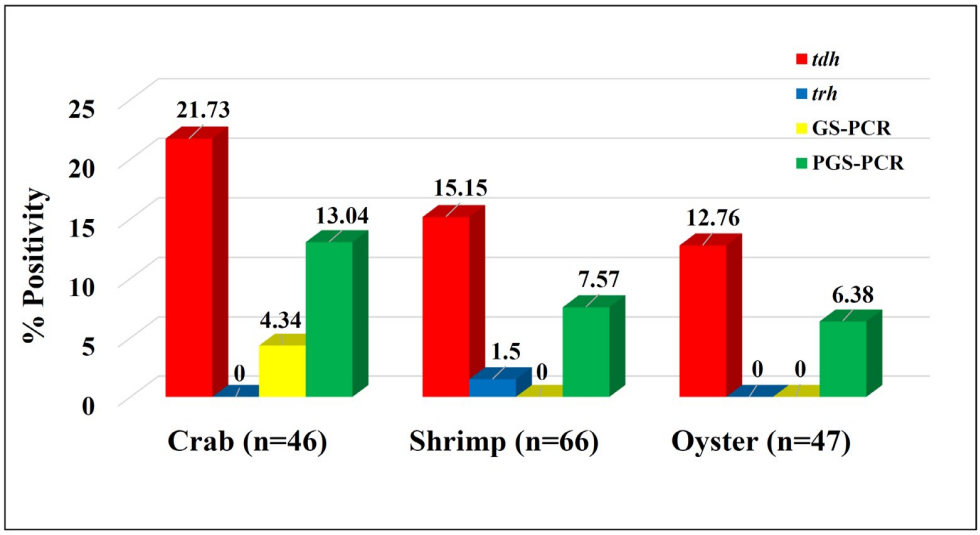
*The values are calculated on the basis of total number of samples examined in each category of shellfishes

Fig. 8: Occurrence of *V. parahaemolyticus* in shellfishes



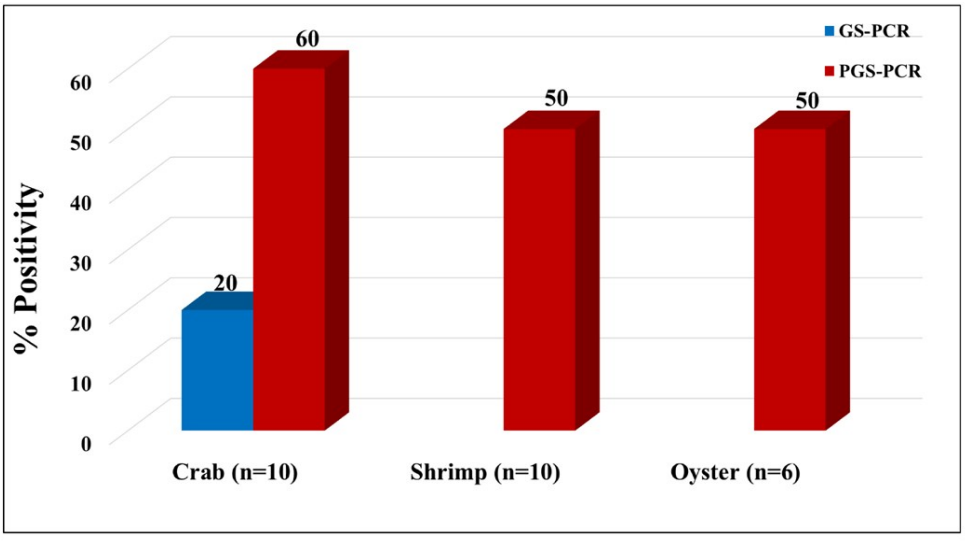
*The values are calculated on the basis of total number of samples examined in each category of shellfishes

Fig. 9: Occurrence of pathogenic and pandemic *V. parahaemolyticus* in shellfishes



*The values are calculated on the basis of total number of VP-*toxR* positive isolates in each category of shellfishes

Fig. 10: Distribution of virulence and pandemic gene in *V. parahaemolyticus* isolates (Vp-*toxR* $^{+}$)



*The values are calculated on the basis of total number of *tdh*$^{+}$ *V. parahaemolyticus* isolates in each category of shellfishes

Fig. 11: Distribution of pandemic gene in virulent (*tdh*$^{+}$) *V. parahaemolyticus* isolates

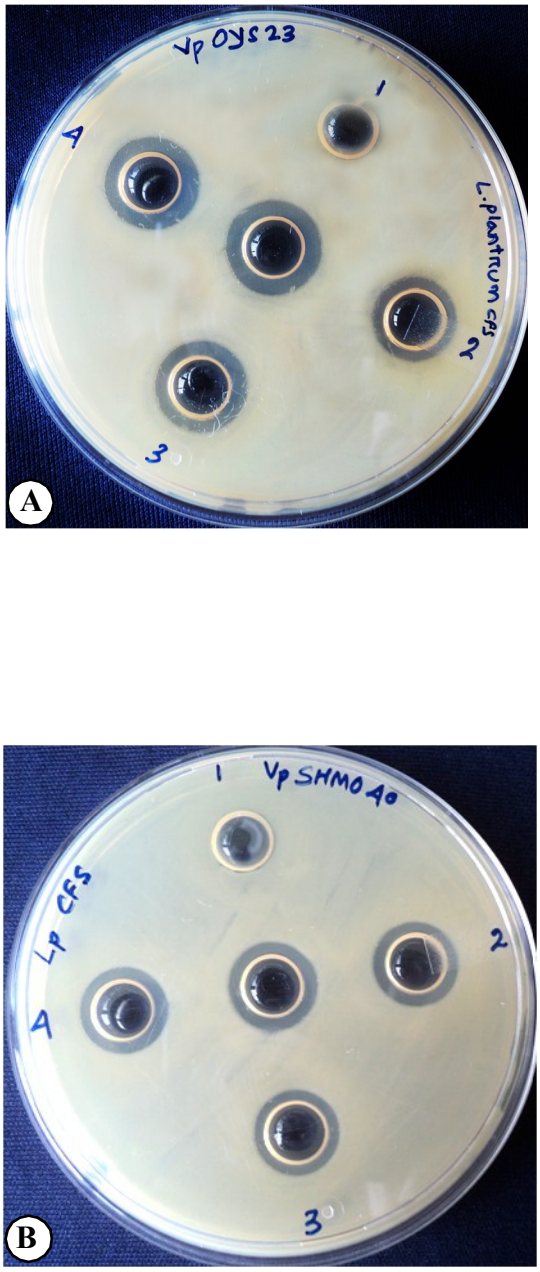
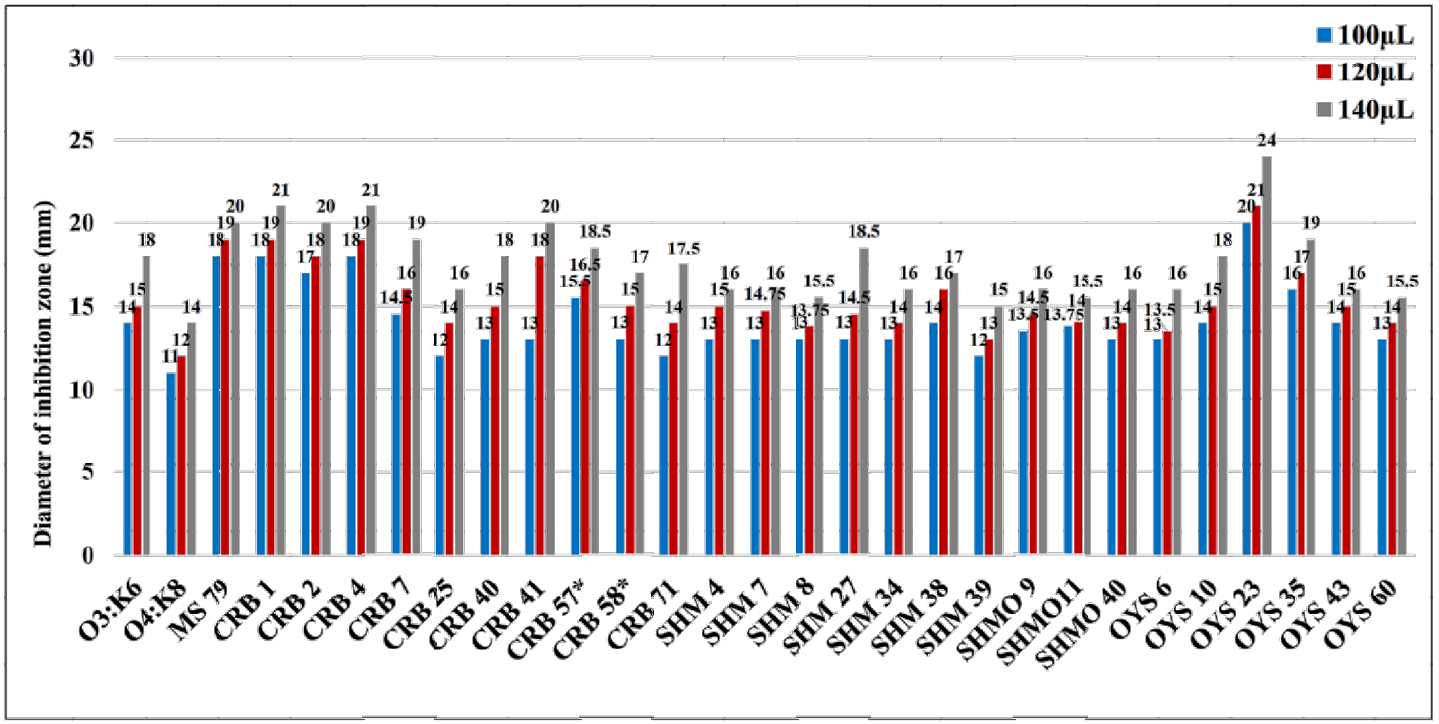


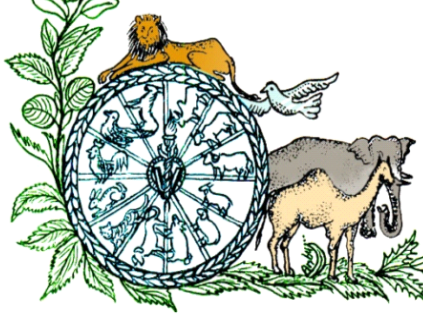
Fig. 12: Agar well diffusion assay



*GS-PCR $^{+}$ isolates

Fig. 13: Antagonistic activity of *L. plantarum* against pathogenic *V. parahaemolyticus* isolates in agar well diffusion assay

Discussion



5 DISCUSSION

V. parahaemolyticus is a normal inhabitant of the marine and esturine environments of coastal areas with worldwide distribution. The presence of the organism has traditionally been limited to warm and temperate geographical areas (DePaola *et al*., 2000). It is an important cause of seafood-borne gastroenteritis (toxi-infection) associated with the ingestion of contaminated raw or improperly cooked saline water origin fish and shellfishes (Yeung and Boor, 2004), mainly characterized by mild diarrhoea, abdominal cramps, nausea, vomition, headache and low grade fever (Honda and Iida, 1993). *V. parahaemolyticus* is also associated with septicemia and occasional wound infection when an open wound exposed to marine animals or warm coastal waters especially seen in South-east Asia (Wong *et al.,* 2000a). Septicemia may be life threating leading to death in the persons with immune-compromisation, underlying liver diseases and old age (Morris and Black, 1985). A short warm period (temperature range from 10ºC - 43ºC, optimum is 37ºC) is sufficient for growth of this bacteria which has short generation time (8-9 min) to attain its infectious levels (Daniels *et al.,* 2000).

The focus of attention in the present study was to evaluate the potentials of saline water origin shellfishes mainly crustacean and molluscs available in the coastal area of the states of West Bengal and Odisha i.e. in and around Kolkata and Bhubaneswar for occurrence of *V. parahaemolyticus.* It was also aimed at to determine the pathogenic and pandemic characters of such *V. parahaemolyticus* isolates with respect to their public health significance i.e. transmission through food chain. Additionally the study was designed to evaluate the *in-vitro* growth inhibitory activity of *Lactobacillus plantarum* (MTCC no. 1407), a probiont, against the isolated pathogenic *V. parahaemolyticus*.

Primarily this organism was considered for study because since 1996, *V. parahaemolyticus* has frequently associated in human diarrhoeal cases in Kolkata environment and has gained a new global dimension in its pathogenicity by virtue of their increasing incidences in virulence (*tdh*$^+$) and pandemic characters (*toxRS* region/ new GS PCR, orf-8, PGS-PCR). Moreover, they are capable of infecting wide host range of marine animals including marine shellfishes which still remains to be the main source of *V. parahaemolyticus* infection in these areas. The perusal of literature suggested that past studies on occurrence of this pathogen were mainly centered on the clinical cases and a very few studies with saline water fishes. However, occurrence of this pathogen in shellfishes available in coastal areas particularly in Odisha and West Bengal has not been addressed attentively where seafood especially crab and shrimps are included in the daily dishes by a considerable population.

To address the unexplored research questions, the present investigation was carried out for isolation of *V. parahaemolyticus* from saline water origin shellfishes like crab, shrimp and oyster of these areas and were characterized by molecular tools for their pathogenicity and pandemic properties. The isolates were characterized biochemically by Kaper's multi test medium (Kaper *et al*., 1980) and confirmation of species was done by adopting species specific Vp-*toxR* PCR assay (Kim *et al*., 1999). Further, virulence genes characterization was done by PCR designed for amplification of *tdh* and *trh* gene responsible for expression of thermostable direct haemolysin (TDH) and TDH related haemolysin (TRH) (Tada *et al*., 1992). TDH is associated with clinical strains and considered as the important virulence factor and is responsible for the induction of β-hemolysis on Wagatsuma blood agar containing human erythrocyte (Wagatsuma, 1968). The *tdh*$^+$ *V. parahaemolyticus* isolates were further characterized for the pandemic potentials by GS-PCR and PGS-PCR. (Matsumoto *et al*., 2000; Okura *et al*., 2004).

A total of two hundred and twenty seven (227) saline water origin shellfishes mainly crab, shrimp and oyster were collected from retail markets and fishing harbours of Kolkata and Bhubaneswar area. The samples were screened by adopting standard bacteriological method for isolation and identification of *V. parahaemolyticus*. In this method, a loopful of overnight cultured broth of the sample in APW was streaked on TCBS agar (selective medium)

where typical round (2-3 mm in diameter), green or blue center colonies were produced by *V. parahaemolyticus*. Among all the two hundred and twenty seven (227) saline water shellfish samples, 194 (85.46%) yielded characteristic sucrose non-fermenting green colour colonies on TCBS agar plate and were subjected for presumptive identification using a multi-test medium (Kaper's medium) (Kaper *et al*., 1980). It was found that 159 (70.04%) of 194 presumptive isolates (characteristic colonies on TCBS agar) produced purple alkali slant and yellow acidic butt (K/A) reaction and thereby, presumed that 159 (70.04%) shellfish samples were positive for *V. parahaemolyticus*. Such isolates were further examined for confirmation of species by determining the presence of species specific signature gene i.e. *toxR* gene (amplicon 368bp) in Vp-*toxR* PCR assay. The Vp-*toxR* assay revealed that all the 159 K/A^{+} isolates yielded positive amplicon. The result focused that of the 227 samples from saline water shellfishes from Kolkata and Bhubaneswar fish market and fishing harbours, 159 (70.04%) were harbouring the *V. parahaemolyticus* with the highest percentage present in oyster 47 (78.33%) followed by shrimp 66 (77.64%) and crab 46 (56.09%) (Table 3; Fig. 8). Such rate of incidence of this pathogen in common shellfishes in these areas revealed its potentials as a food borne problem. The findings was in concordance with earlier studies that reported the presence of this organism in about 50-70% of seafoods (Fletcher, 1985; Annick *et al.*, 2002; Ward and Bej, 2006). Moreover, such increasing occurrence of *V. parahaemolyticus* might be partially linked to rising trend of environmental temperature (*El-nino* effect) that favours the multiplication, propagation and maintenance of this organism in aquatic environment.

The major virulence genes associated with pathogenesis of *V. parahaemolyticus* are *tdh* and *trh* that encode haemolysins production namely thermostable direct haemolysin (TDH) and TDH-related haemolysin (TRH), respectively (Honda *et al.*, 1992). DNA probe studies on *tdh* or *trh* or both genes of *V. parahaemolyticus* strains showed a strong relationship between clinically significant strains and presence of either of these genes, suggesting that both *tdh* and *trh* genes are primarily associated in virulence of this organism and pathogenesis, thereby (Shirai *et al*., 1990).

PCR was used to detect both *tdh* and *trh* gene using DNA primers that are specific for the genes encoding TDH or TRH (Tada *et al*., 1992). These two genes are unanimously

recognized in almost all virulent isolates of *V. parahaemolyticus*; however, *tdh* and *trh* like genes are seldom reported in some strains of other *Vibrio* species such as *V. mimicus*, *V. cholerae* and *V. hollisae* (Nishibuchi and Kaper, 1995). Hence, in this investigation isolates were first confirmed as *V. parahaemolyticus* by Vp-*toxR* PCR assay and thereafter *tdh* was screened to determine the pathogenic population. Accordingly, *tdh* and *trh* were screened for only 159 isolates that have been identified as *V. parahaemolyticus* by observation of Kaper's reaction as well as Vp-*tox*R PCR assay and 26 out of 227 (11.45%) saline water shellfishes were found to harbour the *tdh* gene; however, *trh*-gene was recorded only in one sample (0.44%) that belonged to shrimp (Table 3). The incidence (11.45%) of pathogenic (*tdh*-positive) *V. parahaemolyticus* in routine dietary shellfishes in these areas was alarming and indicating the potentials of these shellfishes for causing gastroenteritis. The findings for occurrence of this pathogen in the shellfishes were in agreement with previous studies (Hara–Kudo *et al*., 2003; Mohammad *et al*., 2005; Sujeewa *et al*., 2009) where *tdh*-positive *V. parahaemolyticus* was recovered in 10%, 11% and 15% of shellfishes like oyster and shrimps. Further, the higher incidence (74%) of *tdh*-positive population of this pathogen in environmental isolates was reported with Alaskan oysters, United States (McLaughlin *et al*., 2005). On the contrary, lower frequency ranging from 0 to 17% of *tdh* positive *V. parahaemolyticus* isolates in environmental samples and seafoods were reported by group of studies (Kiiyukia *et al*., 1989; Ogawa *et al*., 1989; Cook *et al*., 2002; DePaola *et al*., 2003; Deepanjali *et al*., 2005). Such variation in presence of pathogenic population of *V. parahaemolyticus* in the reported studies may be associated with the differences in factors prevailing in different geographical areas that influences the harbouring of pathogenic genotype in the part population of this pathogen and perpetuation, thereby.

The high frequency of occurrence of pathogenic (tdh^+) *V. parahaemolyticus* in saline water shellfishes in these areas indicates the potentials of common market shellfishes for causing food-borne gastroenteritis linked to food chain. The present findings also highlighted the real magnitude of public health risk in terms of *V. parahaemolyticus* borne gastroenteritis attributable to routine intake of these common shellfishes that carry pathogenic *V. parahaemolyticus* in their considerable population (>10%). In reality, in Indian situation, gastroenteritis to the

consumers of these shellfishes are not reported so frequently; hopefully, it is the boon of our Indian cuisine that attained much higher temperature than the thermal death point of this pathogen. More precisely, Indian tradition for adoption of proper cooking temperature and cook ready to eat are strongly in favour of community health by restricting the survivability of almost all food borne aerobic bacteria. Daniels *et al*., (2000) stated that warmer sea temperatures (the El Nino effect) have resulted in the emergence of more virulent *V. parahaemolyticus* in USA. Since 1996, occurrence of *V. parahaemolyticus* in endemic and epidemic situations has been increasingly reported in many Asian countries including India. Regular rise of ambient and aquatic environment temperature may be associated in abetting such increasing incidences of virulent *V. parahaemolyticus* (Martinez-Urtaza *et al.,* 2005 and 2008). Increasing environmental temperature in these area may facilitate to cope up and propagate the pathogenic strains of this organism.

Recent past studies reported induction of fluid accumulation in suckling mice by non-pathogenic *V. parahaemolyticus* strains i.e. no evidence for production of TDH or TRH (Kothary *et al*., 2000). Lee *et al*. (2002) identified a heat-labile protein (serine protease) as potential virulence factor in clinical strain lacking both *tdh* and *trh* gene which had significant effects on the growth of cell line *viz.* Chinese hamster ovary, Vero, HeLa and Caco-2 cells as well as lytic effect on RBCs and also caused tissue haemorrhage and death in mice in both intraperitoneal and intravenous injection. This is a new horizon in pathogenicity selfie of *V. parahaemolyticus* where the tdh^- and trh^- population of *V. parahaemolyticus* will not be discarded rather have to allow the same laboratory screening as adopted for their tdh^+ and trh^+ counter population. In this study, *V. parahaemolyticus* was detected in 70.04% shellfish samples of which 11.45% and 0.44% were harbouring the commonly recognised pathogenicity marker genes (*tdh* and *trh*). As per the approved commitment, the present study was restricted to deal with the *tdh* and *trh* positive isolates; however, if the total *V. parahaemolyticus* isolates obtained in this study is considered, the probability and potential of shellfishes in these areas for causing gastroenteritis and diarrhoea may attain remarkable degree.

Epidemiological study revealed that most of the reported outbreaks of *V. parahaemolyticus* infection were due to consumption of raw or insufficiently cooked sea

foods especially crustacean and molluscs (Daniels *et al*., 2000). The linkage in transmission of this pathogen through food chain among the consumers of shellfishes in places like Kolkata and Bhubaneswar, India and probability for incidence of gastroenteritis are seems to be almost similar with the earlier studies because lower income group people of these areas prefer these shellfish in their daily diet that serves the low cost common dietary affordable protein sources. In this point, there is probability of exception with the earlier published studies conducted abroad where considering the nutritional point of view and fast food practices, it is not uncommon to take insufficiently cooked seafoods. On the contrary, in Indian context, usually the consumers prepare these marine source foods with proper heating that spare feeble chances for this pathogen to remain alive. However, in modern era of fast food practice, some groups of Indian people skip the recommended cooking temperature and time protocol that may accidentally allow entry of this pathogen in food chain. In Indian circumstances, contamination of freshwater fishes at the market level through shellfishes implicated with *V. parahaemolyticus* and subsequently, cross contamination of other foods in the kitchen by *V. parahaemolyticus* contaminated shellfish brought from markets are believed to be the possible sources of entry of this organism in food chain (Sudha *et al*., 2012 and 2014).

With the enormous increase in human population and growing demand of more food vis-à-vis depletion of traditional terrestrial resources, the aquatic domain has been considered as an alternative source of dietetic protein to meet up the growing need of the community. The indiscriminate use of water bodies for disposal of waste products has led to marked alterations in their physical and chemical characteristics, especially of microbial community status with the replacement of autochthonous species by allochthonous forms and many of which are turn to be pathogenic to man. Because of increasing protein hunger, the community intended to adopt the non-conventional food protein sources like seafoods such as crustaceans, scallops, molluscs, octopus etc. in their dietary dishes that fetched the main source of *V. parahaemolyticus* in food chain. Okuda *et al*. (1997) reported an increase in hospital admissions of patients with *V. parahaemolyticus* gastroenteritis in Kolkata from the beginning of February 1996 and analysis of the strains revealed that a unique serotype, O3:K6 [$tdh^{+,}$ trh^{-}, urease^{-} and *toxRS* (GS-PCR)$^{+}$] accounted for 50 to 80% of infections and this serotype was not previously isolated during surveillance in Kolkata.

In the present study, *trh* gene was detected in one (0.44%) sample only that belonged to shrimp. This finding was in accordance with previous study that reported only one *trh*$^+$ (0.87%) isolate from 114 fresh market seafood samples (Vuddhakul *et al*., 2000). On the other hand, in a recent past study, Deepanjali and coworkers reported markedly higher incidence (59.3%) (29/49) of *trh*$^+$ *V. parahaemolyticus* from oysters in southwest coast of India (Deepanjali *et. al.,* 2005). Details of published literature say that incidence of *trh* gene in *V. parahaemolyticus* is variable with place of study. Interestingly, *trh* positivity among the isolates in this study was negligible. The finding was in support of the published statements suggested that presence of *tdh* and *trh* in virulent *V. parahaemolyticus* are contrary to each other. From Kolkata and Bhubaneswar samples the presence of *trh* gene in *V. parahaemolyticus* isolates was comparatively very low to the *tdh* gene. It reflects that screening of *tdh* should be preferred to *trh* for identification of pathogenic *V. parahaemolyticus* in Kolkata environment.

PCR based assay, known as group-specific PCR (GS-PCR), has detected nucleotide variations within the 1,364-bp *toxRS* region that are unique to the pandemic clone. The *toxRS* operon of *V. parahaemolyticus* encodes the trans-membrane proteins involved in the regulation of virulence-associated genes. Utilizing this highly conserved region, a PCR-based detection system (GS-PCR) was formulated specifically to detect the *V. parahaemolyticus* isolates of pandemic genotype by identifying the presence of *toxRS* sequence as well as to differentiate the pandemic isolates from the non-pandemic population of this organism (Matsumoto *et al*., 2000). Bhoopong *et al*. (2007) defined pandemic strains as those carrying the *tdh* gene, lacking the *trh* gene and yielding GS-PCR positive reactions.

In this study, the *tdh* and *trh* positive isolates of *V. parahaemolyticus* from shellfish samples were selected for screening of the *toxRS*-new region (GS-PCR). It was observed that the *trh* positive only isolate was not carrying the *toxRS*-new region gene and out of twenty six (26) *tdh*$^+$ isolates, 2 (7.69%) were harbouring the *toxRS* sequence of the new pandemic clone which belonged to crab 2 (20%) only (Table 5; Fig. 11). On total collected sample basis, the distribution of *toxRS*-new region gene in different marine shellfish samples were 2 (0.88%) (Table 3). Although the frequency of GS-PCR$^+$ *V. parahaemolyticus* was limited in this study; however, occurrence of such pandemic potential *V. parahaemolyticus* in the common

saline water shellfishes in this area (Kolkata) poses the ample potential risk of gastroenteritis that have record of global occurrence.

Okura *et al*. (2004) developed a PCR assay to identify the pandemic group of *V. parahaemolyticus* using a marker derived from the group-specific sequence of an arbitrarily primed-PCR (AP-PCR) fragment (930bp) that encodes for a "hypothetical protein". A number of putative pandemic group-specific primer pairs were also designed and tested for their applicability in identification of isolate of pandemic group. Of these, only the primer pair PGS-1 and PGS-2 was found to be specific to the pandemic sequence and was recommended for use in identification of pandemic *V. parahaemolyticus* from clinical source. With this primer pair, they performed PCR on genomic DNAs of 82 *V. parahaemolyticus* strains, including 38 pandemic and 44 non-pandemic strains of various serotypes. They found that all 38 pandemic strains yielded a 235 bp amplicon while all 44 non-pandemic group strains did not. They concluded that this PGS-PCR assay can be a useful molecular tool not only for identification of pandemic *V. parahaemolyticus* strains but also for direct detection of this organism contaminating food and environmental samples. Keeping this observation in view, all the *tdh*$^+$ (26) and *trh*$^+$ (1) isolates recovered in this study were screened by PGS-PCR for presence of pandemic character besides *toxRS*-new region (GS-PCR). Out of twenty seven (27) isolates, 14 (53.8%) isolates were found positive for PGS PCR and belonged to crab 6 (60%), shrimp 5 (50%) and oyster 3 (50%) samples (Table 5; Fig. 11). All the above 14 isolates produced the defining amplicon (235 bp) in the PGS PCR assay. The findings suggested to conclude that besides the GS-PCR, this PGS-PCR assay may be suitable for the identification of pandemic *V. parahaemolyticus* from shellfishes in these areas. The findings also indicated occurrence of considerably high percentage of pandemic *V. parahaemolyticus* in the marine environment in these areas that needs attention for adoption of appropriate measures to limit the dissemination of such pathogens of public health importance with pandemic potential. It is interesting to note that pathogenic and pandemic *V. parahaemolyticus* was detected in a considerable population of shellfishes in the study areas; although, clinical detection of this organism in routine as well as epidemic gastroenteritis has been reported at an average frequency. These facts forward to presume that the indigenous Indian cooking practices may have indomitable role to annihilate

such pathogenic and pandemic *V. parahaemolyticus* in food and limit the occurrence of gastroenteritis in these areas. On the analogy, the growing practice for fast food habit is also an important factor in this regard. Altogether, a constant surveillance on occurrence of this pathogen in index clinical cases and thereafter, linking to suspected foods may be beneficial to a large extent to combat the health problems arise with this pathogen. Furthermore, inculcation of the utmost effective measure i.e. to educate the people routinely about fundamentals of public health and hygiene will be certainly contributory to minimize the incidence of this pathogen and to project a healthy community life.

The concept of biological control for health maintenance has received widespread attention during the last few years, driven in large part by consumers and the lay press. The first report of the inhibitory effect of a seawater bacteria against a *Vibrio* spp. has been attributed to De-Giaxa (1889). Subsequently, Rosenfeld and ZoBell (1947) described an antibiotic-producing marine microorganisms, and since then research has begun to develop biological control strategies based on the application of bacteria both Gram-positive and Gram-negative including algae, fungi as probiotic against halophilic Vibrios in aquaculture system to reduce the mortality as well as to protect the consumer health.

Many mechanisms have been suggested by which probiotics enhance the health of host organisms such as creation of an antagonistic environment for pathogens by the production of inhibitory compounds (bacteriocins, lysozymes, proteases and hydrogen peroxide), competition for essential nutrients and adhesion sites, supply of essential nutrients and enzymes resulting in enhanced nutrition of the cultured animal, direct uptake of dissolved organic material mediated by the bacteria, modulating interactions with the environment and development of beneficial immune responses (Gatesoupe, 1999; Gomez-Gil *et al.*, 2000; Irianto and Austin, 2002; Balcazar *et al.*, 2006).

Antimicrobial activity is one of the most important selection criteria for probiotics. The application of probiotics for disease control in aquaculture is a growing area of interest vis-a-vis the increased incidence of antibiotic resistance. Probiotics have been defined by the World Health Organization and Food and Agriculture Organization as "live microorganism which

when administered in adequate amount, confers a health benefit on the host". In the past, several Gram negative and Gram positive bacteria have been evaluated *in-vitro* and *in-vivo* for their potential to inhibit pathogenic organisms and overcome infections in aquaculture (Itoh *et al.,* 1995).

Antimicrobial effects of lactic acid bacteria exhibited by production of substances such as organic acids (lactic, acetic, propionic acids), carbon dioxide, hydrogen peroxide, diacetyl and low molecular weight antimicrobial substances i.e. bacteriocin (Dunne *et al.,* 2001; Balcazar *et al.,* 2008). Bacteriocins are small cationic, membrane permeabilizing peptides synthesized by ribosome having antimicrobial activity (Klaenhammer, 1993; Jack *et al*., 1995). Any protein secreted by bacteria that inhibits growth of other bacteria is classified as a bacteriocin irrespective of their varying chemical structure, mode of action and host specificity (Rammelsberg and Radler, 1990). Bacteriocins and their producer organisms have potential as natural preservatives in food and feed industry and pharmaceutical industry as probiotics (Green *et al*., 1999; Cleveland *et al*., 2001). Probiotics including *Lactobacillus*, Bifidobacterium and *Streptococcus* spp. are known to be inhibitory to the growth of a wide range of intestinal pathogens in human. In addition to the favorable effects against upsets and imbalances in gut microflora in different clinical situations, several experimental observations have shown a potential protective effect of probiotic bacteria against the development of colon tumors (Savadogo *et al.,* 2004).

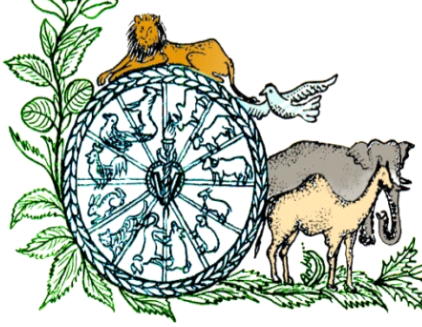
In the present study, antagonistic activity of *Lactobacillus plantarum* was evaluated against pathogenic isolates of *V. parahaemolyticus* (26 *tdh*$^{+}$ *V. parahaemolyticus* isolates and three clinical isolates) by adopting the agar well diffusion assay. The growth of all the tested *V. parahaemolyticus* isolates were inhibited by the CFS of *L. plantarum* producing inhibition zone around well in agar well diffusion assay (AWDA) where 24 mm was observed as highest zone of inhibition and 11 mm as the lowest after 12 hour of incubation (Table 7; Fig. 13). The diameter for zone of inhibition remains almost same when the culture plates were incubated further for 24 h, 36 h and 72 h. Moreover, similar pattern of results were observed in using LBA in place of MHA in agar well diffusion assay. Further, while using *L. plantarum* culture supernatant without filtration; similar zone of inhibition was observed with the tested *V.*

parahaemolyticus isolates. Hence, it is presumed that in AWDA, use of *L. plantarum* culture supernatant and LBA may replace the CFS and MHA for yielding the diagnostic zone of inhibition by *L. plantarum* on pathogenic *V. parahaemolyticus.* Moreover, the highest zone of inhibition was noted against the isolate Vp-Oys23 recovered from oyster harbouring the pathogenic (*tdh*$^+$) and pandemic genotype (PGS-PCR$^+$) (Fig. 12). The observation strongly supports the lucid probiotic effect of *L. plantarum* on distinctly pathogenic *V. parahaemolyticus*. The result was in agreement with the study by Nuphet *et al.* (2003) where *L. plantarum* showed antagonistic effect on pandemic strain of *V. parahaemolyticus* in agar spot method with zone of inhibition ranging between 11mm to 15mm. *Lactobacillus* exhibited inhibitory effect on clinically important pathogens such as Enterotoxigenic *E. coli*, S. Typhimurium and *L. monocytogenes* with zone of inhibition 4.2 mm, 4.3 mm and 5.0 mm, respectively (Osuntoki *et al.,* 2008). In another study by Chowdhury *et al*. (2012) three *L. plantarum* isolates from buffalo milk yoghurt showed antagonistic activity against *V. parahaemolyticus* with a higher zone of inhibition ranging from 25 mm to 45 mm. Ravishankar *et al*. (2012) isolated bacteriocin producing *L. plantarum* strain from raw cow milk that showed antibacterial activity in well diffusion assay against important food borne pathogens *viz. S. aureus*, *E. fecalis*, *E. coli* and *L. monocytogenes* with inhibition zone of 21 mm, 17 mm, 18 mm and 11 mm, respectively. The inhibition zone formed against pathogenic *V. parahaemolyticus* isolates in the present study may be linked with production of inhibitory substances such as bacteriocin or hydrogen peroxide or organic acids (Vazquez *et al*., 2005; Sugita *et al*., 2007).

The commonly available shellfishes in the coastal part of West Bengal and Odisha that are accepted part of daily dishes for a considerable size of population being affordable protein source but have so far attained least attention to study the presence of pathogenic *V. parahaemolyticus*. In this regard, findings of the present study focused that shellfishes in the coastal part of West Bengal and Odisha harbour the pathogenic *V. parahaemolyticus* in a considerable significant occurrence (11.46%); moreover, a part population (14/26) of this pathogen carry the signature gene for pandemic potential which is remarkable considering its past propensity for spreading in global spectrum. The result suggested that the shellfishes of

these study areas are equally potential source like sea-foods (shellfishes, other marine fishes and aquatic bodies) that are explored in previous studies in respect to the presence of pathogenic *V. parahaemolyticus* (Sujeewa *et al*., 2009; Anjay *et al*., 2014). So, there is reason enough to pay heed to shellfishes of these areas in the event of screening and particulating environmental sources for zoonotic transmission of this pathogen. Moreover, the injudicious use of antibiotics to combat this pathogen has invited a health issue of increasing antibiotic resistance. In recent past, a list of probiotics including *Lactobacillus* spp. are explored as alternate of antibiotics as a tool of biological control. With this concept, *L. plantarum* was employed in this study for its growth inhibitory effect on pathogenic *V. parahaemolyticus* in laboratory condition. It was observed that this species of *Lactobacillus* expressed the discriminatory growth inhibiting effect on pathogenic *V. parahaemolyticus* that led to re-affirm such probiotic effect in *in-vivo* condition to generalize and recommend for use of this probiont as a biological control agent against *V. parahaemolyticus* in aquaculture of these areas.

Summary and Conclusions



6 SUMMARY AND CONCLUSIONS

Vibrio parahaemolyticus was first acknowledged in late 1950 as a food borne pathogen in Osaka, Japan in context to an outbreak related to consumption of semidried sardines (Shirasu). Over the years, it has emerged globally as one of the leading pathogen for diarrhoea and gastroenteritis (toxi-infection) resulting from the consumption of raw or under cooked seafoods. This halophile is ubiquitous in marine and estuarine environments and commonly isolated from various sea-foods *viz*. marine fishes, crab, shrimp, oysters, mussels, scallops, octopus, clam, mackerel, sardines, codfish etc. all over the world. Till 1995, occurrence of *V. parahaemolyticus* gastroenteritis was thought to be a problem of limited countries and was never associated with a pandemic like the ones caused by toxigenic *V. cholerae* O1 and O139 sero-groups. However, the past few years have witnessed an unusual change in the pattern of incidence of *V. parahaemolyticus* infections primarily due to the emergence and dominance of unique serovar (O3:K6) and its other clonal serovars in causing diarrhoeal disease in India and other parts of the world. In Kolkata, the O3:K6 serovar was found to be the cause of 50-80% of the *V. parahaemolyticus* infections from February 1996 to August 1996 and was subsequently found responsible for gastroenteritis in other countries *viz.* Japan, Bangladesh, Indonesia, Thailand, Singapore, Taiwan, Laos, Korea, Texas, Mexico, Chile, Peru, Russia, Mozambique and China indicating the pandemic spreads of pathogenic *V. parahaemolyticus* into Asian, African, American and European continents.

The present study was envisaged to proximate the occurrence of pathogenic and pandemic *V. parahaemolyticus* in saline water origin shellfishes (crustaceans and molluscs) retailed in Eastern part of India mainly in and around Kolkata and Bhubaneswar by characterizing

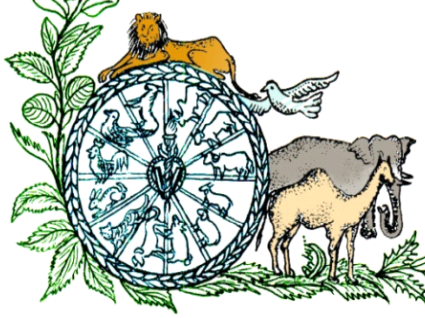
their virulence and pandemic genotypes. It was also explored to evaluate the *in-vitro* antagonistic effect of *L. plantarum* on the isolated pathogenic *V. parahaemolyticus* in order to define this probiont as a suitable probiotic candidate in aquaculture as well as in fish food industry.

A total of two hundred twenty seven (227) saline water shellfish samples including crab, shrimp and oyster collected from the study areas were screened by conventional bacteriological culture and isolation method i.e. overnight pre-enrichment in APW followed by streaking on selective agar (TCBS). The characteristic 5 colonies, representative of each sample, were screened by Kaper's multi-test medium and such positive colonies were considered as presumptive *V. parahaemolyticus* and stored. Of the total 227 samples, 159 (70.04%) yielded the presumptive *V. parahaemolyticus* (K/A^+ Colony: yellow acidic butt and purple alkaline slant). All the K/A^+ isolates were subjected to species specific Vp-*toxR* PCR assay wherein all presumptive isolates generated the determinative amplicon (368bp) and resulting occurrence of this pathogen was highest percentage in oyster (78.33%) followed by shrimp (77.64%) and crab (56.09%).

All the Vp-*toxR* PCR positive isolates (n=159) were tested for the cardinal virulence genes (*tdh* and *trh*). About 11.45 % (26/227) of the samples yielded the confirmatory amplicon (199bp) for *tdh* and detected in different shell fishes *viz.* crab (12.2%), shrimp (11.76%) and oyster (10%). Similarly, *trh* gene was screened by PCR assay yielded 250bp amplicon in one sample that belonged to shrimp source. Such high percentage of pathogenic *V. parahaemolyticus* (tdh^+) in shellfishes of saline water origin poses public health risk in consumption of improperly processed shellfishes. The health risk arising with cross contamination by such shellfishes to other marine and fresh water market fishes in these areas may be an additional point of risk. Pandemic potentials of the pathogenic *V. parahaemolyticus* (tdh^+ and trh^+) isolates were assessed by GS-PCR as well as by PGS-PCR assay as the latter assay found more determinative with environmental samples. In total, 0.88% of the samples harbouring *toxRS*-new region revealed GS-PCR positivity and belonged to crab samples (2.4%) only. Similarly, 6.16% of the samples were positive in PGS-PCR that belonged to crab (7.31%) followed by shrimp (5.88%) and oyster (5%). The study highlighted the presence of pandemic potential *V. parahaemolyticus* in considerable population of crab, shrimp and oyster in these areas.

Antagonistic activity of *L. plantarum* was studied by employing all *tdh*$^+$ as well as three clinical isolates in agar well diffusion assay using MHA. All the *V. parahaemolyticus* isolates were inhibited by the CFS of *L. plantarum* with volume ranging from 100 µl to 140 µl where 24 mm was observed as highest zone of inhibition and 11 mm as the lowest after 12 hour of incubation. Optimum observation noted on use of 140 µl CFS. Testing with more incubation hours, LBA as growth medium and non-filtered culture supernatant showed no difference in zone of inhibition. The highest zone of inhibition was noted against the isolate Vp-Oys23 (*tdh*$^+$ and PGS-PCR$^+$) recovered from oyster. The observation strongly support the distinct probiotic effect of *L. plantarum* on pathogenic *V. parahaemolyticus*. This finding corroborates in favour of the efficacy of *L. plantarum* as a potential probiont for use in aquaculture and marine food industry to combat public health consequences of *V. parahaemolyticus* origin.

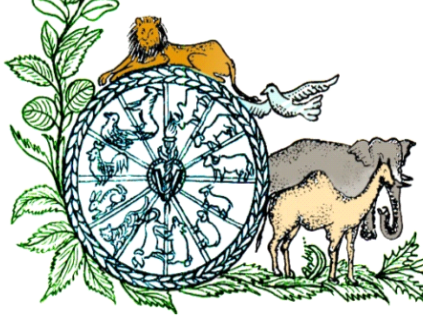
Mini Abstract



7 MINI ABSTRACT

The present study was designed to know the occurrence of pathogenic and pandemic *V. parahaemolyticus* in saline water shellfishes in Eastern coastal areas particularly in and around Kolkata and Bhubaneswar and their potentials for public health hazards. Besides, *in-vitro* antagonistic effect of *L. plantarum* (MTCC No. 1407) was studied against the pathogenic isolates of *V. parahaemolyticus*. Out of 227 different saline water shellfishes (crab n=82, shrimp n=85, oyster n=60) samples examined by cultural isolation; 159 (70.04%) were found to harbor *V. parahaemolyticus* (K/A^+ and Vp-$toxR^+$). All the 159 isolates were subjected to their virulence gene repertoire i.e. *tdh* and *trh*. Only one found positive for *trh* and belonged to shrimp; however, 26 (11.45%) samples found to harbour *tdh* gene and belonged to crab 10 (12.2%), shrimp 10 (11.76%) and oyster 6 (10%). Pandemic potentials of the pathogenic *V. parahaemolyticus* (tdh^+ and trh^+) isolates were assessed by GS-PCR as well as by PGS-PCR assay and the latter assay found more determinative with environmental samples. Of the 27 isolates, 2 (0.88%) gave amplicon for pandemic gene (*toxRS*-new region) and recovered from crab (2.4%) only. Similarly, in PGS-PCR, 14 (6.16%) samples were detected to carry the pandemic isolates and belonged to crab (7.31%) followed by shrimp (5.88%) and oyster (5%). The study findings focused that a considerable population of common shellfishes in these areas harbor the pathogenic and pandemic *V. parahaemolyticus* reflecting the potentials of public health risk as well as probability of dissemination through food chain. Besides, the data also concurred the possibility of cross contamination of other market fishes both of fresh and saline water origin that usually are being marketed along with such contaminated shellfishes. Growth inhibitory effect of *L. plantarum* was assessed against all tdh^+ *V. parahaemolyticus* isolates including three clinical isolates in agar well diffusion assay and revealed that both filtrate and non-filtrate CFS of *L. plantarum* exhibited equal growth inhibition on MHA as well as on LBA with the zone of inhibition ranging from 11 mm to 24 mm. Its highest inhibition zone was noted against the isolate from oyster harbouring the pathogenic (tdh^+) and pandemic genotype (PGS-PCR^+). The observation strongly supports the *in-vitro* probiotic effect of *L. plantarum* on distinctly pathogenic *V. parahaemolyticus* and this led to re-affirm in *in-vivo* to generalize and recommend for use of this probiont as a biological control agent against *V. parahaemolyticus* in aquaculture in these areas.

लघु सारांश



8 लघु सारांश

वर्तमान अध्ययन पूर्वी तटीय क्षेत्रो विशेषतः कोलकाता और भुवनेश्वर और इनके आसपास के नमकीन पानी शैलफिशों में विब्रियोपैराहिमोलाइटिकस की रोगजनकता और महामारी की घटना और उनके जन स्वास्थ्य संबंधी खतरों को जानने हेतु डिजाइन किया गया। इसके अलावा लेक्टोबेसिलस प्लानटेरम (एमटीसीसी नम्बर 1407) का विब्रियो पैराहीमोलटिकस के खिलॉफ विरोधी प्रभाव का अध्ययन भी किया गया। 227 विभिन्न नमकीन पानी शैलफिशों (केकड़ा–82, झींगा–85, सीप–60) के नमूने संवर्धन पृथककरण द्वारा परीक्षित किये गये, उनमें से 159 (70.09 प्रतिशत) में विब्रियोपैराहिमोलाइटिकस पाया गया। सभी 159 नमूनों बिरूलेन्स जीनो, जैसे टी.डी.एच. और टी.आर.एच. हेतु परीक्षण किये। केवल झींगा से एक सकारात्मक टी.आर.एच जबकि 26 (11.45 प्रतिशत) नमूने टी. डी.एच. सकारात्मक थे, जो कि 10 (10.2 प्रतिशत) केकड़ो से, 10 (11.76 प्रतिशत) झींगा से और 6 (10 प्रतिशत) सीप से थे। रोगजनक वी. पैराहिमोलाइटिकस (टी.डी.एच.$^{+}$, टी. आर.एच.$^{+}$) की महामारी क्षमता का परीक्षण जी.एस.–पीसीआर और पी.जी.एस.–पीसीआर परखों द्वारा किया गया, और बाद के परख वातावरणीय नमूने के साथ ज्यादा निर्धारक था। 27 नमूनों से, 2 (0.88 प्रतिशत) एम्पिलीकॉन महामारी जीन जो कि केकड़ों (2.4%) से प्राप्त हुये। इसी प्रकार पीजीएस–पीसीआर द्वारा 14(6.16 प्रतिशत) नमूने महामारी नमूने हेतु संसूचित हुये जो केकड़ो 7.31 प्रतिशत झींगा 5.88 प्रतिशत और सीप से 5% संबन्धित थे। अध्ययन निष्कर्षो से पता चला कि इन क्षेत्रों में मुख्य शैलफिशों की काफी आबादी रोजनक और महामारी के विब्रियो पैराहेमोलटिकस को धारण करती है जो कि जन स्वास्थ्य के जोखिम और खाद्य श्रृंखला के माध्यम सेप्रसार की संभावना दर्शाता है। इसके अलावा, डाटा यह भी सहमति दर्शा रहा है कि बाजार की साफ और नमकीन पानी की मछलियों के मध्य क्रॉस संदूषण हो सकता है जो सामान्यतः इन संदूषित सेलफिसेस के साथ बेची जाती है। इन टीडीएच सकारात्मक विब्रियो पैराहिमोलाइटिकस खिलाफ लेक्टोबेसिलस प्लानटेरम का प्रभाव; जिसमें तीन क्लिनिकल नमूने भी थे अगार वेल प्रसाारण परख द्वारा किया गया और इसमेंदेखा गया कि लेक्टोबेसिलस प्लानटेरम के सीएफएस में वृद्धि में अवरोध दोनों ही एमएचए और एलबीए पर दर्शाया और अवरोध जोन 11 मिमी0 से 24 मिमी0 तक पाया गया। अधिकतम अवरोध जोन सीप से पृथक नमूने के खिलाफ था जो रोगजनक (टीडीएच$^{+}$) और महामारी (पीजीएस–पीसीआर$^{+}$) था। अवलोक दृढ़ता से रोगजनक वि. पैराहीमोलाइटिकस पर इन–बिट्रो प्रोबायोटिक का प्रभाव दर्शाता है, और इसके सामान्यीकरण के लिए इन–बिवों परखों द्वारा पुष्टि की जानी चाहिए और इस प्रोबायोट का प्रयोग ऐक्वाकल्चर में वि. पैराहिमोलाइटिकस के खिलाफ जैविक नियंत्रक के रूप में संस्तुति की जा सकती है।

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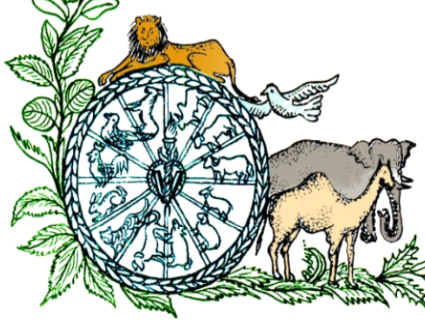

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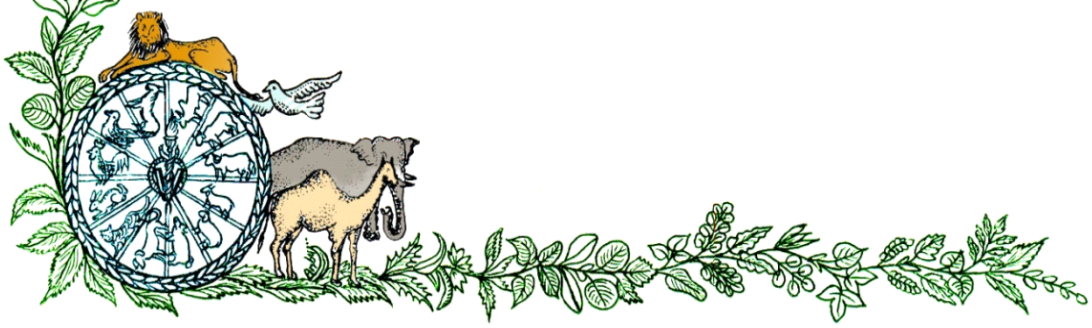
Appendix



APPENDIX-I

MEDIA USED FOR BACTERIOLOGICAL PROCEDURES

Alkaline Peptone Water

Peptone	1 g
Sodium chloride	3 g
D/W upto	100 ml

pH adjusted to 8.5 with 1 N NaOH

Ingredients were mixed properly by mild heating in a conical flask, evenly distributed 30 ml in tubes (50 ml capacity) and sterilized by autoclaving at 15 psi for 15 minutes. The tubes were stored at 4ºC.

TCBS agar

Yeast Extract	5.0 g
Proteose Peptone No. 3	10.0 g
Sodium Citrate	10.0 g
Sodium Thiosulphate	10.0 g
Oxgall	8.0 g
Saccharose	20.0 g
Sodium Chloride	10.0 g
Ferric Ammonium Citrate	1.0 g
Bromo thymol Blue	0.04 g
Thymol Blue	0.04 g
Agar	15.0 g
D/W upto	1000 ml

Ingredients were suspended in 1000 ml distilled water, mixed thoroughly then heated with frequent agitation and boiled for 1 minute to completely dissolve the ingredients, not autoclaved. Cooled to 45-50°C and poured into sterile petri plates, allowed to solidify and kept over night in incubator at 37°C for sterility checking. Then plates were stored at 4ºC.

Kaper's slant

Glysate Peptone	5.0 g
Yeast extract	3.0 g
Tryptone	10.0 g
Sodium chloride	30.0 g
Sucrose	20.0 g
Lactose	20.0 g
Mannitol	1.0 g
Arginine hydrochloride	5.0 g
Sodium thiosulphate	0.3 g
Ferric Ammonium Citrate	0.5 g
Bromo cresol purple	0.04 g

Agar	20.0 g
D/W upto	1000 ml

pH adjusted to 6.7 with HCl

Ingredients were mixed properly by mild heating, then 5 ml evenly distributed in 15 ml tubes, sterilized by autoclaving at 15 psi for 15 minutes and kept in slant position to solidify and was stored at 4ºC.

LB (Luria-Bertani) broth

Pancreatic Digest of Casein	10.0 g
Yeast Extract	5.0 g
Sodium Chloride	30.0 g
Distilled water upto	1000 ml

Ingredients were mixed properly by mild heating in a conical flask. 5 ml of the mixture was evenly distributed 15 ml capacity test tubes. Tubes were sterilized by autoclaving at 121ºC, 15 psi for 15 minutes and was stored at 4ºC.

LB agar

Bacteriological Agar (1.5%) in LB medium

Sterilized by autoclaving at 15 psi for 15 min. The media allowed to cool to 45°C - 50°C, mixed well and poured to sterile petri plates. The plates were allowed for solidification at room temperature followed by overnight incubation at 37°C for sterility checking and then stored at 4°C.

Nutrient broth

Peptone	5.0 g
Beef extract	1.5 g
Sodium chloride	3.0 g
Yeast Extract	1.5 g
Distilled water upto	1000 ml

pH 6.8 ± 0.2

The ingredients were dissolved in distilled water by slight heating and then sterilized by autoclaving after pH adjustment.

Nutrient agar

The nutrient agar was prepared by adding 1.5 g agar in 100 ml nutrient broth (prepared above) and was autoclaved. The media allowed to cool to 45°C -50°C, mixed well and poured to sterile petri plates. The plates were allowed for solidification at room temperature followed by overnight incubation at 37°C for sterility checking and then stored at 4°C.

Nutrient agar stab

Nutrient Broth	0.8 g
Bacteriological agar	1.0 g
NaCl	3.0 g
D/W upto	100 ml

Ingredients were mixed properly by mild heating, evenly distributed 3 ml in test tubes (10-12 ml capacity), sterilized by autoclaving at 15 psi for 15 minutes and were stored at 4°C.

APPENDIX-II

REAGENTS FOR AGAROSE GEL ELECTROPHORESIS

0.5M EDTA (pH 8)

Disodium EDTA-$2H_2O$	186.1 g
Distilled water upto	1000 ml
Adjusted the pH with NaOH	

Tris-acetate- EDTA (TAE) buffer 50X

Tris base	242.0 g
Glacial acetic acid	57.1 ml
0.5 M EDTA (pH 8.0)	100 ml

Distilled water was added to make the final volume upto 100 ml. Sterelized by autoclaving. A working solution of 1X was used.

Ethidium bromide (0.5µg/ml)

Ethidium bromide	5 µg
Nuclease free water	10 ml

Ingredients were mixed properly in 15ml tube and tube was stored away from light.

APPENDIX-III

MEDIA FOR AGAR WELL DIFFUSION ASSAY

Muller Hinton Agar

Beef Extract	2.0 g
Acid hydrolysate of Casein	7.5 g
Starch	1.5 g
Agar	17.0 g

Final pH (at 25°C) 7.3±0.1

38 grams suspended in 1000 ml distilled water. Heated to boiling to dissolve the medium completely. Sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes. The media allowed to cool to 45°C -50°C, mixed well and poured to sterile petri plates. The plates were allowed for solidification at room temperature and kept in incubator at 37°C overnight for sterility checking and then stored at 4°C.

MRS Broth

Proteose peptone	10.0 g
Beef extract	10.0 g
Yeast extract	5.0 g
Dextrose	20.0 g
Polysorbate 80	1.0 g
Ammonium citrate	2.0 g
Sodium acetate	5.0 g
Magnesium sulphate	0.1 g
Manganese sulphate	0.05 g
Dipotassium phosphate	2.0 g

The ingredients were dissolved in 1000 ml distilled water and the pH was adjusted at 6.5±2 and then medium was sterilized at 121°C for 15 minutes, under 15lb pressure.

MRS Agar

MRS agar was prepared by adding 1.5% agar in MRS broth (prepared above) and was then sterilized by autoclaving at 121°C for 15 minutes, under 15 lb pressure. The media allowed to cool to 45°C-50°C, mixed well and poured to sterile petridishes. The plates were kept at room tempetature for solidification followed by overnight incubation at 37°C for sterility checking. Finally plates were stored at 4°C

1% Agar

Bacto agar	1.0 g
Distilled water	100 ml

Ingredients were mixed properly by mild heating, sterilized by autoclaving at 121°C, 15 psi for 15 minutes and was stored at 4°C.

I N NaOH

Sodium hydroxide pellets	4.0 g
Distilled water	100 ml

4 gm of sodium hydroxide accurately weighed and properly mixed in 100 ml of distilled water to obtain 1 Normal NaOH.

Glucose phosphate broth

Peptone	0.5 g
K_2HPO_4	0.5 g
Glucose	0.5 g
Distilled water upto	100 ml

The ingredients were dissolved in 100 ml distilled water. The pH was adjusted to 7.5 and the medium was dispensed in 5 ml amounts in test tubes. Test tubes were sterilized by autoclaving.

VITAE

Name	:	**Dr. S. Parthasarathy**
Fathers Name	:	Mr. Sudhansu Bhusan Swain
Mothers Name	:	Mrs. Tarulata Swain
Date of birth	:	07.07.1983
Gender	:	Male
Marital Status	:	Married
Permanent Address	:	AT- Byree (Swain sahi) P.O- Byree Dist- Jajpur Pin- 754082, Odisha
E-mail	:	parthasarathyvet@gmail.com
Nationality	:	Indian
Mother Tongue	:	Odia

Educational Qualifications

S. NO.	Degree	O.G.P.A. Secured	University
1	B.V.Sc & A.H	8.14/10	OUAT
2.	M.V.Sc. (VPH)	8.675/10	IVRI

Member of Societies:

1. Member of Veterinary Council of India.
2. Member of Odisha Veterinary Council.
3. Member of Indian Association of Veterinary Public Health Specialists.

Achievements:

1. NCC 'B' certificate
2. Qualified UGC NET