"DETECTION AND CONTROL OF ANTIMICROBIAL RESISTANCE IN INLAND SALINE SHRIMP CULTURE AREAS WITH SPECIAL REFERENCE TO *Vibrio parahaemolyticus*"

Thesis

Submitted to the Guru Angad Dev Veterinary and Animal Sciences University in partial fulfillment of the requirements for the degree of

MASTER OF FISHERIES SCIENCE
in
AQUATIC ENVIRONMENT MANAGEMENT
(Minor Subject: Aquaculture)

By

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2022

CERTIFICATE – I

This is to certify that the thesis entitled, **"DETECTION AND CONTROL OF ANTIMICROBIAL RESISTANCE IN INLAND SALINE SHRIMP CULTURE AREAS WITH SPECIAL REFERENCE TO *Vibrio parahaemolyticus*"** submitted for the degree of **M.F.Sc.,** in the subject of **Aquatic Environment Management** (Minor Subject: **Aquaculture**) of the Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, is a bonafide research work carried out by **Prapti Sudan (L-2019-F-08-M)** under my supervision and that no part of this thesis has been submitted for any other degree.

The assistance and help received during the course of investigation have been fully acknowledged.

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CERTIFICATE – II

This is to certify that the thesis entitled, **"DETECTION AND CONTROL OF ANTIMICROBIAL RESISTANCE IN INLAND SALINE SHRIMP CULTURE AREAS WITH SPECIAL REFERENCE TO *Vibrio parahaemolyticus*"** submitted by **Prapti Sudan (L-2019-F-08-M)** to the Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, in partial fulfillment of the requirements for the degree of **M.F.Sc.,** in the subject of **Aquatic Environment Management** (Minor Subject: **Aquaculture**) has been approved by the Student's Advisory Committee after an oral examination on the same, in collaboration with an external examiner.

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ABSTRACT

The present study was conducted to evaluate the potential pathogenicity and antimicrobial resistance of *Vibrio* spp., including *V. parahaemolyticus,* in inland saline shrimp culture farms. Among 109 *Vibrio* isolates obtained from 109 shrimp/water samples, 48 isolates were confirmed as *V. parahaemolyticus*. During PCR screening of virulence-associated genes, 32 and 6 isolates of *V. parahaemolyticus* were found positive for *tlh* and *trh* genes, respectively. No other virulence-associated gene was detected in any other isolates. Antimicrobial susceptibility testing against 12 antibiotics revealed highest resistance against cefotaxime (91.6%), ampicillin and amoxiclav (90.9%) in all *Vibrio* and *V. parahaemolyticus* isolates. Also, high (>50%) resistance was observed against ampicillin, amoxiclav, cefotaxime and ceftadizime. The multiple antibiotic resistance (MAR) index values of all *Vibrio* isolates ranged from 0.00 to 0.75, whereas the MAR index values of *V. parahaemolyticus* isolates ranged from 0.00 to 0.58. Among all the *Vibrio* and *V. parahaemolyticus* isolates, 87.4% and 86.3% isolates, respectively were resistant to 3 or more antibiotics. No significant difference in the antibiotic resistance patterns was observed between water and shrimp samples. These results indicate that multidrug resistant vibrios could act as the reservoirs of antibiotic resistance genes in the shrimp culture environment. Twelve previously isolated *V. parahaemolyticus* phages showed very limited lytic activity against *V. parahaemolyticus* isolates from the present study. These phage host range findings indicate high diversity among the stains of *V. parahaemolyticus* prevalent in inland saline shrimp culture farms. To the best of our knowledge, this is the first study evaluating the antimicrobial resistance of potentially pathogenic vibrios in non-coastal inland saline shrimp farms as well as their susceptibility to various phages. The presence of multiple drug resistance in *Vibrio* isolates including *V. parahaemolyticus* emphasize on the need for their routine monitoring in rapidly expanding aquaculture areas for risk assessment and mitigation.

Keywords: Shrimp culture, *Vibrio* spp.; *Vibrio parahaemolyticus*; virulence genes; antimicrobial resistance; phage

Signature of Major Advisor **Signature of the Student**

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LIST OF ABBREVIATIONS

µg	:	Microgram
µl	:	Microlitre
µM	:	Micromolar
AHPND	:	Acute hepatopancreatic necrosis disease
APS	:	Alternative protein Source
APW	:	Alkaline peptone water
BMPs	:	Best management Practices
bp	:	basepair(s)
Ca^{+2}	:	Calcium ion
cfu	:	Colony forming unit
CSSRI	:	Central Soil Salinity Research Institute
CV	:	CHROM agar *Vibrio*
CYMA	:	Casein yeast magnesium agar
DNA	:	Deoxyribonucleic acid
dsDNA	:	Double strand deoxyribonucleic acid
dsRNA	:	Double strand ribonucleic acid
EDTA	:	Ethylene diamine tetra acetic acid
ELISA	:	Enzyme-linked immunosorbent assay
EMS	:	Early mortality syndrome
EPA	:	Environmental protection Agency
FAO	:	Food and agricultural organization
FDA	:	Food and Drug Administration
g	:	Gram(s)
GADVASU	:	Guru Angad Dev Veterinary and Animal Sciences University
GRAS	:	Generally recognized as safe
HCl	:	Hydro chloric acid
hr(s)	:	Hour(s)
ICTV	:	International committee on taxonomy of viruses
LAMP	:	Loop mediated isothermal amplification
LB	:	Luria bertani broth
M	:	Minute(s)
M	:	Molar

MAM	:	Multivalent Adhesion Molecules
mg	:	Magnesium ion
Mg I^{-1}	:	Milligram per litre
Mg^{2+}	:	Magnesium ion
Ml	:	Milliliter
mm	:	Millimeter
mM	:	Millimolar
MOI		Multiplicity of infection
MPEDA	:	Marine products exports development authority
MTCC	:	Microbial type culture collection microgram
NaCl	:	Sodium chloride
NCBI	:	National center for biotechnology information
NFW	:	Nuclease free water
ng	:	Nanogram
NGS	:	Next-generation-sequencing
nm	:	Nanometer
^{o}C	:	Celsius
OCED	:	Organization for economic co-operation and development
OD	:	Optical density
PBS	:	Phosphate buffer saline
PCR	:	Polymerase chain reaction
pH	:	Power of hydrogen
Pir	:	Photorhabdus insect-related
Ppm	:	Parts per million
QC	:	Quality check
RPM	:	Rotations per minute
RT	:	Room Temperature
S	:	Second
scFvs	:	Single-chain variable antibody fragments
SPB	:	Salt polymyxin Broth
SPF	:	Specific Pathogen Free
ssDNA	:	Single strand deoxyribonucleic acid
ssRNA	:	Single strand ribonucleic acid

ST	:	Sodium taurocholate
TAE	:	Tris acetate EDTA
TCBS	:	Thiosulfate citrate bile salts sucrose agar
TDH	:	Thermostable direct hemolysin
TLH	:	Thermolabile hemolysin
TRH	:	Thermostable related hemolysin
TSA	:	Tryptone soya agar
TSB	:	Tryptone soya broth
U	:	Units
USA	:	United States of America
UV	:	Ultraviolet
VopS	:	*Vibrio parahaemolyticus* serotype
w/v	:	Weight by volume
WHO	:	World Health Organization

CHAPTER I

INTRODUCTION

The global economic and political stability is strongly dependent on the access to nutritious, affordable and safe food for the rapidly growing populations. Besides other food producing sectors, capture and culture fisheries productions have been playing the important roles in global food and nutritional security. However, due to almost stagnation of capture fisheries production in recent years, onus has shifted on aquaculture sector to meet the rapidly increasing food demands. Consequently, due to horizontal and vertical expansion, the global aquaculture production has increased by >500% between 1990 and 2018; and at the current production value of 82.1 million tonnes, its current contribution to global fish production is ~46% (FAO, 2020). Among various aquaculture species, white leg shrimp *Litopenaeus vannamei*, henceforth referred as vannamei shrimp, is the important one. With total production of 4.97 million tonnes, vannamei shrimp constitutes 6.1% and >10% of global aquaculture in terms of quantity and trade value, respectively (FAO, 2020). In India too, shrimp is the major aquaculture commodity. In year 2019-20, frozen shrimp was the largest contributor to the India's total fisheries export earnings of Rs. 46,662.85 crore, both in terms of quantity (51%) and trade value (73%) (Handbook of Fisheries Statistics, 2020). Due to high global demand, export potential and low salinity tolerance of vannamei shrimp, inland saline areas, previously considered as wastelands, have emerged as attractive destinations for shrimp farming. Besides the diverse freshwater aquatic resources, Punjab also has about 0.15 million ha salt affected and waterlogged lands in six South-West districts of Fazilka, Ferozepur, Sri Muktsar Sahib, Bhatinda, Faridkot and Mansa. Due to consistent efforts of various stakeholders, the area under shrimp farming in Punjab has rapidly increased to approximately 350 acres in last five years (CSSRI, 2010; Singh et al., 2019; Singh et al., 2021).

Despite the rapid growth, frequent outbreaks of devastating diseases often threaten the growth/sustainability of aquaculture industry, and shrimp farming is no exception to it. Due to lack of adaptive immune system, shrimp is prone to >20 types of viral, bacterial, fungal and parasitic diseases. Because of high input cost and disease risk, shrimp farming is considered a "risky business", in which the failure of

even one crop may lead to severe economic setback for the farmer (Joffre et al., 2018). Though viral diseases constitute the most serious problem in shrimp culture, the bacterial infections caused by *Vibrio* species are also the important cause of mortalities. These Gram-negative, rod-shaped *Vibrio* species (family *Vibrionaceae*) are ubiquitous in marine, estuarine and freshwater aquatic systems, worldwide. Among various shrimp pathogenic *Vibrio* species viz. *V. harveyi*, *V. fluvialis*, *V. parahaemolyticus*, *V. anguillarum*, *V. damsela* and *V. vulnificus* are the important ones. *V. parahaemolyticus* carrying thermostable direct hemolysin (*tdh*) and/or thermostable direct hemolysin-related hemolysin (*trh*) genes has often been found as the causative agent of acute gastroenteritis in humans resulting from the consumption of poor quality, undercooked or raw seafood (Tyagi et al., 2009). Recently, one strain of *V. parahaemolyticus* carrying a plasmid encoding for *PirA* and *PirB* toxins has also been found responsible for a devastating shrimp early mortality syndrome (EMS), also known as Acute Hepatopancreatic Necrosis Disease (AHPND) (Tran et al., 2013). Losses due to AHPND have significantly increased from reported value of approximately US$ 26 million in 2015 to the present estimated value of billions of US$ (Shinn et al., 2018). Though *V. parahaemolyticus* is primarily a halophile bacterium, and natural inhabitant of estuarine and coastal marine environments, its existence in low salinity inland saline areas has also been reported recently (Singh et al., 2018).

In addition to prophylactic measures such as maintenance of optimum culture conditions, probiotics and immunostimulants, antibiotics often become the last resort for farm/hatchery personnel to prevent staggering production losses. Indiscriminate use of antibiotics in aquaculture without following the recommended withdrawal periods, not only leads to the emergence of antibiotic resistant bacteria but also results in occurrence of antibiotic residues in fishery products (Cabello, 2006). According to a report from Organization for Economic Co-operation and Development (OCED) released during November 2018, antimicrobial resistance (AMR) will cost almost 2.4 billion lives in Europe, North America and Australia within next 30 years with an annual economic cost of US$ 3.5 billion (Hofer, 2019). Though not much information about developing countries like India is available, the report also stated that resistance rates in low- and middle-income countries are between 40-60% (Hofer, 2019). Moreover, "hammer like" like approach of antibiotics kills even the beneficial

microbiota of host and environment, thus causing the microbial disturbance in the culture system (Cabello, 2006; Igbinosa, 2016). Similar to humans and terrestrial animals, maintenance of stable microbiota in aquatic organisms and the environment is very much crucial. Thus, there an urgent need of the alternative methods for the control of potentially pathogenic antibiotic resistant bacteria in the aquaculture environment.

Among the various strategies to combat antibiotic resistance bacterial pathogens, bacteriophage (or phage) based innovations constitute a promising solution. Due to their specificity, phages kill only target bacteria without affecting the other beneficial microbiota of the habitat. Moreover, phages are naturally present and remarkably stable in various environments (Rai et al., 2019; Rai et al., 2020). Due to rapidly spreading AMR, phage application has been suggested to control the flow of antibiotic resistance in the environment by targeted killing of multi-drug resistant bacteria (Parmar et al., 2017). Phage therapy has also been successfully applied to control the pathogenic members of *Vibrio* species in the aquaculture environment (Vinod et al., 2006; Jun et al., 2018).

As, shrimp culture in inland saline aquaculture areas of Punjab is expanding at rapid rate, there is high risk of emergence of potentially pathogenic multi-drug resistant *Vibrio*. Thus, the proposed study was designed to use the *V. parahaemolyticus* as candidate bacterium to assess the current status of antibiotic resistance in inland saline shrimp culture farms of Punjab. Further, the lytic efficacy of previously isolated phages (available with Department of Aquatic Environment, College of Fisheries) (Dubey et al., 2021) against antibiotic resistant *V. parahaemolyticus* isolates, detected in the present study, was also tested. Below are the specific objectives of the present study:

- Isolation and identification of *Vibrio parahaemolyticus* from inland saline shrimp culture farms.
- Molecular characterization of virulence and antimicrobial susceptibility testing of *V. parahaemolyticus* isolates.
- Evaluation of phage lytic activity against antimicrobial resistant *V. parahaemolyticus* isolates.

CHAPTER-II

REVIEW OF LITERATURE

2.1 Significance of Aquaculture

Fisheries sector has been playing a crucial role in food security, livelihood, employment generation and social development in developed and developing countries. The contribution of fisheries sector in global food and nutritional security could be understood from the fact that between year 1961 and 2017, the average annual increase of 3.1% in global fish consumption has been higher than other foods of animal origin. In year 2018, 87.6% of total global fish production of 178.5 million tonnes was utilized for human consumption. At the per capita consumption of 20.5 kg, fish account for 17% and 7% of animal proteins and all proteins consumed across the globe, respectively (FAO, 2020). Fish is the primary source of omega-3 fatty acids in the human diet. Omega-3 fatty acids are critical nutrients for normal brain and eye development of infants, and have preventive roles in a number of human illnesses, such as cardiovascular disease, lupus, depression, and other mental illnesses (Pradeepkiran, 2019). Fish is also an important source of essential amino acids, vitamins (particularly A, B and D) and minerals such as iron, calcium, zinc and selenium. This unique nutritional composition makes fish a valuable source for healthy dietary diversification, even in relatively small quantities.

Besides their contribution in global food and nutritional security, fisheries and aquaculture activities are also important for the development and improvement of fisheries resources and revitalization of ecosystems (Prabu et al., 2017). Besides, these activities also play important roles in social and economic development through the use of unutilized and underutilized resources (Jayasankar, 2018). During 2018, the total value of global fish exports was US$ 164.1 billion (FAO, 2020). Due to almost stagnation of capture fisheries production, recently aquaculture sector has emerged as major driving force behind the overall growth of fisheries sector. For example, between year 1986 and 2018, capture fisheries production only increased marginally from 86 .9 million tonnes to 96.4 million tonnes. On the other hand, aquaculture production showed the rapidly increased from 14.9 million tonnes to 82.1 million tonnes within the same period (FAO, 2020).

With total fish production of 14.16 million tonnes during the year 2019-20, India currently ranks at 4th and 2nd spot in global capture fisheries and aquaculture production, respectively. During 2018-19, the contribution of fisheries sector in overall Indian economy and agriculture sector economy was 1.24% and 7.28%, respectively. India also exported 1.29 million tonnes of fishery products, worth Rs. 46,662.85 crore, during year 2019-20 (FAO, 2020; Handbook of Fisheries Statistics, 2020). With a national per capita consumption of 11 kg, fish is the important component of the domestic food security in India, and demand for fishery products is further expected to increase (Jayasankar, 2018).

2.2 Indian shrimp culture scenario

Though global as well Indian aquaculture scenario is dominated by the finfish in terms of volumes, shrimp is considered as one of the important aquaculture commodities due export potential and overall trade value (de Lacerda et al., 2021; Naylor et al., 2021). At present, contribution of shrimp culture in global aquaculture is >6.1% in terms of volumes, whereas the contribution in terms of value is >10% (FAO, 2020). Out of total fisheries export of India worth Rs. 46,662.85 crore, the contribution of frozen shrimp export was Rs. 34,152.03 Crore (Fig. 1 a & b, Table 1). With imported quantity of 2,85,904 tonnes, USA is the largest importer of frozen shrimp from India, followed by China (1,45,710 tonnes), European Union (74,035 tonnes) and Japan (38,961 tonnes) (Handbook of Fisheries Statistics, 2020).

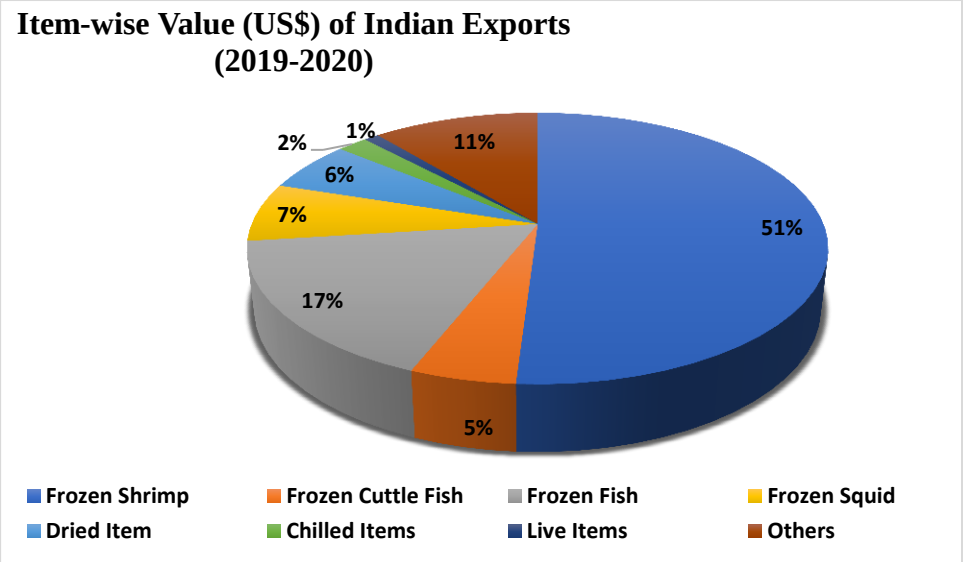

Fig. 1(a): Graphical representation of overall Indian fisheries exports in terms of value

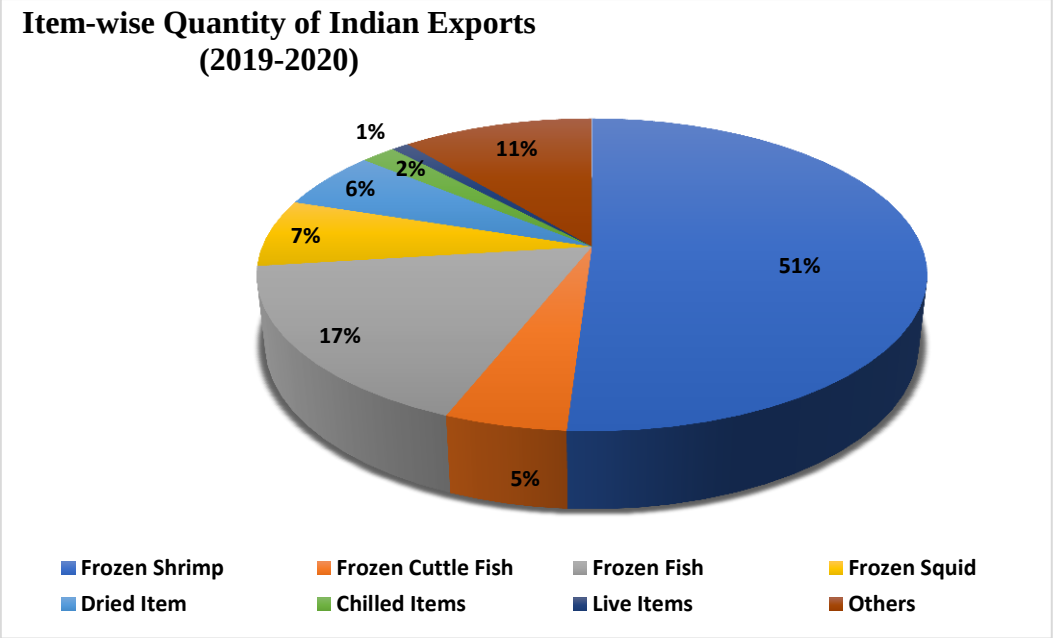

Fig. 1(b): Graphical representation of overall Indian fisheries exports in terms of quantity

Table 1: Current status and annual growth of Indian frozen shrimp export

Export criteria	Share in overall exports (%)	FY 2018-2019	FY 2019-2020	Annual growth (%)
Quantity (tonnes):	50.58	6,14,145	6,52,253	6.20
Value (Rs. Crore):	73.19	31,800.51	34,152.03	7.39
Value (US$ million)	73.21	4,610.59	4,88.912	6.04
Average price (US$/kg)		7.51	7.50	-0.15

Due to continuously increasing demand, high export potential and consequent better economic returns for the farmers, shrimp culture in India is expanding at a very rapid rate in India. Between year 2009-10 and 2020-21, the estimated area under shrimp culture in India has increased by 62.6% from 1,02,542 ha to 1,66,722 ha. Within the same period, the estimated shrimp production has increased 8.64 times from 97,650 tonnes to 8,43,361 tonnes. Being a brackishwater species, shrimp farming in India is mostly concentrated in coastal regions of 9 states from West Bengal to Gujarat. However, inland saline areas of Punjab, Haryana, Uttar Pradesh and Rajasthan are fast emerging as attractive destinations for shrimp farming (to be discussed later) (ICAR-CIBA, 2020; MPEDA, 2021).

Similar to the global scenario, tiger shrimp (*Penaeus monodon*) had been the dominant cultured species in India throughout the 1990s as well as in the first decade of 21st century. Due of fear of disease outbreaks, decreased growth rate in captive lines, difficulties in captive breeding and production of specific pathogen free (SPF) post-larvae (PL), and consequent poor economic returns, the culture of tiger shrimp has been gradually declining throughout the world (Chris loew, 2017; Nisar et al., 2021). In the last decade, the estimated area under monodon farming in India has steadily declined from the peak of 1,14,370 ha in year 2011-12 to 58,196 ha in year 2020-21. Since its introduction in year 2010, white leg shrimp (*Litopenaeus vannamei*), henceforth referred as vannamei shrimp, has emerged as major candidate species for shrimp farming India. At present, with an estimated culture area of 1,08,526 ha, vannamei shrimp constitutes >95% of total shrimp production in India (MPEDA, 2021). Following factors have been playing important role in the dominance of vannamei shrimp in global as well as Indian shrimp farming scenario (Funge-smith & Briggs, 2002).

- In comparison to tiger shrimp, vannamei has higher growth rate. Under optimal conditions the growth rate can be as higher as 3 g/week.
- Vannamei shrimp can be cultured at high stocking density of 60-150 nos./m^2 (up to 400 nos./m^2) (Esparza-Leal et al., 2020).
- Due to wider range of salinity (0.5–45 ppt) and temperature tolerance (15-35 ^{O}C) (Wang et al., 2019), vannamei shrimp is more amenable to culture in low salinity inland saline areas with higher temperature fluctuations.
- The dietary protein requirement (20-35%) of vannamei shrimp is lower than the monodon shrimp (36-42%) (Lee & Lee, 2018).
- Due to gonad development under captive conditions, the domestication of vannamei brooders is relatively easy.
- Being an open thelycum species, the captive breeding of vannamei shrimp is relatively easy with higher larval survival rates.
- Though not proven conclusively, the vannamei shrimp is considered to be more resistant to white spot syndrome virus (WSSV) infection (Trang et al., 2019).

2.3 Inland saline shrimp culture in Punjab – Prospects and challenges

The geography of Punjab state can be clearly divided in two regions as per the hydrology and topography. The northern and central regions of the state consist of highly productive lands with plenty of underground freshwater. On the other hand, south-western region with underground saline water is largely unsuitable for any agricultural activity, and these areas are usually referred as the "wastelands". Out of total geographical area of 5 million ha, 60.2% area is semi-arid. Nineteen percent of this semi-arid area has poor underground water with salinity issues. This area has saline (22%), sodic (54%) and saline-sodic (24%) soils (Jain et al., 2002; Kumar & Sharma, 2020). Due to unique north-east to south-west drainage topography of Punjab, the south-west low-lying and poorly drained regions have high accumulation of salts in soils. Poor rainfall and leaching of salt also contribute to the soil salinization (Ansal & Singh, 2019; Kumar & Sharma, 2020).

Salt affects 1.51 million hectares of land in the south-west districts of Punjab, including Sri Muktsar Sahib, Ferozepur, Fazilka, Faridkot, Bathinda, and Mansa (CSSRI, 2010). The saline underground water in these salt affected districts is neither suitable for human consumption, nor for any kind of agricultural or industrial use. Non-extraction of underground water in these low-lying poorly drained areas is also resulting in annual rise of water table at the alarming rate of 15-20 cm (Saoud et al., 2003). Soil salinization and consequent lack of any profitable agricultural activity is the major hinderance in social and economic development of these regions. Measures to control soil salinization by pumping out the groundwater and evapotranspir*a*tion are quite costly, and beyond the reach of poor and marginal farmers (Kumar & Sharma, 2020). In the current scenario where, conventional agriculture has less or no scope, aquaculture is the best option. In the past, significant efforts have been made to reclaim and utilize these inland saline areas for aquaculture. From freshwater carps to brackishwater finfish (mullets, milk fish, pearl spot and sea bass) and shrimp have been successfully cultured in inland saline waters (Pathak et al., 2013).

In Punjab, the current success of inland saline shrimp in south-west districts is the culmination of efforts of several agencies viz., The College of Fisheries at Guru Angad Dev Veterinary and Animal Sciences University (GADVASU), Ludhiana, Punjab State Fisheries Department and the Regional Centre of ICAR- Central Institute

of Fisheries Education (ICAR-CIFE), Rohtak, Haryana. In the year 2007, the College of Fisheries, GADVASU took on the challenge of developing the salt-affected waterlogged areas of Punjab through aquaculture with funding support from the Punjab State Farmers Commission (2007-2010). Based on the success of above project, the college was awarded the ICAR's prestigious 'Niche Area of Excellence' programme – Inland Aquaculture in Punjab. During the initial stage, the focus was on culture of freshwater carps in low saline (<5 ppt) areas. During the extensive off-campus research trials in village Shajrana (District Fazilka), average annual productivity of 4.75 tonne/ha was achieved through technological interventions with respect to water quality, stocking density, species combinations and integration strategies. These technological interventions enabled the poor farmers to earn up to Rs. 1,50,000/ha annually from these otherwise unproductive lands. GADVASU's success in developing and demonstrating aquaculture technologies for inland salt affected waterlogged waste lands in the State's south west districts has not only inspired villagers to start fish farming on their zero-income lands, but it has also enabled the farmers to get substantial financial assistance from the State Government for the construction of aquaculture units. Area under aquaculture increased from 20 acres in 2012-13 to 45 ½ acres in 2013-14, and further to 69 acres in 2014-15 and 85 acres in 2015-16, in village Shajrana itself (Dhawan et al., 2010; Ansal et al., 2013; Ansal et al., 2016; Dhawan et al., 2016).

After the successful demonstration of carp farming in low salinity areas, the preliminary trial of vannamei shrimp farming was conducted in village Shajrana in district Fazilka in year 2013. However, the first commercial farming trial of vannamei shrimp was conducted year 2014 in Painchanwali village of district Fazilka by a team of scientists from College of Fisheries, GADVASU, Ludhiana. This unique trial with very successful harvest created a positive impact on the farmers in the regions as well as on the State government authorities. Impressed with the success of Painchanwali village trials, the State Fisheries Department, in year 2016, conducted another vannamei culture demonstration project in Rattakhera village of district Sri Muktsar Sahib with technical support of College of Fisheries, GADVASU and regional center of ICAR-CIFE, Rohtak. This commercial trial was also an absolute success. Buoyed by these successes Government of Punjab announced 50% subsidy on shrimp farming with quantum to subsidy up to 90% for limited period in selected villages (Ratta

Khera, Tappa Khera, Fethepur Mania, Ghagga, Bahadur Khera and Shajrana) of district Shri Muktsar Sahib and Fazilka. Due to intensive efforts of all stakeholders and high entrepreneurial spirit of farmers, shrimp culture area in Punjab has rapidly increased from one acre in year 2016-17 to 410 acres in year 2019-2021. During the year 2021, the area under shrimp culture is expected to be >500 acres (Ansal & Singh, 2019; Sahib, 2019; Singh et al., 2021).

Shrimp farming has turned wastelands into gold mines, with productivity ranging from 8 to 10 tonnes per ha per crop cycle of 4 months, and a corresponding net profit of US$14,345 to $17,216 (INR 1-1.2 million), especially for large farmers with relatively stronger economic backgrounds and higher investment and risk-taking capacity. The winter season (Nov-Dec to Feb-Mar) limits shrimp culture to seven to eight months in non-coastal northern states, but two crops of shrimp can be produced and harvested in Punjab between April and November (before the onset of winter), making it highly profitable in comparison to any other agriculture and livestock enterprise (Ansal & Singh, 2019).

In spite of rapid growth of shrimp farming in Punjab and its tremendous potential in the socio-economic development of rural folks, the concerns regarding the long-term sustainability cannot be overlooked. It is well-known that shrimp farming is a “high cost, high risk and high profit” enterprise. Several threats related to poor seed quality, biosecurity and disease outbreaks may affect the profitability, which in turn may dampen the growth of shrimp farming by demotivating the prospective farmers. Due to their unique geography and water sources, inland saline areas are considered as “virgin” in terms of pathogen/disease prevalence. Thus, proper implementation of is biosecurity measures is very much important to minimize the incidences of potential disease outbreaks in these areas (Allan et al., 2009; Debroy et al., 2020).

At present, shrimp farmers of Punjab are dependent on coastal states for important inputs (seed and feed) required for farming. Due to poor quality inputs and inadequate management practices, incidences of diseases outbreaks have recently been reported from inland saline shrimp culture farms of Punjab (Kumar et al., 2011). Thus, there is urgent need of regulatory authority for policy formulation and monitoring to support the shrimp culture in inland areas. Besides, awareness generation and pathogen detection/disease surveillance measures should be

implemented to ensure the long-term sustainability and economic viability of shrimp farming in inland saline areas of Punjab.

2.4 Economic significance of diseases in shrimp aquaculture

Due to culture intensification and consequent environmental degradation, aquaculture has recently become highly susceptible to frequent disease outbreaks, shrimp farming is no exception to it (Karunasagar & Ababouch, 2012). Globally as well as in India, frequent disease outbreaks have been playing havoc in various aquaculture sectors. According to an old report from World Bank, global economic impact of disease outbreak in aquaculture was estimated to be approximately US$ 6 billion per annum. The same study also pegged the economic losses of approximately US$ 10 billion to shrimp farming due to disease outbreaks since 1990 (WorldBank, 2014). In India too, economic losses of Rs. 1022.1 Crore were estimated annually to shrimp farming due to frequent disease outbreaks. However, recent studies show that current scale of economic loss due to disease outbreaks in shrimp farming is actually much higher. For example, in China, Maxico, Thailand, Vietnam and Malaysia, the economic losses in shrimp farming, between year 2010 and 2016, due to a single acute hepatopancreatic necrosis disease (AHPND) itself were US$ 44 billion (Tang & Bondad-Reantaso, 2019). A recent study from ICAR-Central Institute of Brackishwater (ICAR-CIBA) estimated 49% probability of infectious disease outbreaks in Indian shrimp farming. The annual shrimp production loss was estimated to be 0.21 million tonnes worth US$ 1.02 billion. Besides, loss of 1.65 million man-days of employment worth approximately US$ 7.07 million was also estimated due to disease outbreaks in shrimp farming. Among all the shrimp pathogens, highest production loss (2.58 ± 0.32 tonnes/ha per crop) was reported due to WSSV followed by *Enterocytozoon hepatopenaei* (EHP) (1.80 ± 0.24 tonnes/ha per crop). Among the bacterial diseases, probability of occurrence of *Vibrio*sis was determined to be 2% with estimated production loss of 0.97 ± 0.42 tonnes/ha per crop (Patil et al., 2021). Above studies clearly show the impact of disease outbreaks on shrimp farming.

2.5 Important *Vibrio* spp. of shrimp and human health significance associated with shrimp farming

In aquaculture systems, diseases can be caused by both indigenous and introduced pathogens in aquaculture systems, and their occurrence is determined by their ecology, source, and survival (Thompson et al., 2005). Large numbers of aquatic bacterial pathogens are opportunistic pathogens and these are generally natural microbiota of the aquatic environment. On the other hand, human pathogenic bacteria are not generally found in aquatic environments, and their presence indicates that aquatic resources have been contaminated. Once inside the aquatic environment, these pathogenic bacteria survive, grow, and accumulate through direct or indirect contact. Pathogen abundance in the environment is further increased through activities such as bio-concentration, faecal contamination, and the creation of growth-promoting conditions. They are frequently bacterial species that are facultatively pathogenic for both aquatic animals and humans, and they can be isolated from aquatic animals with no visible symptoms of disease (Novotny et al., 2004). In marine environments, filter-feeding shellfish are effective bio-concentrators of small particles and pathogenic contaminants. Shellfish samples have been found to contain marine contaminants such as enteric bacteria (Watkins et al., 1992), *Campylobacter* spp. (Abeyta et al., 1993; Endtz et al., 1997) and *Listeria* spp. (Colburn et al., 2011) as well as potentially pathogenic indigenous flora diversity, sources, and detection of 37 human bacterial pathogens, including *Vibrio* spp. (Olafsen et al., 1993). Thus, it is not surprising that shellfish have long been recognized as a potential source for marine-acquired illness. A surprising number of pathogens have been identified in marine environments, and the likelihood of transmission to humans is linked to factors that influence their distribution. Pathogen transmission to humans through marine environments is most commonly accomplished through the consumption of contaminated fish products, but it can also occur through other means such as water contact or exposure to aerosols and zoonoses. The risk of contracting human diseases through aquatic environments is determined by a number of factors, including the susceptibility of the human host, the degree of exposure to a pathogen population, and the virulence of the pathogenic agent. Infections can occur in healthy people as well (Novoslavskij et al., 2016).

Though viral diseases are the most serious issue in shrimp culture, bacterial infections caused by *Vibrio* species are also the leading cause of death in shrimp

hatcheries and grow-out systems. Global climate change and rising temperatures have resulted in an increase in the frequency of *Vibrio*-associated infections (Roux et al., 2017). *Vibrio* spp. belonging to family *Vibrionaceae* under the class Gammaproteobacteria are autochthonous to diverse freshwater, brackishwater and marine environments; and these are ubiquitous in various types of aquafarming systems. Among 65 well-characterized heterotrophic species of *Vibrio*, at least 14 species (*Vibrio harveyi, V. parahaemolyticus*, *V. vulnificus*, *V. alginolyticus*, *V. fischeri V. anguillarum, and V. campbelli* etc.) are known shrimp pathogens (de Souza Valente & Wan, 2021). In shrimp culture these *Vibrio* pathogens cause a variety of diseases collectively known as '*Vibrio*sis' (de Souza Valente & Wan, 2021). At least, 11 members of genus *Vibrio* including *V. cholerae*, *V. parahaemolyticus*, *V. vulnificus* and *V. anguillarum* are also important human pathogens (Baker-Austin et al., 2018).

2.5.1 *Vibrio parahaemolyticus*

Similar to other members of family *Vibrionaceae, V. parahaemolyticus* is a Gram-negative, motile, curved rod-shaped bacterium. *Vibrio parahaemolyticus* is a human pathogen as well as an emerging shrimp. Being a halophile, *V. parahaemolyticus* is common in brackishwater and marine environments, and it is also an important part of microbiota of shrimp production systems (Gopal et al., 2005; Letchumanan et al., 2014). For the first time, Tsunesaburo Fujino identified *V. parahaemolyticus* from semidried sardine samples as the cause of a foodborne disease outbreak in Japan in 1950. The serotyping of *V. parahaemolyticus* has been done based on somatic (O) and capsular (K) antigens. Being a multiserotype bacterium, more than 12 O antigen and more than 70 types of K antigens have been reported in *V. parahaemolyticus*. Currently, more than 20 *V. parahaemolyticus* serovariants have been reported worldwide, including several pandemic strains (03:K6, 04:K68, 01:K25, and 01:KUT) (Nair et al., 2007; Wang et al., 2015).

Among various aquatic organisms, *V. parahaemolyticus* is most commonly associated with shellfish such as shrimps, crabs, lobsters and oysters etc. These shellfish accumulate higher concentrations of *V. parahaemolyticus* than the surrounding waters due to their filter-feeding habits. Furthermore, these shellfish are frequently consumed raw or undercooked, often resulting in foodborne infection with *V. parahaemolyticus* (Newton et al., 2012; Zarei et al., 2012). In tropical climates, *V.*

parahaemolyticus infection are usually reported in the summer season during the months of June to October. *V. parahaemolyticus* contaminated seafood is responsible for 20-30% of food poisoning cases in Japan and other Asian countries. In addition, it is the leading cause of seafood-associated human gastroenteritis in the United States. *V. parahaemolyticus* was linked to 1.3 percent to 23.9 percent of acute diarrheal cases in India (Pal et al., 1985; Pazhani et al., 2014). Severe abdominal pain with dysentery and vomiting, nausea, chills, fever, and watery stool are all common symptoms of illness. In severe cases, unconsciousness and even death may occur (Wang et al., 2015). In rare cases, wound infection, ear infection, or septicemia can be fatal to immunocompromised people (Zhang & Orth, 2013). Due to significance of foodborne disease risk due to *V. parahaemolyticus*, US-Food and Drug Administration (FDA) and the Environmental Protection Agency (EPA) have set the standard limits on maximum tolerance limits of *V. parahaemolyticus* in various fishery products. In fresh or frozen raw shellfish (clams, mussels, oysters, and scallops) the *V. parahaemolyticus* counts should not be > $1x10^4$/g. On the other hand, ready to eat fresh or frozen fishery products, requiring minimum cooking by the consumers, the *V. parahaemolyticus* counts should be less than 30/g (FDA, 2011). Recently, a strain of *V. parahaemolyticus* carrying a plasmid encoding for *PirA* and *PirB* toxins was discovered to be the cause of a devastating shrimp early mortality syndrome (EMS), also known as acute hepatopancreatic necrosis disease (AHPND) (Tran et al., 2013; Shinn et al., 2018).

2.5.2. Isolation and identification of *V. parahaemolyticus*

Although the isolation and identification of *V. parahaemolyticus* from clinical samples is well standardized, there are some ambiguities regarding the protocols to be followed in the case of fishery products and other food samples. After biochemical enrichment, isolation, and identification, highly sensitive and rapid molecular and immunological methods are available for species and serotype level confirmations (Motarjemi et al., 2013).

In the case of food samples, the density of *V. parahaemolyticus* may be very low, and would need a selective enrichment step to improve the efficiency of subsequent isolation and identification steps. Being a halophilic and alkalophilic bacterium, *V. parahaemolyticus* prefers Na^+ and alkaline (pH >8.5) conditions due to

its origin in the natural marine environment. Thus, for selective enrichment, alkaline media (pH 8.6-9.4) with a NaCl concentrations varying from 1 to 7% is typically prepared. Other *Vibrio* selective agents, such as bile salts, sodium thiosulphate, sodium citrates, and colistin or polymyxin B antibiotics are also usually added to culture media for selective isolation of *V. parahaemolyticus* (Donovan & van Netten, 1995; Tyagi et al., 2009). Alkaline peptone water (APW) has been recommended by the FDA for the enrichment of all *Vibrio* species, including *V. parahaemolyticus*. The alkaline 8.5-9.0 pH of APW with high NaCl concentration inhibits the growth of other bacteria while allowing *Vibrio* to grow at very rapid rate. Sodium taurocholate (ST) broth, salt polymyxin broth (SPB), alternative protein source (APS) broth, glucose salt teepol broth, and salt colistin broth, in addition to APW, have been used for selective enrichment of *V. Parahaemolyticus.* The enrichment is typically performed for 6-8 hours at 37^{o}C after inoculation with sample, as longer incubation periods may result in the growth of non-specific bacteria resulting in poor results during further identification (Tyagi et al., 2009; Bisha et al., 2012; Letchumanan et al., 2014).

Thiosulfate Citrate Bile Salts Sucrose (TCBS) agar is the most commonly used plating media for isolation and presumptive identification of *V. parahaemolyticus* and other *Vibrio* spp. The selective components of TCBS agar (such as ox bile, NaCl, and alkaline pH) inhibit the growth of interfering Gram-positive bacteria. Furthermore, the sucrose/bromothymol blue system of TCBS agar aids in distinguishing sucrose-fermenting *Vibrio* like *V. harveyi* and *V. cholerae* from sucrose-negative *Vibrio* spp. like *V. parahaemolyticus*. On the TCBS agar, *V. parahaemolyticus* form opaque, round, green or bluish-green colonies of 2-3 mm diameter, whereas *V. harveyi* and *V. cholerae* form yellow colour colonies of similar morphology (Di Pinto et al., 2011; Bisha et al., 2012). However, practical issues arise when isolating *V. parahaemolyticus* on TCBS agar from seafood samples. Because TCBS is a general media for the isolation of all *Vibrio*, several other *Vibrio* spp. from seafood form yellow or green colonies on it, making the isolation of *V. parahaemolyticus* difficult. Thus, a new CHROMagar *Vibrio* (CV) agar media containing -galactosidase substrate was developed to distinguish ortho-nitrophenyl-galactoside-positive *V. parahaemolyticus* from other *Vibrio* spp. *V. parahaemolyticus* is easily distinguished from other *Vibrio* on CV agar due to its mauve colour colonies (Hara-Kudo et al., 2001). Wagatsuma agar with rabbit or human blood is another

medium used to detect thermostable direct hemolysin (TDH) producing *V. parahaemolyticus*. A clear halo (Kanagawa Phenomenon) is observed around the colonies of thermostable hemolysin producing *V. parahaemolyticus* strains using this method (Nishibuchi & Kaper, 1995). However, this media cannot detect the *V. parahaemolyticus* strains producing TDH-related hemolysin (TRH) (Su & Liu, 2007; Bisha et al., 2012).

Following the presumptive isolation, suspected *V. parahaemolyticus* colonies are routinely identified using biochemical testing. However, these tests are time consuming and laborious (Motarjemi et al., 2013). For the detection of *V. parahaemolyticus*, DNA-based molecular assays such as colony hybridization, PCR, real-time PCR, loop mediated isothermal amplification (LAMP), and others are now widely used. These methods are generally based on the detection of virulence-associated genes such as *tdh* (Tada et al., 1992; Bej et al., 1999; Tyagi et al., 2009), *trh* (Tada et al., 1992; Bej et al., 1999), *toxR* (Bej et al., 1999; Paydar et al., 2013; Suffredini et al., 2014; Yi et al., 2014).

Immunological methods based on monoclonal antibodies have also been used for the rapid detection and quantification of bacterial pathogens in seafood. In the case of *V. parahaemolyticus*, enzyme-linked immunosorbent assays (ELISA) have been used to detect these toxins in clinical strains using monoclonal antibodies against TDH, TRH, and thermolabile hemolysin (TLH) proteins (Honda et al., 1995; Kumar et al., 2011). However, ELISA-based assay may often give false positive results due to cross-reactivity of monoclonal antibodies against the non-target antigens from different bacteria (Prompamorn et al., 2013). Besides the monoclonal antibodies generated by classical hybridoma technology, single-chain variable antibody fragments (scFvs) displayed on the surface of phags have also been used to detect the TLH virulence factor of *V. parahaemolyticus*. However, when compared to natural full-length antibodies, ScFvs, on the other hand, had lower affinity, sensitivity, stability, and solubility. This makes the ScFvs unsuitable for clinical or diagnostic applications (Wang et al., 2012; Wang et al., 2013).

2.5.3 Virulence factors of *V. parahaemolyticus*

Virulent *V. parahaemolyticus* strains typically produce TDH and/or TRH. Both TDH and TRH have been used as the molecular marker of the pathogenicity of

V. parahaemolyticus. (Saito et al., 2015). Adhesins, two different type III secretion systems (T3SS1 and T3SS2) are also important virulence factors of *V. parahaemolyticus* (Makino et al., 2003). Recently components of type VI secretion systems (T6SSs) have also been detected in large numbers of *Vibrio* spp. including *V. parahaemolyticus*. The T6SSs are present in both the clinical as well as environmental isolates of *V. parahaemolyticus* (Boyd et al., 2008). However, not all virulence factors are present in each strain of *V. parahaemolyticus* (Meador et al., 2007). The *tdh* and *trh* were previously reported to be expressed in very low percentages of nonclinical isolates, but a recent study discovered the prevalence of *tdh* and *trh* genes in 48% and 8.3% of environmental isolates of *V. parahaemolyticus*, respectively (West et al., 2013). Though little is known about actual animal hosts and the evolution of these toxins, the evolutionary conserved nature of effectors and targets has been observed. Insights into these virulence factors are required for a thorough understanding of *V. parahaemolyticus* pathogenesis (Zhang & Orth, 2013; Letchumanan et al., 2014).

2.5.3.1 Adhesins

Attachment to the host cell is the first and one of the most crucial steps of bacterial pathogenesis. For attachment purpose, bacteria have specific proteins on its surface. These proteins are known as multivalent adhesion molecules (MAMs). In Gram-negative bacteria several MAM subtypes are found and *V. parahaemolyticus* expresses MAM7 type constitutively. MAM7's N terminus contains a hydrophobic stretch of 44 amino acids, which is required for proper protein localization and anchoring. MAMs form a tripartite complex with fibronectin protein on the host's surface and plasma membrane phospholipid phosphatidic acid to facilitate bacterial attachment to host cells (Zhang & Orth, 2013; Letchumanan et al., 2014).

2.5.3.2 Hemolysins

TDH and TRH, two key virulence factors of *V. parahaemolyticus*, were discovered in the late 1980s and early 1990s (Honda et al., 1988; Nishibuchi et al., 1992). The hemolytic activity of nearly all the clinical isolates of *V. parahaemolyticus* is due to presence of either TDH or TRH or both. TDH is one of the major pathogenic factors in *V. parahaemolyticus*. According to an epidemiological study, it was found in nearly all (95%) clinical isolates. TDH is a pore-forming tetramer toxin with a central pore that aids in the diffusion of small molecules (Bechlars et al., 2013). Due

to its property to lyse red blood cells, TDH can produce specific types of hemolysis rings on the Wagatsuma blood agar, and this particular phenomenon is known as "Kanagawa phenomenon" (Miyamoto et al., 1969; Joseph at al., 1982; Nishibuchi et al., 1992; Honda & Lida, 1993) Purified TDH is heat stable for 10 minutes at 100°C. TDH is involved in a variety of biological processes, including hemolysis, cytotoxicity, and enterotoxicity (Shimohata & Takahashi, 2010). Relatively large sized pores formed by TDH allow water and ions to diffuse through membranes, resulting in colloidal osmotic lysis. During an infection, TDH primarily affects epithelial and intestinal cells resulting in diarrhoea. TDH is also cytotoxic and forms a channel in the cell membrane, increasing extracellular Ca^{2+} concentration and Cl^{-} secretion (Matsuda et al., 20I0). Morphological changes such as cell expansion and even death may occur as a result of changes in the cell's osmotic pressure (Shimohata & Takahashi, 2010; Ceccarelli et al., 2013)

TRH is immunologically similar to TDH, with approximately 70% homology at gene level (Raghunath, 2014). Compared to TDH, the heat stability of TRH is less and it get denatured when exposed to 60 °C for 10 minutes. Even though the ability of TRH to lyse blood cells is almost comparable to TDH, TRH plays a minor role in the pathogenicity of *V. parahaemolyticus* (Ham & Orth, 2012). Though the roles of TDH and TRH in the pathogenicity of *V. parahaemolyticus* are well established, it should be noted that these toxins are not present in all clinically pathogenic strains. Even in the absence of these hemolytic toxins, some clinical strains of *V. parahaemolyticus* remain pathogenic (Ceccarelli et al., 2013; Raghunath, 2014).

V. parahaemolyticus may also contain a thermolabile homolysin (TLH) encoded by *tlh* gene (Wang et al., 2013). TLH is a lecithin-dependent phospholipase that also lyses human erythrocytes (Broberg et al., 2011). However, in case of *V. parahaemolyticus*, TLH is not considered a virulence factor. On the other hand, *tlh* gene is considered as a reliable species-specific marker of *V. parahaemolyticus*, and it has been used as target gene in several PCR-based assays for identification of *V. parahaemolyticus* (Bej et al., 1999; Su & Liu, 2007; Klein et al., 2014).

2.5.3.3 Type III secretion system (T3SS)

T3SS is needle-like bacterial machinery that injects bacterial proteins known as effectors directly into the membrane and cytoplasm of eukaryotic cells, bypassing the external environment (Cornelis, 2006). T3SS structural elements are usually conserved among bacteria, and they consist of 20-30 proteins that form a basal body that spans the inner and outer membranes of bacteria. Needle is also an important part of the T3SS secretion system. It is formed by polymerization of multiple proteins and extend into to the extracellular space. On the top of the needle, there is a tip complex. It has been proposed that tip complex is involved in the activation of T3SS system (Izore et al., 2011; Letchumanan et al., 2014). T3SS targets and activities can range from the actin cytoskeleton to innate immune signaling and autophagy (Zhang & Orth, 2013). Depending on the needs of the bacteria, the T3SS may be up-regulated or down-regulated during pathogenesis (Broberg et al., 2011).

T3SS1 and T3SS2 are the two types of T3SS systems detected in *V. parahaemolyticus*. T3SS1 genes are found on chromosome 1 in all clinical and environmental strains of *V. parahaemolyticus* (Ono et al., 2006; Paranjpye et al., 2012). The *V. parahaemolyticus* T3SS1 gene cluster shares sequence homology with *Yersinia* spp., implying ancestral acquisition and evolutionary conservation (Ono et al., 2006; Zhang & Orth, 2013). Three interacting proteins (ExsC, ExsD, and ExsE) that control the activity of the master transcriptional regulator ExsA, and play an important role in T3SS1 regulation. ExsA is bound to ExsD and rendered inactive in non-inducing conditions, whereas ExsC, the system's anti-anti-activator, is bound to ExsE. When ExsE is secreted during inducing conditions, ExsC is released and binds to ExsD, allowing ExsA to be released and the transcription of T3SS1 genes to be activated (Zhou et al., 2010). T3SS1 initiates a series of events involving autophagy, membrane blebbing, cell rounding, and cell death within three hours of infection during tissue culture infection studies. These repeatable events are mediated by three T3SS1 effectors: VopQ, VPA0450, and VopS. VopQ, for example, prevents infecting bacteria from being phagocytosed, VPA0450 causes cell destabilisation by detaching the plasma membrane from the actin cytoskeleton, and VopS targets the actin cytoskeleton, causing cell rounding and shrinkage (Broberg et al., 2011; Ceccarelli et al., 2013; Sreelatha et al., 2013).

The T3SS2 gene cluster, found in clinical isolates of *V. parahaemolyticus*, is found on the pathogenicity island Vp-PAI (VPaI-7) on chromosome 2, and it has been linked to pandemic strains (Paranjpye et al., 2012; Zhang et al., 2012).

The G+C content of VPaI-7 is lower than the genomic average, and it contains a greater number of genes unique to each *Vibrio* spp., implying that it was acquired by *V. parahaemolyticus* via lateral transfer (Ham & Orth, 2012). T3SS2 genes are primarily regulated by two transcriptional regulators, *VtrA* and *VtrB*. (Kodama et al., 2010). Among the seven types of T3SS2 effector proteins identified till date, VopA/P suppresses the host's innate immune response, and VopL nucleates actin and induces stress fibres, facilitating bacterial uptake into the host cells. VopC promotes bacterial invasion, whereas VopT and VopV contribute to cytotoxicity and enterotoxicity, respectively. VopZ mediates the pathological phenotypes, whereas the function of VPA1380 is yet unknown (Zhang & Orth, 2013; Ghenem et al., 2017).

2.5.3.4 *PirA* and *PirB* toxins

Frequent outbreaks of acute hepatopancreatic necrosis disease (AHPND), formerly known as early mortality syndrome (EMS), have recently become a serious threat to the global shrimp industry. A specific strain of *V. parahaemolyticus* was identified as the AHPND causative agent in 2013. (Tran et al., 2013). A 69-kb extrachromosomal plasmid was discovered through whole genome sequencing and comparison of AHPND-causing and non-virulent strains of *V. parahaemolyticus*. This plasmid, later known as pVA1, was found in all AHPND-causing strains but not in non-virulent strains. The plasmid pVA1 contains two genes that encode for the toxin *PirA* and *PirB*, which are homologous to the *Photorhabdus* insect-related (Pir) toxin (Yang et al., 2014; Lee et al., 2015). *PirA* and *PirB* toxin genes are 336 bp and 1317 bp in length, respectively (Han et al., 2015). In addition to *PirA* and *PirB* genes, plasmid pVA1 contains transposons and conjugative transfer genes, which may facilitate plasmid transfer to other bacteria strains or species (Yang et al., 2014). The pathogenesis process begins with AHPND causing *V. parahaemolyticus* colonising the stomach of infected shrimp (Tran et al., 2013; Lai et al., 2015). *PirA* and *PirB* toxins produced by bacteria reach the hepatopancreas, where they cause sloughing of tubule epithelial cells, resulting in pale hepatopancreas coloration (Tran et al., 2013).

AHPND may result in 70-100% mortalities within a span of few days (Lin et al., 2017).

2.6 Antibiotics in aquaculture

The stress of keeping aquatic species in limited holding areas increases their susceptibility to disease in commercial intensive aquaculture setups. Depending on the susceptibility of the fish and the environmental conditions, a potential pathogen can cause disease (Pillay, 1990). While available preventive strategies such as environmental control, ensuring optimum water quality, good diet, and stocking densities are used to control disease outbreaks, commercial pressure to increase production and uncontrollable variables make antibiotics play an important role in aquaculture (Michel & Alderman, 1992). Gutshell (1946) was the first to report the use of nitrofurazone to control furunculosis in trout. Several antibiotics used in human and veterinary medicine have been tested for potential use in aquaculture. Smith (1950) reported that chloramphenicol was effective against *Aeromonas* and *Pseudomonas* infections. Tetracyclines, particularly oxytetracyclines, have been shown to be extremely effective against Gram-negative pathogens (Snieszko et al., 1952). In Japan, 4-quinolones were first used in the 1970s (Endo et al., 1973). Due to lack of proper surveillance and monitoring systems, the exact amount of antibiotics used worldwide is very difficult to estimate. However, some indicator data from several countries showing use/misuse is available. In USA various production systems used 13,000 tons of antibiotics in year 2009. During the year 2016, 382.5 tons of antibiotics were used in Chilean salmon culture (Miranda et al., 2018). No data about the use antibiotics in Indian aquaculture is currently available.

2.6.1 Emergence of antibiotic resistant *Vibrio* spp.

Historically, regulatory approval and use of antibiotics in aquaculture have given little thought to the environmental fate of these drugs. Various treatment methods, such as dips, flushes, and injection, are used in aquaculture (Austin & Austin, 1993), but the most common method is drug mixing with feed (Rae, 1992). However, it is becoming clear that a large proportion of antibiotics delivered to cultured animals, typically in the form of feed additives, are not absorbed and retained by the animal, but are instead released into the aquatic environment via one of the three routes listed below. First, a portion of the medicated feed is not ingested and

instead enters the substratum. This feed loss is most likely to occur during disease outbreaks, when cultured fish feed slowly and in large quantities (Thorpe et al., 1990). Second, a portion of the antibiotics consumed are not absorbed during the digestive process and are excreted into the environment. Depending on the type of antibiotic used, 60-80 percent of the drug can be excreted unchanged through the faeces (Cravedi et al., 1987). Finally, some antibiotics are excreted in an unmetabolized, microbially active form through the urine and bile fluid (Bjorklund et al., 1990). Besides, the antibiotics used in human and veterinary medicine may also find their ways into the aquatic environments. For residues of antibiotics used indiscriminately in the human and veterinary medicine, aquatic environment may serve ultimate sink (Watts et al., 2017).

In any environment, the presence of antibiotics puts a selection pressure on natural microbial communities. The bacteria having metabolic capabilities to withstand the antibiotics-mediated selection pressure thrives, whereas the other communities sensitive to antibiotics may stop growing or become extinct. The metabolic capabilities of bacteria to resist antibiotics are mediated by certain genetic elements known as antibiotic resistance genes (ARGs) (Zhang et al., 2009; Xiong et al., 2015; Preena et al., 2020). In any microbial community, the selection pressure due to antibiotics promote the evolution and spread of hundreds of antibiotic resistance genes (ARGs) that confer resistance to various bacteria, regardless of their origins (Zhang et al., 2009; Allen et al., 2010). Moreover, large numbers of antibiotics are naturally produces by bacteria/fungi in their native environments and these strains carry genes encoding resistance to the antibiotics that they produce (Hopwood, 2007). Many of the known antibiotic resistance genes are found on transposons, integrons or plasmids, which can be mobilized and transferred to other pathogenic/non-pathogenic bacteria of the same or different species through the horizontal gene transfer process (Rhodes et al., 2000; Allen et al., 2010). Any acquisition of antibiotic resistance gene by human pathogenic bacteria, already containing highly virulent genes, often results in failure/efficiency reduction of antibiotic treatment and severe health/economic consequences (Rhodes et al., 2000). According to a report from Organization for Economic Co-operation and Development (OCED) released during November 2018, antimicrobial resistance will cost almost 2.4 billion lives in Europe, North America and Australia within next 30 years with annual economic cost of US$ 3.5 billion.

Though not much information about developing countries like is available, the report also stated that resistance rates in low- and middle-income countries are between 40-60% (Hofer, 2019).

Members of *Vibrio* spp., including *V. parahaemolyticus*, are autochthones marine microflora. Several studies have looked into its AMR patterns in aquatic environment. Being the natural microbiota, members of *Vibrio* spp. also give important insights into the patterns of resistance originating from environment rather that clinical conditions (Baker-Austin, 2015). Conventionally, most antibiotics of veterinary and human significance (cephalosporin, fluoroquinolone, tetracycline, doxycycline, ciprofloxacin, aminoglycosides and trimethoprim-sulfamethoxazole) have been reported usually effective against *Vibrio* spp. However, resistance against majority of these antibiotics has recently been reported in several *Vibrio* spp. isolates (Elmahdi et al., 2016). In USA, intermediate to high resistance against ampicillin, penicillin and chloramphenicol was observed in clinical and environmental isolates of *V. parahaemolyticus* (Shaw et al., 2014). In a study to investigate *V. parahaemolyticus* antimicrobial resistance in a shrimp mariculture environment in China, 53.5 - 78.9% isolates were resistant to streptomycin, ampicillin, and gentamicin antibiotics. Furthermore, 61.4% of isolates exhibited multi-drug resistance patterns (Zhao et al.,2018). Sperling et al. (2015) investigated *Vibrio* spp. prevalence and AMR in farm and retail shrimps in Ecuador. *V. parahaemolyticus* was found in 80.8% of the 219 samples that tested positive for *Vibrio* spp. Despite the absence of virulence-related thermostable direct hemolysin (TDH) and TDH-related hemolysin (*trh*) in *V. parahaemolyticus* isolates, high resistance to ampicillin (92.2%), tetracycline (51.3%), and amikacin (22.1%) was observed. Sixty-eight *V. parahaemolyticus* isolates were resistant to three antibiotics, while two isolates were resistant to seven antibiotics (Sperling et al., 2015). Because inland saline areas have recently emerged as potential shrimp farming areas, the prevalence of *Vibrio* spp. and their AMR patterns have also been investigated to generate baseline data. Singh et al. (2019) studied the prevalence, potential pathogenicity, and AMR of *Vibrio* spp. in Punjab's inland saline areas. Antimicrobial susceptibility testing against seven commonly used antibiotics revealed that ampicillin had the highest resistance (50.4%). Intermediate resistance to erythromycin was also found to be very high (87.4%). Almost 20% of the *Vibrio* isolates were resistant to two or more antibiotics.

However, *V. cholerae* dominated the *Vibrio* isolates in this study (91.6%), with only a 1% prevalence of *V. parahaemolyticus*. However, samples in this study were collected from inland saline carp culture ponds. As shrimp culture in Punjab is expanding at **rapid rate, there is urgent need to investigate the AMR patterns of *V. parahaemolyticus*** isolates from these farms.

2.7 Control of potentially pathogenic antibiotic resistant bacteria in the aquaculture

Though fish/shrimp farmers follow lots of best management practices (BMPs) and biosecurity measures of such as the use of seeds produced from specific pathogen free (SPF) broodstock, regular screening for pathogens, routine use of disinfectants and maintenance of optimum culture conditions, environmental degradation during the culture period cannot be avoided. High stocking densities and inputs (feed, fertilizers etc) leads to the buildup of organic waste and toxic metabolites, which in turn may lead to stress in aquatic animals. Thus, negative interactions involving stress, reduced host immunity and presence of pathogen may lead to disease outbreaks (Gatesoupe, 1999; Joffre et al., 2018). Indiscriminate use of antibiotics may lead to rapid spread of ARGs in the environment. The situation may further become complicated due to acquisition of these ARGs by pathogenic bacteria. Recently, application of bacteriophages (phage therapy) has been suggested as important tools to control the flow of ARGs in the environment by targeted killing of multi-drug resistant bacteria with affecting the natural microbiota (Parmar et al., 2017).

2.7.1 Bacteriophages

Bacteriophages are viruses that infect bacteria, and they are commonly referred to as phages. Phages are everywhere and require a bacterial host to survive. Phages are the most dominant biological entity in the biosphere, with an estimated number of 10^{31} belonging to 10^{6} distinct species. Phages play an important role in bacterial control in nature, killing nearly half of the bacterial population every 48 hours (Brüssow & Hendrix, 2002). Scientists have used bacteriophage-bacterial host interactions as tools to better understand basic molecular biology, genetic recombination events, horizontal gene transfer, and how phages drive bacterial evolution (Henry & Debarbieux, 2012). Since their discovery in 1915 by Frederick

W. Twort and in 1917 by Felix dHerelle, several trials demonstrating the therapeutic potential of phages against bacterial pathogens have been conducted from the 1920s to the 1950s. In one such trial, dHerelle successfully reported the treatment of cholera with *V. cholerae* lytic phages, bringing phage therapy to the attention of the world (D'Herelle & Malone, 1927). During the early stages of phage research, however, a lack of understanding of phage physiology and genomics frequently resulted in inconsistent and unreliable results. These factors, combined with the introduction of antibiotics as "magic bullets," resulted in a decline in interest in phage therapy in Western countries (Cisek et al., 2017). However, progress in phage research and therapy continued in several Eastern European countries and the former Soviet Union. The availability of advanced technological tools for phage characterization, combined with the global emergence of antibiotic-resistant pathogens, has rekindled interest in phage research over the last decade. Phage research for human clinical applications is advancing rapidly. A new human phage therapy centre was recently established in the United States. The centre has treated several patients (including intravenous phage administration) with remarkable success; all patients tolerated the therapy well and no side effects were observed (UCSD-2019 Human applications of phage therapy, on the other hand, must still go through lengthy regulatory approval processes. This has prompted researchers to concentrate on agricultural, food safety, and industrial phage applications (Goodridge & Bisha, 2011; Cisek et al., 2017).

2.7.2 Phage therapy in aquaculture

Due to the rapid spread of AMR, phage application has been proposed as a means of controlling the spread of antibiotic resistance in the environment through the targeted killing of multi-drug resistant bacteria (Parmar et al., 2017). Phage therapy has also been used successfully to control pathogenic *Vibrio* species in aquaculture environments. Vinod et al. (2006) isolated and characterised a double-stranded DNA phage with lytic activity against 50 V. harveyi isolates from India's east and west coasts. During the shrimp hatchery trials, the phage was able to control the growth of luminous bacteria for the entire 17-day trial period. Furthermore, larval survival in phage-treated tanks was 86 percent, compared to 40 percent and 17 percent in antibiotic-treated (Oxytetracycline 5 ppm and Kanamycin 10 ppm daily respectively) and control tank. In another study, use of bacteriophage against *V. harveyi* in hatchery systems increased postlarval survival by 40-60% (Patil et al.,

2014). Silva and colleagues isolated and characterized two phages specific to *V. anguillarum* and *V. parahaemolyticus* from sewage. Within 6 hours of application, these phages were able to reduce bacterial counts by 3.9 log CFU/ml in the laboratory (Silva et al., 2014). Several lytic phages have recently been identified for pre- and post-harvest control of *V. parahaemolyticus*.

Jun and colleagues isolated pVp-1, a Siphoviridae family phage with lytic activity against 90.9 percent of AHPND-causing *V. parahaemolyticus* strains. This particular phage was also able to reduce mortality rates in shrimps suffering from AHPND by 50% during phage therapy (Jun et al., 2018). Zhang and colleagues isolated and characterized an oyster-specific phage OMN from *V. parahaemolyticus*. The genome of phage OMN was 42,202 bp long, and it was able to lyse 67 percent of the *V. parahaemolyticus* isolates tested in the lab. OMN phage was applied to oysters for 72 hours and was able to reduce *V. parahaemolyticus* counts by 99 percent (Zhang et al., 2018).

As stated above, the use of lytic phages for pre- and post-harvest control of bacterial pathogens has become a promising area of research, with several commercial phage preparations achieving Generally Recognized As Safe (GRAS) status from the United States Food and Drug Administration (Lu & Koeris, 2011). However, safety concerns, a limited host range, and the development of phage resistance in the host bacterium remain. Prior to using phages for therapeutic purposes, these issues should be thoroughly investigated and addressed.

CHAPTER – III

MATERIALS AND METHODS

3.1 Collection and transportation of water and shrimp samples

During the present study, water and shrimp samples were collected from inland saline shrimp farms located in the south-west district of Mansa and Bhatinda (Fig.2). In case of water, sub-surface (one feet below surface) water samples were collected in sterile bottles at a distance of approximately 2 meters from the pond's dyke. Shrimps sample were collected from the feeding check trays in sterile pouches. All the samples were transported to laboratory in cool gel packs and processed on the same day.

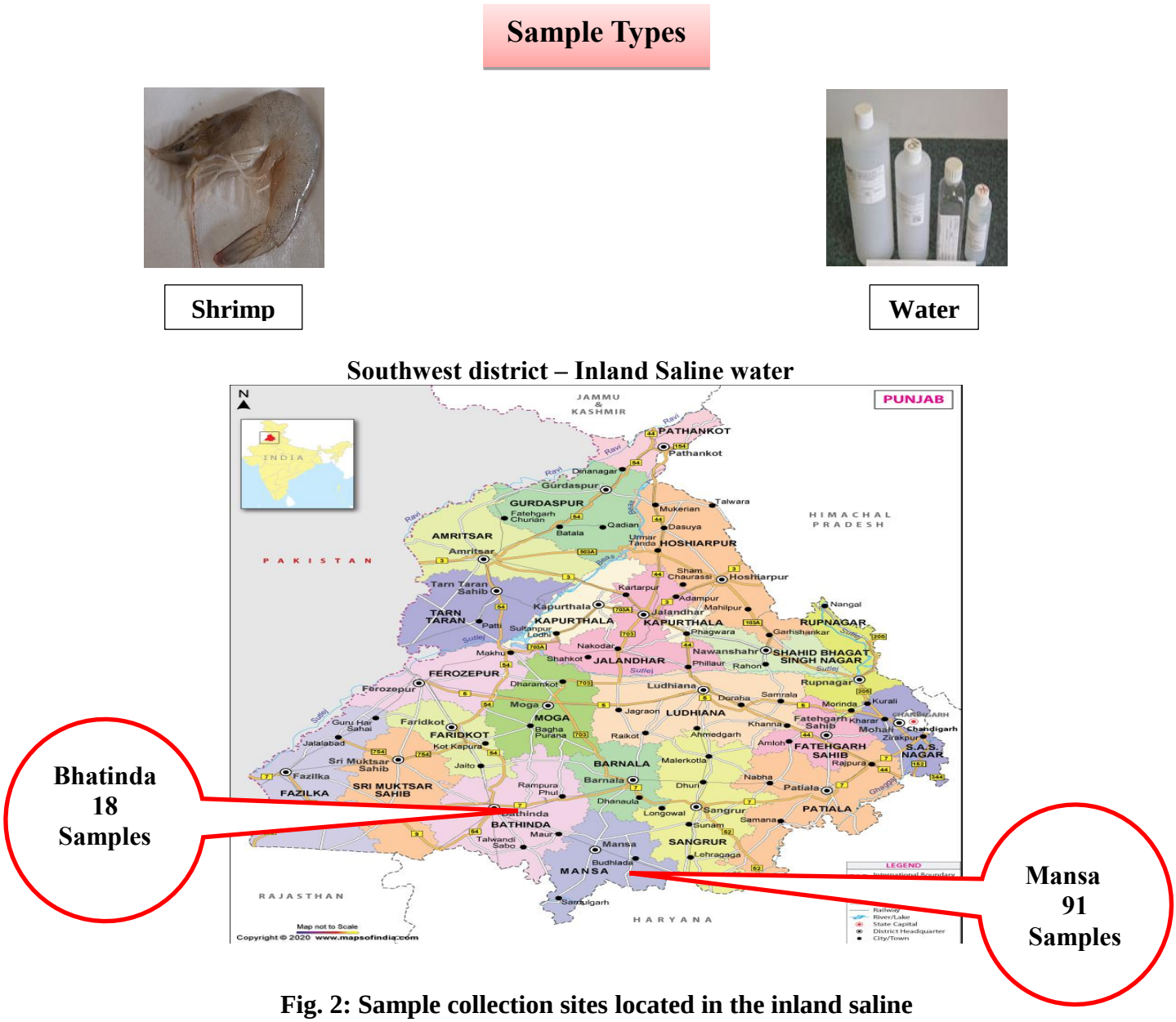

Fig. 2: Sample collection sites located in the inland saline areas of Punjab

3.2 Enrichment and isolation of presumptive *Vibrio* spp.

Once in the laboratory, the samples were processed as per previously described protocols (Singh et al., 2018) with minor modifications, as and when required. As *V. parahaemolyticus* is a halophilic bacterium, all the culture media were supplement with NaCl to the final concentration of 3% instead of usual 1%, unless stated otherwise. Below is the step-by-step protocol for enrichment and presumptive *Vibrio* isolation:

1. Depending upon sample type (water or shrimp), 25 ml or g were transferred into 225 ml of alkaline peptone water with 3% NaCl (APW, pH 8.5) and mixed by vigorous shaking/homogenizing.

2. After incubation at 35 ^{o}C for 6-8 h with shaking at 150 rotation per minute (RPM), a loopful of enriched culture was streaked on the thiosulfate-citrate-bile salts-sucrose (TCBS) agar.

3. After overnight (16-18 h) incubation at 35 ^{o}C, 2-4 colonies showing typical yet distinct *Vibrio* morphologies were picked up from each TCBS plate and subcultured on tryptone soy agar (TSA) agar plates for further downstream identification and characterization. Aliquots of these cultures were also stored in 30% glycerol broth at -80^{o}C.

Alkaline Peptone Water (APW)

Ingredients	Quantity
Peptone	1 g
Sodium chloride	1 g
Distilled water	100 ml
Final pH (at 25 ^{o}C)	8.5±0.2

The required ingredients were mixed together and pH was adjusted with 10 N NaOH. The media was sterilized by autoclaving at 121 ^{o}C for 15 min.

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

Ingredient	Quantity
Proteose peptone	1 g
Yeast extract	0.5 g
Sodium thiosulphate	1 g

Sodium citrate	1 g
Oxgall	0.8 g
Sucrose	2 g
Sodium chloride	1 g
Ferric citrate	0.1 g
Bromo thymol blue	0.004 g
Thymol blue	0.004 g
Agar	1.5 g
Sterile distilled water	100 ml
Final pH (at 25°C)	8.6±0.2

The required ingredients were mixed followed by boiling to dissolve the medium. No autoclaving was performed.

Tryptone Soy Broth (TSB)

Ingredients	**Quantity**
Tryptone	1.5 g
Peptone	0.5 g
Sodium chloride	1.0 g
Dipotassium phosphate	0.25 g
Distilled water	100 ml

For preparation of tryptone soy agar (TSA), 1.5 g of bacteriological grade agar powder was added into TSB. Required ingredients were mixed together and sterilized by autoclaving at 121^{o}C for 15 min.

Glycerol Broth

Ingredients	**Quantity**
Tryptone	1 g
Yeast extract	0.5 g
Sodium chloride	1 g
Glycerol	30 ml
Distilled water	70 ml

The media was sterilized by was autoclaving at 121^{o}C for 15 min.

3.3 Confirmatory genus-level identification of presumptive *Vibrio* isolates

The bacterial isolates, presumptively identified as *Vibrio* spp. due to their TCBS agar growth characteristic, were further subjected to Gram staining and biochemical tests for confirmatory genus-level identification as per Bergey`s Manual of Systematic Bacteriology (Brenner et al., 2005).

3.3.1 Gram staining

1. Bacterial cultures were grown overnight in tryptone soy broth (TSB) with 3% NaCl. Following morning, one loopful of bacterial culture was placed on clean glass slide and spreaded into an area approximately the size of a dime.
2. The slide was air dried and the smear was heat fixed by quickly passing the slide over the Bunsen burners flame two or three times.
3. The slide containing bacterial smear was flooded with the primary stain (crystal violet) and allowed to stand for 1 min.
4. The slide was gently washed with running tap water and flooded by Gram`s iodine mordant followed by incubation for 1 min.
5. The slide was gently washed with running tap water, and decolourized with 95% ethanol. The decolourizer was added drop by drop until no colour was running off the slide.
6. The slide was gently washed with running tap water and counterstained with safranin for 45 seconds.
7. The slide was gently blot dried with bibulous paper and observed with light microscope under 10x, 40x and 100x (oil immersion) objectives.

3.3.2 Oxidase test

1. Bacterial cultures were inoculated in to 4.5 ml of nutrient broth with 3% NaCl. After overnight incubation at 35 ^{o}C with shaking at 150 rpm, 0.2 ml of 1% α-naphthol (Gaby and Hadley reagent A) and 0.3 ml of 1% *p*-aminodimethylaniline oxalate (Gaby and Hadley Reagent B) were added into the test tubes.
2. The tubes were observed for any colour change. Development of blue colour within 10 to 30 seconds was taken as the indication of positive oxidase test.

3.3.3 Glucose fermentation test

Previously described sugar fermentation media was used to detect the glucose fermentation ability of bacterial isolates (MacFaddin, 2000). Test tubes containing 4 ml of sterilized sugar fermentation media were inoculated with bacterial culture. After incubation at 35 ^{o}C for 24 h, the change of culture media colour from purple to yellow was taken as the indication of positive fermentation reaction with acid production.

Ingredients	**Quantity**
Peptone	1 g
Sodium chloride	0.5 g
Glucose	0.5
Bromocresol purple	0.0016 g
Distilled water	100 ml
Final pH	7.2 ± 0.1

Required media ingredients were mixed together and sterilized by autoclaving at 121 ^{o}C for 15 min.

3.3.4 Salt requirement test

The ability of bacterial isolates to growth in the absence of NaCl was also evaluated. The bacterial cultures were inoculated in nutrient broth with 0%, 1% and 3% NaCl concentration. After overnight incubation at 35 ^{o}C, the development of visible turbidity was taken as the indication of positive reaction.

3.4 Confirmatory species-level identification of *V. parahaemolyticus* isolates

After the confirmation of *Vibrio* spp. isolates by biochemical testing, the polymerase chain reaction (PCR)-based molecular biology approach was used for confirmatory species-level identification of *V. parahaemolyticus* isolates.

3.4.1 Isolation of bacterial genomic DNA

From the overnight grown bacterial cultures, genomic DNA was using the HiPurA® Bacterial Genomic DNA purification Kit (HiMedia, India) as per below mentioned protocol:

1. From the overnight grown bacterial cultures, 1.5 ml were centrifuged at 12,000 *x g* for 2 min. After discarding the supernatant, the bacterial pallet was resuspended in 180 µl of Lysis solution (AL).

2. For complete lysis of bacterial cells, 20 µl of Proteinase K (of 20 mg/ml concentration) was added followed by incubation at 55 ^{o}C for 30 min.

3. After incubation, 200 µl of lysis solution (C1) was added into the centrifuge tube followed by brief vortexing to mix the contents.

4. Further, 200 µl of molecular biology grade absolute ethanol (HiMedia, India) was added and the contents were mixed by gently inverting the tube 5 to 6 times.

5. The entire content was then transferred into the HiElute miniprep spin silica column followed by centrifugation at 10,000 *x g* for 1.5 min. The flow-through was discarded.

6. To the silica column, 500 µl of prewash solution was added followed by the centrifugation at 10,000 *x g* for 1 min. The flow-through was discarded and the wash process was repeated in the similar manner with 500 µl of wash solution.

7. After discarding the flow-through, the empty silica column was again centrifuged at 10,000 *x g* for 2 min. The silica column was transferred into a fresh 1.5 ml centrifuge tube and allowed to dry at room temperature for 1-2 min.

8. For elution of purified genomic DNA, 100 µl of elution buffer was added directly on the silica membrane without wetting the rim followed by incubation at room temperature for 5 min. After centrifugation at 10,000 *x g* for 1 min, the flow-through containing purified genomic DNA was collected and stored at -20 ^{o}C till further use.

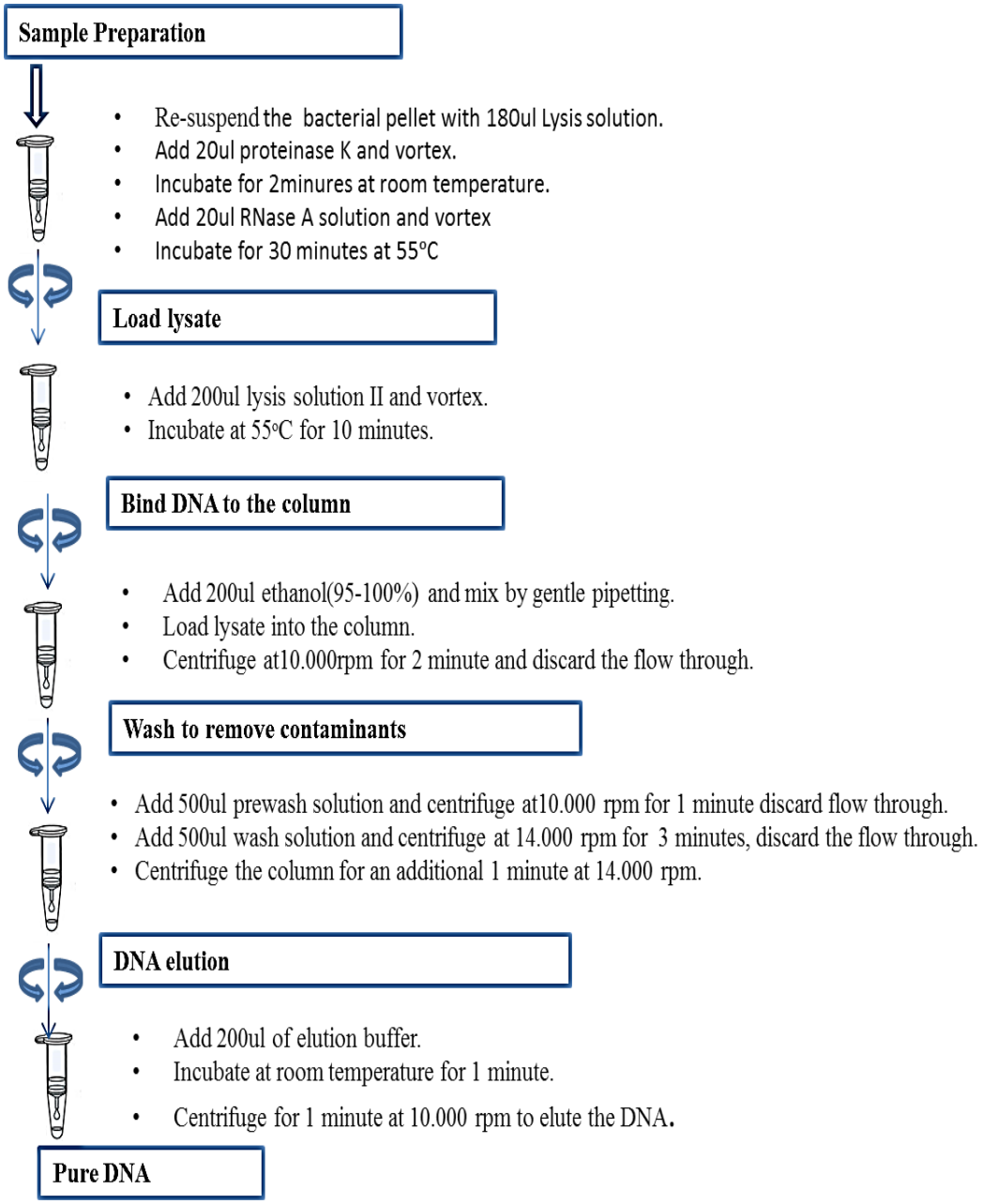

Fig. 3: Diagrammatic representation of isolation of DNA

3.4.2 PCR reaction set-up

The PCR for species level confirmation of *V. parahaemolyticus* was performed as previously described protocol (Kim et al., 2015). Briefly, the 25 µl PCR reactions consisted of 2.5 µl of 10x Taq buffer A (10 mM Tris-HCl, pH 9.0; 15 mM $MgCl_2$, 50 mM KCl, 0.01% Gelatin) (GeNei, India), 0.24 µM of each of *V. parahaemolyticus*-specific forward (VPF 5`-AGCTTATTGGCGGTTTCTGTCGG-3`) and reverse (VPR 5`-CKCAAGACCAAGAAAAGCCGTC-3`) primers, 1 unit of Taq DNA polymerase (GeNei, India), 200 µM of each dNTP (GeNei, India) and 20 -

50 ng of bacterial genomic DNA. The PCR thermal cycler (T100, Bio-Rad, USA) conditions consisted of initial denaturation at 95 ^{o}C for 5 min followed by 30 cycles of denaturation at 95 ^{o}C for 1 min, primer annealing at 60 ^{o}C for 1 min, and extension at 72 ^{o}C for 1 min. The final extension was performed at 72 ^{o}C for 10 min. A previously isolated confirmed *tdh* positive isolate of *V. parahaemolyticus* (Vp7PE), obtained from College of Fisheries, Mangalore, Karnataka, was used as positive control during the PCR reaction.

3.4.3 Agarose gel electrophoresis

The PCR products were detected by agarose gel electrophoresis. Two percent (w/v) agarose gels were prepared in 1X TAE buffer. The molten agarose was cooled to below 65°C, ethidium bromide was added to a final concentration of 0.5 μg/ml, mixed and poured to gel mould and allowed to set or post staining was done after electrophoresis. 10 μl of the PCR products was mixed with 2 μl of 6X loading buffer and loaded into the wells. 100 bp DNA ladder (GeNei, India) was used as a molecular weight marker. Electrophoresis was carried out at 100-120 V and the bands were visualized under UV transilluminator (Syngene, USA).

Tris Acetate EDTA (TAE) buffer (50X)

Tris base	242 g
Glacial acetic acid	57 ml
0.5 M Ethylene Diamine Tetra acetic acid (pH 8.0)	100 ml

The solution was made up to a final volume of 1 liter with distilled water. The working solution (1000 ml of 1X TAE) was prepared by diluting 20 ml of 50X stock solution to 1000 ml with distilled water.

TAE buffer working solution (1X)

Tris-acetate	0.04M
EDTA	0.001M
Distilled water	100 ml

This solution was stored at room temperature (28° ± 1ºC).

Sample loading buffer (6X)

Bromophenol blue	0.3 g
Sucrose	40 g
Distilled water	100 ml

Ethidium bromide (HiMedia, India)

A stock solution was prepared by dissolving 5 mg of ethidium bromide (Sigma, U.S.A) in 1 ml of distilled water. The solution was either added to agarose gel during preparation to yield a final concentration of 0.5 μg/ml.

3.5 PCR detection of virulence-associated genes in *V. parahaemolyticus*

After confirmatory identification, *V. parahaemolyticus* isolates were screened for the presence of known virulence-associated genes. The general PCR master mix composition was similar to the one described above. Except the variations in primer specific annealing temperatures and expected amplicon length specific extension times, rest of the PCR conditions were similar to ones described in Table 2. The identity of initial PCR amplicons was established by Sanger sequencing to ensure specific target detection. The details of virulence-associated genes screened, primers used, annealing temperature and amplicon size have been provide in the Table 3.

Table 2: PCR thermal cycler conditions for amplification of virulence-associated genes

Sr No.	Steps	Temperature (°C)	Time
1.	Initial denaturation	94	5 min
2.	Denaturation	94	1 min
3.	Annealing	Acc. to primers	1 min
4.	Extension	72	30 – 45 s (as per amplicon length)
5.	Repeat step 2 to 4 For 29 times	-	-
6.	Final extension	72	10
7.	Incubate	4	10

Table 3: Details of virulence-associated genes screened, primers used, annealing temperature and amplicon size for *V. parahaemolyticus*

Gene	Primer name/sequence (5`- 3`)	Annealing temperature (^{o}C)	Amplicon size (bp)	Reference
tdh	D3- CCACTACCACTCTCATATGC D5- GGTACTAAATGGCTGACATC	55	251	(Tada et al., 1992)
trh	R2- GGCTCAAAATGGTTAAGCG R6- CATTTCCGCTCTCATATGC	55	250	(Tada et al., 1992)
tlh	F- GCTGGTTCTTAGGTCACTTCTC R- GCATAATCCGCTTTCACTTCTG	55	277	(Singh et al., 2018)
VopC	F- CTGTCCAAGAGGCGTACCA R- CAGCGTGGTGGTTAGTGAAT	55	331	(Singh et al., 2018)
PirA and *PirB* plasmid	F 5'- ATGAGTAACAATATAAAACATGAAAC-3' R5' - ACGATTTCGACGTTCCCCAA-3'	52	1269	(Dangtip et al., 2015)

3.6 Antibiotic susceptibility testing of *Vibrio* isolates

Antimicrobial susceptibility of confirmed *Vibrio* isolates (including *V. parahaemolyticus*) was tested by disk diffusion method against 12 commonly used antibiotics in aquaculture and veterinary medicine per Clinical Laboratory Standards Institute (CLSI) guideline M45 (CLSI, 2016a). Antibiotics disks of ampicillin (AMP; 10 µg), gentamicin (GEN; 10 µg), tetracycline (TE; 30 µg), chloramphenicol (C; 30 µg), ciprofloxacin (CIP; 5 µg), sulfisoxazole (SF; 300 µg), kanamycin (K; 30 µg), amoxiclav (AMC; 30ug), imipenem (IPM; 10ug), cefotaxime (CTX; 30ug), co-trimoxazole (COT; 25ug), ciftazidime (CAZ; 30ug) (HiMedia, India) were used during the study.

For antibiotic susceptibility testing, bacterial cultures were grown in TSB with 1% NaCL A previously standardized viable count versus OD_{600} curve was used to achieve the required 1.5 x 10^8 CFU/ml counts for log phase (3-4 hour old) test cultures. These standardized inoculums were used for lawn preparation on Mueller Hilton agar (HiMedia, India). Up to five to six antibiotics disks were applied on each lawn followed by incubation at 35^{o}C for 16 h. Following morning, diameters of zone of inhibition around each disk were recorded (Fig. 4). These zone sizes were used to make resistant (R), intermediate (I) and susceptible (S) calls as per Clinical Laboratory Standards Institute (CLSI), USA guideline M45 (CLSI, 2016a)

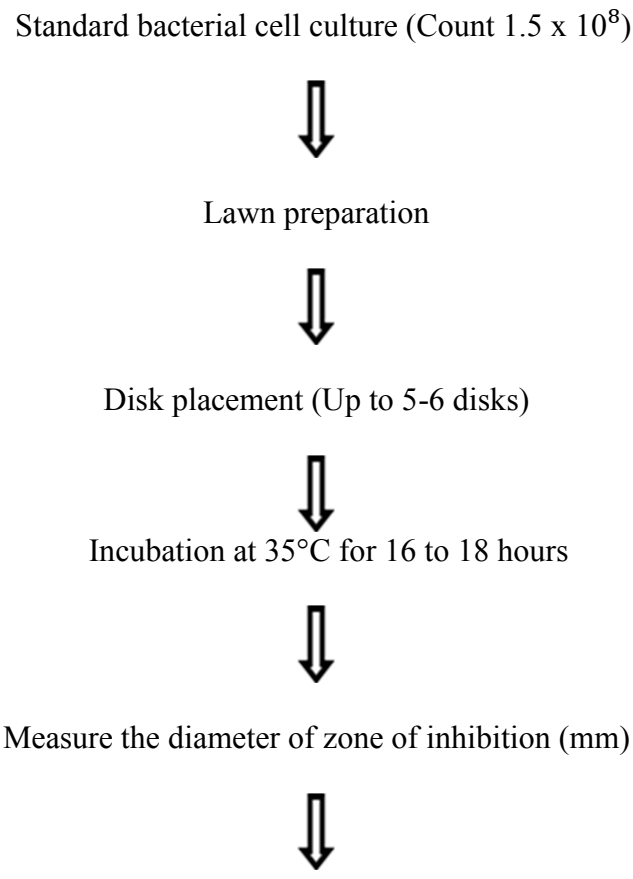

Fig. 4: Diagrammatic representation of antimicrobial susceptibility test

3.7 Evaluation of phage lytic activity against *V. parahaemolyticus* isolates

Lytic activity of 12 previously isolated *V. parahaemolyticus* specific phages (available with Department of Aquatic Environment, College of Fisheries) was tested against *V. parahaemolyticus* isolates by lawn assay as per previously described

protocols. For phage propagation and lysate preparation, *V. parahaemolyticus* (Vp7PE) was used as the host for phages AMN2, FT2, FT3, KD1 and V1. On the other hand, *V. parahaemolyticus* (Vp25), isolated from water sample collected from shrimp farm located in village Bhadur Kheda in district Fazilka, was used as host for phages AMN1, AMN3, PL1, V2, V4, V5 and V6. Four millilitres of log phage host cultures in Luria Bertani (LB) broth with 3% NaCl (HiMedia, India) were inoculated with 25 µl phage stock followed by overnight incubation at 35 ^{o}C with shaking at 100 RPM. Following morning, the host bacterial cells/debris were removed by centrifugation followed by filtering the supernatant through 0.45 µm PES filter (Cole-Parmer, USA). The filtrate containing phage was stored at 4 ^{o}C until further use. *V. parahaemolyticus* host lawns were prepared on TCBS agar with 3% NaCl plates (HiMedia, India) followed by the spotting of 10 µl of phage filtrates on these lawns. For negative control, overnight grown *V. parahaemolyticus* culture was centrifuged and filtered as described above. Ten microliters of negative controls were also spotted on each plate. After overnight incubation at 35 ^{o}C, the plates were inspected for any zones of lysis/clearing around the spots (Dubey et al., 2021).

Luria Bertani (LB) broth, Miller

Ingredient	**Quantity**
Tryptone	1.0 g
Yeast extract	0.5 g
Sodium chloride	3.0 g
Distilled water	100 ml
Final pH (at 25°C)	7.5±0.2

For preparation of LB agar, 1.5 g of bacteriological grade agar powder was added into LB broth. The media was sterilized by autoclaving at 121 ^{o}C for 15 min.

CHAPTER – IV

RESULTS AND DISCUSSION

4.1 Isolation of *Vibrio* spp. from Inland saline shrimp culture farms

In the present study a total of 109 samples were collected from 21 shrimp farms located in the 15 villages of district Mansa and Bhatinda. Out of these, 44 were shrimp samples and 65 were water samples (Table 4). After enrichment in alkaline peptone water and biochemical testing of presumptive yellow/green colonies picked from TCBS agar, a total of 109 bacterial isolates were confirmed as belonging to Genus *Vibrio*.

Table 4: Details of area wise sample collection from inland saline shrimp culture ponds

District	Village	Total Samples
Mansa	Korewala	11
	Akanwali	16
	Mian	6
	Birewala Jattan	26
	Jheryan Wali	11
	Jandwala	2
	Nangla	6
	Bhalaike	8
	Bhammekalan	3
	Kanwali	2
	Kaler Kolta	2
Bhatinda	Sangat Mandi	8
	Giana	2
	Kishanpura Urf Kutti	4
	Talwandi, Fatta Balu	2

4.2 Confirmatory species level identification of *V. parahaemolyticus*

PCR based approach was used for confirmatory identification of *V. parahaemolyticus* from the total 109 *Vibrio* spp. isolates obtained from inland saline shrimp culture farms of district Mansa and Bhatinda. During PCR testing, a total of 48 isolates were confirmed as *V. parahaemolyticus* (Fig. 5). Presence of *V. parahaemolyticus* was confirmed in 40 out of 91 samples and 8 out of 18 samples from Mansa and Bhatinda districts, respectively (Fig. 6).

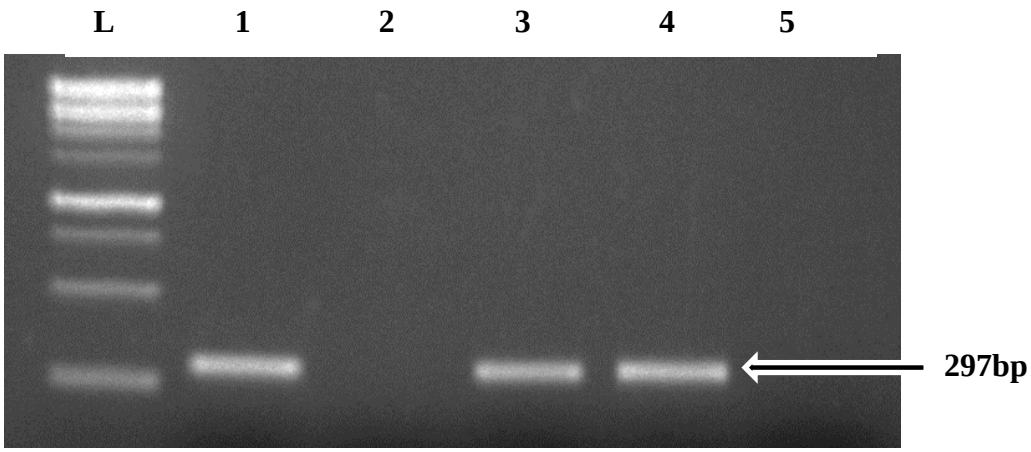

Fig. 5: PCR confirmation of *Vibrio parahaemolyticus*, Lane L: 1 kb DNA ladder (SMOBIO DM3100 ExcelBrand™ 1KB (0.25-10 kb) DNA ladder), Lane 1 & 3: Samples positive for *V. parahaemolyticus*, Lane 2: Sample negative for *V. parahaemolyticus*, Lane 4: Positive control, Lane 5: Negative control

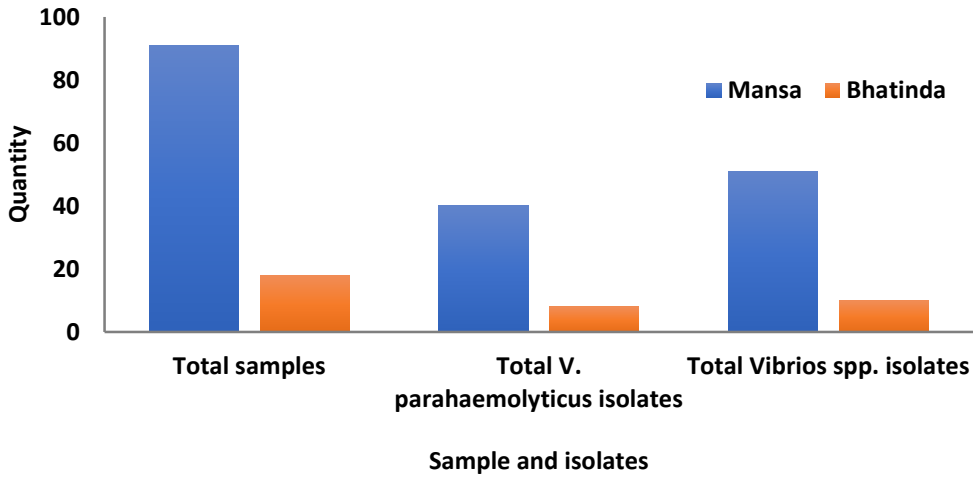

Fig. 6: Graphical representation of total sample collection, *Vibrio* spp. isolates and *V. parahaemolyticus* isolates

Heterotrophic and metabolically diverse *Vibrio* spp. are the indigenous microflora of diverse aquatic environments. Though primarily marine halophile, several members of *Vibrio* spp. are well adapted to grown in brackishwater/estuarine as well as freshwater environments (Letchumanan et al., 2014; Takemura et al., 2014). Besides, studies have also reported the presence of *Vibrio* spp. in low salinity inland saline farms in India and abroad (Yano et al., 2014; Singh et al., 2018). Among various pathogenic species of *Vibrio*, *V. parahaemolyticus* is the important one from the human and shrimp health point. Though halophilic and primarily a resident of marine environment, *V. parahaemolyticus* has also been isolated from the low salinity

inland areas. In an study by Yano et al. (2014), 38% of shrimp samples, collected from low salinity (1-5 ppt) inland saline farms of Thailand, were found to be positive for *V. parahaemolyticus* (Yano et al., 2014). In a previous study, Singh et al. (2018) reported 1% prevalence of *V. parahaemolyticus* in inland saline areas of Punjab (Singh et al., 2018). However, in the present study, the prevalence was found to be much higher at 53.9%. This could be due to the fact the Singh et al. collected the sample from low salinity (≤9 ppt) farms practicing freshwater carp culture in inland saline areas, whereas in the present study samples were collected from inland shrimp culture farms. The dominance of various *Vibrio* (including *V. parahaemolyticus*) in shrimp culture facilities across the globe is well established. These finding also suggest that with expanding shrimp culture, the *V. parahaemolyticus* prevalence and associated risk is also increasing in the inland saline areas.

4.3 Presence of virulence-associated genes in *V. parahaemolyticus*

Previously standardized and validated PCR methods were used for screening of virulence-associated genes in all 48 *V. parahaemolyticus* isolates as well as other *Vibrio* isolates. Among these, 32 and 6 isolates of *V. parahaemolyticus* were found positive for *tlh* and *trh* genes, respectively (Fig. 7 and 8). No presence of *tdh* and *VopC* genes as well as *PirA* and *PirB* toxin encoding plasmid was detected in any isolate.

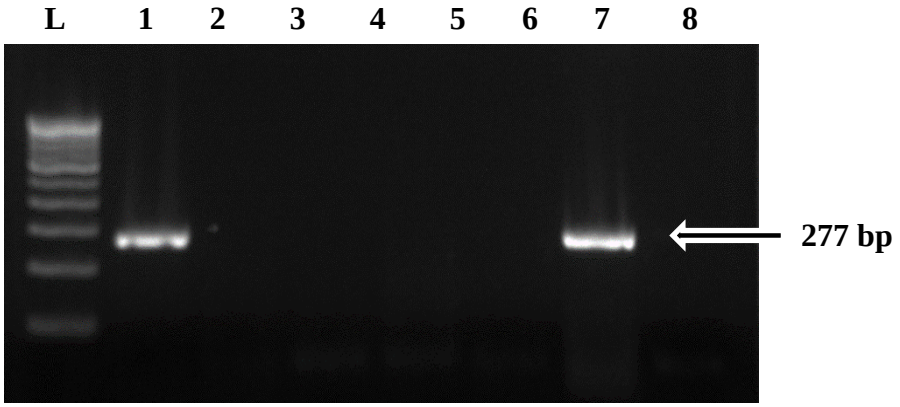

Fig. 7: PCR Detection of *tlh* gene in *Vibrio parahaemolyticus*, Lane L: StepUp™100 bp DNA Ladder (GeNei), Lane 1: *tlh* positive sample, Lane 2-6: *tlh* negative samples Lane 7: Positive control, Lane 8: Negative control

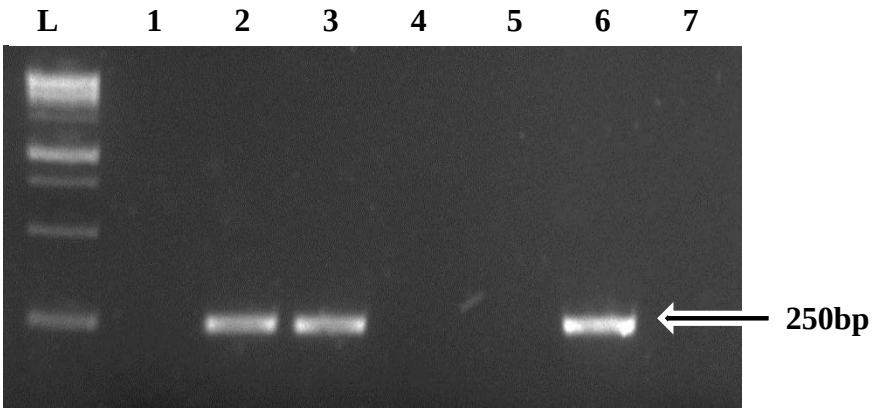

Fig. 8: PCR detection of *trh* gene in *Vibrio parahaemolyticus*, Lane L: 1 kb DNA ladder (SMOBIO DM3100 ExcelBrand^{TM} 1KB (0.25-10 kb) DNA ladder), Lane 2 & 3: *trh* positive samples, Lane 4 & 5: *trh* negative sample, Lane 6: Positive control, Lane 7: Negative control

In *V. parahaemolyticus, tdh* and *trh* genes are important virulence factors that cause erythrocyte haemolysis, cardiotoxicity, and enterotoxicity (Wang et al.,2015; Paria et al., 2019). Almost all the clinical isolates of *V. parahaemolyticus* contain either both or one of these virulence factors. The immunogenicity of TRH toxin is similar to TDH, and there is homology of 70% between both the genes. This suggests that *trh*-positive *V. parahaemolyticus* induces infection in the same way that *tdh*-positive *V. parahaemolyticus* does (Saito et al., 2015). Even though role of *trh* and *tdh* genes in pathogenicity of *V. parahaemolyticus* is well established, their presence is not absolute in all the clinical isolates of *V. parahaemolyticus*. Studies have reported that environmental isolates of *V. parahaemolyticus* lacking *trh* or *tdh* genes may still cause cytotoxicity in human gastrointestinal cells (Mahoney et al., 2010; Raghunath, 2014; Ghenem et al., 2017).

Thermolabile hemolysin (TLH) is another hemolysin of *V. parahaemolyticus*, that causes lysis of red blood cells and it is encoded by *tlh* gene (Shinoda et al., 1991; McCarthy et al., 1999; Wang et al., 2013). Clinical and environmental strains of *V. parahaemolyticus* expresses *tlh* gene (Bej et al., 1999). The *tlh* gene is significantly upregulated under simulated intestinal infection conditions (Gotoh et al., 2010). TLH also lyses human erythrocytes and has typical lecithin-dependent phospholipase activity (Broberg et al., 2011). Therefore, *tlh* positive strains may have biological roles similar to those of the TDH and TRH toxins, and may play a crucial part in the *V. parahaemolyticus* infection. In case of *V. parahaemolyticus*, *tlh* gene has also been used as a species-species marker for confirmatory PCR identification (Bej et al., 1999;

Dickinson et al., 2013). However, recent studies have indicated that not all the isolates of *V. parahaemolyticus* harbour *tlh* gene (Zaher et al., 2021).

All *Vibrio* isolates samples were screened for *PirA* and *PirB* toxin encoding plasmid by PCR method. However, none of the isolates tested positive for this virulence genes. AHPND is a newly emerging shrimp disease that has severely damaged the global shrimp industry. AHPND is caused by toxic strains of *V. parahaemolyticus* that have acquired a "selfish plasmid" encoding the deadly binary toxins $PirAB^{vp}$ (Li et al., 2017). Till date, AHPND has not been reported in India, and negative results in the present study are as per expectations.

4.4 Antimicrobial susceptibility testing

In this study, confirmed *Vibrio* isolates, including 48 *V. parahaemolyticus* isolates, were subjected to antimicrobial susceptibility testing against 12 commonly used antibiotics in aquaculture. Antibiotics disks (HiMedia, India) ampicillin (AMP; 10 µg), gentamycin (GEN; 10 µg), tetracycline (TE; 30 µg), chloramphenicol (C; 30 µg), ciprofloxacin (CIP; 5 µg), sulfisoxazole (SF; 300 µg), kanamycin (K; 30 µg), amoxiclav (AMC; 30 µg), imipenem (IPM; 10 µg), cefotaxime (CTX; 30 µg), co-trimaxozole (COT; 25 µg) and ceftazidime (CAZ; 30 µg) were used. Depending upon the antibiotics and sample types, either clear zones of lysis or confluent bacterial growth were observed around the antibiotic discs (Fig 9).

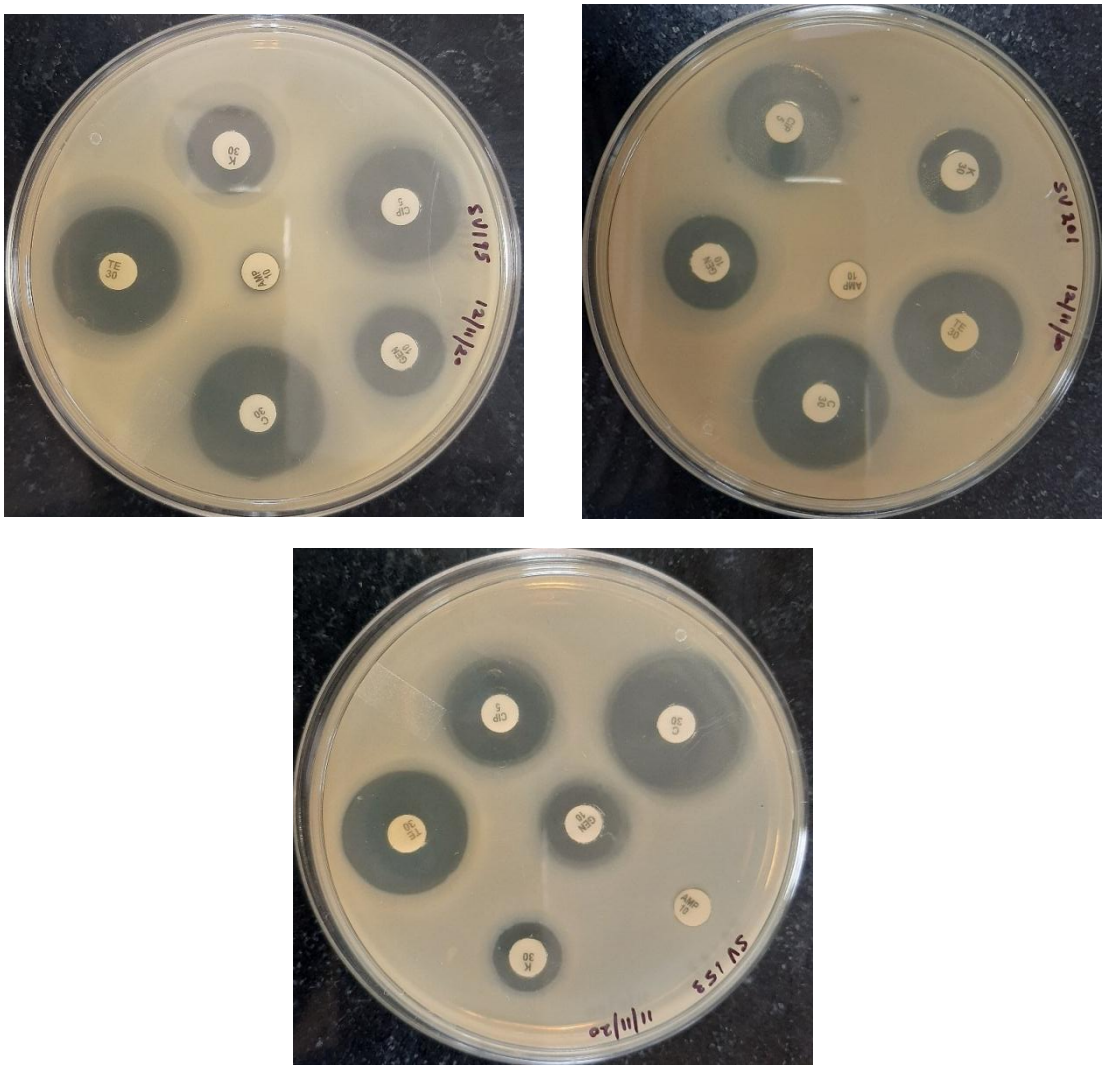

Fig. 9: Representative images of antibiotics susceptibility testing of *Vibrio* from inland saline shrimp culture farms

4.4.1 Overall patterns of antibiotic resistance in *Vibrio* isolates

During the evaluation of antibiotic resistance patterns in all *Vibrio* isolates, highest resistance was observed against cefotaxime (91.6%) followed by amoxiclav (87.4%), ampicillin (85.3%) and ceftadizime (69.5%). The susceptibility of all *Vibrio* isolates was found to highest against chloramphenicol (98.9%), followed by tetracycline (96.8%), co-trimoxazole (86.3%), sulfisoxazole (75.8%) and imipenem (54.7%) (Fig. 10).

During the separate evaluation of antibiotic resistance patterns in 48 *V. parahaemolyticus* isolates, highest resistance (90.9%) was observed against ampicillin and amoxiclav followed by cefotaxime (86.4%) and ceftazidime (70.5%) The susceptibility of all *V. parahaemolyticus* isolates was found to highest (100%) against chloramphenicol and tetracycline followed by co-trimoxazole (93.2), sulfisoxazole

(72.7%) and imipenem (72.7%) (Fig.11). However, there was not significant difference in the distribution of antibiotic resistance patterns between all *Vibrio* isolates and *Vibrio parahaemolyticus* isolates. Also, no significant difference was observed in the distribution of resistance patterns of *V. parahaemolyticus* isolates and *Vibrio* spp. isolates excluding *V. parahaemolyticus* (Chi-square test, $p<0.05$).

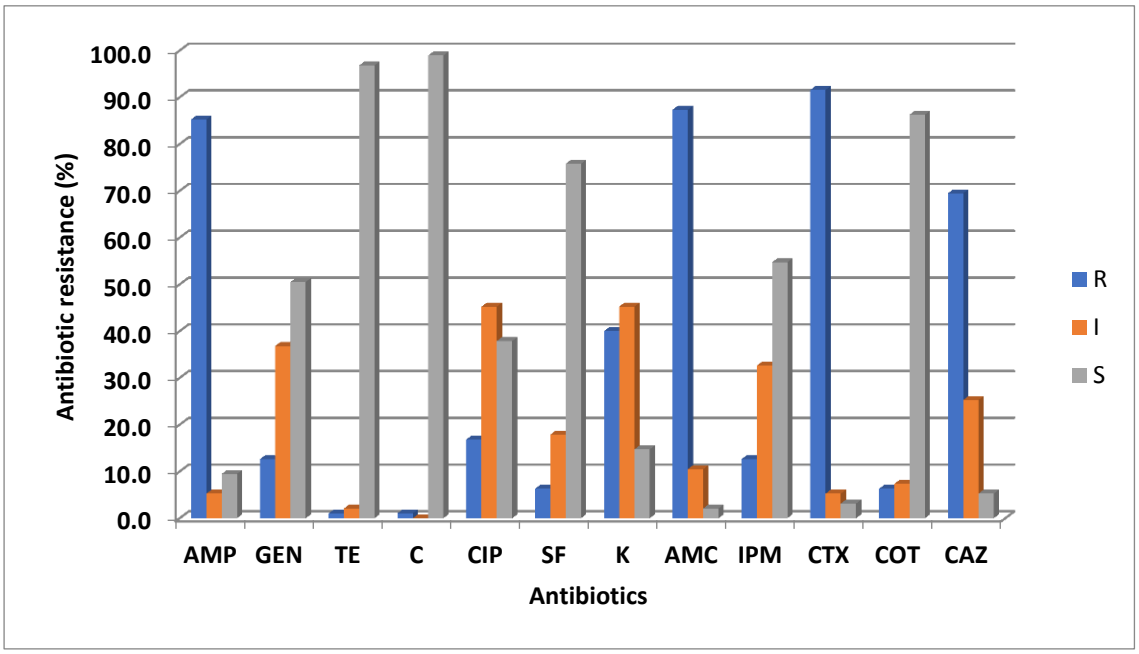

Fig. 10: Antibiotic resistance profiles of all *Vibrio* isolates

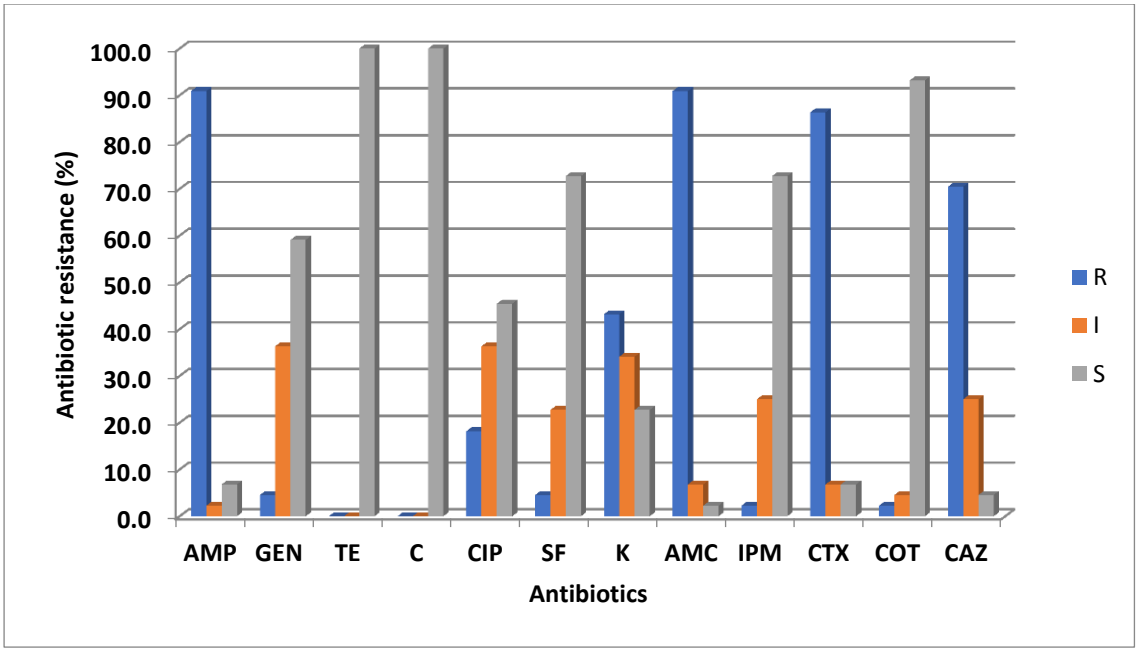

Fig. 11: Antibiotic resistance profile of all *Vibrio parahaemolyticus* isolates

Other than routine applications in human and animal therapy, antibiotics have been also used at sub-therapeutic levels in poultry, livestock production and in

aquaculture to stimulate the growth and to prevent infection (Landers et al., 2012). Antibiotics can enter through both agricultural and urban sources and persist in aquatic environment causing resistant bacteria to survive. Hundreds of antibiotic resistance genes (ARGs) have evolved and spread as a result of this selection pressure, conferring resistance to bacteria of various origins (Allen et al., 2010). Being the natural autochthonous microbiota of diverse aquatic systems, environmental *Vibrio* often act a natural reservoir of antibiotic resistance genes, which can often be horizontally transferred to other bacterial species (Elmahdi et al., 2016). Higher prevalence of resistance against ampicillin, colistin, ceftazidime, vancomycin, amoxicillin, midecamycin and furazolidonhas previously been reported in several environmental *Vibrio* spp. including *V. parahaemolyticus* (Sudha et al., 2014; Yano et al., 2014; Deng et al., 2020). In the present study too, high (>50%) resistance was observed against ampicillin, amoxiclav, cefotaxime and ceftadizime. The widespread use of ampicillin and amoxicillin in human, veterinary, and aquaculture over the last several decades may explain why environmental *Vibrio* isolates have developed significant resistance against these antibiotics (Ibrahim et al., 2020). Recently, rate of resistance to third-generation cephalosporins has also increased in Gram-negative bacteria. In case of enteric pathogens belonging to family *Enterobacteriaceae*, extended-spectrum β-lactamases (ESBLs) mediate the resistance against third generation cephalosporins. As these enteric bacteria can coexist with *Vibrio* in the same ecological niche, the horizontal transfer of resistance genes from between these bacterial communities cannot be ruled out. This could explain the emerging resistance against third-generation cephalosporins in *Vibrio* (Ceccarelli et al., 2016; Obaidat et al., 2017). High susceptibility of environmental *Vibrio* isolates against chloramphenicol and tetracycline has also been previously reported (Letchumanan et al., 2015). These findings imply that antimicrobial resistance trends are influenced by environmental factors and geographic locations.

4.4.2 Sample-wise distribution of antibiotic resistance

During the evaluation of antibiotic resistance as per sample types, all the *Vibrio* isolates from shrimp and 96.15% of *Vibrio* isolates from water samples were resistant to one or more antibiotics. Besides, no distinct patterns of antibiotic resistance distribution were observed between shrimp and water samples (Chi-square test, $p<0.05$). Isolates from both shrimp and water, showed similar patterns of high

(>50%) resistance against ampicillin, amoxiclav, cefotaxime and ceftadizime, whereas >50% of isolates were susceptible to chloramphenicol, tetracycline, co-trimoxazole, sulfisoxazole and imipenem (Fig 12 and 13). Due to indiscriminate use and contamination of aquatic resources, antimicrobial compounds may remain in sediment and aquatic habitats, causing environmental degradation and conferring antimicrobial resistance to microorganisms present in water and sediment. As shrimp connects with the water column and sediments in the culture system, the transfer of these antimicrobial resistant potential pathogens to shrimp is very much feasible. Thus, shrimps could serve as delivery vehicles of antimicrobial resistance to pathogenic bacteria from aquatic environments to humans and from one country to another (Hossain et al., 2012). The long-term effects are demonstrated by the fact that, even in the absence of selective pressures, when the antibiotic used was banned from an aquaculture system, genes conferring low susceptibility to that antibiotic persisted in the environment (Tamminen et al., 2011)

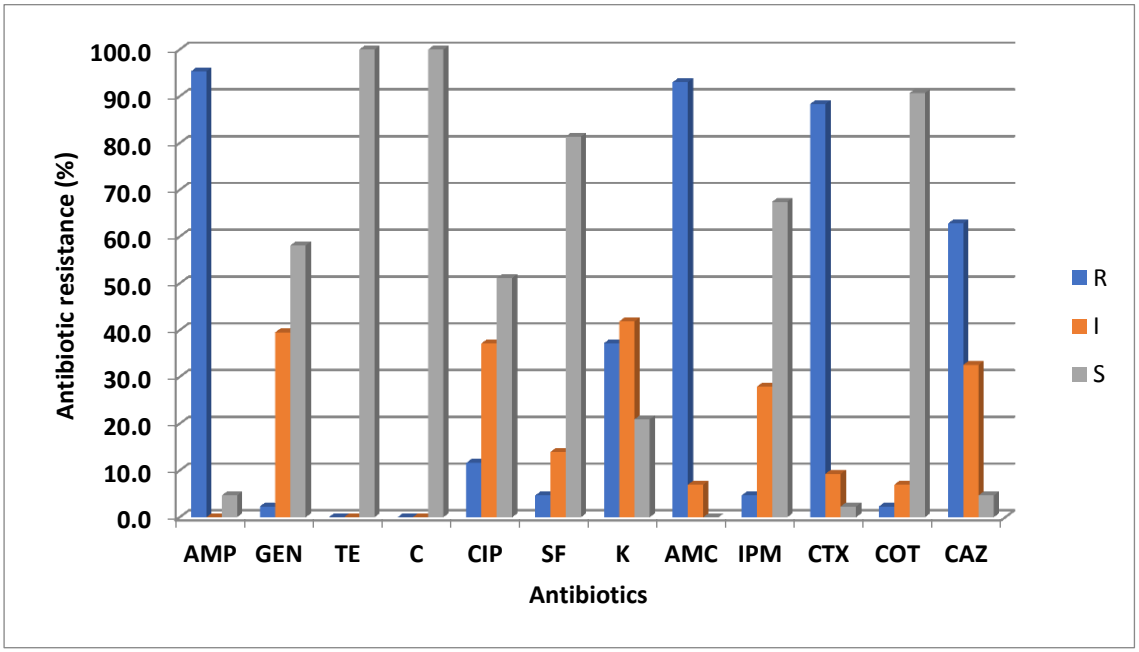

Fig. 12: Antibiotic resistance profile of all *Vibrio* isolates from shrimp samples

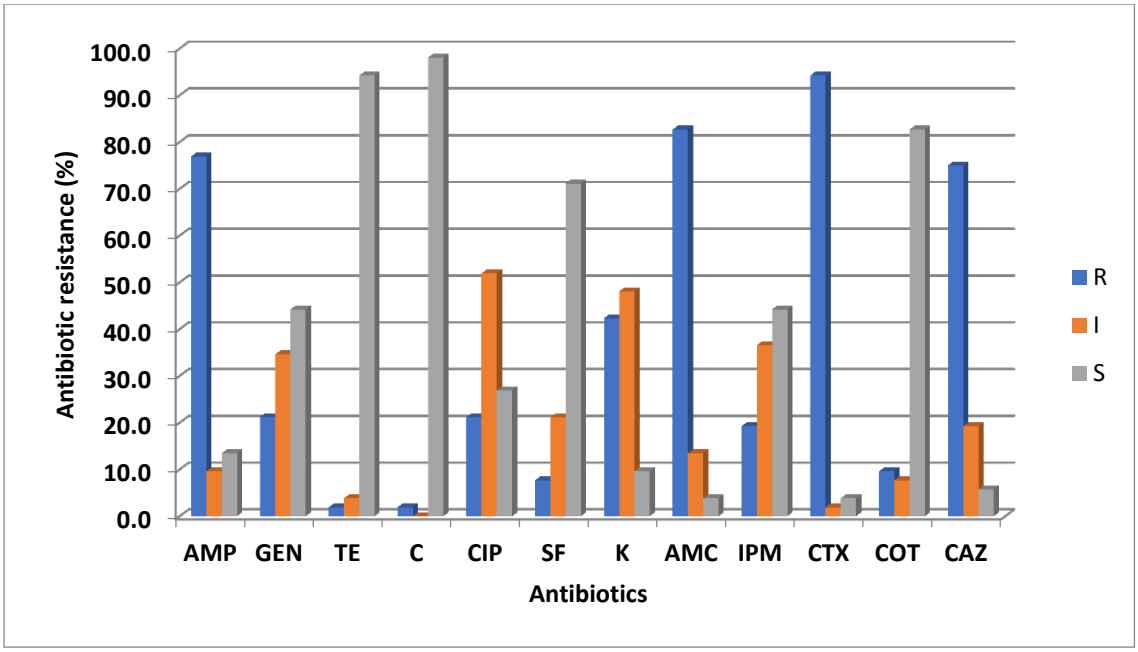

Fig. 13: Antibiotic resistance profile of all *Vibrio* isolates from water samples

4.4.3 Patterns of multiple antibiotics resistance (MAR) in *Vibrio* isolates

In this study, the MAR index values of *Vibrio* isolates ranged from 0.00 to 0.75. Among the all the *Vibrio* isolates, only 2 isolates were susceptible to all the tested antibiotics. Among all the *Vibrio* isolates, 95.8% isolates were resistant to 2 or more antibiotics, whereas 87.4% isolates were resistant to 3 or more antibiotics with MAR index value of ≥0.25. Highest MAR index value of 0.75 with resistance against 9 antibiotics was observed against one *Vibrio* isolate (Fig.14).

In case of only *V. parahaemolyticus* isolates, MAR index values ranged from 0.00 to 0.58. Among all *V. parahaemolyticus* isolates, 86.3% were found to be resistant to 3 or more antibiotics with MAR index value of ≥0.25. Highest MAR index value of 0.58 with resistance against 7 antibiotics was recorded for 3 *V. parahaemolyticus* isolates (Fig. 15). Though overall MAR index range of 0.00 – 0.75 for all *Vibrio* isolates was higher than the MAR index range of 0.00 - 0.58 for *V. parahaemolyticus* isolates, no significant difference was observed in the distribution of MAR index patterns between these two groups (Chi-square test, $p<0.05$). Besides, the distribution of MAR index values of *V. parahaemolyticus* isolates was also statistically indifferent from MAR index values of *Vibrio* spp. isolates excluding *V. parahaemolyticus* (Chi-square test, $p<0.05$).

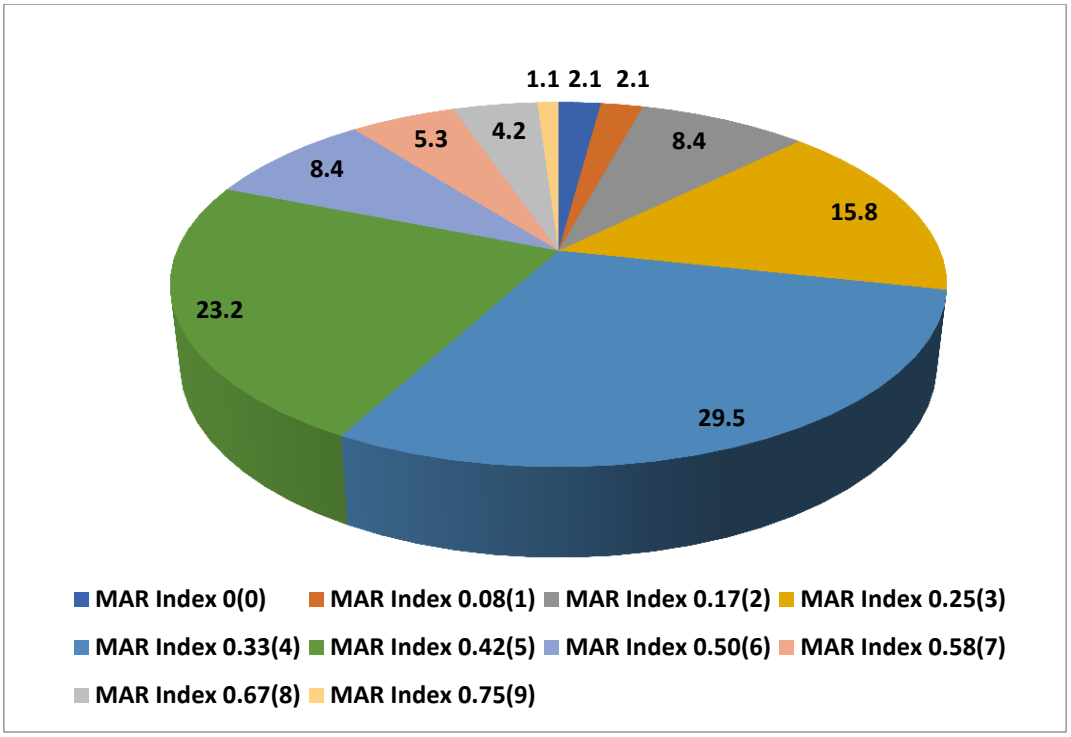

Fig. 14: Overall percentage distribution of (MAR) index values of *Vibrio* isolates. Values in parentheses indicate the number of antibiotics against which resistance was observed for each MAR index.

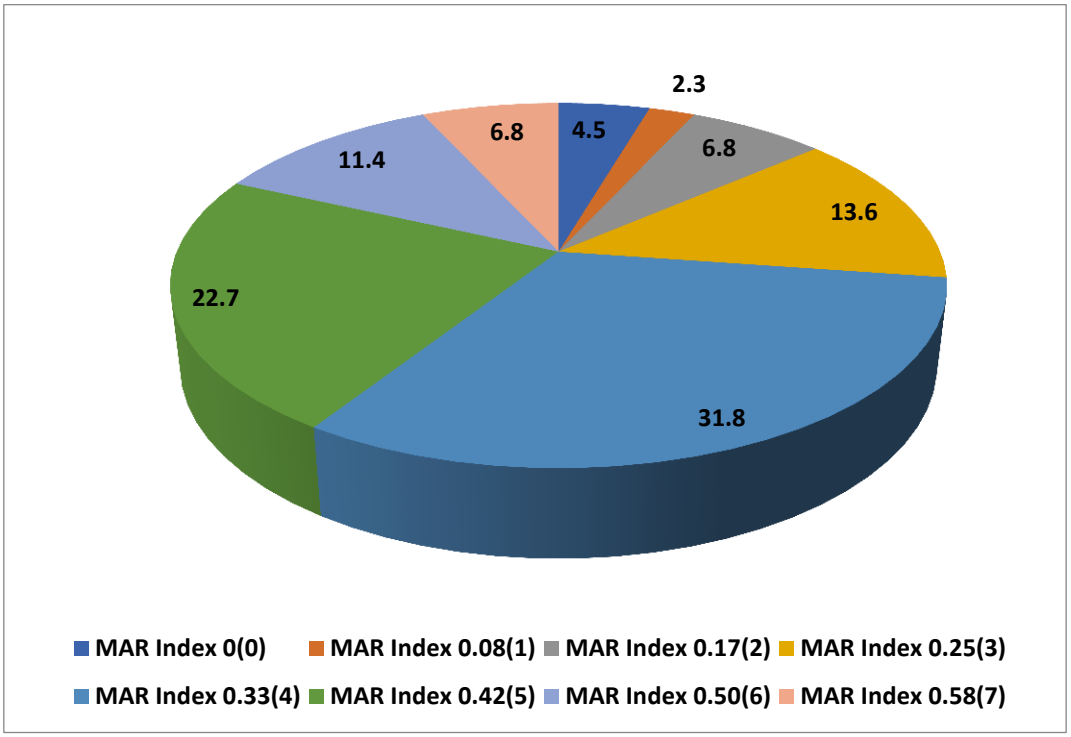

Fig. 15: Percentage distribution of (MAR) index values of *V. parahaemolyticus* isolates. Values in parentheses indicate the number of antibiotics against which resistance was observed for each MAR index

Multiple antibiotic resistance in the *Vibrio* isolates from shrimp and water samples was also analyzed separately (Fig 16 and 17). However, no significant difference between in the MAR of *Vibrio* isolates from shrimp and water was observed (Chi-square test, $p<0.05$).

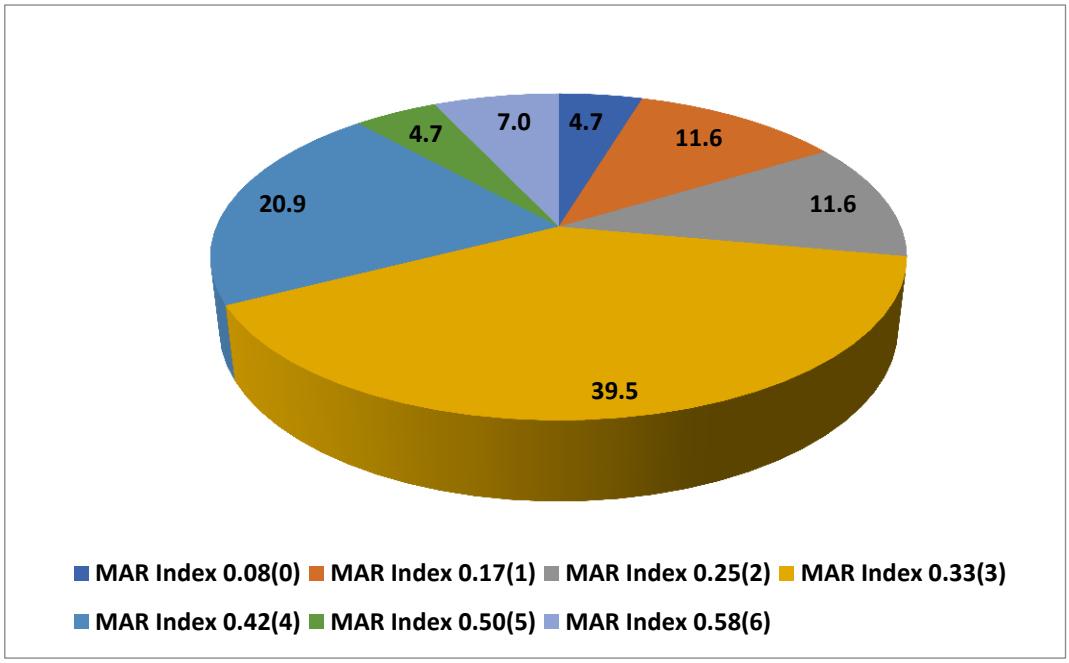

Fig. 16: Percentage distribution of (MAR) index values of *Vibrio* isolates from shrimp samples. Values in parentheses indicate the number of antibiotics against which resistance was observed for each MAR index

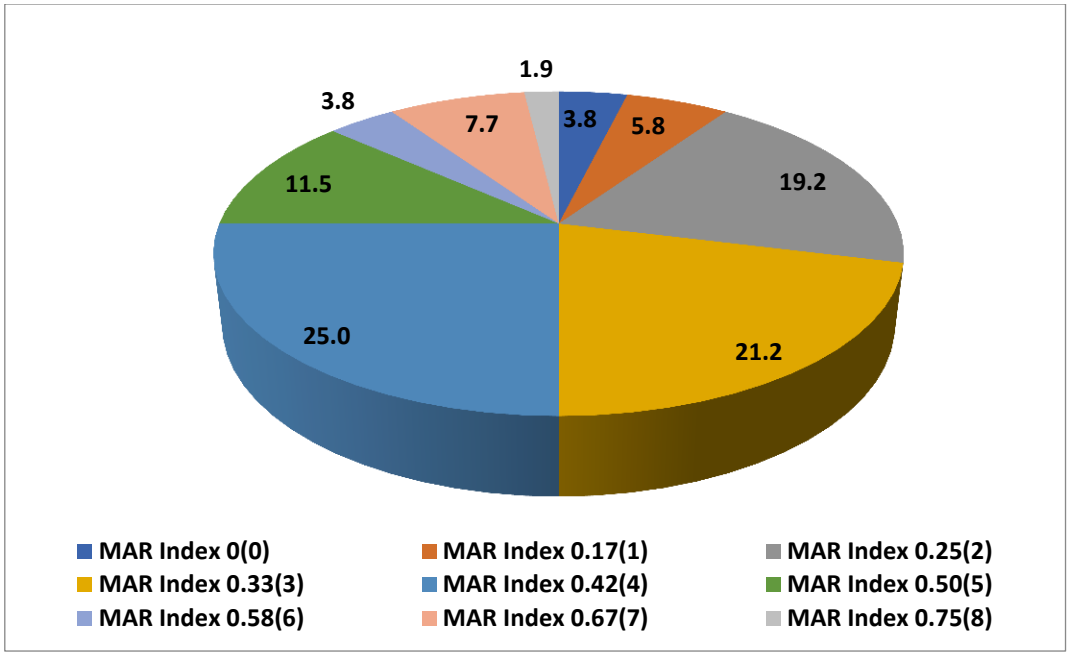

Fig. 17: Percentage distribution of (MAR) index values of *Vibrio* isolates from water samples. Values in parentheses indicate the number of antibiotics against which resistance was observed for each MAR index

The MAR index value is used to evaluate the prevalence of antibiotic resistance in specific environment. It also serves as indicator of potential risk to human health originating from such environments. It has also been suggested that seafood samples with MAR index value of higher than 0.2 originate from the sources with potential risks associated with antibiotic resistance (Krumperman, 1983; Preena et al., 2020). Though multiple antibiotic resistance is more common in the clinical isolates of *Vibrio*, environmental *Vibrio* isolates showing resistance to multiple antibiotics have also been described (Chomvarin et al., 2015; Igbinosa, 2016). The presence of multidrug resistant *Vibrio* spp. has been reported across the globe (Elmahdi et al., 2016). In a study in Korea, 51% of *V. parahaemolyticus* isolates from oyster samples were resistant to five or more antibiotics. The MAR index value for majority of isolates was 0.32. However, one *V. parahaemolyticus* isolate with MAR index value of 0.69 (resistant to 11 antibiotics) was also detected (Kang et al., 2018). Multidrug resistant *Vibrio* in the environment could act as the reservoirs of antibiotic resistance genes. As majority of resistance antibiotic resistance genes are present on the mobile genetic elements (plasmids, transposons and integrons etc.), horizontal gene transfer to ecologically/evolutionarily related or unrelated bacteria may result in severe consequences for aquatic animals and human health (Liu et al., 2013; Preena et al., 2020). Thus, routine monitoring of antimicrobial resistance status in diverse aquaculture environments including the rapidly growing inland saline shrimp culture is very much necessary. To the best of our knowledge, this is the first Indian study evaluating potentially pathogenic *Vibrio* spp. and their antimicrobial resistance in non-coastal inland saline shrimp farms.

4.5 Lytic activity of phage against *V. parahaemolyticus*

In the present study, the lytic activity of twelve previously isolated *V. parahaemolyticus* phages (available with Department of Aquatic Environment, College of Fisheries) was also tested against all 48 *V. parahaemolyticus* isolates by lawn assay (Fig. 18). Among all the phages, only one phage (AMN1) showed activity against 4 isolates of *V. parahaemolyticus*, whereas the lytic activities of other phages were limited to only the host strain (Table 5). With estimated 10^{31} particles belonging to 10^{6} species, phages form the most dominant biological entity on the earth species (Brussow & Hendrix, 2002). Though having been around for several decades, phage-based biocontrol of potential bacterial pathogens has reemerged as an attractive

alternative due to rapid emergence of antimicrobial resistance. Besides, phage intervention has also been suggest as important approach for control the flow of multi-drug resistance genes in the environment (Parmar et al., 2017). However, the activity of phage against potential bacterial pathogens is specifically dependent on its host range. Being the virus, each phage has its own host range. Some phages may show the lytic activity against only or few bacterial isolates, whereas the others can infect a large number of bacterial isolates belonging to distinct species or even genera. This difference in phage host range is determined the ability of various phages to bind the specific receptors on the bacterial cell wall (Ross et al., 2016). Within the same bacterial species, different strains may carry different receptors for phage binding. Besides the therapeutic application, host specificity of phage can also be used for characterization of bacteria through phage typing approach. During phage typing, distinctive phage sensitivity patterns are used to understand the prevalence of different strains of same bacterial species. Phage typing is an important tool for pathogen characterization and surveillance (Haq et al., 2012; Ferrari et al., 2017). In the present study, the limited host range of phage against *V. parahaemolyticus* isolates emphasize on the need of isolation and characterization of more phages from inland saline shrimp culture areas for future therapeutic use. The host range findings also indicated the there is great diversity among the stains of *V. parahaemolyticus* prevalent in inland saline shrimp culture farms of Punjab.

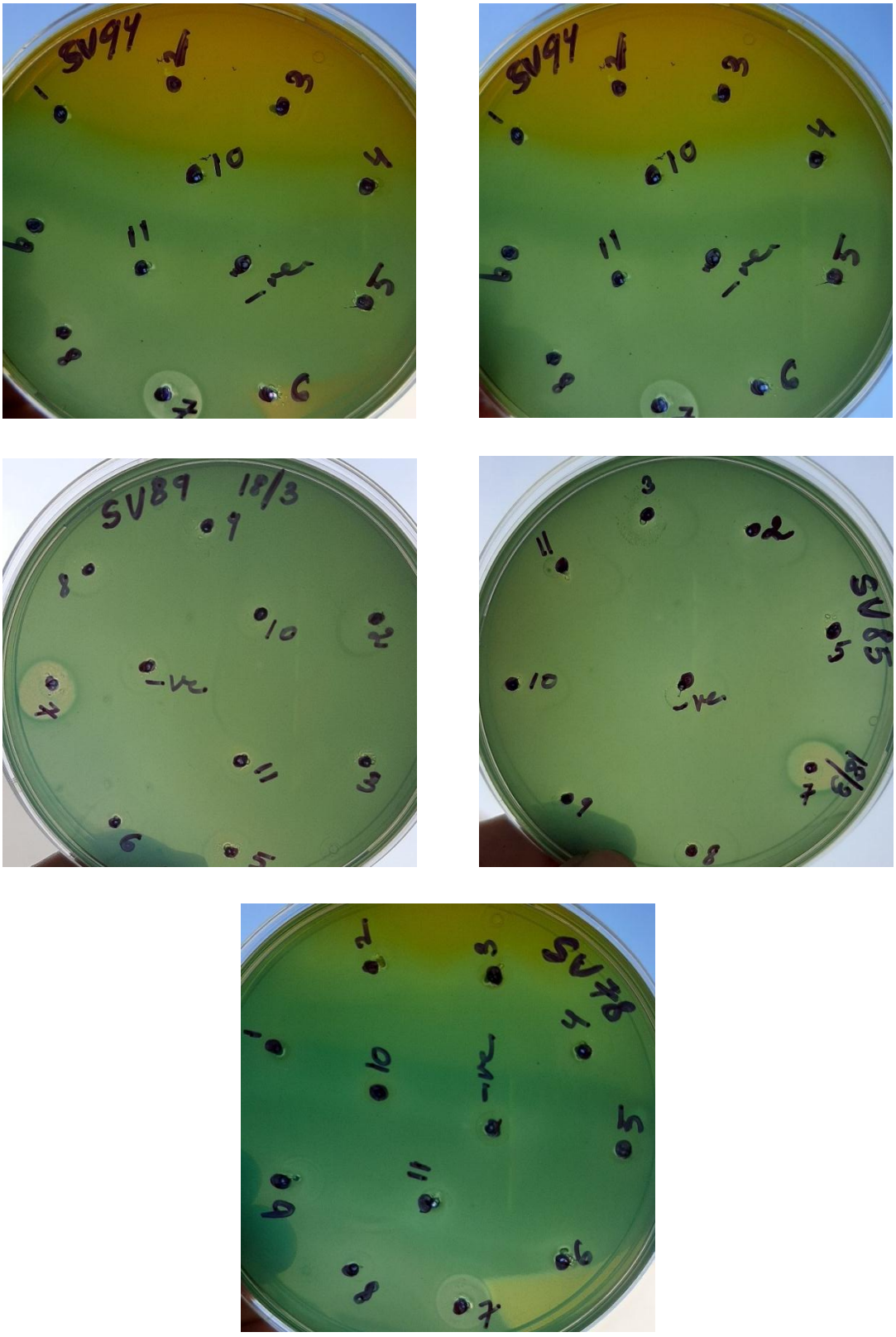

Fig. 18: Lytic activity of various phages against *V. parahaemolyticus* isolates. Negative control is the filtered extract of the overnight grown host bacterium without any phage.

Table 5 : Host range analysis of *V. parahaemolyticus* phages

S. No.	Bacterial Strain	Isolate Name	Lytic activity of phage isolated against *V. parahaemolyticus* Vp25 host							Lytic activity of phage isolated against *V. parahaemolyticus* Vp7PE host				
			AMN1	AMN3	PL1	V2	V4	V5	V6	AMN2	FT2	FT3	KD1	V1
A. Lytic activity against *V. parahaemolyticus* isolates														
1	*V. parahaemolyticus*	VP7PE	-	-	-	-	-	-	-	+	+	+	+	+
2	*V. parahaemolyticus*	VP-25	+	+	+	+	+	+	+	-	-	-	-	-
3	*V. parahaemolyticus*	SV-31	-	-	-	-	-	-	-	-	-	-	-	-
4	*V. parahaemolyticus*	SV-32	-	-	-	-	-	-	-	-	-	-	-	-
5	*V. parahaemolyticus*	SV-33	-	-	-	-	-	-	-	-	-	-	-	-
6	*V. parahaemolyticus*	SV-34	-	-	-	-	-	-	-	-	-	-	-	-
7	*V. parahaemolyticus*	SV-35	-	-	-	-	-	-	-	-	-	-	-	-
8	*V. parahaemolyticus*	SV-36	-	-	-	-	-	-	-	-	-	-	-	-
9	*V. parahaemolyticus*	SV-57	-	-	-	-	-	-	-	-	-	-	-	-
10	*V. parahaemolyticus*	SV-58	-	-	-	-	-	-	-	-	-	-	-	-
11	*V. parahaemolyticus*	SV-65	+	-	-	-	-	-	-	-	-	-	-	-
12	*V. parahaemolyticus*	SV-66	+	-	-	-	-	-	-	-	-	-	-	-
13	*V. parahaemolyticus*	SV-72	+	-	-	-	-	-	-	-	-	-	-	-
14	*V. parahaemolyticus*	SV-76	-	-	-	-	-	-	-	-	-	-	-	-
15	*V. parahaemolyticus*	SV-77	-	-	-	-	-	-	-	-	-	-	-	-
16	*V. parahaemolyticus*	SV-80	-	-	-	-	-	-	-	-	-	-	-	-

S. No.	Bacterial Strain	Isolate Name	Lytic activity of phage isolated against *V. parahaemolyticus* Vp25 host							Lytic activity of phage isolated against *V. parahaemolyticus* Vp7PE host				
			AMN1	AMN3	PL1	V2	V4	V5	V6	AMN2	FT2	FT3	KD1	V1
17	*V. parahaemolyticus*	SV-113	-	-	-	-	-	-	-	-	-	-	-	-
18	*V. parahaemolyticus*	SV-114	-	-	-	-	-	-	-	-	-	-	-	-
19	*V. parahaemolyticus*	SV-115	-	-	-	-	-	-	-	-	-	-	-	-
20	*V. parahaemolyticus*	SV-116	-	-	-	-	-	-	-	-	-	-	-	-
21	*V. parahaemolyticus*	SV-122	-	-	-	-	-	-	-	-	-	-	-	-
22	*V. parahaemolyticus*	SV-135	-	-	-	-	-	-	-	-	-	-	-	-
23	*V. parahaemolyticus*	SV-137	-	-	-	-	-	-	-	-	-	-	-	-
24	*V. parahaemolyticus*	SV-139	-	-	-	-	-	-	-	-	-	-	-	-
25	*V. parahaemolyticus*	SV-140	-	-	-	-	-	-	-	-	-	-	-	-
26	*V. parahaemolyticus*	SV-145	-	-	-	-	-	-	-	-	-	-	-	-
27	*V. parahaemolyticus*	SV-148	-	-	-	-	-	-	-	-	-	-	-	-
28	*V. parahaemolyticus*	SV-149	-	-	-	-	-	-	-	-	-	-	-	-
29	*V. parahaemolyticus*	SV-150	-	-	-	-	-	-	-	-	-	-	-	-
30	*V. parahaemolyticus*	SV-152	-	-	-	-	-	-	-	-	-	-	-	-
31	*V. parahaemolyticus*	SV-154	-	-	-	-	-	-	-	-	-	-	-	-
32	*V. parahaemolyticus*	SV-155	-	-	-	-	-	-	-	-	-	-	-	-
33	*V. parahaemolyticus*	SV-158	-	-	-	-	-	-	-	-	-	-	-	-
34	*V. parahaemolyticus*	SV-160	-	-	-	-	-	-	-	-	-	-	-	-

S. No.	Bacterial Strain	Isolate Name	Lytic activity of phage isolated against *V. parahaemolyticus* Vp25 host							Lytic activity of phage isolated against *V. parahaemolyticus* Vp7PE host				
			AMN1	AMN3	PL1	V2	V4	V5	V6	AMN2	FT2	FT3	KD1	V1
35	*V. parahaemolyticus*	SV-161	-	-	-	-	-	-	-	-	-	-	-	-
36	*V. parahaemolyticus*	SV-163	-	-	-	-	-	-	-	-	-	-	+	-
37	*V. parahaemolyticus*	SV-165	-	-	-	-	-	-	-	-	-	-	-	-
38	*V. parahaemolyticus*	SV-166	-	-	-	-	-	-	-	-	-	-	-	-
39	*V. parahaemolyticus*	SV-167	-	-	-	-	-	-	-	-	-	-	-	-
40	*V. parahaemolyticus*	SV-179	-	-	-	-	-	-	-	-	-	-	-	-
41	*V. parahaemolyticus*	SV-180	-	-	-	-	-	-	-	-	-	-	-	-
42	*V. parahaemolyticus*	SV-182	-	-	-	-	-	-	-	-	-	-	-	-
43	*V. parahaemolyticus*	SV-184	-	-	-	-	-	-	-	-	-	-	-	-
44	*V. parahaemolyticus*	SV-187	-	-	-	-	-	-	-	-	-	-	-	-
45	*V. parahaemolyticus*	SV-188	-	-	-	-	-	-	-	-	-	-	-	-
46	*V. parahaemolyticus*	SV-189	-	-	-	-	-	-	-	-	-	-	-	-
47	*V. parahaemolyticus*	SV-191	-	-	-	-	-	-	-	-	-	-	-	-
48	*V. parahaemolyticus*	SV-218	-	-	-	-	-	-	-	-	-	-	-	-
49	*V. parahaemolyticus*	SV-219	-	-	-	-	-	-	-	-	-	-	-	-
50	*V. parahaemolyticus*	SV-231	-	-	-	-	-	-	-	-	-	-	-	-
Subtotal of phage lytic activity against *V. parahaemolyticus* isolates			4	1	1	1	1	1	1	1	1	1	2	1

CHAPTER – V

SUMMARY AND CONCLUSIONS

Among various aquaculture species, white leg shrimp *Litopenaeus vannamei* is the important one. Due to high global demand, export potential and low salinity tolerance of vannamei shrimp, inland saline areas, previously considered as wastelands, have emerged as attractive destinations for shrimp farming. Due to lack of adaptive immune system, shrimp is prone to >20 types of viral, bacterial, fungal and parasitic diseases. The bacterial infections caused by *Vibrio* species are also the second important cause of mortalities. Among various shrimp pathogenic *Vibrio* species, *V. parahaemolyticus* is the important one. *Vibrio parahaemolyticus* is a human pathogen as well as an emerging shrimp. *V. parahaemolyticus* carrying thermostable direct hemolysin (*tdh*) and/or thermostable direct hemolysin-related hemolysin (*trh*) genes has often been found as the causative agent of acute gastroenteritis in humans resulting from the consumption of poor quality, undercooked or raw seafood. Recently, one strain of *V. parahaemolyticus* carrying a plasmid encoding for *PirA* and *PirB* toxins has also been found responsible for a devastating shrimp early mortality syndrome (EMS), also known as Acute Hepatopancreatic Necrosis Disease (AHPND). *V. parahaemolyticus* is primarily a halophile bacterium, and natural inhabitant of estuarine and coastal marine environments, its existence in low salinity inland saline areas (like some districts of Punjab) has also been reported recently. Antibiotics are often used for control of bacterial pathogens human, veterinary and aquaculture. As, shrimp culture in inland saline aquaculture areas of Punjab is expanding at rapid rate, there is high risk of emergence of potentially pathogenic multi-drug resistant *Vibrio*. The present study was carried out to assess the current status of antibiotic resistance in inland shrimp culture farms of Punjab. For control of potentially pathogenic *V. parahaemolyticus*, lytic efficacy of previously isolated phages against antibiotic resistant *V. parahaemolyticus* strains were also evaluated.

In the present study, a total of 109 samples (shrimp – 66 samples, water – 44 samples) were collected from 21 inland saline shrimp farms located in the 15 villages of district Mansa and Bhatinda of Punjab. After enrichment in alkaline peptone water (APW, pH 8.5 with 3% NaCl) and biochemical testing of presumptive yellow/green

colonies picked from TCBS agar, a total of 109 bacterial isolates were confirmed as belonging to Genus *Vibrio*. After PCR testing as per previously described protocol, a total of 48 isolates were confirmed as *V. parahaemolyticus*. Halophilic nature and primarily a resident of marine environment, *V. parahaemolyticus* has already been isolated from the low salinity inland areas in previous studies. In the present study, the prevalence was found to be much higher at 53.9%.

Previously standardized and validated PCR methods were used for screening of virulence-associated genes in all 48 *V. parahaemolyticus* isolates as well as other *Vibrio* isolates. Among these, 32 and 6 isolates of *V. parahaemolyticus* were found positive for *tlh* and *trh* genes, respectively. No presence of *tdh* and *VopC* genes as well as *PirA* and *PirB* toxin encoding plasmid was detected in any isolate. These results were agreement to the previous studies.

Then Antimicrobial susceptibility of all confirmed *Vibrio* isolates (including *V. parahaemolyticus*) was tested by disk diffusion method against 12 commonly used antibiotics in aquaculture and veterinary medicine per Clinical Laboratory Standards Institute (CLSI) guideline M45. Antibiotics disks of ampicillin (AMP; 10 µg), gentamicin (GEN; 10 µg), tetracycline (TE; 30 µg), chloramphenicol (C; 30 µg), ciprofloxacin (CIP; 5 µg), sulfisoxazole (SF; 300 µg), kanamycin (K; 30 µg), amoxiclav (AMC; 30ug), imipenem (IPM; 10ug), cefotaxime (CTX; 30ug), co-trimoxazole (COT; 25ug), ciftazidime (CAZ; 30ug) (HiMedia, India) were used during the study. Among all *Vibrio* isolates, highest resistance was observed against cefotaxime (91.6%) followed by amoxiclav (87.4%), ampicillin (85.3%) and ceftadizime (69.5%). The susceptibility of these *Vibrio* isolates was found to highest against chloramphenicol (98.9%), followed by tetracycline (96.8%), co-trimoxazole (86.3%), sulfisoxazole (75.8%) and imipenem (54.7%). Also, separate evaluation of antibiotic resistance patterns in 48 *V. parahaemolyticus* isolates were done, in which highest resistance (90.9%) was observed against ampicillin and amoxiclav followed by cefotaxime (86.4%) and ceftazidime (70.5%). The susceptibility of all *V. parahaemolyticus* isolates was found to highest (100%) against chloramphenicol and tetracycline followed by co-trimoxazole (93.2), sulfisoxazole (72.7%) and imipenem (72.7%). No significant difference was observed in the distribution of resistance patterns of *V. parahaemolyticus* isolates and *Vibrio* spp. isolates excluding *V. parahaemolyticus* (Chi-square test, $p<0.05$). While evaluating antibiotic resistance as

per sample types, isolates from both shrimp and water, showed similar patterns of high (>50%) resistance against ampicillin, amoxiclav, cefotaxime and ceftadizime, whereas >50% of isolates were susceptible to chloramphenicol, tetracycline, co-trimoxazole, sulfisoxazole and imipenem. No distinct patterns of antibiotic resistance distribution were observed between shrimp and water samples (Chi-square test, $p<0.05$). To evaluate the potential risk due to multi-drug resistant bacterial isolates, the multiple antibiotic resistance (MAR) index values were also calcultated. The MAR index values of *Vibrio* isolates ranged from 0.00 to 0.75. Among the all the *Vibrio* isolates, only 2 isolates were susceptible to all the tested antibiotics. 95.8% isolates were resistant to 2 or more antibiotics, whereas 87.4% isolates were resistant to 3 or more antibiotics with MAR index value of ≥0.25. Highest MAR index value of 0.75 with resistance against 9 antibiotics was observed against one *Vibrio* isolate. In case of only *V. parahaemolyticus* isolates, MAR index values ranged from 0.00 to 0.58. Among all *V. parahaemolyticus* isolates, 86.3% were found to be resistant to 3 or more antibiotics with MAR index value of ≥0.25. Highest MAR index value of 0.58 with resistance against 7 antibiotics was recorded for 3 *V. parahaemolyticus* isolates. No significant difference was observed in the distribution of MAR index patterns between these two groups (Chi-square test, $p<0.05$). Also, Multiple antibiotic resistance in the *Vibrio* isolates from shrimp and water samples was also analyzed separately and no significant difference between in the MAR of *Vibrio* isolates from shrimp and water was observed (Chi-square test, $p<0.05$).

In the present study, the lytic activity of twelve previously isolated *V. parahaemolyticus* phages (available with Department of Aquatic Environment, College of Fisheries) was tested against all 48 *V. parahaemolyticus* isolates by lawn assay. Among all the phages, only one phage (AMN1) showed activity against 4 isolates of *V. parahaemolyticus*, whereas the lytic activities of other phages were limited to only the host strain. The limited host range of phage against *V. parahaemolyticus* isolates emphasize on the need of isolation and characterization of more phages from inland saline shrimp culture areas for future therapeutic use. The host range findings also indicated the there is great diversity among the stains of *V. parahaemolyticus* prevalent in inland saline shrimp culture farms of Punjab.

Conclusions

- A total of 109 *Vibrio* isolates were obtained from 109 shrimp and water samples collected from inland saline shrimp farms of district Mansa and Bhatinda. Among these, 48 isolates were confirmed as *V. parahaemolyticus*.
- During PCR screening of virulence-associated genes, 32 and 6 isolates of *V. parahaemolyticus* were found positive for *tlh* and *trh* genes, respectively. No other virulence-associated gene was detected in any of the isolates.
- During antibiotic susceptibility testing, highest resistance (91.6%) and susceptibility (98.9%) was observed against cefotaxime and chloramphenicol, respectively in all *Vibrio* isolates. Among 48 *V. parahaemolyticus* isolates, highest resistance (90.9%) was observed against ampicillin and amoxiclav and susceptibility (100%) against chloramphenicol and tetracycline. High (>50%) resistance was also observed against ampicillin, amoxiclav, cefotaxime and ceftadizime in all *Vibrio* and *V. parahaemolyticus* isolates.
- No significant difference was observed in the distribution of antibiotic resistance patterns between all *Vibrio* isolates and *Vibrio parahaemolyticus* isolates. Also, no significant difference was observed in the distribution of resistance patterns of *V. parahaemolyticus* isolates and *Vibrio* spp. isolates excluding *V. parahaemolyticus*. No distinct patterns of antibiotic resistance distribution were observed between shrimp and water samples.
- The MAR index values of *Vibrio* isolates ranged from 0.00 to 0.75. Highest MAR index value of 0.75 with resistance against 9 antibiotics was observed against one *Vibrio* isolate. In case of only *V. parahaemolyticus* isolates, MAR index values ranged from 0.00 to 0.58. Highest MAR index value of 0.58 with resistance against 7 antibiotics was recorded for 3 *V. parahaemolyticus* isolates. During statistical analysis, MAR index values between overall *Vibrio* isolates and *V. parahaemolyticus* isolates were not significantly different.
- The lytic activity of twelve previously isolated *V. parahaemolyticus* phages was also tested against all 48 *V. parahaemolyticus* isolates. Only one phage (AMN1) showed activity against 4 isolates of *V. parahaemolyticus*, whereas the lytic activities of other phages were limited to only the host strain.

- The presence of multiple drug resistance in *Vibrio* isolates including *V. parahaemolyticus* emphasize the need for their routine monitoring in rapidly expanding aquaculture areas for risk assessment and mitigation.
- The data provided from such kind of studies will help the government to formulate the regulatory policies for appropriate use of antibiotics in the shrimp culture systems.

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