DISTRIBUTION OF ANTIBIOTIC RESISTANCE AND PUBLIC HEALTH SIGNIFICANT VIRULENCE GENES AMONG VIBRIONACEAE ASSOCIATED WITH *Scylla olivacea* (HERBST, 1896)

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2015-09-007

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KERALA, INDIA

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by

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(2015-09-007)

THESIS

Submitted in partial fulfilment of the

requirement for the degree of

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KERALA, INDIA

2020

DECLARATION

I, hereby declare that the thesis entitled **"Distribution of antibiotic resistance and public health significant virulence genes among *Vibrionaceaee* associated with *Scylla olivacea* (Herbst, 1896)"** is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

Place: Vellayani
Date: 03/07/2020

Mariya Sony
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CERTIFICATE

Certified that this thesis entitled **"Distribution of antibiotic resistance and public health significant virulence genes among *Vibrionaceaee* associated with *Scylla olivacea* (Herbst, 1896)"** is a record of research work done independently by **Ms. MARIYA SONY (2015-09-007)** under my guidance and supervision and that this has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

Place: Kochi
Date: 03.07.2020

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Mariya Sony

DEDICATED TO

MY BELOVED FAMILY

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ADH	Arginine decarboxylase test
BLAST	Basic Local Alignment Search Tool
bp	Base pair
^{0}C	Degree Celsius
cm	Centimetre
CFU	Colony forming unit
DNA	Deoxyribonucleic acid
dNTP	Deoxy-nucleotide triphosphates
EDTA	Ethylenediamine tetra-acetic acid
Fig.	Figure
Frd	Forward
g	Gram
h	Hour
H_2O_2	Hydrogen peroxide
IU	International units
KOH	Potassium hydroxide
L	Litre
LB	Luria Bertani
LDH	Lysine decarboxylase test
$MgCl_2$	Magnesium chloride
MHA	Mueller Hinton Agar

µg	Microgram
µL	Microlitre
mg	Milligram
mL	Millilitre
mM	Millimolar
min	Minutes
M	Molar
N	Normality
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NCBI	National Centre for Biotechnology Information
NFW	Nuclease free water
ODT	Ornithine decarboxylase test
per cent	Per cent
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
pH	Log hydrogen ion concentration
rpm	Rotations per minute
sec	Second(s)
SD	Standard Deviation
spp.	Species (plural)
Taq	*Thermus aquaticus*

TCBS	Thiosulfate citrate bile salts sucrose agar
Tris HCL	Tris Hydrochloric acid
U	Unit(s)
UV	UltraViolet
V	Volts
ZMA	Zobell Marine Agar

INTRODUCTION

1. INTRODUCTION

The family *Vibrionaceaee* containing marine originated halophilic Gram-negative bacteria, comprise both pathogenic and non-pathogenic species and, are widely distributed in diverse aquatic environments (Gennari *et al*., 2012). Three species namely, *Vibrio cholerae, V. parahaemolyticus* and *V. vulnificus* are the major human pathogens in this family and form a major concern by causing diseases ranging from seafood-borne gastroenteritis, wound infections to severe septicaemia. Species namely, *V. anguilarum, V. harveyi, V. alginolyticus, V. mimicus, V. metschnikovii, V. parahaemolyticus, V. vulnificus, V. fluvialis* and *Photobacterium damselae* are the major economically significant fish pathogens of this family causing infections in many fish and shellfish species (Lightner, 1993; Mohajeri *et al*., 2011). It has been recognised that the frequency and spread of vibrio related infections in both humans and aquatic animals will become more rigid in the future times due to ocean warming and climate change (Paillard *et al*., 2004).

Earlier concept was that only the clinical isolates will be harbouring the virulence genes where as the environmental bacterial strains usually lack those genes. On contradiction to those findings, recent researches have indicated that virulence genes and their homologues, can be present in environmental strains due to acquisition/horizontal gene transfer of such genes from clinical isolates that might have taken in aquatic environments (Sechi *et al*., 2000; Caburlotto *et al*., 2009). The acquisition of mobile genetic elements namely, bacteriophages, transposons, integrative and conjugative elements plasmids and genetic islands (GEIs) are important processes in bacterial evolution and allows bacteria to enhance their acclamatisation and proliferation under different environmental conditions and their virulence potential. GEIs encode different functions, of which the most important being virulence and resistance to antibiotics (Gennari *et al*., 2012). Several such mobile elements carrying virulence genes have been detected in human pathogenic *Vibrio* species and transfer of these mobile genes could facilitate emergence of virulent or more virulent strains, even in a species defined as non-pathogenic (Boyd *et al*., 2009). Therefore, analysis of possible presence of

virulence genes in environmental strains of pathogens and in non-pathogenic species has become mandatory in this bacterial family (Gennari *et al*., 2012).

Emergence and dissemination of antimicrobial resistant bacterial strains has become an international health crisis (WHO, 2014). Setting up a monitoring system to identify antimicrobial resistance patterns and genetic basis of resistance have become an indispensable area in both clinical and environmental research fields. As vibrio strains are autochthonous microbial communities in many fish and shellfish, they can be useful indicators for antimicrobial resistance development in various environments and aquaculture practices (Eun-Gyoung *et al*., 2011). Additionally, in specific ecological niches, such as fish farms, where antibiotics are frequently used for disease control, they might become a reservoir of many antibiotic-resistant bacterial forms (Alcaide *et al*., 2005; Pedersen *et al*., 2008). Such resistant vibrio strains could be transmitted through the food chain to humans *via* horizontal gene transfer constituting a major risk to public health (Etinosa, 2016). In view of these facts, presence of *Vibrionaceaee* in aquaculture candidate animals especially human and fish/shellfish pathogenic vibrios, distribution of antibiotic resistance and public health significant virulence genes in these bacteria are the indispensable arena to be investigated (Law *et al*., 2015).

Orange mud crab (*Scylla olivacea* Herbst, 1896) represents a valuable component of coastal fisheries in several tropical and subtropical Asian countries and fetches a high price in the international seafood market. They are candidate species for aquaculture owing to their winsome qualities like faster growth, larger size, high fecundity, disease resistance, marketability, and adaptability to farming systems (Waiho *et al*., 2015). Knowledge on abundance and composition of microbes in different organs including haemolymph is essential to predict their health status and also for disease diagnosis. However, very limited data is available on the microbiology of these crabs. Assessment of the abundance and distribution of *Vibrionaceaee* in different organs coupled with studying the antibiotic resistance and public health significant virulence genes in these bacteria can be valuable in both aquaculture and public health perspectives.

Keeping all these facts in view, the objectives of the present study was kept as follows targeting its final applications in disease management studies of *S. olivacea* and for finding out the public health significance of *Vibrionaceaee* associated with this supreme aquaculture candidate.

- To analyze diversity of *Vibrionaceaee* associated with *S. olivacea*
- To check the presence of typical and non-typical virulent genes of zoonotically significant vibrios
- To identify the pattern of antibiotic resistance and to depict the genetic basis

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

2.1. BACKGROUND

Fish and other marine foods are a huge source of protein and many essential nutrients; thus, are recognised as potentially vital to food security and good nutrition in both developed and developing countries (Allison, 2011; Bene *et al*., 2015). Consequently, there has been an increase in the worldwide seafood consumption, leading to a global rise in the production of seafood from aquaculture and capture fisheries (FAO, 2014). On the other hand, increased use of aquatic products led to a remarkable global increase of seafood-borne infections, in which vibrios especially *V. vulnificus*, *V. parahaemolyticus* and *V. cholerae*, are the major culprits (Bonnin - Jusserand *et al*., 2019). Apart from acting as aetiology of diseases, vibrios are regarded as the prime and most diverse seafaring heterotrophic microbial clusters for establishing the relationship between ecological adaptation and genome progression as they are widespread in marine environment, showing versatile ecologies and metabolisms (Sawabe *et al*., 2013). Vibrios can also be used as indicators for development of antimicrobial resistance in different environments and aquaculture practices, due to the fact that these strains are the common flora in many fishes and shellfishes (Eun-Gyoung *et al*., 2011). Since the resistant vibrio strains could be transferred *via* the food chain to humans through horizontal gene transfer, antibiotic resistant vibrios in the environment pose a risk to public health (Etinosa, 2016). Hence, an indepth study of abundance, diversity and antimicrobial resistance pattern of *Vibrionaceaee* in haemolymph and various internal organs of *S. olivaceae,* one among the most consumable sea food is targeted in the present investigation. The results can be used to predict the vigor prominence and ailment diagnosis of this supreme aquaculture candidate. In short, the study is targeted to serve as a baseline reference for further public health perceptive and disease management investigations of *S. olivacea*.

2.2. *Scylla olivacea* (Herbst, 1796)/ ORANGE MUD CRAB

Mud crabs belonging to the genus *Scylla* signify a valuable element of customary, small scale coastline fisheries in quite a lot of tropical and subtropical

Southeast Asian countries that also fetches a high price in the international seafood market (BOBP, 1992). Owing to their attractive potentials like larger size, faster growth, disease resistance, higher reproductive ability (fecundity), adaptiveness to farming set-up and marketability, the mud crabs are considered as 'candidate species for aquaculture' (Viswanathan and Raffi , 2015). They are also considered to have a high content value of essential amino acids, minerals, vitamins, and fatty acids (Wan Yusof *et al*., 2019) and are extensively distributed throughout the coastal zones, estuaries and mangroves of the Indo-Pacific region and inhabit areas with reduced salinity and fluctuating water levels (Lebata *et al*., 2007). Reviewed categorisation of the genus *Scylla* through molecular methodology has evidenced the existence of four species (*Scylla. olivacea, S. tranquebarica, S. serrate* and *S. paramamosain*) (Keenan *et al*., 1998). Out of these four species, *S. olivacea* is prevalently chosen for aquaculture because of its tough nature, tolerance to the changes in temperature and salinity and ease of capture. However, most of the husbandry undertakings of *S. olivacea* encompass only crab stuffing and the creation of soft-shelled crabs, with the usage of captured wild juveniles (Ikhwanuddin *et al*., 2013). This dependence on mud crab juveniles caught from the wild, is due to the unobtainability of sufficient awareness about the biology, microbiology, diseases, nutrition and larval nurturing of mud crabs (Williams and Primavera, 2001).

Major identifying morphological features of *S. olivacea* from *S. serrata* include; 1. 'Elbow' possesses either one small blunted spine or no spines at all. 2. Claws are light brownish orange in colour with blunt or reduced prominences on claw spines. 3. Short, broad lobes between the eyes and 4. Walking legs may have very faint pattern. Their eyes that are set on stalks which enables a vision of 360-degree. They can detect slight variations in water movement and chemistry by a pair of antennae between their eyes. Additionally, their tiny hair covered dactyls (the tips of the legs) are highly sensitive to taste and touch. Their last pair of legs are flattened for enabling the swimming behaviour. Majority of their adults find shelter inside the mud or in shallow places below the low tide mark or in the burrows in the intertidal zone during daytime. Due to the strength, speed and big

size of the claws, once it locks within its claw, even if the claw is detached from the crab, it still stays as if gripped between a vice. Therefore, safety precautions are necessary to distract the claws while handling a mud crab. Their food mainly includes the plant material and bottom-dwelling creatures such as smaller crabs, worms and molluscs, which are slow-moving or stationary. It is during the night-time that they come out of burrows and search for food. The food is crushed using their large claw and cut using their smaller claw. *Scylla olivacea* can attain a size of 1.5 kg and a shell width of 150 mm (Department of fisheries, Australia, 2013).

2.3. FAMILY: *Vibrionaceaee*

The family *Vibrionaceaee* encompasses a group of metabolically and genetically diverse heterotrophic bacteria. They are categorised in the order *Vibrionales* of class Gammaproteobacteria and phylum Proteobacteria. The family *Vibrionaceaee* have undergone extensive revision (Baumann *et al*., 1980). Type genus for the family is *Vibrio* and the type species is *V. cholerae*, a microbe that has killed millions of people during several shattering epidemics of cholera (Farmer, 2006). A total of 143 species are described in this family till date, which can be classified into six genera. They are *Vibrio* (Pacini, 1854), *Photobacterium* (Beijerinck, 1889), *Salinivibrio* (Mellado *et al*., 1996), *Enterovibrio* (Thompson *et al*., 2002), *Grimontia* (Thompson *et al*., 2003a) and *Aliivibrio* (Urbanczyk *et al*., 2007). The studies focusing on evolution of bacterial species through rRNA-DNA homology and quantitative micro-complement fixation has revealed that families *Vibrionaceaee* and *Enterobacteriaceae* are sharing a common ancestor (Baumann and Baumann, 1981). The family *Vibrionaceae* are mainly differentiated from *Enterobacteriaceae*, based on the motility through single polar flagellum and possession of cytochrome oxidase (Véron, 1965).

Vibrios are found in all the environments of the ocean from shoreline to open areas and exterior to deep water (Thompson *et al*., 2004; Thompson and Polz, 2006). Further, some of *Vibrio* species are also seen in briny and environments of fresh water (Thompson *et al*., 2004). Additionally, they are the prime entities of bacterial flora present in marine fish, and planktons. Vibrios play a vital role in the

degradation of biological stuff in these environments (Damir *et al*., 2013). Thus, it adjusts the dissolved carbon-based matters to higher trophic levels of the marine food web (Grossart *et al*. 2005; Al-saari *et al*. 2015). However, some members of this family are critical pathogens for many animals and humans (Austin, 2010).

Bacteria of the family *Vibrionaceae* are Gram-negative motile bacteria with straight or curved-rod shape. When they are grown on solid media, they may develop additional lateral flagella ranging from a few to over 100 flagella/cell which has a different wavelength and antigenicity from that of polar flagellum. Normally, these are chemoorganotrophs and do not form microcysts or endospores. They are facultative anaerobes showing both respiratory and fermentative metabolism. Majority of the species are oxidase positive and reduce nitrate to nitrite. Additionally, they use D-glucose as the main source of energy as well as carbon, and ammonium as the only source of nitrogen. They can ferment and use simple carbohydrates, various complex molecules and several other sources of carbon. Several species of vibrios need a seawater base for Na^+ growth and optimal concentration of NaCl is 0.5–3 per cent. There are some bioluminescent species in the family. Even-though phenotypic characterisation was the traditional identification method of vibrio, they have got various limitations like inconsistencies in the results sometimes with the same strain itself, inability to reproduce the results in inter-laboratories, vague phenotypes in dissimilar species and distinctive phenotypes within strains of similar species *etc*, necessitating molecular methods in their characterisation (Amaral *et al*. 2014). The G + C content of the DNA is 38–51 mol per cent. (Sawabe *et al*. 2007) developed a multilocus sequence analysis (MLSA) scheme for the family *Vibrionaceaee* using nine gene sequences (*rpoA*, *topA*, *16S rRNA*, *ftsZ*, *gapA*, *gyrB*, *mreB*, *pyrH* and *recA*). The analysis comprised the whole sequence sets of nine genes from 58 vibrio taxa, and this discovered 14 monophyletic clades with a prominent bootstrap support. The species within each clade shared >20 per cent DDH (DNA-DNA hybridization), <5 per cent G+C (mol per cent), >85 per cent MLSA sequence similarity, and >89 per cent average amino acid identity (Sawabe *et al*., 2007).

2.4. DIVERSITY OF VIBRIOS IN MARINE ANIMALS

Vibrios are ubiquitous marine bacteria associated with varied members of planktonic and animal populations (Thompson *et al*., 2004; Takemura , 2014). They have been called as 'opportunitrophs' (Polz *et al*., 2006) due to their high metabolic flexibility and genetic variability coupled with chemotaxis and quorum sensing, all of which allow greater colonisation potential (Reen *et al*., 2006; Hazen *et al*., 2010). Investigations on population biology, diversity and genetics of vibrios is an emerging field in microbiology. Until now, several studies on vibrio ecology focused on the specific taxon, resulting in a widespread frame of literature on their ecology and genetics (Rehnstam *et al*., 1993; Eilers *et al*., 2000; Heidelberg *et al*., 2002; Chimetto-Tonon *et al*., 2015; Vezzulli *et al*., 2015). However, dynamics and diversity of co-occurring vibrio populations remain seldom addressed. Cumulative evolvement in the study of genomics, evolution and vibrio diversity has enforced bacteriologists to modernise their phylogeny with ecological and evolutionary status. However, taxonomic facts of vibrios are yet unsatisfactory to push forward the distinct interpretation of vibrio diversity, evolution and dynamics (Amin, 2017).

A lot of different vibrios have been associated with different healthy marine organisms. Some of the examples include, *V. corallilyticus, V. maritimus*, *V. shiloi*, *V. stylophorae*, and *V. variabilis* from corals (Kushmaro *et al*., 2001; Ben-Haim *et al*., 2003; Chimetto *et al*., 2011; Sheu *et al*., 2011), *V. rotiferianus* from rotifers (Gomez-Gil *et al*., 2003), *V. comitans*, *V. inusitatus*, *V. neonates*, *V. rarus*, and *V. superstes* from abalones (Hayashi *et al*., 2003; Sawabe *et al*., 2007), *V. alfacsensis*, *V. sinaloensis*, *V. tasmaniensis* from fish (Thompson *et al*., 2003c; Gomez-Gil *et al*.,2008, 2012), *V. zhanjiangensis*, and *V. zhuhaiensis* from crustaceans (Gomez-Gil *et al*., 2004; Cano-Gomez *et al*., 2010; Wang *et al*., 2010; Yoshizawa *et al*., 2012; Jin *et al*., 2013), *V. hippocampi* from sea horses (Balcazar *et al*., 2010), and *V. hemicentroti* from sea-urchin (Kim *et al*., 2013a). The most prominent species associated with Ishigaki coral holobionts comprised of 12 potential novel and 22 known Vibrio species, of which *V. harvey*i, *V. owensii*, and *V. hyugaensis* followed by *V. campbellii, P. rosenbergii*, *V. maritimus/V. variabillis* and *V. coralliilyticus* formed the major species (Amin, 2017). *Vibrio chagasii* sp. nov., *V. pomeroyi* sp.

nov. and *V. kanaloae* sp. nov. were seen ubiquitous in the aquatic environment, while isolates of *V. pomeroyi* were plentiful in *Nodipecten nodosus* larvae cultures in the southern part of Brazil. *Vibrio splendidus* is generally considered as an organism without any pathogenic importance (Baticados *et al*. 1990; Paillard and Maes 1990; Myhr *et al*. 1991; Castro *et al*. 1992), however some studies have shown the involvement of these organisms in infections of turbot, rainbow trout (*Oncorhynchus mykiss*) (Myhr *et al*. 1991; Pazos *et al*. 1993; Angulo *et al*. 1994) and in shellfish (Jeffries 1982). *V. chagasii* isolates were found to be common inhabitants of rotifer cultures in Greece (Verdonck *et al*., 1997). Over the years, *V. splendidus* strains are steadily found associated with cultured oysters (*Ostrea edulis*) in the Mediterranean Sea, which suggests a closer connection between the host invertebrate and the bacterium (Macian *et al*., 2000). However, the same was recognised as the agent responsible for bacillary necrosis of oyster larvae (Sugumar *et al*., 1998). The species *V. chagassi* was proposed to encompass isolates originating from rotifer and fish cultures (Thompson *et al*. 2003c). Interactions between *V. fischeri* and squids and between *V. halioticoli and* abalone have been documented (Ben-Haim *et al*., 2003; Mc Fall-Ngai, 2002; Sawabe *et al*., 2002). *V. scophthalmi* is a newly defined species that is isolated from the gastrointestinal tracts of healthy juvenile turbot (Cerdàg-cuéllar *et al.,* 1997). *V. parahaemolyticus* was found in a healthy mud crab from Pemalang Coast (Lightner *et al*., 1988). *Vibrio anguillarum, V. fumissii and Plesiomonas shigelloides* were also isolated from healthy eels, turbot and rainbow trout (Esteve, 1995). *V. halioticoli*, *V. wodanis, V. gallicus, V. salmonicida, V. logei* and *V. rumoiensis* also were isolated from abalone (Sawabe *et al*., 2004). *V. communis* and *V. owensii, V. natriegens* and *V. jasicida* were isolated from packhorse lobster, eastern rock lobster or green rock lobster; abalone and Atlantic salmon (Yoshizawa *et al*., 2012). The major vibrios associated with the gut of healthy red snapper (*Lutjanus argentimaculatus*) has been reported recently by Reshma *et al*. (2018). In majority of the studies, the dominating *Vibrio* species in association with bivalves from diverse topographical regions (Spain, Canada, Italy or Brazil), all from temperate climates, were either *V. splendidus*, *V. harveyi*, *V. alginolyticus* or any combination of these species

(Montilla *et al*.,1994; Arias *et al*.,1999; Pujalte *et al*.,1999).The other species like *V. fluvialis, V. mimicus* and *V. vulnificus* have been also associated with molluscs (Maugeri *et al*., 2000; Caballo and Stabili, 2002). *V. lentu*s (Macian *et al*., 2001), *V. pomeroyi* (Thompson *et al*., 2003e), *V. fortis* (Thompson *et al*., 2003d), *V. neptunius* (Thompson *et al*., 2003b), *V. kanaloae* (Thompson *et al*., 2003e), *V. xuii* (Thompson *et al*., 2003b), *V. brasiliensis* (Thompson *et al*., 2003b), *V. crassostreae* (Faury *et al*., 2004), *V. ponticus* (Macián *et al*., 2004), *V. gigantis* (Le Roux *et al*., 2005), *V. aestuarianus* (Garnier *et al*., 2008), *V. gallaecicus* (Beaz-Hidalgo *et al*., 2009b), *V. breoganii* (Beaz-Hidalgo *et al*., 2009a), *V. celticus* (Beaz-Hidalgo *et al*., 2010),*V. artabrorum* , *V. atlanticus* (Dieguez *et al*., 2011), *V. cortegadensis* (Lasa *et al*., 2013), *V. crosaei* (Gonzalez-Castillo *et al*., 2014) and *V. ostreicida* (Prado *et al*., 2014), are some of new *Vibrio* sp. associated with bivalve other than the normally found vibrios.

2.5. DIVERSITY OF VIBRIOS IN AQUATIC CRABS

Studies on the microbial communities isolated from different aquatic crabs showed a highly diverse and varied microbial population. However, *Vibrio* species especially *V. parahemolyticus, V. vulnificus* and *V. cholerae* were reported as the predominant bacteria isolated from haemolymph and external carapace of blue crab, *Callinectes sapidus* (Krantz *et al*., 1969; Tubiash and Krantz, 1970; Sizemore *et al*., 1975; Davis and Sizemore, 1982; Huq *et al*., 1986). Isolation of *Bdellovibrio* sp. From the gill tissues of blue crab have been made by Kelley and Williams (1992). The animals caught from the Galician coast (including wild and captive crabs) shared more species of bacteria than with those from the southern region of Canary Islands, where the *Vibrio* species found were mostly more tropical-temperate (e.g. *V. alginolyticus, V. harveyi*) (Maeda *et al*., 2003). It had been discovered that the species composition of vibrios alters as the seawater temperature level varies (Miguez and Combarro, 2003). The members of the Harveyi clade preponderate when the temperature is above 20^0 C and those from the Splendidus clade when it is below that (Maeda *et al*. 2003). According to a study done in spider crabs (*Maja brachydactyla*) the vibrios diversity was dominated by three clades, Harveyi,

Orientalis and Splendidus clade. Within the Harvey clade *V. harrveyi (*Lavilla and Peña, 2004; Shanmuga, 2008; Sarjito *et al*., 2014;), *V. vulnificus (*Lavilla and Peña, 2004; Shanmuga, 2008, Wang, 2011), *V. splendidus,* and *V. Orientalis (*Lavilla and Peña, 2004), *V. parahaemolyticus* (Lavilla and Peña., 2004; Najiah *et al*., 2010; Wang, 2011), *V. vulnificus, V. splendidus, V. orientalis* (Lavilla and Peña, 2004), *V. nereis, V. fischeri* and *V. campbelli* (Shanmuga, 2008), *V. nereis, V. fischeri* and *V. campbelli* (Shanmuga, 2008), *V.atypicus, V.rotiferianus* and *V.campbelli* were more dominant (Gomez-Gil *et al*., 2010). *V.cholerae* and *V. alginolyticus (*Najiah *et al*., 2010, Wang, 2011) *V. ordalii* (Sarjito *et al*., 2014) have been reported in apparently healthy crabs. Occurrence of *V. parahaemolyticus (*Lavilla and Peña, 2004; Sarjito *et al*., 2009; Najiah *et al*., 2010; Chen *et al*., 2011), have been mentioned in mud crabs. The fattening mud crabs from Pemalang coast, Central Java, Indonesia were associated with *V. parahaemolyticus, V. harveyi, V. fischeri, V. alginolyticus* and *V. cholera.* Diversity of vibrios associated with the haemolymph of 4 different healthy marine crab species from Indian waters has been documented by Sumithra *et al*. (2019).

2.6. VIBRIOS AS HUMAN PATHOGENS

Twelve pathogenic vibrios species are recognised till date which causes human sicknesses (Summer *et al*. 2001). Vibrios are principally transmitted to the food cycle through the ingestion of inadequately cooked or raw seafood (Matte *et al*., 1994). Due to the failure to report infections, lack of international systems of epidemiology, limitations in existing surveillance systems and differences in reporting procedures, the exact number of *Vibrio* spp. infections across the world are not certain. The most studied human pathogens are *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* (Baker-Austin *et al*., 2018). Although less severe, other *Vibrio* species like *V. alginolyticus, V. mimicus, V. fluvialis* and *V. furnissii* were also identified as human pathogens (Baker-Austin *et al*., 2018). Data on isolation of three species namely *V. metschnikovii, V. cincinnatiensis* and *V. carchariae* were asymptomatic colonisation instead of infection (Jean-Jaques *et al*. 1981; Bode *et al*. 1986; Pavia *et al*. 1989; Dalsgaard *et al*. 1996; Morris Jr. and

Acheson, 2003). Hence, their criticality as human pathogens demands further detailed research. Similarly, investigations are required in order to find out the pathogenicity of *V. metoecus*, a close companion to *V. cholerae* which was initially considered as a non-pathogenic environmental variant of *V. cholerae* (Choopun, 2004; Kirchberger *et al*., 2014) nevertheless has recently been isolated from leg, ear, wound, stool samples and blood (Orata *et al*., 2015). Because of the continuous interactions with *V. cholerae, V. metoecus* is assumed to become a pathogen, which allows amplified rate of interspecies gene exchange including genes involved in pathogenicity from *V. cholerae* to *V. metoecus* (Orata *et al*., 2015). Hence, the significance of *V. metoecus* as a human pathogen is worthy to be studied further. The pathogenic vibrios need to elaborate a set of virulence factors to cause infections in humans. Vibrio infections are usually developed either through intake of contaminated raw or poorly cooked foods and water, or through the exposure of skin lesions, such as cuts, open wounds and abrasions, to aquatic environments and marine animals as a part of recreational or occupational subjection to natural aquatic environments (especially above 20°C) (West, 1989; Toti *et al*., 1996; Lee and Younger, 2002).

Because of its importance and antiquity as a pathogen of global significance, *V. cholerae* is the extremely researched *Vibrio* species. WHO (World Health Organization) has mentioned 172,454 cases of cholera in the year 2015 only involving 1304 deaths across 42 countries (World Health Organization, 2016). Because of the criticality of septicity and the sensitive reaction it creates with public and medical authorities, *V. cholerae* was mostly involved in the information and research regarding the human pathogenic vibrios. *V. parahaemolyticus* is another human pathogenic *Vibrio* species and is considered as the most common reason of seafood-borne bacterial sickness in Japan, Taiwan and the United States (Strom *et al*., 2013). The emergence of a novel, extremely virulent serovar (O3:K6) of *V. parahaemolyticus* that can cause larger outbreaks than classical strains (Chiou *et al*., 2000) is reported. Since its emergence in 1996, the O3:K6 serovar is identified as the dominant serovar in Asia (Chiou *et al*., 2000; Matsumoto *et al*., 2000) and in the United States (Daniels *et al*., 2000). The O3:K6 serovar is currently categorised

as a pandemic strain. The third significant human pathogenic vibrio namely, *V. vulnificus* is reported as the most invasive *Vibrio* species because of its capability to cause lethal septicaemia (Harwood *et al.*, 2004; Levin, 2005). Mortality rates among the population with low immune, can exceed 50per cent (Harwood *et al.*, 2004). Consumption of raw or under-cooked oysters are the main route for *V. vulnificus* infection (Hlady, 1997). Involvement of *V. fluvialis* in human diseases especially diarrhoea was first reported in 1977. Since then, it has occurred as a potential entero-pathogen in natural aquatic surroundings (Huq *et al*., 1980; Spellman *et al*. 1986). *V. alginolyticus* is also categorised as a weak pathogen for humans mostly as involved in diversified bacterial infections of extraintestinal wounds (Bonner *et al*. 1983).

2.7. VIBRIOS AS AQUATIC PATHOGENS

It is noteworthy that vibrio infections are not restricted to humans. At least fifteen species of vibrios are identified as pathogens of marine animals. Among coral pathogens, *V. shiloi* and *V. coralliilyticus* are accountable for significant bleaching events (Ben-Haim *et al*., 2003; Mc Fall-Ngai, 2002; Sawabe *et al*., 2002; Thompson *et al.*, 2003c). Among pathogens of animals reared for aquaculture, *V. salmonicida, P. damseale, V. anguillarum, V. alginolyticus, V. harveyi, V. vulnificus* and *Aliivibrio salmonicida* are reported to cause mass mortalities in fin fish aquaculture facilities (Coleman *et al.*, 1996; Colquhoun and Sorum 2001; Thompson *et al.*, 2003d; Sharma *et al*., 2016; Rameshkumar *et al*., 2017; Shen *et al*., 2017; Sumithra *et al*., 2019). Apart from these, *V. carchariae* was obtianed from a chronic skin ulcer of a shark (Bertone *et al*. 1996), gastro-enteritis in moribund black sea bream (*Acanthopagrus schlegeli)*, yellow fin sea bream (*Acanthopagrus latus*), Japanese sea bass (*Lateolabrax japonicus*) and red drum (*Sciaenops ocellatus*) (Lee *et al*., 2002).

Members of vibrios have also been defined as the foremost aetiological means of diseases influencing stages of life of molluscan shellfish (Liu *et al*., 2000; Allam *et al*., 2002; Waechter *et al*., 2002; Lee *et al*., 2003; Anguiano-Beltran *et al*., 2004; Estes *et al*., 2004; Gay *et al*., 2004; Paillard *et al*., 2004; Gomez-Leon *et al*., 2005;

Prado *et al*., 2005; Labreuche *et al*., 2006; Garnier *et al*., 2007, 2008;) even though vibriosis primarily affects nursery cultures of juvenile bivalves. Major pathogenic vibrios associated with bivalve molluscs include *V. alginolyticus*, *V. tapetis, V. crassostreae,* and *V. pectenicida* (Paillard *et al*., 1994; Nicolas *et al*., 1996; Lambert *et al*., 1998). *V. splendidus* strains have been identified as the agent that causes bacillary necrosis of oyster larvae (Sugumar *et al*., 1998). *V. alginolyticus* was commonly reported as a bacterial pathogen associated with abalone (Sizemore *et al*., 1985; Liu *et al*., 2001). *V. lentus* was associated with diseased wild octopus (*Octopus vulgaris*) (Farto *et al*., 2003).

In case of shrimps, the major pathogens include *V. fluvialis*, *P. damselae* and *V. vulnificus* (Chythanya *et al*., 2002), *V. harveyi* (Thongkao, 2005; Setiawan *et al*., 2015), *V. alginolyticus* (Liu *et al*., 2017), *V. parahaemolyticus* (Chythanya *et al*., 2002; Liu *et al*., 2017). *V. aestuarianus,* an opportunistic environmental pathogen has remained related with epidemics in cultured shrimp *Penaeus vannamei* (Xu *et al*., 2018). In case of crabs, physiological and pathological effects of vibrio infections are recognised where vibrio infections characteristically cause or produce bacteraemia and shell disease. *V. parahemolyticus* was isolated from lethargic and moribund *C. sapidus* in commercial tanks during ‘‘shedding” of soft crabs in Chesapeake Bay (Krantz *et al*., 1969; Tubiash and Krantz, 1970). Burnett *et al*. (2006) found that *V. campbellii* interfered with the respiratory function of gills in *C. sapidus*. *V. harveyi* is recognised as an important secondary bacterial pathogen in *S. tranquebarica* which are affected primarily by white spot disease (Poornima *et al*., 2012).

2.8. VIRULENT GENES IN FAMILY *Vibrionaceaee*

Extracellular products those are recognised to contribute to the virulence of vibrios comprise phospholipases, siderophores, hemolysins, cytotoxins, proteases, biofilm formation, quorum sensing agents and presence of phages (Ronneseth *et al*., 2017). Swarming motility of vibrios is steadily related with their virulence (Frans *et al*., 2011). Hemolysins are potent toxins playing a vital role in the

virulence of many vibrios of both fish and humans (Austin and Zhang, 2006) especially in *V. harveyi, V. cholera* and *V. parahaemolyticus* (Zhang *et al*., 2001; Chattopadhyay and Banerjee 2003; Rattanama *et al*., 2009). Hemolysins are encoded by different genes such as *V. cholerae*- like haemolysin gene (*hhl*) and *Vibrio haemolysin* gene (*vhh*) (Rattanama *et al*., 2009). Along with its own haemolysin, presence of *V. cholerae* haemolysin, *hlyA* within some Harveyi clade isolates suggesting horizontal gene transfer has been reported (Ruwandeepika *et al*., 2010). An Additional vital factor that influences pathogenicity of vibrios is type III secretion system, which is encoded by a cluster comprising approximately 20 genes, including vibrio calcium response gene (*vcrD*) (Henke and Bassler 2004).

The major virulence-associated factors of *V. cholerae* include cholera toxin (*ctxA*), hemolysin (*hlyA*), non-O1 heat-stable enterotoxin (*stn/sto*), neuraminidase (*nanH*), ToxR regulatory protein (*toxR*), TCP (*tcpA* and *tcpI*), outer membrane protein (*ompU*), zonula occludens toxin (*zot*), Shiga-like toxin (*stx*), and El Tor-like hemolysin (*hlyA*) (Rivera *et al*., 2001). Of which virulence determinants like cholera toxin (Ctx), a toxin-coregulated pilus (Tcp), and accessory colonisation factor (Acf) are regulated by specific environmental conditions (Taylor *et al*., 1987; Peterson and Mekalanos, 1988; Di Rita and Mekalanos, 1989). These virulent determinants are part of a regulon under the control of *toxRS* locus (Miller and Mekalanos, 1984; Miller *et al*., 1989; DiRita and Mekalanos, 1991). ToxR is the trans-membrane protein which specifically binds to cholera toxin encoding operon (*ctxAB*) promoter (Miller and Mekalanos, 1984; Miller *et al*., 1987). Expression of the genes like *tcpA, tepl, akL4* and *tagA* are under the control of ToxR regulon. ToxR controls the expression of outer membrane proteins which are important for surviving in the small intestine and is also involved in the regulation of *toxT* by binding to a site upstream of the TcpP binding site on the *toxT* promoter (Rivera-Cancel and Orth, 2017). *ctxAB* encoding for cholerae toxin (CT) and *V. cholerae* pathogenicity island (PAI) namely VPI are associated with epidemic strains in *V. cholerae* (Waldor and Mekalanos 1996; Zhang *et al*., 2003). *ctxAB* exists in in the lysogenic filamentous bacteriophage CTX. VPI, which encodes the CTX receptor is found on another filamentous bacteriophage, designated VPI (Karaolis *et al*.,

1999). Virulence genes namely *tcpP* and *tcpH* are encoded in vibrio pathogenicity island (VPI). They regulate the expression of cytoplasmic transcription factors ToxT, ToxR and ToxS. Four PAI are already identified in *V. cholerae* serogroups O1 and O139 associated with epidemic and pandemic cholera. They are VPI-1 and VPI-2, vibrio seventh pandemic island-I (VSP-I) and VSP-II (Karaolis *et al*., 1998; Dziejman *et al*., 2002; Faruque and Mekalanos, 2003; Jermyn and Boyd, 2005; Murphy and Boyd, 2008). The genes those are responsible for the survival and persistence of vibrios in the environment are called as fitness genes. In *V. cholerae* the major fitness genes include *flrA* (gene responsible for regulation of *V. cholerae* flagella synthesis and response to environmental changes), *vpsR* (a transcriptional regulator responsible for biofilm formation and environmental persistence) and *vpsR* (a putative two-component response regulator protein that modifies the transcriptome to create a biofilm-competent state under specific environmental conditions (Beyhan and Yildiz, 2007). A gene namely *luxA* gene in the lux operon is involved in bioluminescent expression and quorum sensing (Gennari *et al*., 2012a).

ToxR, transmembrane transcription regulator playing a vital role in the regulation of virulence gene expression in *V. cholerae* (Miller and Mekalanos 1988) is reported to be existing in other vibrios including *V. parahaemolyticus* (Lin *et al*. 1993), *V. fischeri* (Reich and Schoolnik 1994), *Photobacterium* sp. (Welch and Bartlett 1998), *V. alginolyticus, V. mimicus, V. fluvialis* and *V. hollisae* (Osorio and Klose 2000), *V. anguillarum* (Okuda *et al*. 2001), *V. vulnificus* (Lee *et al*., 2006) and *V. harveyi* (Franco and Hedreyda, 2006).

Major virulence genes contributing to the virulence of *V. parahaemolyticus* are *tdh*, coding thermostable direct haemolysin (TDH); *trh*, coding TDH-related haemolysin (TRH); and *tlh*, coding thermolabile haemolysin (Iida *et al*. 1998; McCarthy *et al*. 1999). ToxRS, a highly homogeneous locus among vibrios, mediates environmentally induced regulation of the virulence gene expression including *tdh* and *ctxAB* in different vibrios (Lin *et al*., 1993; Reich and Schoolnik, 1994; Lee *et al*., 2000; Wang *et al*., 2002; Crawford *et al*., 2003). Seven mobile

genomic islands/pathogenic islands (PAI) have already been identified in *V. parahaemolyticus* (Makino *et al*., 2003). Out of these, the Vp-PAI, that carries the *tdh* gene and the genes from the type III secretion system has been subjected to extensive study (Makino *et al*., 2003; Izutsu *et al*., 2008; Okada *et al*., 2009) and were found to be associated with *V. parahaemolyticus* virulence capability.

Three typical virulence genes are usually possessed by *V. harveyi* are *chiA, luxR* and *vhpA*. Typical virulence genes widely distributed among pathogenic *V. harveyi* of both fish and humans include *flaC, hlyA, toxRvc, tlh, vhh, tdh* and *trh* (Mohamad *et al*., 2019). Cysteine protease has been identified as the major exotoxin of *V. harveyi* affecting penaeid shrimps (Liu *et al*. 1997). However, Montero and Austin (1999) recommended that lipopolysaccharide (LPS) might be the lethal toxin that affects penaeid shrimp. Bacteriophages are also identified as virulence agents of these bacteria (Austin *et al*. 2003; Khemayan *et al*. 2006). Prasad *et al*. (2005) has isolated novel bacteriocin-like substances from a pathogenic strain of *V. harveyi*, which aids the survival and domination of the bacteria within the host. Other virulence factors of *V. harveyi* clade include caseinase, gelatinase, phospholipase, lipase, haemolysin, metalloprotease, serine protease and chitinase (Zhang and Austin 2000; Aguirre-Guzman *et al*. 2004; Defoirdt *et al*. 2010). Bacteriophages responsible for transferring the virulence genes are also reported (Austin *et al*. 2003; Khemayan *et al*. 2006). Quorum-sensing in *V. harveyi* contains three channels feeding a common signal transduction cascade of which *luxR* is the main regulatory gene (Henke and Bassler 2004). The *tcpA* gene is reported to encode the major subunit protein of the toxin coregulated pilus (TCP) of *V. aestuarianus* (Xu *et al*., 2018).

The probability for several mobile genetic elements carrying virulent genes to obtain or lose DNA fragments is the basis for strain variability within a bacterial species such as the appearance of nonvirulent, virulent or more virulent strains and also appearance of virulence in a non-pathogenic species (Boyd *et al*., 2009). Consequently, it turns out to be mandatory to analyze the possible prevalence of

virulence genes in environmental non-pathogenic strains of virulent species and in non-pathogenic *Vibrio* species (Gennari *et al*., 2012b).

2.9. ANTIMICROBIAL RESISTANCE: A BURNING HEALTH ISSUE

Antimicrobial resistance (AMR) is the mechanism by which bacteria acquire resistance to a class of antimicrobial drugs. This resistance may be innate or acquired resistance. Innate resistance means that some bacteria may be naturally resistant to a few classes of antimicrobials. Acquired resistance means bacteria that were previously susceptible to a group of drugs become resistant to the same group due to the continuous exposure. Acquired resistance in bacteria to the antimicrobial agents is a much more serious issue and recognised as one of the major threats to medicine in future (Tenover, 2006). Overuse and misuse of antimicrobials is the major cause for the increasing antimicrobial resistance. It is estimated that consumption of antimicrobials has been increased worldwide by 65per cent during 2000-2015 (Klein *et al*., 2018). As a partial consequence, the resistance to drugs is also increasing. As per the present estimates it is forecasted that 10 million human deaths may occur only due to AMR pathogens by 2040 if the present trend continues (O'Neill, 2014). Similarly, a cost of US $3.5 billion per year due to AMR infections in the next 30 years in Europe, North America and Australia only is predicted by the Organisation for Economic Co-operation and Development (OECD) (Taylor and Tracz, 2005).

2.10. AMR IN AQUACULTURE

Accompanying the increase in antibiotic administration in aquaculture as a part of prophylaxis and treatments, AMR has emerged among the bacterial fish pathogens (FAO 2005). AMR bacteria evolved from the selective pressure caused by the continuous use of antibiotics can form an environmental reservoir of AMR genes. Thus, fish farms and aquaculture systems have now been considered as the 'hotspots for AMR genes'. Propagation of AMR pathogens from aquaculture environment into the natural marine environment can result in the development of AMR in wild fishes and the associated dietery suppliments. This also influences

human health due to their direct ingestion and wound contamination (Cizek *et al*., 2010). Thus, valuation of resistome, the AMR gene assemblage in aquaculture, is globally a relevant focus for research (Watts *et al*., 2017).

2.11. MECHANISMS OF AMR IN BACTERIA

Bacteria have long been in this planet and have thrived on multiple environmental conditions that gave them the ability to adapt and survive in their surroundings (Economou and Gousia, 2015). Emergence of acquired AMR to more widely used antimicrobials may be one such adaptation mechanism due to the selective pressure exerted by the drug by the unnecessarily wide spread use (Tenover, 2006). Majority of the antibiotics perform their action broadly by hindering the synthesis or assembly of the cell wall, interrupting the integrity of cell membrane, inhibiting the synthesis of protein, RNA and DNA and by stopping the progress of needed cellular metabolic pathways (Das *et al*., 2020). In order to defend the destruction from these actions, microorganisms have established robust mechanisms through a Darwinian selection process. This may be in the form of (i) chemical modification or hydrolysis, inactivating antibiotic, (ii) modifying target site of antibiotic (iii) replacing antibiotic target (iv) protecting the target site of antibiotic, (v) removing the target site (vi) by altering membrane permeability, prevent the contact to the target site and (vii) by dynamically exporting antibiotics from bacterial cell (Blair *et al*., 2015).

The mechanism of acquired resistance from other resistant microbes are identified as either by vertical or horizontal gene transfer (HGT). Vertical gene transfer involves transfer throughout generations of the same species of bacteria and latter is by any of the genetic transfer processes like conjugation, transformation and transduction which occur between same or different bacterial species (McManus 1997). Conjugation involve the exchange of mobile genetic elements or transposons via sex pilus in case of gram-negative bacteria or by sex pheromones in gram positive microbes. Transformation requires bacteriophage and transduction is uptake of DNA from the surrounding medium. All these methods of gene exchange mechanism in horizontal gene transfer are reported to contribute to the

development of multidrug resistant (MDR) strains which are the microbes resistant to three or more classes of antimicrobial agents. Acquired resistance can also be developed by mutation in existing genes (McManus, 1997) leading to the modification of antimicrobial target protein or its binding site, extensive production of enzymes that render antimicrobials inactive and deactivating an extraneous protein channel required by the drug (McManus, 1997; Tenover, 2006). For the exchange of genetic materials including AMR genes among bacterial species through HGT, certain environmental factors are crucial, particularly the compounds inducing bacterial SOS response and modulating HGT (Pant *et al*., 2016).

2.12. MECHANISM OF AMR IN VIBRIOS

Among the intracellular pathogens, resistance via spontaneous transformation mechanism is common (McGrath *et al*., 2014); whereas in vibrios, the antimicrobial resistance is mainly out of accumulation of resistance genes from distantly or closely related microbial species by HGT (Verma *et al*., 2019). Resistance through genetic alterations in the target gene of antibiotics is commonly observed in *V. cholerae* against routinely prescribed antibiotics. For example, (i) quinolone resistance is restored by influencing *gyrB* and *parC* gene functions in the quinolone resistance-determining region (Hooper, 2001) (ii) rifampicin resistance is by influencing the transcription of *rpoB* encoded protein, (iii) streptomycin resistance by influencing *rpsL* gene function (iv) resistance to β lactam antibiotics by influencing *pbp* gene function and (v) several metabolic enzymes by influencing *katG, mshA, embB* functions. Acquisition of point mutations in *gyrA* and *parC* genes encoding topoisomerase II and IV, respectively is another mechanism responsible for the quinolone resistance in vibrios (Vila *et al*., 1994; Everett *et al*., 1996). Similarly, susceptibility to ciprofloxacin is reduced by the substitution mutations in genes of topoisomerase II and IV (Ser-83-Ile in *gyrA* and Ser-85-Leu in *parC*) of vibrios (Quilici *et al*., 2010).

Modification of targets by acquired functions is recognised as the second mechanism of AMR in vibrios. Resistance against various macrolides and aminoglycosides are can be because of the incidence or lack of RNA modification

enzymes such as rRNA methyltransferase which transfers a methyl group to the specific nucleotides of 16S or 23S rRNA (Holmes *et al*., 2016). Resistance against antibiotics such as lincosamides, phenicols, pleuromutilins, streptogramin-A and oxazolidinones in Gram-negative bacteria is encoded by various genes namely, a*viR, cfr, emtA, ermC* and *ermAM* encoding 23S rRNA methyltransferases. The fact that antibiotic resistance is also conferred by the absence of certain rRNA methyltransferases is interesting. In a similar way, resistance to clindamycin, linezolid and tiamulin in vibrios and other enteric pathogens are caused by the inactivation of *rulC* gene (Holmes *et al*., 2016).

Resistance through the reduced permeability of antibiotics into the cells is also described in some vibrios. Many vibrios are made intrinsically defensive to various antibiotics like rifamycin, erythromycin, polymixin B and azithromycin, by the strong permeability barrier formed by lipopolysaccharides in the cell wall. Permeability of antibiotics into different folds is also restricted by membrane lipid barrier modification and changes in the expression pattern of outer membrane porins. For specific antibiotics, permeability can be reduced by substitution, reduction or loss of outer membrane channel proteins, like porins. Resistance to chloramphenicol, carbapenems, tetracycline, fluoroquinolones and aminoglycosides was shown by the loss of porins or functional changes in cell wall (Delcour, 2009)

Active ejection of antibiotics through the efflux pump proteins are omnipresent in vibrios. By using the energy from ATP or transmembrane ion gradients, different antimicrobial compounds are actively transported by these encoded proteins (Abdelwahab *et al*., 2016). The genes responsible for efflux pump proteins may be present in extrachromosomal genetic elements such as plasmids or may also be part of chromosomes. Efflux pumps can be classified into multicomponent transporters or single component transporters, in the Gram-negative bacteria. Like the tetracycline efflux pumps, they can be so specific or may carry across a range of structurally discrete compounds, with respect to the substrate recognition. In vibrios and other bacterial species, six distinct classes of drug efflux proteins are identified so far: (i) ATP-binding cassette (ABC) efflux protein are capable of recognising

and pumping out aminoglycosides, β-lactams, macrolides, tetracyclines, SXT (Trimethoprim/sulfamethoxazole) and nalidixic acid. (ii) The resistance-nodulation-cell division (RND) efflux protein has the potential to specifically remove antibiotics like tetracycline, ampicillin, streptomycin and chloramphenicol. (iii) Major facilitator (MFS) efflux protein is detected in many Gram-negative pathogens like *Shewanella* spp., *Vibrio* spp. and *E. coli*. (iv) small multidrug resistance (SMR) family (v) multidrug and toxic compound extrusion (MATE) families are able to grant resistance against many antimicrobial compounds which are derived from macrolides, β-lactams, chloramphenicol SXT scaffolds, aminoglycosides and nalidixic acid (vi) Proteobacterial antibacterial compound efflux family (PACEF).

Resistance due to inactivation of the antibiotics is another AMR mechanism in vibrios. By transferring a chemical group to the scaffolds or through the hydrolysis of the core structure, vibrios have the ability to destroy or modify antibiotic scaffolds. In pathogenic *V. cholerae*, the usual mechanism of drug resistance is the chemical alteration of antibiotics through the enzymatic function of acquired genetic traits. Normally, vibrios attain these enzymatic functions through HGT and hence acquire resistance against many antibiotics. Inactivation of antibiotics by hydrolysis for example, inactivation of β-lactam antibiotics by β-lactamases, is another AMR mechanism in vibrios and other Proteobacteria (De Pascale *et al*., 2010). All the β-lactam antibiotics such as monobactams, cephalosporins, penicillins and carbapenem have β-lactam rings as a common structural feature. In most of the bacterial species, the β-lactam resistance is offered by the hydrolysis of β-lactam ring by serine-β-lactamase or metallo-β-lactamase. Inactivation of antibiotics like fosfomycin (epoxide ring), macrolide (macrocyclic lactone ring) and bacitracin (amidohydrolysis of bacitracin undecaprenyl pyrophosphate) by hydrolysis has also been reported (De Pascale *et al*., 2010). Antibiotic inactivation through the mechanism of chemical alterations such as enzymatic alteration of antibiotic scaffolds due to the covalent transfer of chemical groups is a very usual AMR mechanism in vibrios and other Proteobacteria. Several enzymes involved in drug resistance by changing antibiotic structure have been detected (De Pascale *et*

al., 2010). These enzymes can cause one of the following seven alterations to inactivate antibiotics (i) Hydroxylation, which is the inactivation of antibiotic due to addition of hydroxyl group, (ii) O-phosphorylation, through the transfer of phosphate group to antibiotic scaffold from ATP or GTP (Resistance through phosphorylation has been identified for fosfomycin, chloramphenicol, viomycin, macrolide and rifampicin) (iii) O-nucleotidylylation, alter the antibiotics by transferring adenosine monophosphate (AMP) (Nucleotidylylation confers resistance against various aminoglycosides and lincosamides) (iv) O- and N-acetylation, is the mechanism of AMR by the transfer of an acetyl group to the antibiotic from acetyl CoA (chloramphenicol, fluoroquinolone, streptothricin and other aminoglycosides can get inactivated due to acetylation) (v) sequestration, that defends the action of antibiotic by a chemical complex production that hinders its accessibility to the target (observed against aminoglycosides) (vi) O-ribosylation, which is the inactivation of rifampin by adding ADP-ribose from NAD and (vii) O-glycosylation, which is the addition of glycosyl moiety to the macrolide and rifampin antibiotics (Blair *et al*., 2015). A widely identified resistance trait in vibrios and other proteobacterial pathogens is N'-acetylation at the 60-position of the aminoglycoside. The acetyl- CoA dependent acetyltransferase catalyses the transfer of acetyl group in the aminoglycoside. The ability of these enzymes to alter fluoroquinolone like ciprofloxacin and norfloxacin is also unveiled through recent researches (McArthur *et al*., 2013). In *V. cholerae*, enzymes that inactivate an antibiotic is reported to spread rapidly through transposons and plasmids (Darley *et al*., 2012).

2.13. GENES ASSOCIATED WITH AMR PHENOTYPES

An enormous quantity of diverse genes can be accountable for antimicrobial resistance. It is vital to identify these genes in order to know the verification of non-susceptible phenotypes, resistance epidemiology, and also for the identification of resistant strains (Cabello *et al*., 2013). A very comprehensive information of AMR related genes are as follows;

The major antibiotic (β-lactam) resistant genes are *blaTEM, blaSHV, blaPER-1* and *blaOXA*. Specific penicillin resistant genes are also reported in bacteria such as *penA, pbp2, pbp1a, pbp1b* and *pbp2* (Srinivasan *et al*., 2005; Zhang *et al*., 2009). The extended-spectrum β-lactamases (ESBLs) are a group of enzymes possessing the capacity to hydrolyse and develop resistance against the oxyimino-cephalosporins and monobactams, but not against the carbapenems or cephamycins. The β-lactamase inhibitors such as clavulanic acid, sulbactam and tazobactam inhibit these enzymes (Peirano and Pitout, 2010). Reports of ESBLs have become more frequent. CTX-M types are the most common ESBLs that have recently emerged (Pitout and Laupland, 2008; Rossolini *et al*., 2008). Plasmids belonging to the narrow host-range incompatibility types (that is, IncFI, IncFII, IncHI2 and IncI) or the broad host-range incompatibility types (that is, IncN, IncP-1-a, IncL/M and IncA/C) encode the genes those are responsible for CTX-M β-lactamases (Peirano and Pitout, 2010). Six distinct groups of enzymes constitute this family of β-lactamases (CTX-M-45, CTX-M-25, CTX-M-9, CTX-M-8, CTX-M-2 and CTXM-1) and shows potent hydrolytic activity against cefotaxime (Rossolini *et al*., 2008). Variants those efficiently hydrolyze ceftazidime, such as CTX-M-15, CTX-M-32 CTX-M-55, TEM-1 and OXA-1 are also reported (Kiratisin *et al*., 2007).

The major chloramphenicol resistant genes are *cat*1, *cat*2, *cat*3, *catA3,catB3 cat*4, *adeb, ceob, mdtl* and *floR* which encode the chloramphenicol acetyltransferases and these genes the most common resistance mechanisms found in aquatic bacteria (Yoo *et al*. 2003; Dang *et al*., 2007, 2008). Major kanamycin resistant genes include *aphA-3* and *norm* (Kim *et al*., 2013b). Among tetracycline resistant genes *tetA, tetB, tetC, tetD, tetE, tetG, tetH, tetJ, tetY, tetZ and* tet(M) are more prevalent and these encode ribosomal binding proteins (Macauley *et al*., 2007; Zhang *et al*., 2009; Kim *et al*., 2013b). The most common oxytetracycline-resistance mechanism in vibrios are reported as an active efflux mechanism through membrane-associated proteins, that is more frequently encoded by one among the six genes, tet(A)–(E) or tet(G) (Chopra and Roberts, 2001; Akinbowale *et al*., 2007; Jacobs and Chenia, 2007; Heepngoen *et al*., 2008). Through transformation, conjugation or transduction, these antibiotic resistant genes can be transferred and

can induce AR among different bacteria (Manjusha and Sarita, 2011). The major genes responsible for macrolide-resistance are *mef*C and *mef*G (Cabello *et al*., 2016). Macrolide efflux through *msrA/msrB* or *mefA/E* and drug inactivation by *reA, ereB* and *mphA* genes are as well reported. Three various mechanisms of erythromycin resistance are (1) targ*et al*teration mediated by an rRNA *erm* methylase that modifies the binding site of antibiotics (macrolides, lincosamides, and streptogramin B antibioticsin) in 23S rRNA (Leclercq and Courvalin, 1993., Weisblum, 1995). (2) production of enzymes (EreA and EreB) that hydrolyze the lactone ring of the macrocyclic nucleus (Ounissi and Courvalin, 1985; Arthur and Courvalin, 1986; Arthur *et al*., 1987; O'Hara, 1996) and (3) production of phosphotransferases type I (*mphA)* (O'Hara *et al*., 1989; Noguchi *et al*., 1995; Kim *et al*., 1996) and type II (Kono *et al*., 1992). Genes belonging to the *ermAM* (*ermB*) and *ermTR* were also reported by Lä *et al*. (1998). Quinolone-resistance genetic determinat possessing region and acquisition of plasmid mediated quinolone resistance genes as *qnrVC* (Hooper, 1999; Ruiz *et al*., 2012; Liu *et al*., 2013); *oqxA* (Hansen *et al*., 2005), *qnrS, qnrA* and *qnrB* (Gay *et al*., 2006), *acc(6')-Ib-cr* (Park *et al*., 2009), *qepA* (Richter *et al*., 2010) and *qnrD* and *qnrC* (Veldman *et al*., 2011) are the major determining factors for quinolone resistance. Various plasmid-mediated quinolone resistance mechanisms of these genes included: (i) protecting the quinolone targets through Qnr proteins (ii) production of Aac(6')-Ib-cr enzyme, which acetylates not only aminoglycosides but also ciprofloxacin and norfloxacin; and (iii) QepA and OqxAB plasmid-mediated efflux pumps (Strahilevitz *et al*., 2009).

Aminoglycoside modifying enzymes like aminoglycoside acetyltransferases (*aac(6')-Ib, aac(3)-I*), nucleotidyl transferase (*ant(2")-I*) and phosphotransferases (*aph(3')-Ia*, *aph(3')-VI*) are identified and these act by the inactivation of aminoglycoside antibiotics (Lee *et al*., 2009). Genes encoding aminoglycoside-modifying enzymes include *ant (2")-Ia*, *aac (6')-Ib*, *aph (3')-Ia*, *aac (3)-Ia* and *aph (3')-VI.* Currently, seven types of plasmid mediated 16S rRNA methyl-transferase genes including *rmtA, rmtB, rmtC, rmtD*, rmtE, *npmA* and *armA*, are found (Huang *et al*., 2012).Genes such as golc, adeb, emre, tolc were also identified as responsible

for aminoglycoside resistance (Deng *et al*., 2019). The essential genetic determinants associated with resistance to vancomycin are v*anA*, *vanH* and *vanX* (Hong *et al*., 2004). Resistant genes for sulfamethoxazole (*sul2*), trimethoprim (*dfrA1*, *dfra17*, dfra26) (Deng *et al*., 2019), and streptomycin (*strA* and *strB*) were reported by Ismail *et al*. (2011).

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1. MATERIALS

3.1.1. EQUIPMENT

Cooling micro centrifuge (Remi, India and Hermle, Germany),Thermal cycler (Proflex, India and ABI Veriti 96 well thermal cycler, Thermofischer scientific), Vortex mixer (Spinix, India), Laminar Air flow system (Labline, India), Class II Biological Safety Cabinet (ESCO), UV transilluminator (Gelstan, India), Microwave oven (Samsung, India),Water bath (Labline) Incubator (Kemi, India and Labline, India), Electronic weighing balance (Shimadzu, India), Hot air oven (Kemi, India), Autoclave (Labline, India), Refrigerator (Samsung, Whirlpool, India) and Ultra low temperature freezer -80ºC (New Brunswick Scientific, India), were used in the present study.

3.1.2. GLASSWARE AND PLASTICWARE

Test tubes, Petri dishes, conical flasks, beakers, glass rods etc. used in this study were procured from reputed firms such as Borosil (India). They were thoroughly washed and sterilised as per standard procedures. Plasticware including microcentrifuge tubes, micropipette tips and centrifuge tubes were sterilised and used.

3.1.3. CHEMICALS AND REAGENTS

The compositions of media are enclosed in Annexure I. Chemicals and reagents used have been mentioned either at appropriate places or listed in the Appendix II, III and IV. Chemicals were from reputed firms such as sigma and Hi Media (India), and others.

3.1.4. PRIMERS

Details of the various primers used in the study are given in Annexure V.

3.1.5. BACTERIAL ISOLATES

Fish pathogenic bacteria belonging to three species namely *Vibrio parahaemolyticus, Vibrio vulnificus, Vibrio harveyi* (six isolates each) were also used in this study apart from the strains isolated from *S. olivacea.* Details of these isolates are given in Annexure VI.

3.2. METHODS

3.2.1. SAMPLE COLLECTION

A total of twelve apparently healthy orange mud crabs (*Scylla olivacea* Herbst, 1896) were used for the study. The animals were collected from Cochin coast through four samplings (each sampling involves three animals) during October-March, 2019. The sampling was done once in 45 days. Twelve crabs of different size and length were collected manually, retained in polyethylene bags and sterilized with the water from which the animals were collected. The bags were then transferred to ice boxes and carried to wet lab within four hours of collection. Morphometry were measured and then, the animals were anaesthetised on ice. Exterior carapace particularly, base of periopods were sterilised with 70 per cent ethanol prior to the collection of haemolymphs, in order to avoid contamination by surface bacteria (Colwell *et al.,* 1975) and haemolymph was aseptically withdrawn. After collecting haemolymph the carapace was opened and systematic dissection was carried out to collect the remaining tissues such as gut, gill, muscle, hepato-pancreas and pleura under aseptic conditions.

3.2.2. ENUMERATION OF PRESUMPTIVE VIBRIOS

For determination of presumptive vibrios (CFU), the collected haemolymph, homogenates of each tissue (gut, gill, adductor muscle and mantle) in sterile normal saline were serially diluted and spread onto Thiosulphate Citrate Bile-salt Sucrose (TCBS) agar (Hi Media, India) plates. All the plates were incubated at 28 ± 2°C. Viable counts of presumptive vibrios (mesophilic *Vibrionaceaee* and other closely associated vibrios) were recorded after 48 hours

of incubation on TCBS agar and expressed as the log colony forming units (CFU) per mL or g (log_{10}CFU) (Bolinches *et al*., 1988).

3.2.3. DETERMINATION OF DIVERSITY OF VIBRIOS

Morphologically different bacteria from the TCBS plates were picked up and aseptically transferred to sterile ZMA plates for further characterisation. The isolates were first checked for their purity by sub-culturing and then characterised by polyphasic taxonomy approach by microbiological and molecular methods. At the same time, glycerol stock of pure culture of the isolates were also made and stored at -80^{0}C.

3.2.4. Morphological characterisation

3.2.4.1. Gram staining

Bacterial smear was prepared from the colony on clean grease free glass slide and heat fixed. The slide was overlayed with crystal violet stain and kept for one min. mildly washed by direct stream of tap water for one min. again. Then the slide was flooded with Gram's iodine solution for one min. The slide was washed and decolourised with 95 per cent ethanol for few seconds, flooded with water and counter stain saffranin was added and allowed to react for one min. The slide was washed further, dried and observed under microscope with 100 X magnification using oil immersion objective.

3.2.4.2. Preliminary biochemical characterisation

A series of biochemical tests were done for the preliminary clustering of the isolates and details of the same are given below.

a. KOH string test: A drop of three per cent KOH was placed on a clean glass slide. Using a loop, a visible amount of fresh bacteria from a well isolated colony was removed and transferred into KOH. This was then mixed continuously on the glass slide for a maximum of one min. and by slowly lifting the loop, formation of a string was observed.

b. Catalase test: A drop of hydrogen peroxide (three per cent) was placed on a microscopic slide. Using loop, the test organism was placed on the slide and observed for effervescence.

c. Oxidase test: Oxidase test was carried out by touching and spreading a well isolated colony with a glass rod on the oxidase disc and the formation of a violet colour was observed within five to ten sec.

d. Indole test**:** Pure bacterial cultures were grown in sterile tryptophan broth for 24-48 h. After incubation, few drops of Kovac's reagent were layered onto the culture broth. A red ring formation was observed on the addition of the reagent.

e. Methyl Red test**:** Isolate was inoculated into MR-VP broth (Hi Media) with a sterile loop. The tube was incubated at 30ºC for two days. This was followed by addition of five drops of pH indicator methyl red to this tube and the colour change was observed.

f. Voges-Proskauer test: Isolate was inoculated into MR-VP broth with sterile loop. The tube was incubated at 30ºC for two days. An aliquot of 0.6 mL of 5 per cent alpha naphthol is added followed by 0.2 mL of 40per cent KOH. The tube was then shaken gently to expose the medium to atmospheric oxygen and allowed to remain undisturbed for 30 to 45 min.

g. Citrate test: The isolate was inoculated into Simmon's citrate agar lightly on the slant by touching the tip of a needle to a colony that is 18 to 24 hours old. Incubated at 35°-37°C for up to 7 days. Observed for growth and the development of blue colour, denoting alkalinization.

After preliminary characterisation by the above methods, the isolates having similar morphological, cultural and biochemical characteristics were clubbed together to one group and representative isolate from each group were then characterised by 16S*rRNA* gene sequencing.

3.2.5. Molecular characterisation by *16SrRNA* gene sequencing

3.2.5.1. Extraction of genomic DNA from bacteria

A well isolated colony was inoculated in five mL of LB broth and kept overnight in a shaker incubator. After incubation, the cells were harvested by centrifugation at 15,000 rpm for 10 min. The pellet was re-suspended in 570 µL TEG buffer with lysozyme and vortexed well. Thirty µL of 10per cent SDS and three µL of proteinase K was added and mixed well. Sample was then incubated at 60ºC for one hour. 100 µL of 5*M* NaCl and 80 µL of CTAB were added and incubated at 65^0C for 15 min. After incubation, equal volume of phenol: chloroform: isoamyl alcohol in the ratio 25:24:1 was added and centrifuged at 20,000 rpm for 20 min. This step was repeated twice. The aqueous phase was then collected in another micro centrifuge tube without disturbing the inter phase and lower phase. To the supernatant, equal volume of chloroform: isoamyl alcohol in the ratio 24:1 was added and centrifuged at 20,000 rpm for 20 min. Then, $1/10^{th}$ volume of 3*M* sodium acetate followed by 80per cent of isopropanol was added and incubated at -20ºC for 20 min so that the DNA got precipitated. This was subjected to centrifugation at 15,000 rpm for 20 min. The supernatant was discarded and the pellet was re-suspended in one millilitre of 70 per cent ethanol and centrifuged at 10,000 rpm for 15 min. Supernatant was discarded and the pellet was air dried for 30 min. Deoxyribonucleic acid samples were then dissolved in 30 µL of DNA dissolving buffer (TE buffer) and stored at 4ºC. Further, purity of DNA was analysed by 0.7 per cent agarose gel electrophoresis.

3.2.5.2. Analysis of genomic DNA by agarose gel electrophoresis

Five microlitre of DNA with one microlitre loading dye (6X) (Takara) was loaded on agarose gel. The agarose gel (0.7 per cent) was run at constant voltage of 100 V and current of 45 A till the bromophenol blue has reached the extreme opposite side of the wells with ladder of gene ruler (Thermo scientific). Viewed the gel in UV trans-illuminator with the safety shield and photographed.

3.2.5.3. PCR amplification of 16SrRNA gene

The method of *16S rRNA* gene amplification was carried out using universal prokaryotic primers; 16SbacF and 16SbacR (Weisburg *et al*., 1991). Each polymerase chain reaction (PCR) mixture consisted of 1 µL of template DNA, 2.5 µL of 1X B takara *Taq* buffer, 0.5 µL of dNTP mix, 0.5 µL of Forward and Reverse Primer and 0.25 U of takara *Taq* DNA polymerase. The PCR programme of each sample included initial denaturation at (95°C for 5 min.) followed by 35 cycles of denaturation (95°C for 30 sec.), annealing (58°C for 1 min.) and extension (72°C for 1.30 min). Final extension was performed at (72°C for 10 min). The PCR products were then characterised by gel electrophoresis (1 per cent agarose gel) using 100 bp DNA ladder (Takara) as molecular weight marker. After electrophoresis, the gel was visualised in a Gel documentation system (Gelstan, India).

3.2.5.4. Sequence Analysis

The PCR products were sequenced at Agrigenome, Kochi. The obtained sequences were subjected to BLAST search (NCBI) and the genus of the bacteria were identified. Sequence identities of 97 per cent and 99 per cent were taken as the benchmarks for genus and species assignments, respectively (Janda and Abbott, 2007). Further species/ sub-species level was confirmed by different biochemical tests based on the genus identified (Bergey *et al*., 2012). Finally, the sequences were submitted to NCBI.

3.2.6. Characterisation up to species level

3.2.6.1. Species identification for the genus *Vibrio*

3.2.6.1.1. Biochemical characterisation

A practical set of biochemical keys designed for the routine identification of *Vibrio* spp. developed by Noguerola and Blanch (2008) was primarily used for the characterisation of the organism up to the species level. The procedures for these tests were as follows:

a. Arginine Decarboxylase Test**:** An inoculum from a pure culture was transferred aseptically to a sterile tube of Arginine Decarboxylase broth (Hi Media). The inoculated tube was incubated at 30°C for 24 h. After inoculation over layered the tube with two to three millilitre mineral oil and yellow to purple colouration was observed.

b. Lysine Decarboxylase Test**:** An inoculum from a pure culture was transferred aseptically to a sterile tube of Lysine Decarboxylase broth (Hi Media). The inoculated tube was incubated at 30°C for 24 h. After inoculation, the tube was over layered with two to three millilitre mineral oil and yellow to purple colouration was observed.

c. Ornithine Decarboxylase Test: An inoculum from a pure culture was transferred aseptically to a sterile tube of Ornithine Decarboxylase Broth (Hi Media). The inoculated tube was incubated at 30°C for 24 h. After inoculation, over layered the tube with two to three millilitre mineral oil and yellow to purple colouration was observed.

d. Urease test: Streak the surface of a urea agar slant (Hi Media) with a portion of a well-isolated colony and incubated at 30°C with ambient aeration for 24 h.

e. Sugar Fermentation Test: Aseptically inoculated a pure culture of the test organism to each labelled bromocresol purple broth (Hi Media) to which various sugar discs (L-arabinose, sucrose, mannitol and melibiose) were added. Incubated the tubes at 18-24 h at 30°C.

f. Growth at different concentration w/v of NaCl: An inoculum from a pure culture was transferred aseptically to peptone broth containing different concentration w/v of NaCl (0 per cent, 6 per cent, 8 per cent and 10 per cent) and inoculated tubes were incubated at 30°C for 24 h.

g. Vibrio 0129 Differential Disc (150 µg): With a sterile swab, a lawn culture of the test organism was made onto Mueller-Hinton agar (MHA).

Aseptically placed both the Vibrio 0129 differential discs (150 μg) on the swabbed plates. Incubate at 30°C for 24 h. Observed for zone of inhibition

h. Ortho-Nitrophenyl-β-galactoside (ONPG) Test: The test disc was placed in a sterile test tube. To that added 0.1 mL of sterile normal saline solution, then the colony under test was picked with a sterile loop and emulsified in a normal saline in the tube containing the disc and incubated at 28°C for 24 h. Tube was observed periodically at an interval of one hour up to six hours.

i. Testing for gelatinase activity: An inoculum from a pure culture was spotted aseptically to gelatine (1 per cent) embedded agar plates and inoculated plate was incubated at 30°C for 24 h. Lugol's iodine was flooded in the plate and observed for zone formation.

j. Growth at different temperature: An inoculum from a pure culture was transferred aseptically to LB broth incubated at different temperature (20°C, 30°C and 40°C) for 24 h.

The analysis of the results of these biochemical tests and the NCBI-BLAST was done in order to precisely identify the species. For the most commonly occurring vibrios, molecular confirmation of species identification was done by species specific PCR (Annexure V).

3.2.6.1.2. Species confirmation of V. alginolyticus

Presence of *gyrB* gene was screened in each isolate using the primer Valg2 (Annexure V Table 1). The protocol for *16S rRNA* gene amplification was followed. Each PCR mixture comprised of 1 μL of template DNA, 2.5 μL of 1X B takara *Taq* buffer, 0.5 μL of dNTP mix, 0.5 μL of Forward and Reverse Primer and 0.25 U of takara *Taq* DNA polymerase. The PCR programme of each sample included initial denaturation (at 95°C for 5 min) followed by 35 cycles of denaturation (at 95°C for 30 sec), annealing (58°C for 1 min) and extension (72°C for 1.30 min). Final extension was carried out at (72°C for 10 min). The PCR products were then characterised by gel electrophoresis (1 per cent agarose gel) and 100 bp DNA ladder (Takara) was used as molecular weight marker. After electrophoresis, the gel was

visualised in a Gel documentation system. A desired amplicon with 773 bps was taken as the benchmark for the confirmation of *V. alginolyticus.*

3.2.6.1.3. Species confirmation of V. parahaemolyticus

Presence of t*oxR* and *collagenas*e based genes were screened in each isolates using primers VP1 and VP2 (Annexure V, Table 1).The protocol for *16S rRNA* gene amplification was followed. Each PCR mixture consisted of 1 µL of template DNA, 2.5 µL of 1X B takara *Taq* buffer, 0.5 µL of dNTP mix, 0.5 µL of Forward and Reverse Primer and 0.25 U of takara *Taq* DNA polymerase. The PCR programme of each sample included initial denaturation at (95°C for 5 min.) followed by 35 cycles of denaturation (95°C for 30 sec.), annealing (58°C for 1 min.) and extension (72°C for 1.30 min). Final extension was carried out at 72°C for 10 min. The PCR products were then analysed by gel electrophoresis (1 per cent agarose gel) using100 bp DNA ladder (Takara) as molecular weight marker. After electrophoresis, the gel was visualised in Gel documentation system. Presence of the desired amplicon with 503 bp for *toxR* and 227 bp for *collagenase* genes was checked for the confirmation of *V. parahaemolyticus.*

3.2.6.1.4. Species confirmation of V. vulnificus

Presence of haemolysin gene (*vvh*) was screened in each isolate using the primer VV1 (Annexure V, Table 1). The protocol for *16S rRNA* gene amplification was followed. The PCR mixture consisted of 1 µL of template DNA, 2.5 µL of 1X B takara *Taq* buffer, 0.5 µL of dNTP mix, 0.5 µL of Forward and Reverse Primer and 0.25 U of takara *Taq* DNA polymerase. The PCR programme of each sample included initial denaturation at (95°C for 5 min) followed by 35 cycles of denaturation (95°C for 30 sec.), annealing (58°C for 1 min.) and extension (72°C for 1.30 min). Final extension was carried out at 72°C for 10 min. The PCR products were then characterised by gel electrophoresis (1 per cent agarose gel) with 100 bp DNA ladder (Takara) as molecular weight marker. After electrophoresis, the gel was visualised in Gel documentation system. A desired amplicon with 501 bp was checked for the confirmation of *V. vulnificus.*

3.2.6.1.5. Species confirmation of V. harveyi

Presence of *topoisomerase* gene of *V. harveyi* was screened in each isolates using the primer VH (Annexure V**,** Table 1). The protocol for *16S rRNA* gene amplification was followed. Each PCR mixture consisted of 1 µL of template DNA, 2.5 µL of 1X B takara *Taq* buffer, 0.5 µL of dNTP mix, 0.5 µL of Forward and Reverse Primer and 0.25 U of takara *Taq* DNA polymerase. The PCR programme of each sample included initial denaturation at (95°C for 5 min.) followed by 35 cycles of denaturation (95°C for 30 sec.), annealing (58°C for 1 min.) and extension (72°C for 1.30 min). Final extension was carried out at 72°C for 10 min. The PCR products were then characterised by gel electrophoresis (1per cent agarose gel) employing 100 bp DNA ladder (Takara) as molecular weight marker. After electrophoresis the gel was visualised in Gel documentation system and a desired amplicon of 121 bp was confirmation as *V. harveyi.*

3.2.6.1.6. Species confirmation of V. diazotrophicus

a. Growth on TCBS: The pure cultures of the bacteria were streaked onto the TCBS agar plates and the colour of the colonies were checked after 24 h. of incubation.

b. Arginine Decarboxylase Test: An inoculum from a pure culture was transferred aseptically to a sterile tube of Arginine Decarboxylase broth (Hi Media). The inoculated tube was incubated at 30°C for 24 h. After inoculation, over layered the tube with two to three millilitre mineral oil.

c. Lysine Decarboxylase Test: An inoculum from a pure culture was transferred aseptically to a sterile tube of Lysine Decarboxylase broth (Hi Media). The inoculated tube was incubated at 30°C for 24 h. After inoculation, over layered the tubes with two to three millilitre mineral oil.

d. Ornithine Decarboxylase Test: An inoculum from a pure culture was transferred aseptically to a sterile tube of ornithine decarboxylase broth

(Hi Media). The inoculated tube was incubated at 30°C for 24 h. After inoculation, over layered the tubes with two to three millilitre mineral oil.

e. Growth at different concentration w/v of NaCl: An inoculum from a pure culture was transferred aseptically to peptone broth containing different concentration w/v of NaCl (0 per cent, 6 per cent, 8 per cent and 10 per cent) and inoculated tube was incubated at 30°C for 24h.

f. Growth at different temperature: An inoculum from a pure culture was transferred aseptically to LB broth and the inoculated tube was incubated at different temperature (20°C, 30°C and 40°C) for 24 h.

g. Urease test: Streak the urea agar slant (Hi Media) surface with a portion of a well-isolated colony and incubated at 30°C in ambient aeration for 24 h.

3.2.7. PHYLOGENETIC ANALYSIS OF CRAB VIBRIO ISOLATES

The sequences obtained after *16SrRNA* gene sequencing were edited and compiled using Editseq program (DNASTAR Lasergene, USA). Pair alignments and sequence identity generation were performed using MegAlign program (DNASTAR Lasergene, USA). The overlapping 726 bp size segment of the *16S rRNA* gene was used for phylogenetic study. The sequences were aligned using ClustalW and Neighbor-joining (NJ) tree was constructed by MEGA version 7 using Jukes-Cantor model (Jukes and Cantor, 1969**).** The confidence in the NJ tree was estimated by 1000 bootstrap replicates.

3.2.8. SCREENING FOR TYPICAL AND NON-TYPICAL VIRULENCE GENES OF PUBLIC HEALTH SIGNIFICANT VIBRIOS

3.2.8.1. PCR amplification of virulence genes

Presence of 11 putative virulence genes of vibrios (*toxR, vhpA* and *chiA* of *V. harveyi*, *yopP*, *tdh* and *trh* of *V. parahaemolyticus*, *ctxA* and *nanH* of *V. cholerae*, *toxR* and *tdh* genes of *V.alginolyticus* and *vvh* of *V. vulnificus*) were screened in each isolate using reported primers (Annexure V, Table 2). The conditions and

composition of PCR mixture were optimised using standard protocol (Sambrook, 2006). The optimised PCR mixture consisted of 1 µL of template DNA, 2.5 µL of 1X Takara *Taq* buffer, 0.5 µL of dNTP mix, 0.5 µL of Forward and Reverse Primer and 0.25 U of Takara *Taq* DNA polymerase. In case of PCR using tdhValg and toxRVh primers, an extra 1 µL $MgCl_2$ was added and for the primer yopVp an extra of 1.5 µL $MgCl_2$ was added to the reaction mix for successful yield of the amplicon. The optimised PCR programme included initial denaturation at (94°C for 5 min.) followed by 35 and 30 cycles cycles of denaturation (94°C for 1 min.), annealing (optimum temperature and time for each set of primers were given in Annexure V and extension (72°C for 2 min. and 1 min). Final extension was carried out at 72°C for 10 min.

3.2.8.2. Analysis of the amplicon by agarose gel electrophoresis

The PCR product five µL was mixed with one µL 6X DNA loading dye (Takara) and loaded in agarose gel (1.5 per cent). The gel was run at constant voltage of 90 V and current of 45A till the bromophenol blue has reached the extreme opposite side of the wells and 100 bp DNA ladder (Takara) was run along with the samples to identify the molecular size of the amplicon. The gel was then visualised and photographed using a gel documentation system (BioRad).

3.2.8.3. Sequence Analysis

Representative PCR products of each virulence gene from each species were sequenced at Agrigenome, Kochi. The obtained sequences were then subjected to BLAST search (NCBI) for the confirmation of the specific amplification.

3.2.9. Assesment of antibiotic resistance pattern

3.2.9.1. Antibiotic sensitivity test by disc diffusion method

In-vitro antibiotic susceptibility of each isolate to 21 antibiotics belonging to six different classes (Hi Media Laboratories, Mumbai, India) was analysed using disc diffusion test (Bauer *et al*., 1966; CLSI, 2018) (Annexure I). Mueller

Hinton Agar (MHA) was prepared and sterilised at standard conditions. Fifteen to twenty mL of MHA was poured to each Petri dish and allowed to solidify. The plates were labelled accordingly. A slight turbid inoculum of the organism was made in 500 µL of sterile PBS by mixing two or three colonies of the selected organism. The suspension was vortexed to result a smooth inoculum. Adjusted the turbidity of the culture to 0.5 McFarland opacity tube (1-2 x 10^8 CFU/mL) using sterile saline. A sterile swab was dipped in the suspension and uniformly swabbed over the MHA plate for three times and ensured even distribution of the inoculum over the entire agar surface. The swab was discarded by heating under flame. Using a sterile forceps, the appropriate antibiotic discs were placed and firmly pressed on to the agar surface to avoid slipping off from the surface. After flame sterilising the forceps, the remaining antibiotic disc were placed carefully by leaving a specific diameter in order to avoid the overlapping between the zones of inhibition of the remaining antibiotic discs. After placing the discs, the plates were incubated at 30°C overnight. After incubation, the zones of inhibition was measured in millimetre using a ruler. The zone size was recorded.

3.2.9.2. Analysis of antibiotic resistance patterns

Established criteria for each *Vibrio sp.* by WHO was explored for result interpretation as either susceptible (S), intermediate (I), or resistant (R) using WHONET (http://www.whonet.org) version 5.6. Further, multiple antibiotic resistance (MAR) index was calculated by the formula MAR index= A/B, where 'A' represented the number of antibiotics for which the isolate showed resistance and 'B' represented the total number of antibiotics against which the isolate was assessed (Krumperman, 1983).

3.2.10. Description of genetic basis underlying antibiotic resistant phenotypes

3.2.10.1. PCR amplification of genes responsible for antibiotic resistance

A total of 18 genes responsible for resistance to different antibiotics (β-lactams, quinolones, chloramphenicol, erythromycin, kanamycin and tetracycline)

were screened in each isolate using reported primers (Annexure V). The PCR conditions and mixture were optimised using standard protocol (Sambrook and Russell, 2006). The optimised PCR mixture consisted of 1 µL of template DNA, 2.5 µL of 1X Takara *Taq* buffer, 0.5 µL of dNTP mix, 0.5 µL of Forward and Reverse Primer and 0.25 U of Takara *Taq* DNA polymerase. In case of PCR using *blaSHV, blaTEM, ermB, tetS, tetEHJ* and *aphA3* primers, extra 1µL $MgCl_2$ was added to the reaction mix for successful yield of the amplicon. The optimised PCR programme included initial denaturation at (94°C for 5 min.) followed by 30 cycles of denaturation (94°C for 1 min.), annealing (optimum temperature and time for each set of primers are given in Annexure V) and extension (72°C for 1 min). Final extension was carried out at 72°C for 10 min.

3.2.8.3.2. Analysis of amplicon by agarose gel electrophoresis

The PCR product (5 µL) was mixed with 1 µL 6X DNA loading dye (Takara) and loaded in agarose gel (1.5per cent) and the gel was run at constant voltage of 90 V and current of 45A till the bromophenol blue has reached the extreme opposite side of the wells. The DNA ladder (100bp - Takara) was run along with the samples to identify the molecular size of the amplicon. The gel was then visualised and photographed in a gel documentation system (BioRad).

RESULTS

4. RESULTS

4.1 SAMPLE COLLECTION

The weight and length of different crabs used in the present study are given in table 4. 1. Sampling sites and crabs used in study are depicted in fig. 4.1.

Table 4.1: Morphometrics of crabs used for the study

Group	Weight of individual animals (g)	Average (Mean ±SD) (g)	Length of individual animal (cm)	Average (Mean ±SD) (cm)
I	158	158 ±1	10.2	10.2 ±1
	159		11.9	
	157		9.3	
II	143	149 ±1	9.1	9.1 ±1
	155		9	
	149		10.2	
III	172.8	170.3 ±1	11.4	11.1 ±1
	171		12.1	
	167.3		9.8	
IV	129.8	144.8 ±1	10	10.1 ±1
	151.1		10.8	
	153.5		9.7	

The crabs used in the present study had an average weight of 155.5 ± 1 g with an average length of 10.1 ± 1 cm.

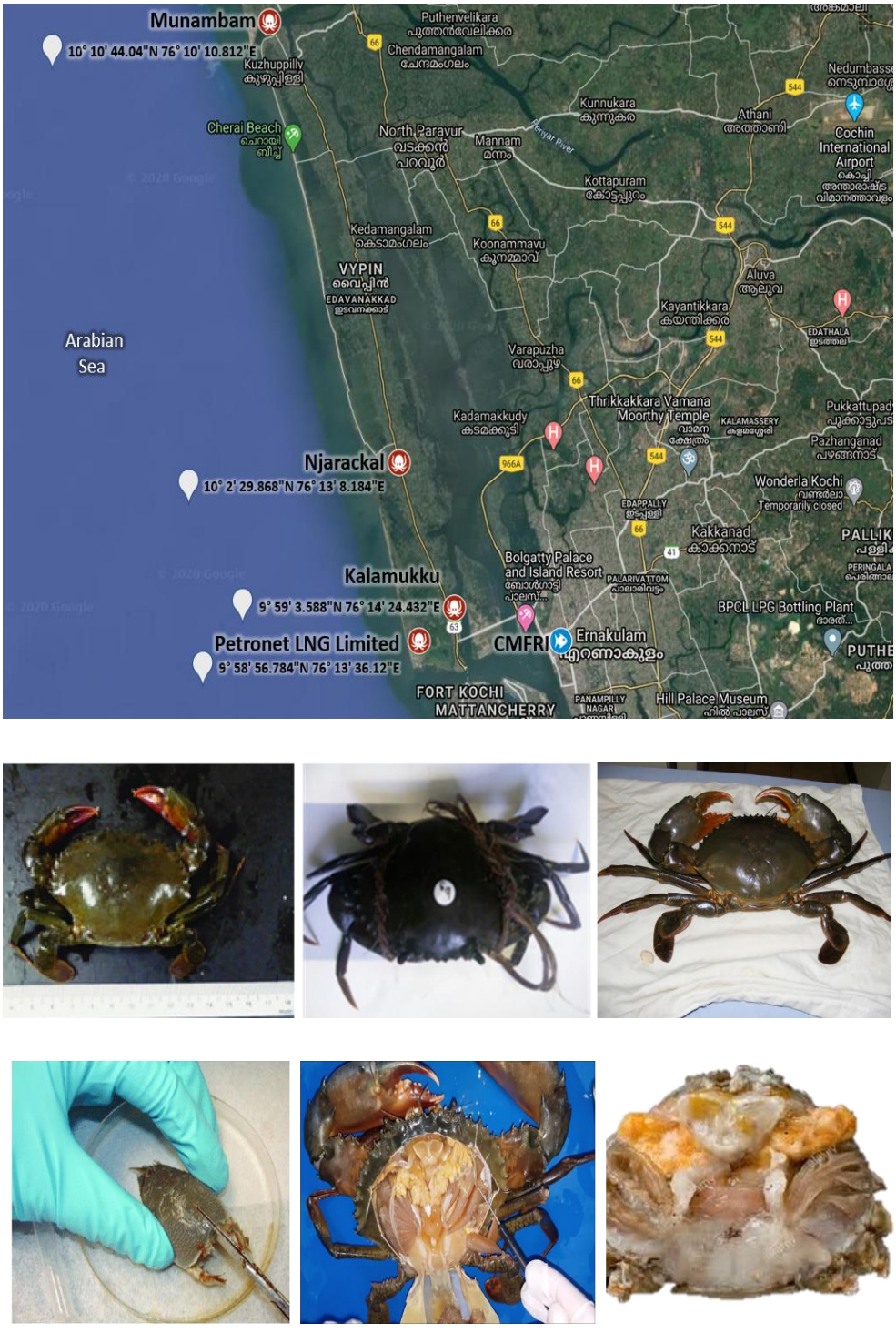

Fig. 4.1 Sampling sites and crabs used in the study

4.2. ENUMERATION OF PRESUMPTIVE VIBRIOS

Load of presumptive vibrios in various tissues of *S. olivacea* was estimated by limiting dilution method in the selective media for vibrios, namely Thiosulfate-citrate-bile salts-sucrose (TCBS) agar (Hovda *et al.*, 2007) and results were represented in terms of log colony forming units per mL/mg (log_{10} CFU/ml or mg) in fig. 4.2 Results showed that all the tissues including haemolymph of *S. olivacea* carried a variable concentration of vibrios even in apparently healthy conditions with the greatest mean concentrations in gut (1.74 X 10^6 CFU/g), immediately followed by gill (1.60 X 10^6 CFU/g). Haemolymph had the lowest mean concentration of vibrios (1.77 X 10^2 CFU/mL). The prevalence of presumptive vibrio was in the order of Gut > Gill > Hepatopancreas > Muscle > Pleura > Haemolymph (Fig 4.3).

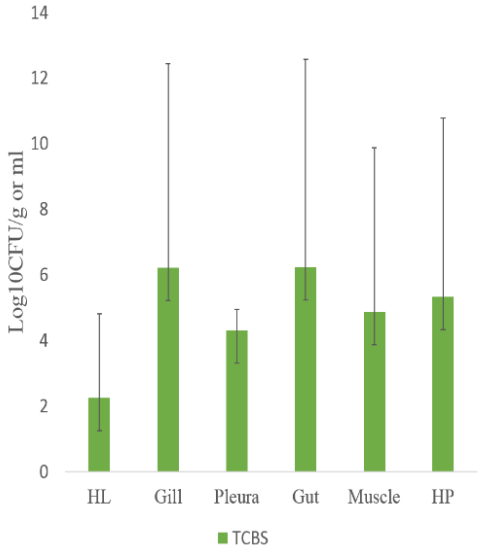

Fig. 4. 2 Density enumeration of bacterial isolates (log_{10} CFU/ml or mg)

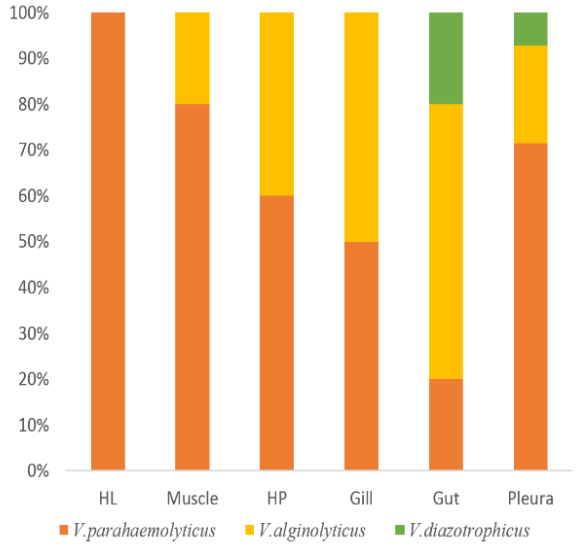

Fig. 4. 3 Relative prevalence of presumptive vibrio in the tissues of crab

4.3. DETERMINATION OF DIVERSITY OF VIBRIOS IN *S. olivacea*

4.3.1. Characterisation of bacterial isolates

Morphologically different bacteria from the various dilutions of TCBS plates were picked up and aseptically transferred to a sterile ZMA slant for further characterisation. The isolates were first checked for their purity by sub-culturing and then characterised by a series of tests. There were a total of 23 morphologically different isolates suggestive of *Vibrio* sp. During morphological characterisation by Gram's staining, all these isolates were observed as pink coloured rods indicative of Gram-negative bacilli (Fig. 4.4). Cultural characteristics of these 23 bacterial isolates in media such as ZMA, TCBS (Fig. 4.4), and Luria Bertani Broth were noted down (Table 4.2). Similarly, biochemical characteristics of the bacterial isolates in the present study are depicted in the table (4.3 and 4.4), respectively.

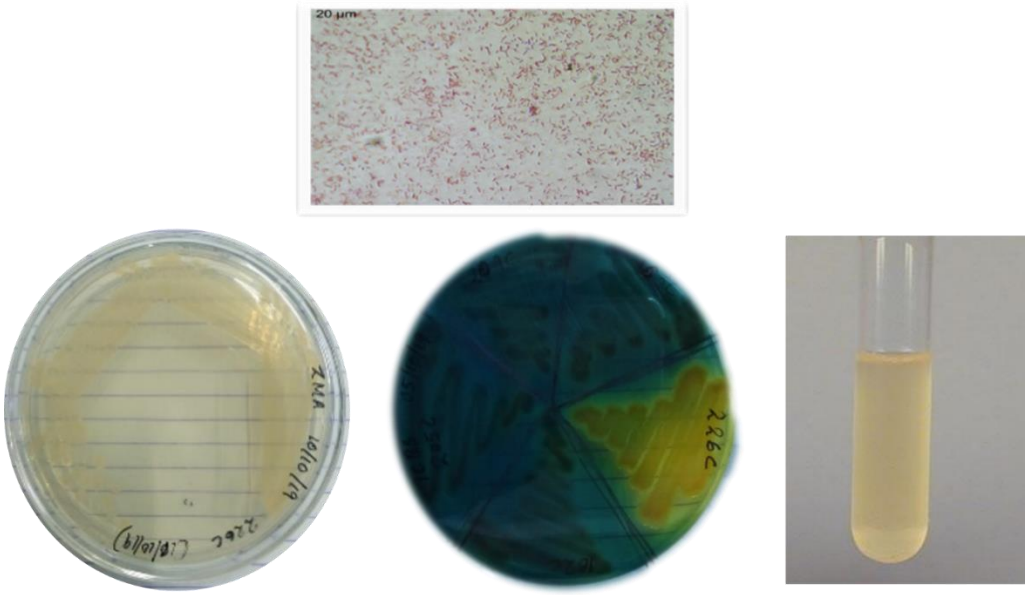

Fig. 4. 4 Cultural characteristics of bacteria

4.3.2. Preliminary biochemical characterisation

Further, a series of seven preliminary biochemical tests (Fig.4.5) were carried out (Bergey *et al*., 2012), the results of the same are given in table.4.3. Criteria for the interpretation of each results were as follows;

a. KOH string test: Formation of viscous bacterial suspension and stringing out of the same when the loop was lifted was considered as positive

b. Catalase test: Positive reaction was evident by the formation of immediate effervescence (bubble formation).

c. Oxidase test: Appearance of a deep blue or purple colour within five to ten sec was considered as positive reaction while no colour change was read as negative.

d. Indole test: Positive reaction was specified by the development of red ring on the surface layer of the broth on addition of Kovac's reagent.

e. Methyl red (MR) test: The positive reaction was observed as the colour change of MR-VP broth from yellow to red on addition of methyl red indicator.

f. Vogues Proskauer (VP) test: Positive test was indicated by the appearance of pinkish red colour on addition of alpha naphthol and potassium hydroxide.

g. Citrate utilisation test: Colour change of the media from green to blue within 48 h. of incubation was regarded as positive reaction.

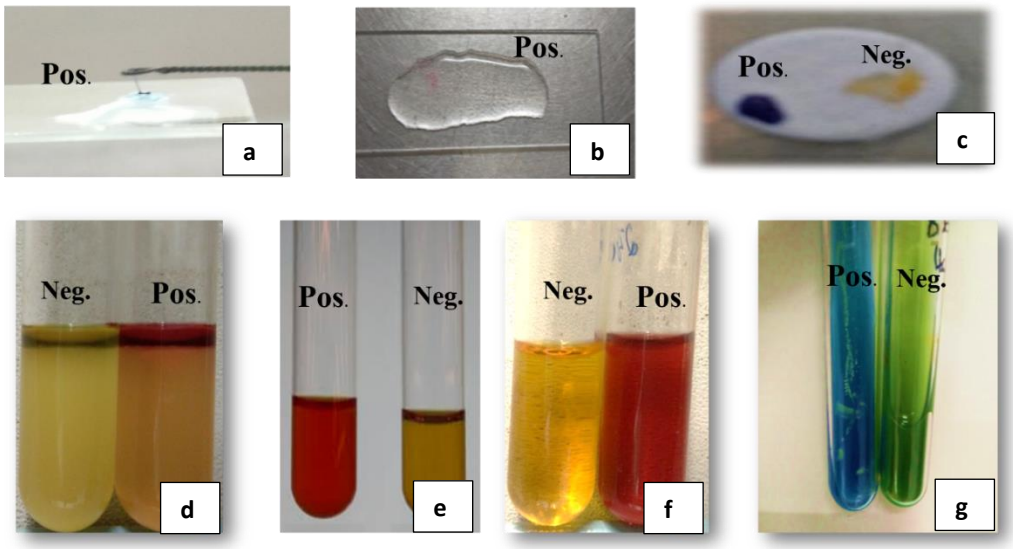

Fig. 4. 5 Preliminary biochemical tests

Table No: 4.2 Cultural characteristics of isolates from *S. olivacea*

SI No.	Tissue source	Culture Code	Isolate ID	MEDIA		
				TCBS	ZMA	LB
1	Muscle	ZMC2MSP	102	GYM*	CO	UG
2	Gill	TGC3B	163	GM*	CO	UG
3	Pleura	TPC4YSR	204	G*	CO	UG
4	HP	THPC4YSR	205	GM	CWO	UG
5	Water	ZH2O4YT	226	Y*	CYO	UG
6	Gill	ZGC-YW	17	YM*	CYO	UG
7	HP	ZHPC2Y	100	YM	CYSM	UG
8	Pleura	ZPC4SP	190	GM	CYO	UG
9	Gut	ZGUC4CMSR	191	G	CT	UG
10	HP	THPC4GY	206	GYM	CYO	UG
11	Gill	ZGC-OW	14	GM	COM	UG
12	Muscle	ZMC4CM	193	Y	CSO	UG
13	Gill	TGC4B	209	G	CSO	UG
14	Gut	TGUC4G	210	G	CO	UG
15	Muscle	BMC2CM	87	GM	CSM	UG

16	Muscle	TMC4G	212	G	CWO	UG
17	Pleura	TPC2Y	109	Y	CYO	UG
18	Muscle	TMC4GMWB	213	G	CYOM	UG
19	Muscle	ZMC2MC	92	GYM	CYOM	UG
20	Pleura	BPC-OWM	53	GM	CWOM	UG
21	Gill	ZGC4CM	200	GYM	CO	UG
22	Gut	TGUC3G	160	GYM	CWOM	UG
23	Gut	ZGUC-OBD	3	GW	CYPT	UG

Abbreviations: GYM: Greenish yellow mucoid; GM : Green mucoid; YM : Yellow mucoid; Y: Yellow; G: Green; GW: Greenish white CO: Creamish opaque; CWO: Creamish white opaque; CYO: Creamish yellow opaque; CYSM: Creamish yellow swarming mucoid; CT: Creamish translucent; COM: Creamish opaque mucoid; CSO: Creamish Swarming opaque; CSM: Creamish swarming mucoid; CYOM: Creamish yellow opaque mucoid; CWOM: Creamish white opaque mucoid; CYPT: Creamish yellow periphery translucent; UG: Uniform growth

Table No: 4. 3: Preliminary biochemical characterisation of bacterial isolates

SL.NO.	Isolate ID	KT*	CT*	OT*	INDOLE	MR*	VP*	CITRATE
1	102	+	+	+	+	+	-	+
2	163	+	+	+	+	+	-	+
3	204	+	+	+	+	+	-	+
4	205	+	+	+	+	+	-	+
5	226	-	+	+	+	-	+	+

6	17	+	+	+	+	+	-	+
7	100	+	+	+	+	-	+	+
8	190	+	+	+	+	+	-	+
9	191	+	+	+	-	-	+	-
10	206	+	+	+	+	+	-	+
11	14	+	+	+	-	-		+
12	193	+	+	+	+	-	+	-
13	209	+	+	+	+	-	+	+
14	210	+	+	+	+	-	+	-
15	87	+	+	-	+	+	-	-
16	212	+	-	+	+	-		+
17	109	-	+	+	+	-	-	-
18	213	+	-	+	+	-		+
19	92	+	+	-	-	-	+	-
20	53	-	+	+	-	-	+	-
21	200	+	+	+	+	+	-	-
22	160	-	+	+	-	-	+	-
23	3	+	+	+	-	-		+

Abbreviations: * KT: KOH test: CT: Catalase test; OT: Oxidase test; MR: Methyl red test VP: Voges–Proskauer test

After the preliminary characterisation by the above methods, the isolates having similar morphological, cultural and biochemical characteristics were clubbed together to one group and one representative isolate from each group were then characterised by *16SrRNA* gene sequencing.

4.3.3. Molecular characterisation by *16S rRNA* gene sequencing

During molecular characterisation by 16S*rRNA* gene sequencing, an amplicon of ~ 1499 bp was obtained for entire isolates used for the study (Fig.4.6) by PCR using universal prokaryotic primers (Weisburg *et al*., 1991). The band size was consistent in all the isolates as analysed by 1.5 per cent agarose gel electrophoresis. The PCR products were then sequenced and the resulted sequences were analysed by NCBI-BLAST for the identification of genus.

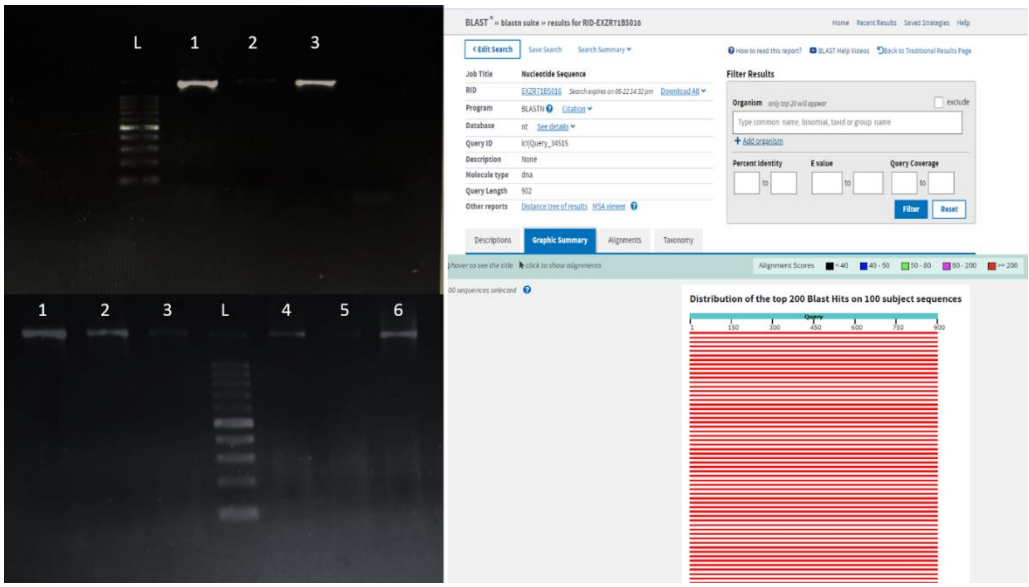

Fig. 4.6 Gel profile of *16S rRNA* gene amplification products and NCBI BLAST results of *16S rRNA* gene sequences

4.3.4. Biochemical characterisation up to species level

The species of each isolate was confirmed by different biochemical tests (Fig. 4.7) according to the genera identified, the results of the same are given in table 4.4. The outcomes were interpreted as follows:

a. Sugar fermentation test: The positive reaction was characterised by a colour change to yellow due to the acid formation by the respective sugar fermentation.
b. Amino acid decarboxylation tests: The positive test was indicated by a colour change from yellow to purple indicating the utilisation of the corresponding amino acids (arginine, ornithine and lysine).
c. Sensitivity to vibrio static agents: (O129) (150 µg): An inhibition zone diametre is observed against the vibrio static agent in the disc differentiating vibrios from non -vibrios.
d. Urease test: The pink colour formation after 48 h. incubation was considered as positive.
e. ONPG test: Formation of yellow colour was taken as positive reaction.
f. Gelatinase test: The positive isolates were identified by the formation of a clear zone around the colonies.
g. Growth of bacteria at different temperatures: The turbidity/pellet/sediment formation at the end of incubation was taken as positive.
h. Salinity tolerance test: The growth of bacteria at the end of incubation was noted down.

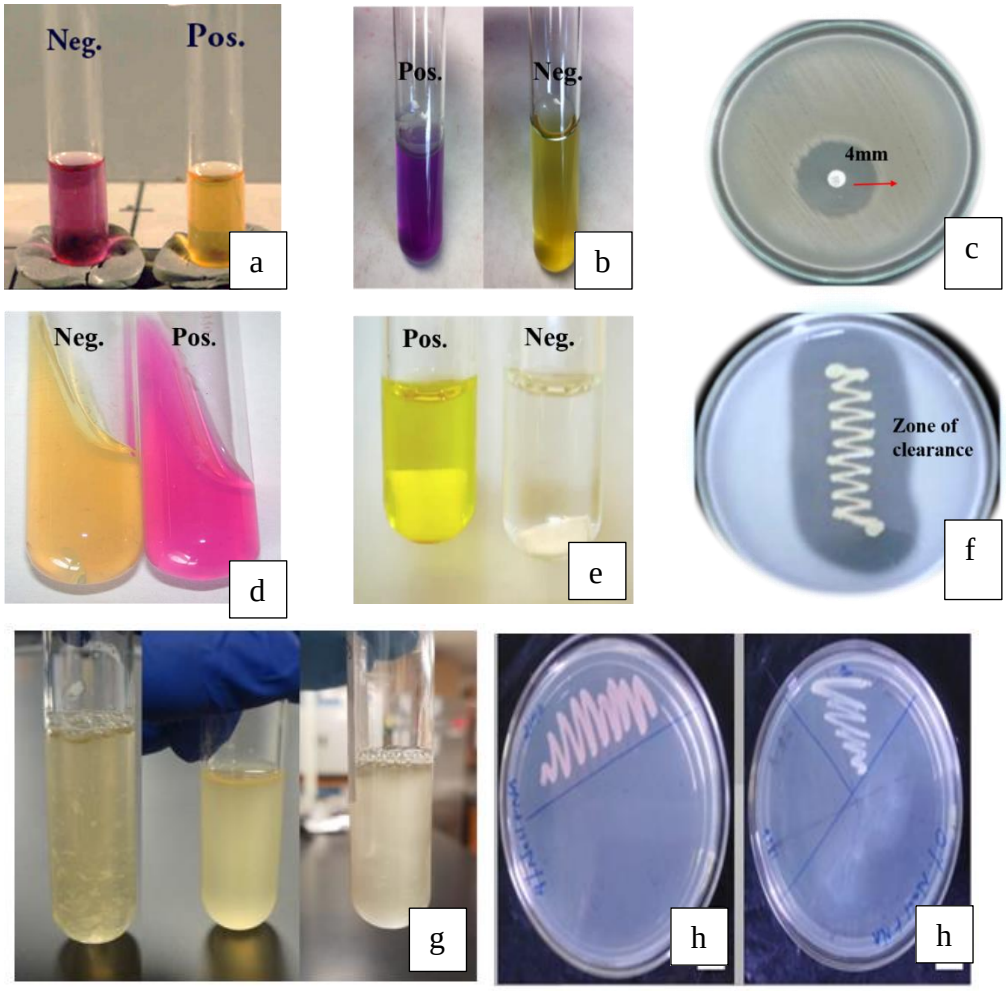

Fig.4.7 Biochemical characterisation up to species level

Table No: 4.4 Biochemical characteristics of the isolates under study

Sl. No	Test	210C	3C	226C	163C	190C	102C	100C	205C	17C	14C	193C	92C	212C	209C	109C	204C	200C	53C	206C	213C	87C	160C	191C
1	ADT	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
2	LDT	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
3	ODT	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
4	Gelatinase	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
5	Urease	+	-	-	+	+	+	+	+	+	+	-	+	+	+	+	+	-	+	+	+	+	+	-
6	Growth at temperature																							
A	20 ºC	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
B	30 ºC	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
C	40ºC	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7	Sugar Fermentation test																							
A	Sucrose	-	+	+	-	-	+	+	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-	+
B	Melibiose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C	L-arabinose	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
D	Mannitol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
8	Growth at different w/v conc. of NaCl																							
A	0 per cent NaCl	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
B	6 per cent NaCl	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
C	8 per cent NaCl	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D	10 per cent NaCl	+	+	-	-	-	-	+	-	-	-	+	-	-	+	+	-	+	-	-	-	-	-	-
9	ONPG Test:	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
10	O/129 (150 mcg)	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
11	Resistant to Ampicillin (10mcg)	R	R	R	R	R	R	R	R	R	S	R	R	R	R	R	R	R	R	R	R	R	R	R

Abbreviations: ONPG- Ortho nitrphenyl hydrazine; ADT- Arginine decarboxylase test; LDT- Lysine decarboxylase test; ODT- Ornithine decarboxylase test; O/129- Vibrio differential disc.

4.3.5. PCR based species confirmation

PCR based species confirmation was done by detecting the presence of *toxR* gene and *collagenase* gene amplification for *V. parahaemolyticus* and *gyrB* gene-based detection for *V. alginolyticus.* Desired amplicons having 503 bp for *toxR* and 227 bp for collagenase were obtained in all the *V. parahaemolyticus* isolates (Fig.4. 8). Out of the 23 vibrio isolates, 14 showed positive amplification for *toxR* and *collagenase* genes. In *gyrB* gene-based detection for *V. alginolyticus*, seven isolates produced positive amplicon having 773 bp (Fig.4.9).

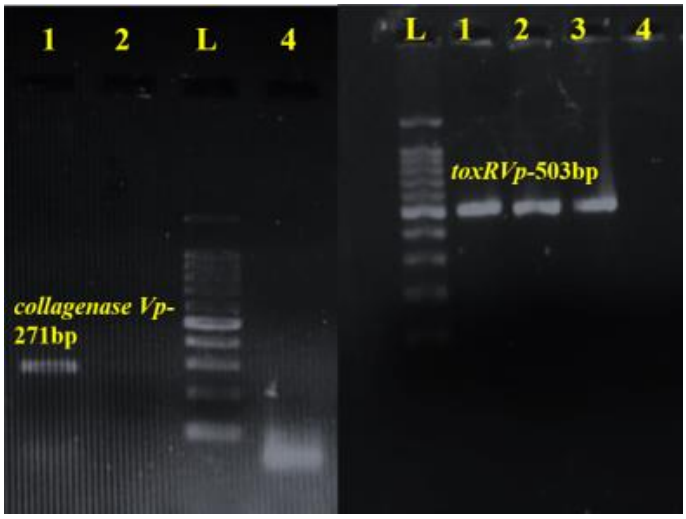

Fig 4.8. Gel profile of positive amplicon for *toxR* and *collagenase* gene of *V. parahaemolyticus*

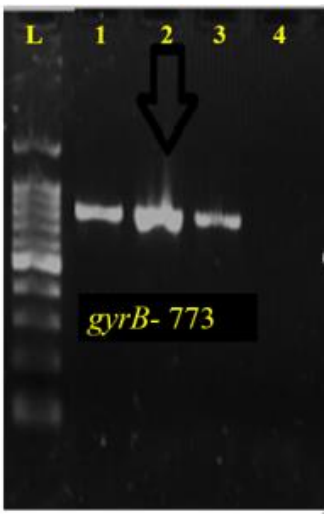

Fig 4.9. Gel profile of positive amplicon for *gyrB* gene of *V. alginolyticus*

4.3.5. Diversity of vibrios in *S. olivacea*

Results of species characterisation of different isolates under study is given in table 4.5. Collectively, three distinct species from a single genus namely *Vibrio* spp. of the family *Vibrionaceae* could be identified. The most prevalant bacterial geneus was *Vibrio* spp. Overall, *V. parahaemolyticus* (56 per cent) was the most abundant species which was followed by *V. alginolyticus* (28 per cent). *Vibrio parahaemolyticus* was the most prevalent bacterium that was present in all the tissues. *Vibrio alginolyticus* was the second most prevalent bacterium which was

present in all the tissues except haemolymph. In the present study diversity of vibrio was maximum in gut tissue and it consisted of *V. parahaemolyticus, V. alginolyticus* and *V. diazotrophicus.*

Table No: 4.5 Results of species identification

Culture ID	Species identified
102C	*V. parahaemolyticus*
163C	*V. parahaemolyticus*
204C	*V. parahaemolyticus*
190C	*V. parahaemolyticus*
205C	*V. parahaemolyticus*
17C	*V. parahaemolyticus*
14C	*V. parahaemolyticus*
92C	*V. parahaemolyticus*
212C	*V. parahaemolyticus*
53C	*V. parahaemolyticus*
206C	*V. parahaemolyticus*
213C	*V. parahaemolyticus*
87C	*V. parahaemolyticus*
160C	*V. parahaemolyticus*
226C	*V. diazotrophicus*
191C	*V. diazotrophicus*
210C	*V. alginolyticus*
100C	*V. alginolyticus*
193C	*V. alginolyticus*
209C	*V. alginolyticus*
109C	*V. alginolyticus*
200C	*V. alginolyticus*
3C	*V. alginolyticus*

4.4. PHYLOGENETIC ANALYSIS OF VIBRIO ISOLATES FROM CRAB

During phylogenetic analysis, it was found that *S. olivacea* contained vibrios belonging to two different clades namely, Harveyi clade (*V. parahaemolyticus* and *V. alginolyticus*) and Diazotrophicus clade (*V. diazotrophicus*) (Fig.4.10). Harveyi clade was the most dominant one. Representative *16srRNA* gene sequences of different species characterised were submitted in GenBank, NCBI and got assigned with accession number (Table . 4.6).

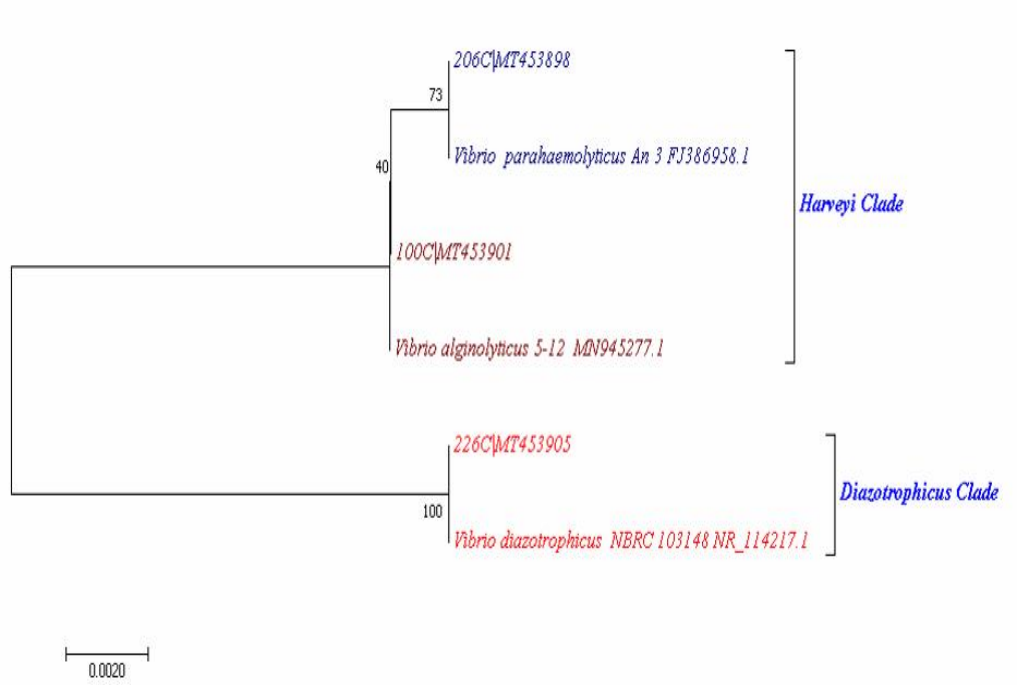

Fig. 4. 10. Phylogenetic analysis of crab vibrio isolates

Table No: 4.6 Sequences submitted in NCBI

Sl. No.	Authors	Submission date	Gene details	Accession No.
SUB7433629	Mariya Sony Reshma K.J. Sumithra T.G. Amala P.V. Sanil N.K.	12-05-2020	cultured Prokaryotic 16S rRNA / *V. diazotrophicus* strain CMFRI/VDi-01 16S ribosomal RNA gene	MT453905
SUB7433576	Mariya Sony Sumithra T.G. Reshma K.J. Anusree V.N. Sanil N.K.	12-05-2020	cultured Prokaryotic 16S rRNA / *V. alginolyticus* strain CMFRI/VAl-40 16S ribosomal RNA gene	MT453901
SUB7433001	Mariya Sony Reshma K.J. Sumithra T.G. Amala P.V. Sanil N.K.	12-05-2020	cultured Prokaryotic 16S rRNA / *V.parahaemolyticus* strain CMFRI/VP-09 16S ribosomal RNA gene	MT453898

4.5. SCREENING FOR TYPICAL AND NON-TYPICAL VIRULENCE GENES OF PUBLIC HEALTH SIGNIFICANT VIBRIO

A total of 14 virulence genes of various vibrios (*toxR, vhpA* and *chiA* of *V. harveyi*, *yopP, tdh, toxR, collagenase* and *trh* of *V. parahaemolyticus*, *ctxA, nanH* and *VPI2* of *V. cholerae*, *toxR* and *tdh* genes of *V. alginolyticus* and *vvh* of *V. vulnificus*) were targeted. Among the 14 target genes, five namely, *chiAVh* of *V. harveyi*, *collagenase* of *V. parahaemolyticus*, *toxR* of *V. parahaemolyticus*, *toxR* of *V. alginolyticus* and *nanH* of *V. cholerae* were found to be present in the isolates. The amplicon size for these genes were consistent in all the isolates irrespective of the species which were 232 bp, 271 bp, 503 bp, 658 bp and 650 bp respectively. Amplification profiles of these virulent genes among vibrio isolates from *S. olivacea* are represented (Fig. 4.11). All the amplicons were sequenced at Agrigenome, India for the confirmation of specific amplification.

It was found that prevalence was in the order of *chiAVh* of *V. harveyi* (29.63 per cent) > *collagenase* of *V. parahaemolyticus* (25.93 per cent) > *toxR* of *V. parahaemolyticus* (22.22 per cent) > *toxR* of *V. alginolyticus* (18.52 per cent)> *nanH* of *V. cholerae* (3.70 per cent) (Fig. 4.12). The genes encoding three important virulence factors seen among clinical isolates of *V. parahemolyticus* namely, thermostable direct haemolysin (TDH), TDH-related haemolysin (TRH) and putative type III secretion effector YopP protein were absent in all the isolates in the present study. At the same time, the genes known to code another two virulence determinants namely, *collagenase* and *toxR* in *V. parahemolyticus* were present in 100 per cent of *V. parahaemolyticus*, 50 per cent of *V. alginolyticus* and 100 per cent of *V. diazotrophicus* isolates tested. Among the three virulence genes of *V. harveyi* screened, two (*toxR* and *vhpAVh*) were absent in all the isolates, while *chitinase* gene of *V. harveyi* was present in all the vibrio isolates in the present study. Of the three virulence-associated factors screened in *V. cholera,* only *nanH* encoding neuraminidase was present in the isolates (in one *V. alginolyticus*). *vvh* gene of *V. vulnificus* encoding haemolysin was absent in all the vibrio isolates from

S. olivacea. Among the virulence genes of *V. alginolyticus* screened, only *toxR* gene was present in the vibrio isolates (62.5 per cent).

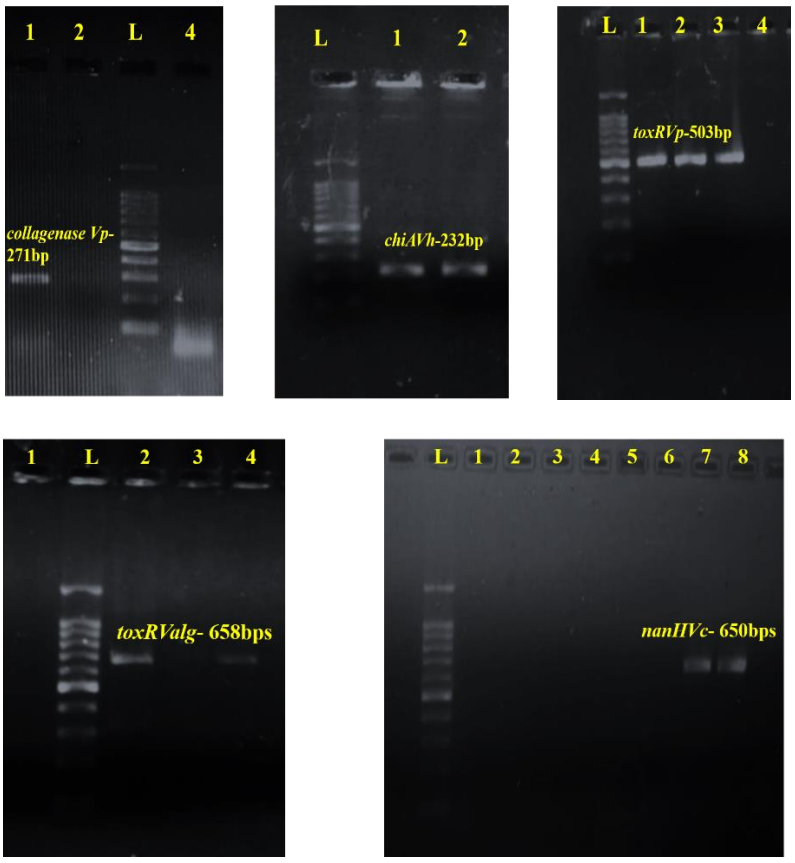

Fig. 4.11. Amplification profiles of virulence genes among vibrio isolates from *S. olivacea*

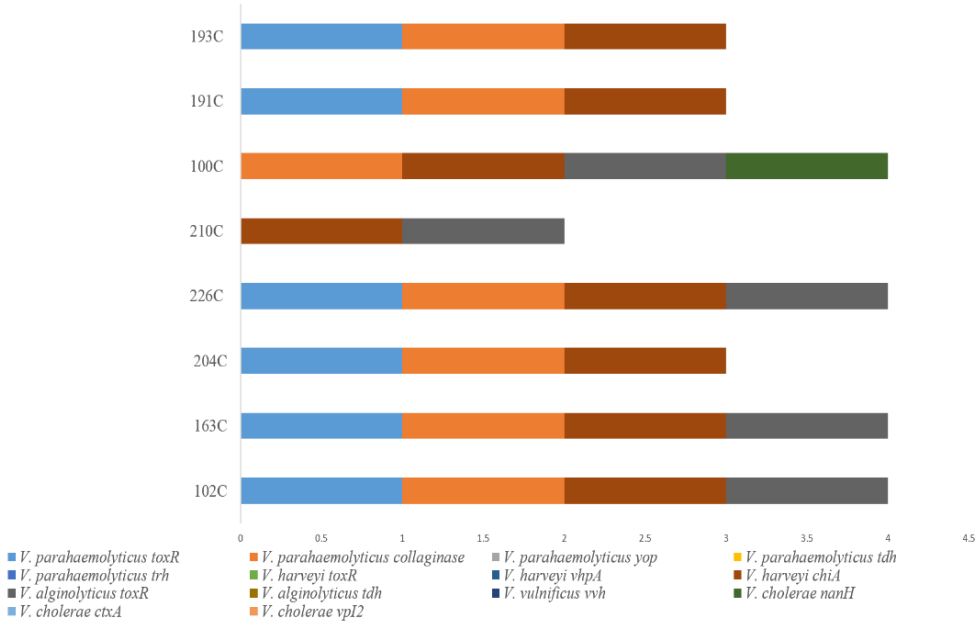

Fig.4.12. Prevalence of the virulence gene among vibrio isolates from *S. olivacea*

4.6. CHARACTERISATION OF ANTIBIOTIC RESISTANCE PATTERN

In-vitro antibiotic susceptibility of each isolate (23 isolates from *S. olivacea* and 18 Fish pathogens) to 21 antibiotics belonging to six different classes was analysed using recommended protocol (Bauer *et al*., 1966; CLSI, 2018). Percentage resistance and susceptibility of each bacterial species to each antibiotic is depicted in fig. 4.13. It was found that all the *V. parahaemolyticus* isolates from *S. olivacea* were resistant to penicillin and first generation cephalosporin (cephalothin), while all of them showed susceptibility to imipenem, nalidixic acid and chloramphenicol (Fig.4. 14). In case of *V. alginolyticus* from *S. olivacea*, 100 per cent resistance was observed against penicillin, methicillin, ampicillin and ciprofloxacin and 100 per cent susceptibility was observed against imipenem, nalidixic acid, chloramphenicol, tetracycline and doxycycline. Except one isolate, all were also susceptible to oxytetracycline and co- trimoxazole (Fig. 4. 15). In case of *V. diazotrophicus* from *S. olivacea*, 100 per cent resistance was observed against penicillin, methicillin, ampicillin, amoxyclav, ciprofloxacin, kanamycin, first and second generation cephalosporin (cephalothin and cefoxitin) and 100 per cent

susceptibility was observed against imipenem, meropenem, nalidixic acid, levofloxacin, chloramphenicol, streptomycin, tetracycline , oxytetracycline and doxycycline (Fig. 4. 16).

In the case of fish pathogens included in the study, resistance pattern to each antibiotic (in terms of individual wise and class wise) is depicted in fig. 4.17. It was found that 100 per cent of *V. parahaemolyticus* isolates were resistant to penicillin, methicillin and ampicillin, while all showed susceptibility to cefoperazone, imipenem, meropenem, nalidixic acid, chloramphenicol, streptomycin, doxycycline and co-trimoxazole (Fig. 4. 18). In case of *V. vulnificus*, 100per cent resistance was observed against penicillin, methicillin and cefoxitin and 100per cent susceptibility was observed against first and third generation cephalosporins (cephalothin and cefoperazone), carbapenems (imipenem and meropenem), first and second generation quinolones (nalidixic acid and ciprofloxacin), chloramphenicol, erythromycin, tetracycline, oxytetracycline, doxycycline and co-trimoxazole (Fig. 4. 19). In case of *V. harveyi*, 100 per cent resistance was observed against penicillin, methicillin, ampicillin and amoxyclav and 100 per cent susceptibility was observed against first and third generation cephalosporins (cephalothin and cefoperazone), carbapenems (imipenem and meropenem), first and third generation quinolones (nalidixic acid and levofloxacin), chloramphenicol, streptomycin, tetracycline, oxytetracycline, doxycycline and co-trimoxazole (Fig. 4. 20).

In short, 87.80 per cent isolates in the present study were found to be multidrug resistant (MDR) as they showed resistance to more than three classes of antibiotics. Further, multiple antibiotic resistance (MAR) index was calculated for all the isolates and results are depicted in table 4.7. Out of all isolates, 92.68 per cent showed a MAR index value more than 0.2. Species wise average MAR index is given in fig. 4.21. Among different species, only *V. alginolyticus* had an average MAR index less than 0.2. However, isolates of the present study were neither extensively drug resistant (XDR) nor Pandrug resistant (PDR).

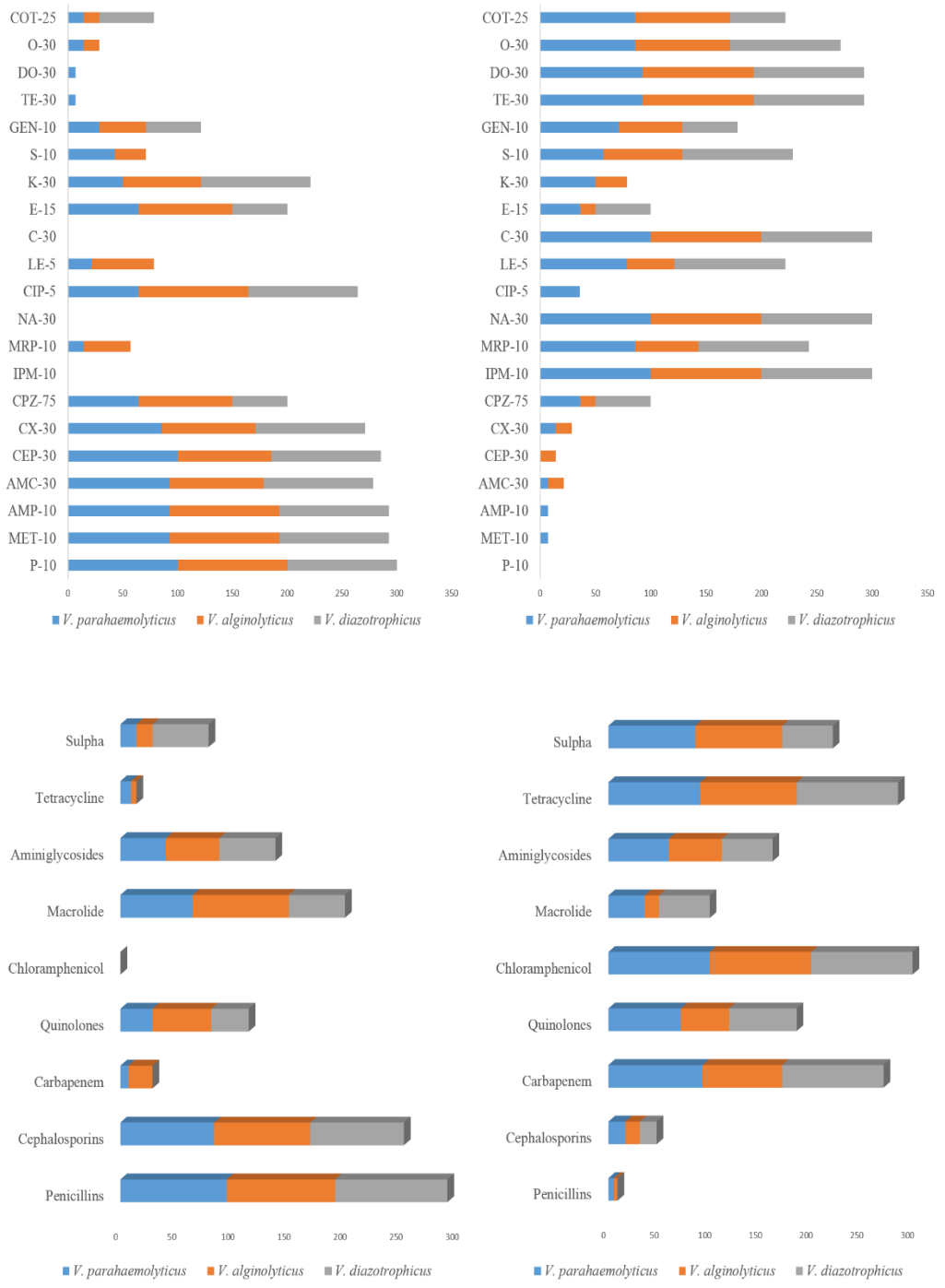

Fig. 4. 13 Percentage resistance and susceptibility of bacterial species to antibiotics used individually and classwise

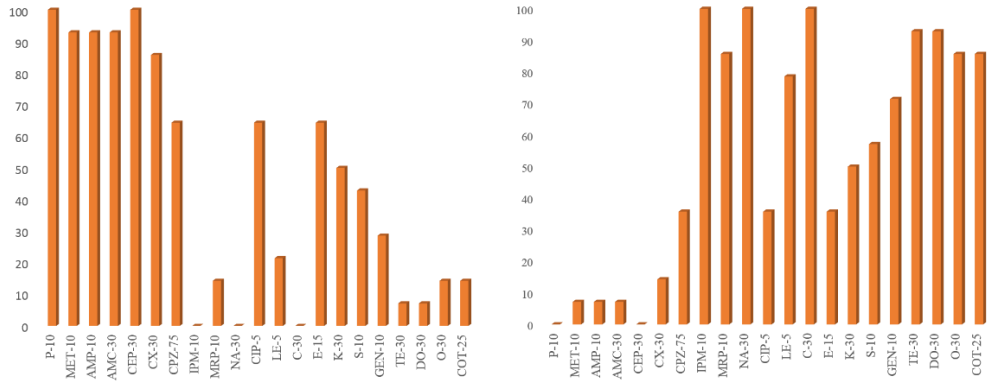

Fig. 4. 14 Percentage resistance and susceptibility of *V. parahaemolyticus* to antibiotics used

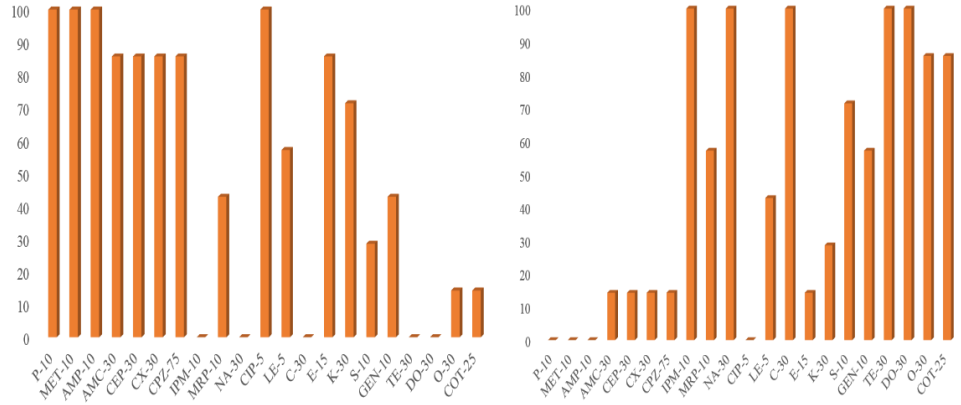

Fig. 4. 15 Percentage resistance and susceptibility of *V. alginolyticus* to antibiotics used

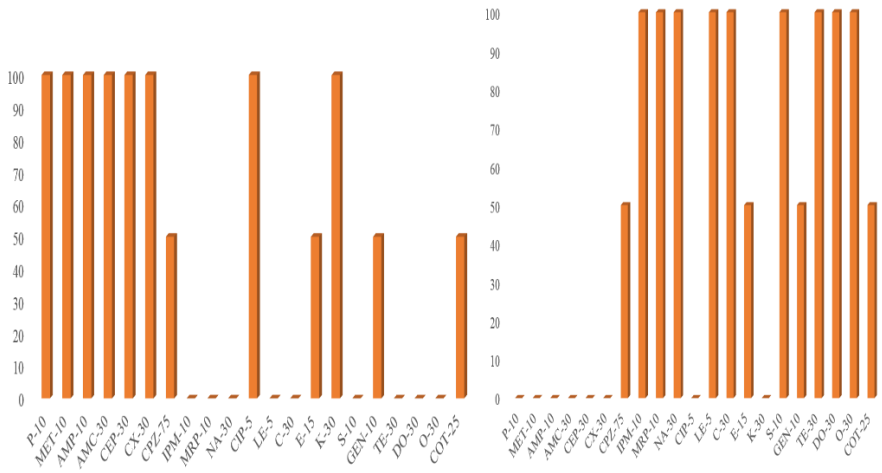

Fig. 4. 16 Percentage resistance and susceptibility of *V. diazotrophicus* to antibiotics used

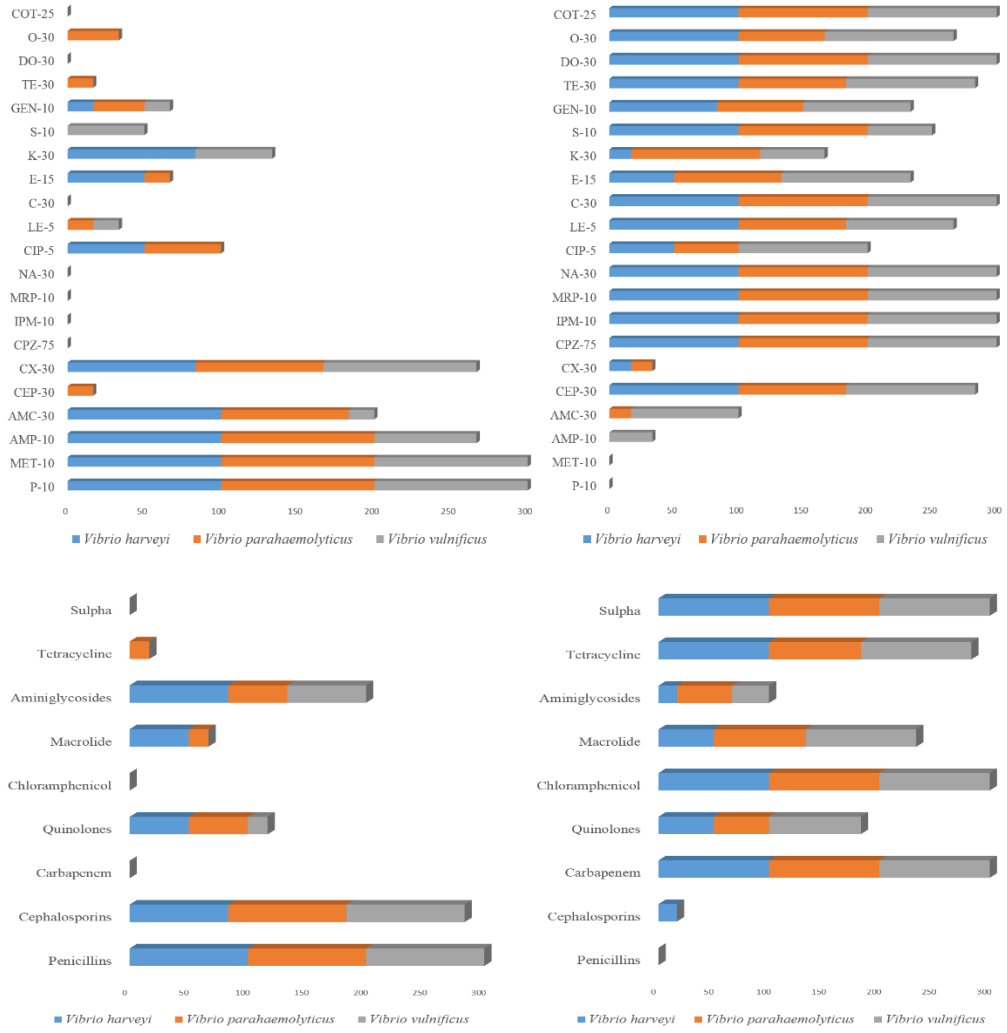

Fig. 4. 17. Percentage resistance and susceptibility of fish pathogens to antibiotics used individually and class wise

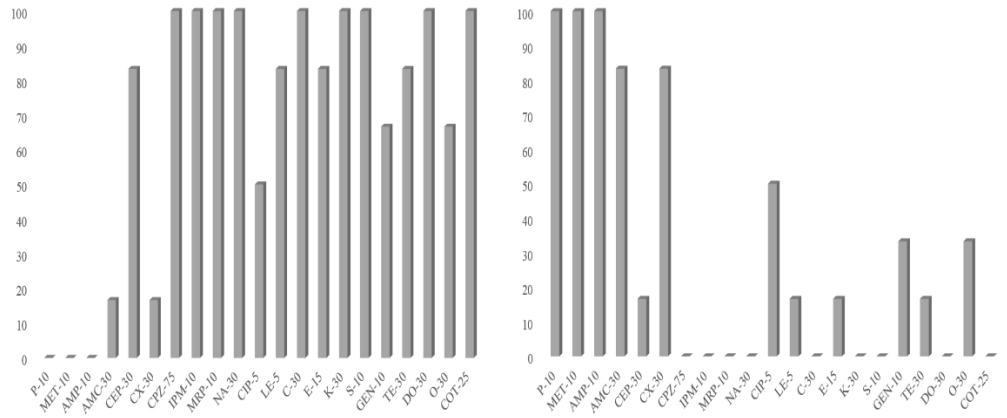

Fig. 4. 18 Percentage resistance and susceptibility of *V. parahaemolyticus* to antibiotics used

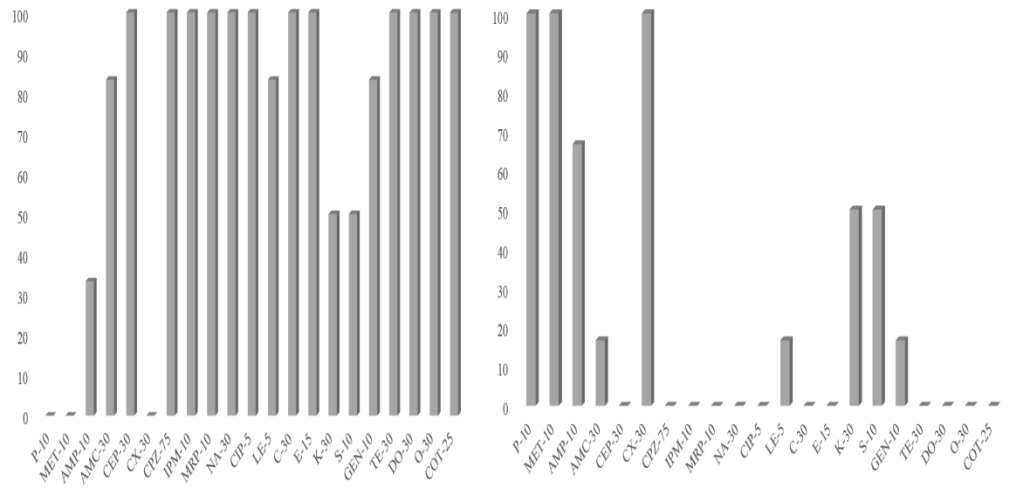

Fig. 4. 19 Percentage resistance and susceptibility of *V. vulnificus* to antibiotics used

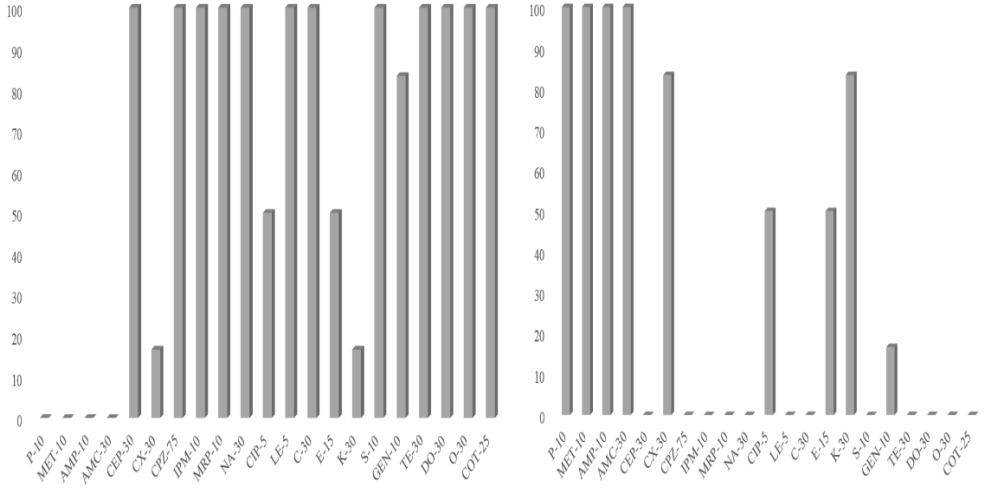

Fig. 4. 20 Percentage resistance and susceptibility of *V. harveyi* to antibiotics used

Table. 4. 7. MAR index of vibrio isolates

ID	Species	MAR index
102C	*V. parahaemolyticus*	0.380952381
163C	*V. parahaemolyticus*	0.476190476
204C	*V. parahaemolyticus*	0.523809524
190C	*V. parahaemolyticus*	0.619047619
205C	*V. parahaemolyticus*	0.523809524
17C	*V. parahaemolyticus*	0.428571429
14C	*V. parahaemolyticus*	0.142857143
92C	*V. parahaemolyticus*	0.523809524
212C	*V. parahaemolyticus*	0.619047619
53C	*V. parahaemolyticus*	0.285714286
206C	*V. parahaemolyticus*	0.476190476
213C	*V. parahaemolyticus*	0.476190476
87C	*V. parahaemolyticus*	0.571428571
160C	*V. parahaemolyticus*	0.333333333
226C	*V. diazotrophicus*	0.476190476
191C	*V. diazotrophicus*	0.476190476
210C	*V. alginolyticus*	0.571428571
100C	*V. alginolyticus*	0.523809524
193C	*V. alginolyticus*	0.666666667
209C	*V. alginolyticus*	0.761904762
109C	*V. alginolyticus*	0.428571429
200C	*V. alginolyticus*	0.285714286

3C	*V. alginolyticus*	0.428571429
2PLT	*V. harveyi*	0.238095
EK2	*V. harveyi*	0.285714
EBL1	*V. harveyi*	0.333333
SLTY	*V. harveyi*	0.428571
PL3	*V. harveyi*	0.333333
CBS4	*V. harveyi*	0.333333
OTK1	*V. parahaemolyticus*	0.571429
SBK4	*V. parahaemolyticus*	0.238095
SZK8	*V. parahaemolyticus*	0.238095
MZLB6	*V. parahaemolyticus*	0.238095
TBL11	*V. parahaemolyticus*	0.333333
SL6	*V. parahaemolyticus*	0.285714
TTL3	*V. vulnificus*	0.285714
TZB1	*V. vulnificus*	0.238095
MZLL4	*V. vulnificus*	0.285714
LK7	*V. vulnificus*	0.142857
ETB3	*V. vulnificus*	0.190476
PBB2	*V. vulnificus*	0.142857

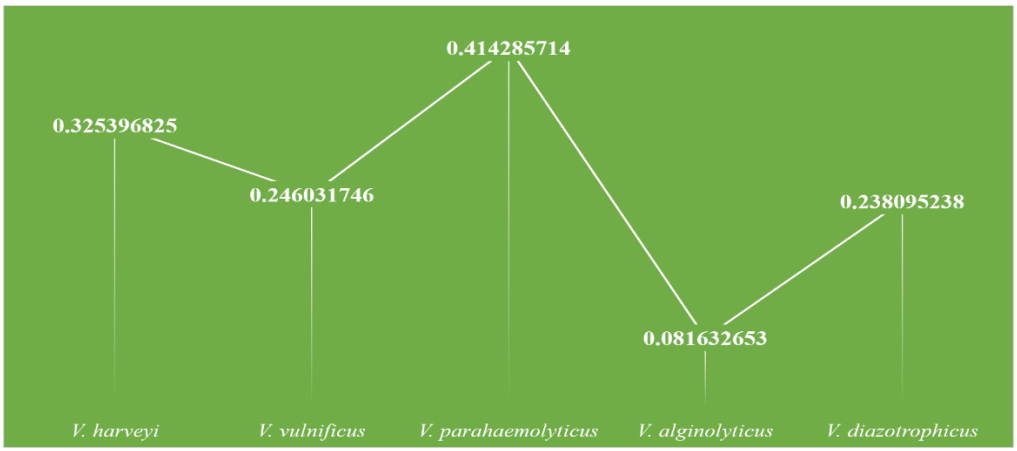

Fig. 4. 21 MAR index value of vibrio species studied

4.7. PCR AMPLIFICATION OF THE GENES RESPONSIBLE FOR ANTIBIOTIC RESISTANCE

A total of 18 genes responsible for resistance to different antibiotics (β-lactams, quinolones, chloramphenicol, erythromycin, kanamycin and tetracycline) were screened in each isolate using reported primers. Among these, two genes namely *tetS* and *aphA3* were found to be present in vibrio isolates from *S. olivaceae* and three genes namely *tetS, tetH* and *tetB* were present in fish pathogens. The amplicon size for these genes were found to be 667 bp (*tetS)*, 600 bp (*aphA3)*, 650 bp (*tetH)* and 960 bp (*tetB*), respectively. The specific amplification of these genes was confirmed by the sequencing of the amplicons (Table 4. 8).

It was noteworthy that even though there was amplification of *ermB*, *blaTEM*, *qnrS*, *catB3*, *qnrA*, *tetGY* and *tetAC* genes (750 bp, 1100 bp, 500 bp, 1000 bp, 300 bp, 500 bp and 417 bp respectively in size), NCBI-BLAST analysis of the sequence showed that amplicon size was not having any relation to corresponding AR gene (Table 4. 9) revealing the low specificity of primers used. Amplification profiles of AR genes among vibrio isolates from *S. olivacea* and fish pathogens are represented in fig. 4.22.

It was found that the prevalence of AR genes in vibrio isolates from *S. olivaceae* was in the order of *tetS* (33.3 per cent) > *aphA3* (8.33 per cent) (Fig.4.23).

In case of fish pathogens the order was *tetS* (19.05 per cent) > *tetH=tetB (*9.52 per cent) (Fig.4.24). Overall prevalence of AR genes among vibrio isolates of the present study was 2.77 per cent. An interesting observation was that resistance and their AR genes against chloramphenicol and quinolones were not found in any of the bacterial isolates tested. Thus, AR genes encoding resistance to tetracycline was the most prevalent one (7.05per cent) among vibrio isolates. Other than *tet* genes only kanamycin gene could be observed in this study. Another interesting observation in the present study was, *tetS* and *tetH* genes were present together in two fish pathogenic isolates.

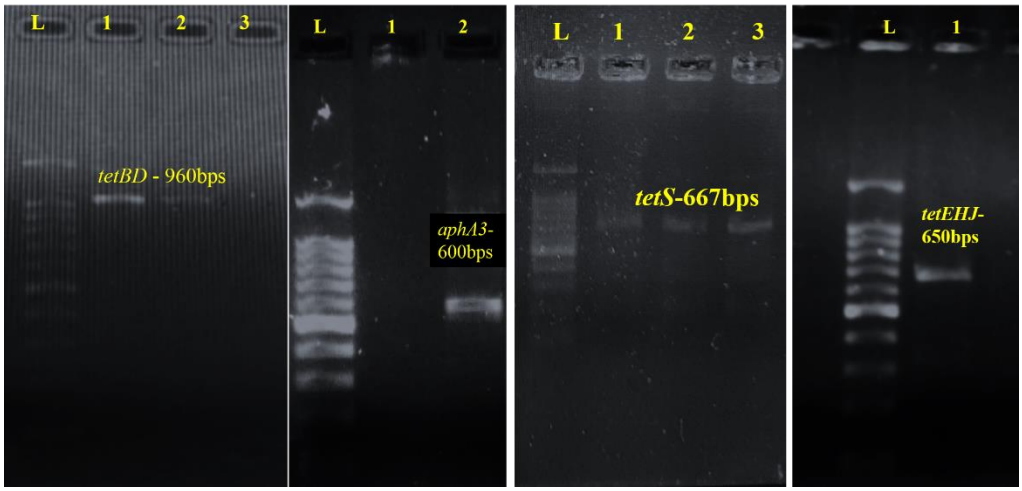

Fig. 4.22. Amplification profiles of AR genes among vibrio isolates from *S. olivacea* and fish pathogens

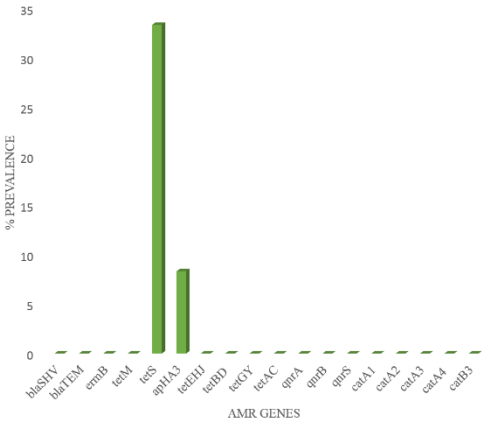

Fig. 4.23. Percentage prevalence of AR genes in vibrio isolates from *S. olivaceae*

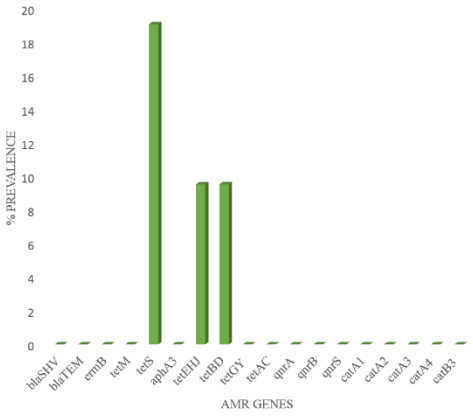

Fig. 4.24. Percentage prevalence of AR genes in fish pathogens

Table:4.8. Similarity of the specific amplicons to the closest GenBank relatives

Isolate	Species identified	Closest relative	Gene identified	Amplicon size	NCBI Accession number	Identity per cent
OTK1	*V. parahaemolyticus (tetEHJ)*	*Histophilus somni* strain UOC-KLM-ATR-014 ICEHsKLM-014 element mobile element, partial sequence	"tetracycline efflux MFS transporter Tet(H)"	614 bp	MN401320.1	99.33
TBL11	*V. parahaemolyticus (tetBD)*	*Escherichia coli* O157:H7 strain 2-6-2 chromosome, complete genome	"tetracycline efflux MFS transporter Tet(B)"	960 bp	CP038419.1	100

Table:4.9. Similarity of the non-specific amplicons to the closest GenBank relatives

Isolate	Species identified	Closest relative	Gene identified	Amplicon size	NCBI Accession number	Identity per cent
EK2	*Vibro harveyi (blaTTEM)*	*Vibrio owensii* strain V180403 chromosome 1, complete sequence	*"extracellular solute-binding protein"*	1100 bp	CP033144.1	96.70

ETB3	*Vibrio vulnificus (qnrS)*	*Vibrio parahaemolyticus* strain 20160303005-1 chromosome II, complete sequence	*"TetR/AcrR family transcriptional regulator"*	500 bp	CP034299.1	99.59
EK2	*Vibro harveyi (tetAC)*	*Vibrio owensii* strain XSBZ03 chromosome 2, complete sequence	"ribulose-phosphate 3-epimerase	417 bp	CP019960.1	96.45
TTL3	*Vibrio vulnificus (ermB)*	*Vibrio vulnificus* strain FDAARGOS_119 chromosome 2, complete sequence	"RNase E specificity factor CsrD"	750 bp	CP014049.2	97.59
2PLT	*Vibro harveyi (tetGY)*	*Vibrio harveyi* strain FDAARGOS_107 chromosome 1, complete sequence	"bifunctional heptose 7-phosphate kinase/heptose 1-phosphate adenyltransferase"	500 bp	CP014038.2	99.42
100C	*V. alginolyticus (qnrA)*	*Vibrio diabolicus* strain FA3 chromosome 2, complete sequence	"dicarboxylate/amino acid:cation symporter"	300 bp	CP042452.1	98.83
100C	*V. alginolyticus (catB3)*	*Vibrio alginolyticus* strain GS_MYPK1 chromosome 1, complete sequenc	"alkaline phosphatase D family protein"	1000 bp	CP054700.1	99.48

DISCUSSION

5. DISCUSSION

The present study was carried out to analyse the density and diversity of *Vibrionaceaee* in different tissues (haemolymph, gills, gut, muscles, pleura, and hepatopancreas) of *Scylla olivacea* (Herbst, 1896) targeting its final applications in disease management studies of this supreme aquaculture candidate. Further, prevalence of typical and non-typical virulence genes of zoonotically significant vibrios. The pattern of antimicrobial resistance in each isolate was also studied to find out the public health significance of *Vibrionaceae* associated with this crab species. Additionally, presence of different antimicrobial resistance genes in each isolate was also screened to depict the genetic basis of antimicrobial resistance (AMR) pattern in *Vibrionaceaee*. Eighteen bacterial strains belonging to three public health significant *Vibrio* species namely, *V. harveyi, V. parahaemolyticus* and *V. vulnificus* (six from each species), isolated from diseased fish were also included in the investigations related to AMR to correlate the findings of environmental isolates with the clinical isolates.

Several investigators have already shown the association of many *Vibrio* species with different tissues of blue crabs collected from temperate waters (Colwell *et al.,* 1975) and haemolymph of commercially significant marine crab species in Indian waters (Sumithra *et al*., 2019). However, studies describing the tissue specific pattern on density and diversity of *Vibrionaceae* in *S. olivacea* were not conducted till date, despite its supreme aquaculture potential. Present study showed that all tissues including haemolymph of *S. olivacea* carried a variable concentration of vibrios even in apparently healthy conditions with the greatest mean concentrations in gut (1.74 X 10^6 CFU/g), immediately followed by gill (1.60 X 10^6 CFU/g). Haemolymph had the lowest mean concentration of vibrios (1.77 X 10^2 CFU/mL) which was an expected result as it was the site where bacteria have to live under the pressure of an active immune system (Weiss and Aksoy, 2011). It was generally assumed earlier, that the circulatory system of healthy animals is sterile, whereas the presence of bacteria was usually considered to be a sign of disease (Lightner and Lewis, 1975). In contrast, it is presently analysed as a natural

phenomenon and haemolymph of many crustacean species including crabs are now known to carry microbes even in apparently healthy conditions (Gomez-Gil *et al*., 1998; Sumithra *et al*., 2019), in parallel to the observations of the present study. The prevalence of presumptive vibrios was in the order of Gut > Gill > Hepatopancreas > Muscle > Pleura > Haemolymph. Analogous to our observation, Faghri *et al*. (1984) had pointed out that the greatest concentration of vibrios occurred in the gut and gill tissues of the crabs. The higher count observed in gills might be attributed to the close proximity of the gills to aquatic environment which will allow harbouring of enormous bacterial load (Zhang *et al*., 2016). An important question in crab microbiology is that whether the symbiotic bacteria related to different tissues could contaminate the muscle tissues, the edible portion of the crab. Direct scanning electron microscopic observations and the viable enumeration procedures indicated that most bacteria of healthy crabs are associated with gills, gut and shell (Faghri *et al*., 1984). The same authors indicated that extensive cellular defense system of rock crabs limits the bacterial contamination of muscle tissue and any impairment of these defence system renders the muscle susceptible to contamination. Non-sterile nature of crab muscle in apparently healthy conditions was already reported (Faghri *et al*., 1984). Density of vibrios in muscle tissue was comparatively lesser than that of gills/gut/hepatopancreas in the present study. However, the load was higher than that was reported in muscle of edible crabs collected from cold water (Faghri *et al*., 1984). Several previous studies have suggested a strong influence of temperature on the concentration of vibrios in marine waters and animals. Therefore, warmer water temperatures and difference in species under study might be responsible for such a high amount of vibrios. Thus, the results of this study suggest the need to improve strategies to prevent the occurrence of vibrio transmitted diseases by consumption of shellfish/ crab.

Morphologically unique colonies from each dilution plate were then purified and characterised up to the species level. There was a total of 23 morphologically different isolates suggestive of *Vibrio* sp. After preliminary characterisation by morphological, cultural and biochemical methods, the isolates having similar morphological, cultural and biochemical characteristics were

clubbed together to one group and one representative isolate from each group was then characterised by *16S rRNA* gene sequencing. Based on the results of NCBI-BLAST analysis, further biochemical tests were conducted to identify the species (Bergey *et al*., 2012). For species confirmation, specific primers were also used at the last step. All together, three distinct species could be identified in the genus *Vibrio*. Overall, *V. parahaemolyticus* (56 per cent) was the most abundant species which was followed by *V. alginolyticus* (28 per cent). *Vibrio parahaemolyticus* was the most prevalent bacterium that was present in all tissues. Occurrence of *V. parahemolyticus* in different species of crabs have already been reported by different authors (Lavilla and Peña, 2004; Najiah *et al*., 2010; Wang, 2011). In parallel to our observation on *V. parahaemolyticus* in *S. olivacea*, *Vibrio* species especially *V. parahemolyticus*, *V. cholerae* and *V. vulnificus* were reported as the predominant bacteria isolated from haemolymph and external carapace of blue crab, *Callinectes sapidus* (Krantz *et al*., 1969; Tubiash and Krantz, 1970; Sizemore *et al*., 1975; Davis and Sizemore, 1982; Huq *et al*., 1986). *Vibrio alginolyticus* was the second most prevalent bacterium which was present in all the tissues except haemolymph. Occurrence of *V. alginolyticus* has also been reported in different apparently healthy crab species (Najiah *et al*., 2010, Wang, 2011). Among mud crabs, microbiology of wild *S. serrata* caught from the Chakoria coast of Bangladesh was studied. The isolated species included *V. alginolyticus, V. cholerae, V. harveyi, V. fluvialis, V. parahaemolyticus* and *V. mimicus* with *V. harveyi* as the most dominant one (Aftabuddin *et al*., 2013). However, Najiah *et al*. (2010) observed *V. parahaemolyticus* followed by *V. alginolyticus* as the predominant *Vibrio* sp. in wild *S. serrata* caught from Setiu Wetland, Malaysia similar to the observation of the present study. Comparison of the present results with microbiology of *S. olivacea* of previous reports could not be done due to the lack of studies in same species. In the present study, gut tissue was found to have maximum diversity of vibrio, containing all three species identified (*V. parahaemolyticus, V. alginolyticus* and *V. diazotrophicus*). During phylogenetic analysis, it was found that *S. olivacea* contained vibrios belonging to two different clades namely, Harveyi clade (*V. parahaemolyticus* and *V. alginolyticus*) and

Diazotrophicus clade (*V. diazotrophicus*). Harveyi clade was the most dominant one. It had been reported that the species composition of vibrios changes as the seawater temperature changes (Miguez and Combarro, 2003). Members of the Harveyi clade usually predominate, when the temperatures is above 20^0C and those from the Splendidus clade when it is below (Maeda *et al*. 2003). As the crabs are poikilothermic animals and their body temperature is similar to that of the surrounding water, temperature above 20^0C might be the reason for the predominance of members of the Harveyi clade in the present study.

Comparative genome analysis in various vibrios has revealed different genomic events like mutation, deletion, gene acquisition through duplication or horizontal transfer and chromosomal rearrangements *etc*. can be the driving forces in evolution and speciation of vibrios (Makino *et al*. 2003). Of these, horizontal gene transfer is an efficient mechanism for introducing new phenotypes into bacterial genome especially in the distribution of virulence genes (Gogarten *et al*. 2002). The distribution/ prevalence of various virulence genes in vibrio isolates rather than mere prevalence of each bacterial species from an apparently healthy animal can give some idea on the significance of these isolates in both animal health as well as public health perspectives (Bai *et al*. 2008). Accordingly, presence of different virulence genes that have been described in different pathogenic vibrios was screened by PCR for the isolates in the present study as the next step. For this, a total of 14 virulence genes of various vibrios (*toxR, vhpA* and *chiA* of *V. harveyi*, *yopP, tdh, toxR, collagense* and *trh* of *V. parahaemolyticus*, *ctxA, nanH* and *VPI2* of *V. cholerae*, *toxR* and *tdh* genes of *V. alginolyticus* and *vvh* of *V. vulnificus*) were targeted.

Among the 14 target genes, five genes namely, *chiAVh* of *V. harveyi*, *collagenase* of *V. parahaemolyticus*, *toxR* of *V. parahaemolyticus*, *toxR* of *V. alginolyticus* and *nanH* of *V. cholerae* were found to be present in the isolates. The amplicon size for these genes were consistent in all the isolates irrespective of the species. All amplicons were sequenced at Agrigenome, India for the confirmation of specific amplification. It was found that prevalence was in the order of *chiAVh*

of *V. harveyi* (29.63 per cent) > *collagenase* of *V. parahaemolyticus* (25.93 per cent) > *toxR* of *V. parahaemolyticus* (22.22 per cent) > *toxR* of *V. alginolyticus* (18.52 per cent)> *nanH* of *V. cholerae* (3.70 per cent). It is intresting to note that even though *V. parahemolyticus* was the most prevalent species among healthy *S. olivaceae*, genes encoding three important virulence factors seen among clinical isolates of *V. parahemolyticus* namely, thermostable direct haemolysin (TDH), TDH-related haemolysin (TRH) and putative type III secretion effector YopP protein were absent in all the present isolates. At the same time, the genes known to code another two virulence determinants namely, *collagenase* and *toxR* in *V. parahemolyticus* were present in 100 per cent of *V. parahaemolyticus*, 50 per cent of *V. alginolyticus* and 100 per cent of *V. diazotrophicus* isolates tested. Among the three virulence genes of *V. harveyi* screened, two (*toxR* and *vhpAVh*) were absent in all the isolates, while *chitinase* gene of *V. harveyi* was present in all vibrio isolates in the present study. Presence of *chiA* gene of *V. harveyi* in other vibrio species of Harveyi clade was reported by Ruwandeepika *et al*. (2010), while presence in Diazotrophicus clade has not been reported till date. Of the three virulence-associated factors in *V. cholerae* screened, only *nanH* encoding neuraminidase was present in the isolates (in one *V. alginolyticus*). In parallel, incidence of *nanH* gene of *V. cholerae* in environmental isolates of *V. alginolyticus* has been reported by Gennari *et al*. (2012b). Galen *et al*. (1992) demonstrated that *nanH* had a modest effect of on cholera toxin function *in-vitro*, while the true role of *nanH* on virulence remains unclear. Future experiments are needed to test the significance of *V. cholerae nanH* gene in other vibrio species. *vvh* gene of *V. vulnificus* encoding haemolysin was absent in all vibrio isolates from *S. olivacea*. Among the virulencce genes of *V. alginolyticus* screened, only *toxR* gene was present in the vibrio isolates (62.5 per cent). *toxR*, one of the primary virulence factors in *V. alginolyticus* (Ravikumar and Vijayakumar, 2017) was found to present in isolates belonging to all vibrio species from *S. olivacea* even though significance of the same remains to be elucidated by future research.

As the next step, i*n-vitro* antibiotic susceptibility of each isolate from *S. olivacea* to 21 antibiotics belonging to six different classes was analysed using

recommended protocol (Bauer *et al*., 1966; CLSI, 2018). Simultaneously, 18 bacterial strains belonging to three public health significant *Vibrio* species namely, *V. harveyi, V. parahaemolyticus* and *V. vulnificus* (6 from each species), isolated from diseased fish were also included to correlate the findings of environmental isolates with clinical isolates. All antibiotics tested in this study namely tetracycline (tetracycline, oxytetracycline and doxycycline), folate pathway inhibitors (trimethoprim-sulfamethoxazole), first, second and third-generation cephalosporins, macrolide group (erythromycin), penicillin group (methicillin, ampicillin and amoxyclav), carbapenems (imipenem and meropenem), aminoglycosides (gentamicin, kanamycin and streptomycin), sulpha drug (co-trimoxazole) and fluoroquinolones (ciprofloxacin, nalidixic acid and levofloxacin), are recommended antobiotics by CDC for the treatment of *Vibrio* sp. infections (Daniels and Shafaie, 2000). It was found that 100 per cent of *V. parahaemolyticus* isolates of the present study (both fish pathogenic isolates and isolates from *S. olivacea*) were susceptible to imipenem, nalidixic acid and chloramphenicol. Results of the previous studies also indicated high susceptibility of *V. parahaemolyticus* against chloramphenicol, nalidixic acid and imipenem (Ottaviani *et al*., 2001; Lee *et al*., 2018). In the case of *V. alginolyticus* from *S. olivacea*, 100 per cent resistance was observed against penicillin, methicillin, ampicillin and ciprofloxacin. High incidence of resistance against penicillin group of antibiotics among *V. alginolyticus* have been reported by various authors (Drais *et al*., 2018). In case of *V. vulnificus*, 100 per cent resistance was observed against penicillin, methicillin and cefoxitin while 100 per cent susceptibility was observed against first and third generation cephalosporins (cephalothin and cefoperazone), carbapenems (imipenem and meropenem), first and second generation quinolones (nalidixic acid and ciprofloxacin), chloramphenicol, erythromycin, tetracycline, oxytetracycline, doxycycline and co-trimoxazole. In a review on AMR pattern of *V. vulnificus*, almost similar pattern of antibiotic resistance among *V. vulnificus* isoaltes were reported worldwide including India (Elmahdi *et al*., 2016). In case of *V. harveyi*, 100 per cent resistance was observed against penicillin, methicillin, ampicillin and amoxyclav and 100 per cent susceptibility was observed against first and third

generation cephalosporins, carbapenems, first and third generation quinolones, chloramphenicol, streptomycin, co-trimoxazole and tetracyclines. High resistance of *V. harveyi* against penicillin groups was reported by previous authors (Rattanama *et al*., 2009). In short, results of the present study placed imipenem, chloramphenicol, and nalidixic acid at the top of scope to resist vibrio infections with 100 per cent susceptibility.

The acquired non-susceptibility to at least one agent in ≥ three antimicrobial categories was defiend as MDR (Multidrug resistant) phenotype. Non-susceptibility to at least one agent in all but two or fewer antimicrobial categories was defined as XDR (Extensively drug resistant) phenotype (i.e., bacterial isolates remain susceptible to only one or two antimicrobial categories). Non-susceptibility to all agents in all antimicrobial categories was defined as PDR (Pandrug resistant) phenotype (Magiorakos *et al*., 2012). As per this criteria 87.80 per cent isolates in the present study were found to be multidrug resistant (MDR). However, isolates of the present study were neither extensively drug resistant (XDR) nor pan-drug resistant (PDR).

Multiple antibiotic resistance (MAR) index is considered as a good cost-effective tool for risk assessment in terms of antibiotic resistance (Paul *et al*., 1997). Multiple antibiotic resistance index is determined as the ratio of number of antibiotics to which organism is resistant to the total number of antibiotics to which organism is exposed (Paul *et al*., 1997; Sandhu *et al*., 2016). Multiple antibiotic resistance index value greater than 0.2 indicate organisms originating from high risk sources of contamination, where antibiotics were frequently used (Osundiya *et al*., 2013). In the study, 92.68per cent isolates showed a MAR index value more than 0.2. Among different species, only *V. alginolyticus* had an average MAR index less than 0.2. Various *Vibrio* spp. from different sources having MAR index > 0.2 have been globally reported (Kurdi Al-Dulaimi and Ariffin, 2019; Mohamad *et al*., 2019).

For depicting the genetic basis of antibiotic resistance among isolates of vibrio, a total of 18 genes responsible for resistance to various antibiotics (β-

lactams, chloramphenicol, quinolones, erythromycin, kanamycin and tetracycline) were screened using reported primers. Among these, two genes namely *tetS* and *aphA3* were found to be present in vibrio isolates from *S. olivaceae* and three genes namely *tetS, tetH* and *tetB* were present in fish pathogens. It was found that prevalence of AR genes in isolates of vibrio from *S. olivaceae* was in the order of *tetS* (33.3 per cent) > *aphA3* (8.33 per cent). In case of fish pathogens, the order was *tetS* (19.05 per cent) > *tetH=tetB (*9.52 per cent) Over all prevalence of AR genes among vibrio isolates of the present study was 2.77 per cent. The genes encoding resistance to tetracycline was the most prevalent (7.05 per cent) among vibrio isolates. Currently, there are thirty eight different *tet* and *otr* genes described among different bacteria which include twenty three genes coding for energy dependent efflux proteins, eleven genes coding for ribosomal protection proteins, three genes coding for inactivating enzyme and one gene with an unknown mechanism of resistance (Roberts, 2005). In this study, *tet* genes encoding both efflux pumps (*tetB*) and *tetH*) and ribosomal protection proteins (*tetS*) were present. In parallel to the observations, *tet* genes namely *tetA, tetB* and *tetD* genes encoding active efflux pumps have been identified previously in *Vibrio* spp. from mariculture environment by Dang *et al*. (2006, 2007). However, this study documents the initial report on occurrence of *tetH* gene among vibrios. Similarly, even though *tetM* was reported in vibrio isolates from fish intestine and seawater at aquaculture sites (Kim *et al*., 2004), occurrence of *tetS* among vibrios has not been reported. During characterisation of antibiotic resistance in *Vibrio* spp. isolated from farmed marine shrimps of Thailand, Kitiyodom *et al*. (2010) showed that none of the vibrio isolates were positive to all the analysed tetracycline genes namely *tetK, tetL, tetM, tetO* and *tetS*. In a study evaluating tetracycline resistance gene diversity among bacteria obtained from various aquaculture sources in Australia, *tetM* was the most prevalent determinant, followed by *tetE, tetA* and *tetD* (Akinbowale *et al*., 2007). Andersen and Sandaa (1994) stated that *tetE* was the most widely spread determinant in isolates of bacterial species sampled from non- polluted and polluted marine sediments of Norway and Denmark. Another interesting observation in the present study was, *tetS* and *tetH* genes were present together in two fish pathogenic

isolates. Jacobs and Chenia (2007) also identified the presence of multiple *tet* resistance genes in single bacterial isolate. The combination of *tet* genes reported earlier included *tetM/tetD, tetA/tetE* together with *tetB* and *tetD/tetH* (Akinbowale and Barton, 2007; Jacobs and Chenia (2007). However, combination of *tet* genes observed in the present study (*tetS/tetH*) has not been reported earlier.

In conclusion, the present study described the tissue specific pattern on density and diversity of *Vibrionaceae* in *S. olivacea*. Results showed that all the tissues including haemolymph of *S. olivacea* carried a variable concentration of vibrios even in apparently healthy conditions with the greatest mean concentrations and diversity in gut. During phylogenetic analysis, it was found that *S. olivacea* contained vibrios belonging to two different clades namely, Harveyi clade and Diazotrophicus clade with Harveyi clade as the dominant one. Among the 14 target virulence genes screened, five genes either from *V. harveyi*, *V. parahaemolyticus*, *V. alginolyticus* or *V. cholerae* were found to be present in the isolates. However, genes encoding three important virulence factors seen among clinical isolates of *V. parahemolyticus* were absent in all the isolates. Results on AR pattern placed imipenem, chloramphenicol, and nalidixic acid at the top of scope to resist vibrio infections with 100 per cent susceptibility. However, 87.8 per cent isolates in the present study were multidrug resistant (MDR), of which 92.7 per cent isolates had a MAR index value > 0.2. Nevertheless, isolates of the present study were neither extensively drug resistant (XDR) nor pan-drug resistant (PDR). In order to depict the genetic basis of antibiotic resistance among vibrio isolates, a total of 18 genes responsible for resistance to different antibiotics were screened. Overall prevalence of AR genes among vibrio isolates was 2.77 per cent. However, only AR genes encoding resistance to tetracycline and aminoglycosides could be detected. Among various *tet* genes, those encoding both efflux pumps (*tetB*) and (*tetH*) and ribosomal protection proteins (*tetS*) were present. The study reported the occurrence of *tetH* and *tetS* among vibrio isolates for the first time. In conclusion, the present study forms the first multi-tissue description on density and diversity of *Vibrionaceaee* of a supreme aquaculture candidate crab species, fetching applications in disease management studies during their aquaculture practices. Further, the generated

ABST pattern, profiles of virulence and AMR genes of vibrio isolates will have additional applications in public health perspectives.

SUMMARY

6. SUMMARY

A study was conducted to analyse the density and diversity of *Vibrionaceaee* in different tissues (haemolymph, gills, gut, muscles, pleura and hepatopancreas) of a supreme aquaculture candidate species of crab namely, *Scylla olivacea*, targeting the final applications in disease management. Further, prevalence of various virulence genes of public health significant vibrios and pattern of antimicrobial resistance in each isolate were investigated to realise the public health significance of *Vibrionaceae* associated with this crab species. Additionally, an attempt was made to depict the genetic basis of antimicrobial resistance (AMR) in *Vibrionaceae.* Eighteen bacterial strains belonging to three public health significant species of *Vibrio* namely, *V. harveyi, V. parahaemolyticus* and *V. vulnificus* (six from each species), isolated from diseased fish were also included in the investigations related to AMR to correlate the findings of environmental isolates with clinical isolates. The major findings of these investigations are summarised as follows.

- All the tissues including haemolymph of *S. olivacea* carried a variable concentration of vibrios even in apparently healthy conditions.
- Order of density of presumptive vibrios was Gut > Gill > Hepatopancreas > Muscle > Pleura > Haemolymph.
- During diversity analysis, three distinct species could be identified in *Vibrio* spp.
- *V. parahaemolyticus* was the most prevalent bacterium that was present in all the tissues.
- *V. alginolyticus* was the second most prevalent bacterium which was present in all tissues except haemolymph.
- Gut was the tissue, where diversity of vibrio was maximum containing all the identified three species.
- *S. olivacea* contained vibrios belonging to two different clades namely, Harveyi clade and Diazotrophicus clade, of which Harveyi clade was the most dominant one.

- Among the 14 virulence genes targeted, five namely, *chiAVh* of *V. harveyi*, *collagenase* of *V. parahaemolyticus*, *toxR* of *V. parahaemolyticus*, *toxR* of *V. alginolyticus* and *nanH* of *V. cholerae* were found to be exisiting in the isolates.
- Prevalence of virulence genes was in the order of *chiAVh* of *V. harveyi* (29.63 per cent) > *collagenase* of *V. parahaemolyticus* (25.93 per cent) > *toxR* of *V. parahaemolyticus* (22.22 per cent) > *toxR* of *V. alginolyticus* (18.52 per cent)> *nanH* of *V. cholerae* (3.70 per cent).
- Genes encoding three important virulence factors seen among clinical isolates of *V. parahemolyticus* were absent in the isolates.
- *nanH* encoding neuraminidase (a virulence-associated factor in *V. cholerae*) was present in *V. alginolyticus*.
- *toxR*, one of the primary virulence factors in *V. alginolyticus* was found to be present in isolates belonging to all *Vibrio* spp. from *S. olivacea.*
- All *Vibrio* spp. isolates in the present study (both fish pathogenic isolates and those from *S. olivacea*) were susceptible to imipenem, nalidixic acid and chloramphenicol.
- All *V. parahaemolyticus* isolates from *S. olivacea* were resistant to penicillin and first-generation cephalosporin (cephalothin).
- *V. alginolyticus* from *S. olivacea* showed 100 per cent resistance against penicillin, methicillin, ampicillin and ciprofloxacin.
- All fish pathogenic *V. parahaemolyticus* isolates were resistant to penicillin, methicillin and ampicillin.
- *V. vulnificus* showed 100 per cent resistance against penicillin, methicillin and cefoxitin.
- *V. harveyi* revealed 100 per cent resistance against penicillin, methicillin, ampicillin and amoxyclav.
- Most of the isolates (87.80 per cent) in the present study were multidrug resistant (MDR). However, isolates of the present study were neither extensively drug resistant (XDR) nor pan-drug resistant (PDR).

- Out of all isolates 92.68 per cent showed a MAR index value more than 0.2. Among different species, only *V. alginolyticus* had an average MAR index less than 0.2.
- Among 18 antibiotics resistance (AR) genes screened, two genes namely *tetS* and *aphA3* were present in vibrio isolates from *S. olivaceae* and three genes namely *tetS*, *tetH* and *tetB* were present in fish pathogens.
- Pervasiveness of AR genes in vibrio isolates from *S. olivaceae* was in the order of *tetS* > *aphA3,* while in case of fish pathogens the order was *tetS* > *tetH=tetB*
- Overall prevalence of AR genes was only 2.77 per cent.
- Among the isolates of present study, *tet* genes encoding both efflux pumps (*tetB*) and *tetH*) and ribosomal protection proteins (*tetS*) were present.
- Another interesting observation in the present study was *tetS* and *tetH* genes were present together in two fish pathogenic isolates.
- The study presents the initial report on occurrence of *tetH* and *tetS* among vibrios.

In brief, the present study reports the first multi-tissue description on density and diversity of *Vibrionaceaee* of a supreme aquaculture candidate crab species, fetching applications in disease management during aquaculture practices. Further, the generated ABST pattern, profiles of virulence and AMR genes of vibrio isolates will have additional applications in public health perspectives.

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7. REFERENCES

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ANNEXURES

ANNEXURE- I

COMPOSITION OF MEDIA USED FOR BACTERIOLOGICAL PURPOSES

1. Zobell Marine Agar

Ingredients	**g L^{-1}**
Peptic digest of animal tissue	5.000
Yeast extract	1.000
Ferric citrate	0.100
Sodium chloride	19.450
Magnesium chloride	8.800
Calcium chloride	1.800
Potassium chloride	0.550
Sodium bicarbonate	0.160
Sodium sulphate	3.240
Potassium bromide	0.080
Strontium chloride	0.034
Boric acid	0.022
Sodium silicate	0.004
Sodium fluorate	0.0024
Ammonium nitrate	0.0016
Disodium phosphate	0.008
Agar	15.000

2. Mueller Hinton Agar

Ingredients	**g L^{-1}**
Meat, infusion solids from 300g	
Casein acid hydrolysate	17.500
Starch	1.500
Agar	17.000
Buffered peptone	7.000
Dextrose	5.000
Dipotassium phosphate	5.000
Final pH (at 25°C)	6.9 ± 0.2
Final pH (at 25°C)	6.9 ± 0.2

3. TCBS Agar

Ingredients	**g L^{-1}**
Proteose peptone	10.00
Yeast extract	5.00
Sodium thiosulphate	10.00
Sodium citrate	10.00
Oxgall	10.00
Sucrose	20.00
Sodium chloride	10.00
Ferric citrate	1.00
Bromo thymol blue	0.040
Thymol blue	0.040
Agar	15.00
Final pH (at 25°C)	8.6 ± 0.2

4. Nutrient Agar

Ingredients	**g L^{-1}**
Beef extract	10.0
Peptone	10.0
Sodium chloride	5.0
Agar	20.0
Distilled water	1000
Final pH	7.2 ± 0.2

5. LB (Luria-Bertani) broth

Ingredients	**g L^{-1}**
Bacto-tryptone	10 g
Bacto Yeast extract	5 g
NaCl	10 g
Deionized Water	

6. Trypton broth for Indole

Ingredients	**g L^{-1}**
Casein enzyme hydrolysate	10.000
Sodium chloride	5.000
Final pH (at 25°C)	7.5 ± 0.2

7. MR-VP Medium (Glucose Phosphate Broth)

Ingredients	**g L^{-1}**
Buffered Peptone	7.000
Dextrose	5000
Dipotassium phosphate	5.000
Final pH (at 25°C)	6.9 ± 0.2

8. Simmon's Citrate Agar

Ingredients	**g L^{-1}**
Yeast extract	0.500
L-Cysteine hydrochloride	0.100
Sodium citrate	3.000
Dextrose	0.200
Monopotassium phosphate	1.000
Sodium chloride	5.000
Phenol red	0.012
Agar	15.000
Final pH (at 25°C)	6.9 ± 0.2

9. Sugar Fermentation test

Ingredients	**g L^{-1}**
Proteose peptone	10.000
Sodium chloride	5.000
Meat extract	1.000
Bromocresol purple	0.100
Final pH (at 25°C)	6.8 ± 0.2

10. Arginine Dihydrolase Broth

Ingredients	**g L^{-1}**
Peptic digest of animal tissue	1.000
Sodium chloride	5.000
Dipotassium hydrogen phosphate	0.300
L-Arginine	10.000
Bromo cresol purple	0.016
Agar	3.000
Final pH (at 25°C)	6.0 ± 0.2

11. Lysine Decarboxylase Broth

Ingredients	**g L^{-1}**
Peptic digest of animal tissue	5.000
Yeast extract	3.000
Dextrose	1.000
L-Lysine hydrochloride	5.000
Bromocresol purple	0.020
Final pH (at 25°C)	6.8 ± 0.2

12. Ornithine Decarboxylase Broth

Ingredients	**g L^{-1}**
L-Ornithine monohydrochloride	5.000
Yeast extract	3.000
Glucose	1.000
Bromo cresol purple	0.015
Final pH (at 25°C)	6.8 ± 0.2

13. Gelatin Agar

Ingredients	**g L^{-1}**
Gelatin	30.000
Casein enzymic hydrolysate	10.000
Sodium chloride	10.000
Agar	15.000
Final pH (at 25°C)	7.2 ± 0.2

14. Urea Agar Base (Christensen)

Ingredients	**g L^{-1}**
Peptic digest of animal tissue	1.000
Dextrose	1.000
Sodium chloride	5.000

Disodium phosphate	12.000
Monopotassium phosphate	0.800
Phenol red	0.012
Agar	15.000
Final pH (at 25°C)	6.8 ± 0.2

15. Normal Saline Solution (NSS)

Ingredients	**g L^{-1}**
Sodium Chloride (NaCl)	8.5
Distilled Water	1000

It was autoclaved at 15 lb pressurefor 20 min.

16. Buffered Peptone Water

Ingredients	**g L^{-1}**
Protease peptone	10.0
Sodium chloride	5.0
Di-sodium phosphate	3.50
Mono-potassium phosphate	1.50
Distilled water	1000
Final pH	7.2 ± 0.2

ANNEXURE- II

COMPOSITION OF REAGENTS USED FOR AGAROSE GEL ELECTROPHORESIS

1. TBE electrophoresis buffer (10x)

Tris base	121.1g
Boric acid	61.1g
EDTA	7.4g
Triple distilled water	1000 mL

2. Ethidium bromide stock solution (10 mg/mL)

Ethidium bromide	100mg
Distilled water	10mL

The solution was mixed and stored at 4ºC. A concentration of 0.5-1 μg/ml was used in preparing agarose gel.

3. **TE buffer**

Tris-HCl (pH 8)	10 m*M*
EDTA (pH 8)	1 m*M*

Sterilized solution by autoclaving

4. 6X gel loading dye

Bromophenol blue	2.5 mg
Sucrose	4 g

Dissolved in 6 ml of TE buffer. 5X TBE was added to make the final volume up to 10 ml.

ANNEXURE III

REAGENTS FOR GENOMIC DNA ISOLATION

1. TE buffer

Tris-Cl (pH 8)	10 m*M*
EDTA (pH 8)	1 m*M*

Sterilize solution by autoclave.

2. 1 *M* Tris-Cl

Tris base	121.1 g
Triple distilled water add upto	1000 mL

Adjust the pH with conc. HCl.

3. 0.5 *M* EDTA (pH 8)

Disodium EDTA-2H2O	186.1 g
Triple distilled water add upto	1000 ml

Adjust the pH with NaOH.

4. 10per cent SDS stock (pH 7.2)

Sodium dodecyl sulphate	200 g
Triple distilled water add upto	1000 mL

Heat to 68°C and stir. Adjust the pH by adding few drops of conc. HCl

5. 5 *M* Sodium chloride

Sodium chloride	292.2 g
Triple distilled water add	1000 mL

6. 7.5 *M* ammonium acetate

Ammonium acetate	578.1 g

Triple distilled water was added up to 1000 ml. Sterilized by filtration

7. CTAB/NaCl solution (10 per cent CTAB in 0.7 *M* NaCl)

Sodium chloride	4.1 g
Triple distilled water	80 mL

Sterilize by autoclaving.

Add CTAB	10 g

Heat to 65°C to dissolve. Adjust the volume to 100 mL.

8. Proteinase K (20 mg/ mL)

Proteinase K	20 mg
Sterile triple distilled water	1 mL

Store at – 20°C.

ANNEXURE IV

ANTIBIOTICS USED IN THE STUDY

Class of antibiotic	Name of antibiotic	Disc code	Concentration (µg)
B-lactams			
Penicillins	Methicillin	MET	10
	Ampicillin	AMP	10
	Amoxyclav	AMC	30
Cephalosporins	Cephalothin	CEP	30
	Cefoxitin	CX	30
	Cefoparazone	CPZ	75
Carbapenem	Imipenem	IPM	10
	Meropenem	MRP	10
Quinolones			
	Nalidixic acid	NA	30
	Ciprofloxacin	CIP	5
	Levofloxacin	LE	5
Chloramphenicol	Chloramphenicol	C	30
Macrolide	Erythromycin	E	15
Aminiglycosides	Kanamycin	K	30
	Streptomycin	S	10
	Gentamycin	GEN	10
Tetracycline	Tetracycline	TE	30
	Doxycycline	DO	30
	Oxytetracycline	O	30
	Co-trimoxazole	COT	25

Annexure V

Details of primers used in the study

Table No.1

Name	Sequence (5'-3')	Amplicon size	Annealing temperture	Annealing time	Reference
toxRvh-F	CGACAACCAAAATACGGAA	131 bp	45ºC	1min.	Ruwandeepika *et al*. (2010)
toxRvh-R	AGAGCAATTTGCTGAAGCTA				
vhpAvh-F	CTGAACGACGCCCATTATTT	201 bp	58ºC	1min.	Ruwandeepika *et al*. (2010)
vhpAvh-R	CGCTGACACATCAAGGCTAA				
chiAvh-F	GGAAGATGGCGTGATTGACT	232 bp	54 ºC	1min.	Ruwandeepika *et al*. (2010)
chiAvh-R	GGCATCAATTTCCCAAGAGA				

tdhvp-F	CCACTACCACTCTCATATGC	250 bp	55ºC	1 min. 30 sec.	Tada *et al.* (1992)
tdhvp-R	ATACGAGTGGTTGCTGTCATG				
yopPvp-F	CGTCCAACTCTATTGTTGTG	393 bp	45ºC	1min.	Caburlotto *et al*. (2009)
yopPvp-R	CAATGTTGGCTATTCGGTTG				
trhvp-F	CATAACAAACATATGCCCATTTCCG	500 bp	58ºC	1 min.	Bej *et al*. (1999)
trhvp-R	TTGGCTTCGATATTTTCAGTATCT				
ctxAvc-F	CGGGCAGATTCTAGACCTCCTG	564 bp	64ºC	1 min	Fields *et al*. (1992)
ctxAvc-R	CGATGATCTTGGAGCATTCCCAC				
VpI2-F	GGATTCGGTCGATACTGTC	1600 bp	54ºC	1min.	Jermyn and Boyd (2002)
VpI2-R	TCGTAGCCTTCCATTGC				
toxRValg-F	GATTAGGAAGCAACGAAAG	658 bp	54ºC	1min.	Xie *et al*. (2005)
toxRValg-R	GCAATCACTTCCACTGGTAAC				
tdhValg-F	CCACTACCACTCTCATATGC	251 bp	45ºC	1min.	Abd-Elghany and Sallam (2013)
tdHValg-R	GGTACTAAATGGCTGACATC				

nanHvc-F	GTTATTCGTGGATGGTCAGC	650 bp	56ºC	45sec.	Jermyn and Boyd (2002)
nanHvc-R	CGTTAGCGTTGTTAGCCTC				
Vvh-F	CCGCGGTACAGGTTGGCGCA	501 bp	58ºC	30sec.	Kaysner and DePaola (2001)
Vvh-R	CGCCACCCACTTTCGGGCC				
Val-F	GAGAACCCGACAGAAGCGAAG	773 bp	58ºC	30sec.	Zhou *et al*. (2007)
Val-R	CCTAGTGCGGTGATCAGTGTTG				
VP1-F	TGTACTGTTGAACGCCTAA	503 bp	58ºC	30sec.	Neogi *et al*. (2010)
VP1-R	CACGTTCTCATACGAGTG				
VP2-F	GAAAGTTGAACATCATCAGCACGA	271 bp	58ºC	30sec.	Zhou *et al*. (2007)
VP2-R	GGTCAGAATCAAACGCCG				
VH-F	TATTTGTCACCGAACTCAGAACC	121 bp	58ºC	30sec.	Cano-Gómez (2012)
VH-R	TGGCGCAGCGTCTATACG				

Table No.2

Name	Sequence (5'-3')	Amplicon size	Annealing temperture	Annealing time	Reference
bla$_{SHV}$-F	TTATCTCCCTGTTAGCCACC	796 bp	55 ºC	1min.	Ahmed *et al*. (2007)
bla$_{SHV}$-R	GATTTGCTGATTTCGCTCGG				
bla$_{TEM}$-F	ATAAAATTCTTGAAGAC	1073 bp	40 ºC	1min.	Speldooren, v. *et al*. (1998)
bla$_{TEM}$-R	TTACCAATGCTTAATCA				
catA1-F	GGTGATATGGGATAGTGTT	300-400 bp	50 ºC	45sec.	Yoo *et al*. (2003)
catA2-F	GATTGACCTGAATACCTGGAA	500-600 bp	55 ºC	45sec.	
catA3-F	CCATACTCATCCGATATTGA	~300 bp	53 ºC	45sec.	
catA4-F	CCGGTAAAGCGAAATTGTAT	500-400 bp	55 ºC	45sec.	
cat-R	CCATCACATACTGCATGATG				
catB3-F	TCAAAGGCAAGCTGCTTTCTGAGC	566 bp	66 ºC	1min.	
catB3-R	TATTAGACGAGCACAGCATGGGCA				

ermB-F	AGACACCTCGTCTAACCTTCGCTC	640 bp	48 ºC	1min.	Raissy *et al*. (2012)
ermB-R	TCCATGTACTACCATGCCACAGG				
aphA3-F	GGGACCACCTATGATGTGGAACG	600 bp	60 ºC	1min.	Letchumanan *et al*. (2014)
aphA3-R	CAGGCTTGATCCCCAGTAAGTC				
tetM-F	GTTAAATAGTGTTCTTGGAG	656 bp	45 ºC	1min.	Aarestrup, F.M., *et al*. (2000)
tetM-R	CTAAGATATGGCTCTAACAA				
tetS-F	CATAGACAAGCCGTTGACC	667 bp	56 ºC	1min.	Ng *et al*. (2001)
tetS-R	ATGTTTTTGGAACGACAGAG				
qnrA-F	ATTTCTCACGCCAGGATTTG	516 bp	53 ºC	45sec.	Ahmed *et al*. (2007)
qnrA-R	GATCGGCAAAGGTTAGGTCA				
qnrB-F	GATCGTGAAAGCCAGAAAGG	469 bp	53 ºC	45sec.	Ahmed *et al*. (2007)
qnrB-R	ACGATGCCTGGTAGTTGTCC				
qnrS-F	ACGACATTCGTCAACTGCAA	417 bp	53 ºC	45sec.	Ahmed *et al*. (2007)
qnrS-R	TAAATTGGCACCCTGTAGGC				

tetAC-F	CGCYTATATYGCCGAYATCAC	417 bp	55 ºC	1min.	Rysz and Alvarez , (2004)
tetAC-R	CCRAAWKCGGCWAGCGA				
tetBDEFHJ-F	GGDATTGGBCTTATYATGCC				Furushita, M *et al*. (2003)
tetBD-R	ATMACKCCCTGYAATGCA	960 bp	39 ºC	1min.	Furushita, M *et al*. (2003)
tetEHJ-R	AWDGTGGCDGGAATTTG	650 bp	48 ºC	1min.	
tetGY-F	TATGCRTTKATGCAGGTC	917 bp	50 ºC	1min.	Furushita, M *et al*. (2003)
tetGY-R	GACRAKCCAAACCCAACC				

Annexure VI

Details of fish pathogenic isolates under the study

Species	Isolate ID	Tissues from which isolated	Fish species	Year of collection
Vibrio parahaemolyticus	SL6	Liver	*Lates calcarifer*	2017
	MZLB6	Liver	*Mugil cephalus*	2018
	OTK1	Kidney	*Astronotus ocellatus*	2018
	TBL11	Liver	*Oreochromis niloticus*	2018
	SBK4	Kidney	*Lates calcarifer*	2017
	SZK8	Kidney	*Lates calcarifer*	2017
Vibrio harveyi	EBL1	Blood	*Abudefduf*	2019
	EK2	Kideny	*Abudefduf*	2019
	2PLT	Lesion	*Trachinotus blochii*	2017
	SLTY	Liver	*Lates calcarifer*	2017
	PL3	Liver	*Trachinotus carolinus*	2017
	CBS4	Spleen	*Amphitrion percula*	2017
Vibrio vulnificus	MZLL4	Liver	*Mugil cephalus*	2018
	LK7	Kidney	*Lates calcarifer*	2018
	PBB2	Blood	*Trachinotus blochii*	2017
	TTL3	Liver	*Ectroplus surratensis*	2018
	TZB1	Blood	*Oreochromis niloticus*	2018
	ETB3	Blood	*Ectroplus surratensis*	2020

ABSTRACT

DISTRIBUTION OF ANTIBIOTIC RESISTANCE AND PUBLIC HEALTH SIGNIFICANT VIRULENCE GENES AMONG VIBRIONACEAE ASSOCIATED WITH *Scylla olivacea* (HERBST, 1896)

by

Mariya Sony

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Abstract of the Thesis

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9. ABSTRACT

Distribution of antibiotic resistance and public health significant virulence genes among *Vibrionaceaee* associated with *Scylla olivacea* (Herbst, 1896)

The study entitled "Distribution of antibiotic resistance and public health significant virulence genes among *Vibrionaceae* associated with *Scylla olivacea* (Herbst, 1896)" undertaken during 2019-20 at Marine biotechnology division, ICAR-Central Marine Fisheries Research Institute, Kochi. The study was performed to evaluate the incidence of vibrio related infections in both humans and aquatic animals continue to rise during the years to come driven by the climate change and ocean warming, demanding detailed studies about vibrios especially in terms of virulence and antibiotic resistance. Orange mud crab (*Scylla olivacea* Herbst, 1896) represents a prime component of aquaculture practices and coastal fisheries. Even though knowledge on abundance and composition of microbes in apparently healthy animals is essential in successful aquaculture practices, data availability on microbiology of these crabs are fewer. Therefore, investigations on *Vibrionaceae* (bacteria that are widely distributed in aquatic environments) in these animals will be valuable in both aquaculture and public health perspectives, which were targeted through the present study. Further, bacteria belonging to three public health significant *Vibrio* spp. (*V. harveyi, V. parahaemolyticus* and *V. vulnificus*) isolated from diseased fish were also included in the study. It was found that all tissues including haemolymph of *S. olivacea* carried a variable concentration of vibrios even in apparently healthy conditions with the greatest mean concentrations in gut (1.74 X 10^6 CFU/g), followed by gill (1.60 X 10^6 CFU/g). Haemolymph had the lowest mean concentration of vibrios (1.77 X 10^2 CFU/mL). There were a total of 23 morphologically different vibrio isolates which were found to be belonging to three distinct species in prevalence order of *V. parahaemolyticus* (56 per cent) > *V.alginolyticus*> *V. diazotrophicus*. Gut was having the maximum diversity. During phylogenetic analysis, there were two clades namely, Harveyi and Diazotrophicus clades, in which Harveyi clade was more dominant. Prevalence order of virulent genes was *chiA* (*V. harveyi*)> *collagenase* (*V. parahaemolyticus*)> *toxR* (*V.*

parahaemolyticus) > *toxR* (*V. alginolyticus*) > *nanH (V. cholerae).* Antibiotic resistance pattern of all these isolates against a total of 21 antibiotics belonging to six classes were also studied and results showed that imipenem, nalidixic acid and chloramphenicol would be the most efficient antibiotics against vibrios. Among 18 antibiotic resistant genes screened, only four genes namely *tetS, tetH, tetB* and *aphA3* were present in vibrio isolates of the present study with an overall prevalence of AR genes as 2.77 per cent. The study forms the initial report on occurrence of *tetH* and *tetS* among vibrios. In conclusion, the present study forms the first multi-tissue description on density and diversity of *Vibrionaceae* of a supreme aquaculture candidate crab species, fetching applications in disease management studies during their aquaculture practices. Furthemore, the generated ABST, profiles of virulence and AMR genes of vibrio isolates can pave the way for its additional applications in public health perspectives.