STUDIES ON ISOLATION AND MOLECULAR CHARACTERIZATION OF PLASMIDS FROM VIBRIOS

Thesis submitted to the
Cochin University of Science and Technology
Under the Faculty of Science
In Partial Fulfillment of the Requirements
For the degree of

Ph.D
IN
BIOTECHNOLOGY

BY

MANJUSHA S.,

Department of Biotechnology
Cochin University of Science and Technology
Cochin 686 022
Kerala, India.

DECEMBER 2006

DEPARTMENT OF BIOTECHNOLOGY
COCHIN UNIVERSITY OF SCIENCE AND TECHNOLOGY
KOCHI-682022, KERALA, INDIA
Ph: 91-484-2576267; Fax: 91-484-2576267
Email: sarit@cusat.ac.in; saritagbhat@gmail.com

Dr. Sarita G Bhat
Senior Lecturer

5-12-2006

CERTIFICATE

This is to certify that the thesis entitled "**Studies on isolation and molecular characterization of plasmids from *Vibrios***" is an authentic record of the research work carried out by Manjusha. S under my supervision and guidance in the Department of Biotechnology, Cochin University of Science and Technology, Cochin, in partial fulfillment of the requirements of the award of the degree of Doctor of Philosophy in Biotechnology under the Faculty of Science of Cochin University of Science and Technology, Cochin. The work presented in this thesis has not been submitted for any other degree or diploma earlier.

Cochin- 22.
6-12-2006

Sarita G Bhat

Dr. Sarita. G. Bhat
(Supervising Guide)

DECLARATION

I hereby declare that this thesis entitled "**Studies on isolation and molecular characterization of plasmids from *Vibrios***" is the authentic record of the research work carried out by me in partial fulfillment of the requirements for the award of the Degree of Doctor of Philosophy in Biotechnology under the supervision and guidance of Dr. Sarita G Bhat, Lecturer, Department of Biotechnology, Cochin University of Science and Technology, Cochin and that no part of it has previously formed the basis for the award of any degree or diploma .

Cochin-22.

6-12-2006

Manjusha. S

ACKNOWLEDGEMENT

At the outset, I express my deep sense of gratitude and indebtedness to my mentor Dr. Sarita. G.Bhat, Senior Lecturer, Department of Biotechnology for valuable guidance, healthy and constructive criticism, inducing a research aptitude in me and molding my research career, assessing my strength and weakness.

I am extremely thankful to Prof. Dr.M. Chandrasekaran, Professor, Department of Biotechnology, Cochin University of Science and Technology, Cochin for facilitating me in my research endeavours and taking keen interest in making my Ph.D life to perfection.

I am indebted to Dr. Elyas. K. K for his timely help and critical views about research ideas to improve my research work, manuscripts and taking keen interest in making my Ph.D life to perfection. Without his inspiration I cannot complete this into perfection.

I take this opportunity to express my gratefulness to Dr. C. S. Paulose, Head, Department of Biotechnology, Cochin University of Science and Technology, Cochin for utilizing the facilities in the Department and special thanks also for utilizing the Gel Doc facilities in Neurobiology lab.

I also express my thanks to other senior Faculties Dr. Padma Nambisan, Dr.E.Vijayan, Department of Biotechnology, Cochin University of Science and Technology, Cochin for their timely help.

I am extremely thankful to Binoy joseph and Girish, for taking all the Gel pictures of my experiments with a pleasant and smiling face. I am also grateful to Soorej. M. Basheer for the timely help done in many ways in tiring situations.

I am grateful to Dr.Sreeja Chellappan, Jasmine, Madhu, Lailaja, Jissa, Beena, ,Dr. Balarama Kaimal, Dr.Santhosh Thomas, Akash George, Riyas Khan, Finla chathu, Savitha, Remya, Anu, Ami, Bijina, Archana Kishore, Bernard Rajeev, Siju.M. Varghese, Jeena and all others in Department of Biotechnolgy for doing the timely help rendered by them.

I also express my thanks to the staff of Department of Biotechnolgy and to the staff of administrative office especially academic and examination wing,Cochin University of Science and Technology, Cochin.

It was the love, blessings and encouragement of my Amma and Vaapa, which made it possible for me to complete this research work and continue my educational career. My heartfelt thanks are due to Ansar, my husband for his support, encouragement and help, without which it wouldn't have been possible for me to complete this work. I am also grateful to my loving kid Ponnu.

My sincere thanks are due to my brother, Dr Manush S.M., JSPS Post Doctoral Fellow, Food Biotechnology Division, NFRI, Japan for his support in all terms during the Ph.D life and his encouragement in tiring situations.

Lastly I would like to thank Cochin University of Science and Technology, Cochin for providing financial support in the form of fellowship during the research tenure.

Above all, I express my gratitude for almighty God for completing this research work.

Manjusha. S.

Dedicated To

MY LOVING Amma,Vappa ,respected Teachers,

Dr.Manush S.M., Ansar.M.S, Ponnu

and Above all to the almighty GOD.

CONTENTS

ABBREVIATIONS

λ	Lambda
%	Percentage
µg	Microgram
µg/ml	Microgram per millilitre
µl	Microlitre
α	Alpha
µm	Micrometer
@	At the rate of
<	Less than
>	Greater than
2/3	Two third
bp	Base pair
$CaCl_2$	Calcium Chloride
cfu	Colony Forming Units
DNA	Deoxy ribonucleic acid
dNTPs	Deoxy Nucleotide Tri phosphate
^0C	Degree Celsius
E.coli	*Escherichia coli*
EDTA	Ethylene diamine tetra acetic acid
Fig.	Figure
g	gram
i.e	That is
int	Integrase
KCl	Potassium chloride
kb	Kilobase
LB	Luria Bertani
lbs	Pounds
M	Molar
MAR	Multiple antibiotic resistance
MRIs	Multiple resistant integrons
mg	Milligram
mg/ml	Milligram per milliliter
$MgCl_2$	Magnesium Chloride
mM	Millimolar
mm	Millimetre
mv	Millivolts
MOF	Medium of Oxidation and Fermentation
M Da	Mega Dalton
N	Normal
NaCl	Sodium Chloride
nm	Nanometre
No.	Number
O.D	Optical density

pmole	Picomole
pH	Potency of hydrogen
pUC	Plasmid discovered by University of California
PCR	Polymerase chain reaction
R	Resistant
rpm	Revolution per minute
S	Sensitive
SDS	Sodium Dodecyl Sulphate
Sl.no	Serial number
spp	Species
SXT	Sulphamethaxazole Trimethoprim
Taq	*Thermus aquaticus*
TCBS	Thiosulphate Citrate Bile salt Sucrose agar
TAE	Tris Acetate EDTA Buffer
TE	Tris EDTA solution
U	Units
UV	Ultra Violet
V	Volts
w/v	Weight / volume
WHO	World Health Organization
Amoxycillin	Ac
Ampicillin	A
Amikacin	Ak
Carbenicillin	Cb
Cefuroxime	Cu
Chloramphenicol	C
Ciprofloxacin	Cf
Chlortetracycline	Ct
Cotrimaxazole	Co
Doxycycline hydrochloride	Do
Furazolidone	Fr
Gentamycin	G
Meropenem	Mr
Netilmycin	Nt
Nalidixic acid	Na
Norfloxacin	Nx
Neomycin	Ne
Rifampicin	R
Streptomycin	S
Sulphafurazole	Sf
Trimethoprim	Tr
Tetracycline	T

List of Tables

List of Figures

Chapter 1

INTRODUCTION

Aquaculture is considered world over as a means to meet the ever-increasing demand for fish and shellfish for human consumption. The practice of growing aquatic animals such as shrimps in high density has resulted in increasing infections by microorganisms leading to the use of antibiotics and chemicals for prophylaxis or control of microbial diseases. Antibiotics and other chemotherapeutic agents are commonly used in fish farms either as feed additives or immersion baths to achieve either prophylaxis or therapy. This leads to the increasing antibiotic resistance in the bacterial pathogens of the aquaculture system. The indiscriminate use of drugs with total disregard to the severe environment leads to the development of resistance that would have affect on the resident autochthonous microflora, is never been addressed. Antibiotics have long been considered the "magic arrow" that may end infectious disease. Although they have improved the health of countless numbers of humans and animals, many antibiotics have lost their efficiency since the beginning of the antibiotic era. Bacteria have adapted to defend against these antibiotics and continue to develop resistance, even as we develop new antibiotics. Therefore, much attention has been given to the increase in antibiotic resistance in the recent years. As more microbial species and strains are becoming resistant, many diseases have become difficult to treat, a phenomenon frequently ascribed to both indiscriminate and inappropriate use of antibiotics in human medicine. However, the use of antibiotics and antimicrobials in rearing food animals has also contributed significantly to the pool of antibiotic resistant organisms globally and antibiotic resistant bacteria are now found in large numbers in virtually every ecosystem on earth. There is no doubt that the use of antibiotics provides selective pressure that result in the emergence of

antibiotic resistant bacteria and resistance genes. While some resistant bacteria are found naturally in the environment, pathogens and non-pathogens are released into the environment in several ways, contributing to a web of resistance that includes humans, animals, and the environment, essentially the biosphere.

The loss of drug by leaching is particularly important in species, such as shrimp, which feeds very slowly. Some antibiotics and their metabolites are also excreted via faeces by aquatic animals. The undigested medicated faeces or feed containing high residues may serve as contaminants of natural waters. More over, the extensive use of these drugs has resulted in an increase of drug-resistant bacteria as well as R-plasmids (Son *et al.*, 1997). Further, many species of halophilic *Vibrios* were recognized as potential human pathogens causing serious gastroenteritis or severe wound infection upon exposure to contaminated seafood and seawater (French *et al.*, 1989).

Plasmids are circular DNA molecules that exist independently and are found in many bacteria, with their own replication origins and are autonomously replicated and stably inherited. Plasmids have relatively few genes, often in the range of 25-30. These genes code for a number of macromolecules like toxins, drug resistance factors, degradative enzymes, etc., which render the bacterium better equipped to establish itself in the adverse environment in the host. Single copy plasmids produce only one copy per host cell, while multi copy plasmid may be present at concentrations of 40 or more per cell.

The genes for drug resistance are found in both the bacterial chromosome and plasmids. Spontaneous mutations in bacterial chromosome, although do not occur very often, will make bacteria drug resistant. Frequently, a bacterial pathogen is drug resistant because it has a plasmid bearing one or more resistant genes (R factors),

which often code for enzymes that destroy or modify drugs. Once a bacterial cell possesses plasmids with 'R' factor; it may be transferred to the other cell quite rapidly through normal gene exchange processes; conjugation, transduction and transformation. Naturally occurring gene expression elements, called *integrons,* have been described as another efficient genetic mechanism by which bacteria can acquire resistance genes (Hall and Collis, 1995). Sulphamethaxazole Trimethoprim element (SXT) is yet another family of conjugative transposon similar to mobile genetic elements that encode multiple antibiotic resistance genes. Of the different roles played by plasmids in the lives of bacteria, virulence factors encoded by their plasmids, by which the bacteria become pathogenic, assume importance. As a single plasmid may carry genes for resistance to several drugs, a pathogen population can become resistant to several antibiotics simultaneously.

Plasmids have been reported to be mediators for resistance to a number of drugs in bacterial pathogens. This has led to an interest in the study of plasmid profile and its association with drug resistance. A number of epidemiologically important characters of bacteria are reported to be plasmid dependant and therefore, characterization of the bacteria in respect of its plasmid profile assumes importance. Recently, a reliable way of assessing the plasmid profile is by microbial molecular genetics such as isolation of plasmids and their characterization using transformation, conjugation, and integron screening and observing their restriction digested profiles in the laboratory.

More over, seafoods such as shrimps and molluscan that grow in aquatic system with drugs or their residues, when consumed may result in serious health consequences for consumer. In addition to development of resistance in other organisms, pathogenic and innocuous, these are directly hazardous for human health.

Growing public and scientific awareness about the risks to consumers ingesting drug residues have forced several countries to impose the strict regulations on the use of antibiotics.

OBJECTIVE OF THE STUDY

The family *Vibrionaceae* harbors the largest number of human as well as fish and shellfish pathogens. Bacteria of the genus *Vibrio*, under this family, are commonly found in coastal and estuarine waters. Some *Vibrio* strains are pathogenic and can cause Vibriosis, a serious infectious disease in both wild and cultured finfish and shellfish (Austin and Austin, 1999). In recent years, Vibriosis has become one of the most important bacterial diseases in cultured organisms, affecting a large number of species of fish and shellfish (Wu and Pan, 1997). The consumption of raw or improperly cooked seafood, contaminated with pathogenic *Vibrios* becomes a great threat to human health. In this context, the present study was conducted to assess the antibiotic resistance in the *Vibrios* isolated from different samples and to characterize the plasmids harbored by these Multiple Antibiotic Resistant (MAR)*Vibrios*.

The objectives of the study are as follows

1. Isolation and characterization of *Vibrios* from the coastal environment of Kerala.
2. To evaluate the antibiotic sensitivity or resistance of the *Vibrio* isolates.
3. To screen for the presence of plasmids in the antibiotic resistant *Vibrios* and
4. Molecular characterization of the resistance plasmids.

Chapter 2

REVIEW OF LITERATURE

The members of the family Vibrionaceae constitute a predominant heterotrophic bacterial group in aquatic environments (Simidu *et al.*, 1977). *Vibrio* is one of the most important aquatic bacterial genera that are widely distributed in marine, estuarine, and fresh waters. *Vibrio* species are commonly observed in shrimp hatcheries; grow out ponds and sediments (Otta *et al.*,1999). The numbers of *Vibrio* species were increasing year by year now the 63 species of *Vibrio* has been recognized (Thompson *et al.*,2001;Thompson *et al.*, 2005a;Thompson *et al.*,2005b).

2.1. Historical perspective- *Vibrio* research

Vibrios are among the most abundant cultivable microbes in aquatic environments (Heidelberg *et al.*, 2002a). *V. cholerae*, the causative agent of cholera is one of the most studied of the *Vibrio* species and one which is amenable to genetic analysis and gene level research (Guidolin and Manning, 1987). Cholera is a devastating and ancient disease, occurring even today in epidemic form, in many parts of the world, claiming hundreds of thousands of lives each year (WHO Report, 2001). First described by Pacini (Pacini, 1854), the cholera *Vibrio* was extensively studied and Koch properly characterized the disease as a waterborne disease in 1884 (Jones,1984). It is well established that the disease is seasonal and studies are beginning to elucidate the role of the coastal environment and the ecology of *Vibrio cholerae* in transmission of the disease.

The family Vibrionaceae consists of ubiquitous halophilic facultative anaerobes, which are gram-negative, motile rods. The fish/shellfish disease associated

with this genus is called Vibriosis. It affects both marine and freshwater fishes/shellfishes. *Vibrios* comprise of some extremely virulent organisms, because to their capacity to infect a wide range of aquatic organisms such as penaeid shrimp (Lightner, 1993), fish (Austin and Austin, 1999) and molluscan (Rheinheimer, 1992); while 11 other species are known to cause diseases in man (Bullock, 1987).

Members of the genus *Vibrio* are known to be marine bacteria with the exception of *Vibrio cholerae*, which is terrestrial (Sakazaki, 1981).The marine *Vibrios* are also postulated to play some roles in the degradation of organic pollutants (West *et al.,* 1984) and nitrogen fixation (West, 1985).They exhibit halophilism or halo tolerance (Baumann *et al.,* 1984) and harbor a wealth of diverse genomes and represents cosmopolitan and endemic species that are yet to be described (Thompson *et al.,* 2001). The exact ecological roles of many of these groups are unknown till date.

Some of the *Vibrio* sp., viz., *V.parahaemolyticus* and *V. anguillarum,* are known to be pathogenic for fish (Blake, *et al.,* 1980). However, other species of *Vibrios* capable of causing disease in humans have received greater attention in the last decade, which include *Vibrio parahaemolyticus*, *Vibrio vulnificus*, *Vibrio alginolyticus*, *Vibrio damselae*, *Vibrio fluvialis*, *Vibrio furnissii*, *Vibrio hollisae*, *Vibrio metschnikovii* and *Vibrio mimicus* (Chakraborty *et al.,* 1997). At present, it is impossible to provide realistic figures concerning the incidence of illnesses caused by *Vibrio* species worldwide; surveillance programmes, where they exist, mostly collect information on only a limited number of incidences. In the USA, *Vibrio* species have been estimated to be the cause of about 8000 illnesses annually (Mead *et al.,* 1999). With regards to *V. cholerae*, morbidity and mortality are likely to be grossly under reported, in part owing to surveillance difficulties, but also for fear of economic and social consequences. Moreover, several cholera endemic countries are not included in the WHO report (Colwell ,1996).. Globally, an estimated 120,000 deaths are caused by cholera each year (WHO, 2001).

The studies on both *V. cholerae* and other human pathogenic *Vibrios* have revealed the existence of these bacteria as free-living or in association with phytoplankton, zooplankton, crustaceans and molluscans in coastal and estuarine environments (Vanderzant *et al.*, 1971; Colwell and Huq, 1994; Otta *et al.*, 1999; Lipp *et al.*, 2002). *Vibrio* and *Photobacterium* are also reported to be attached to the external surface of zooplankton and there exists a close partnership between these bacteria and zooplankton (Heidelberg *et al.*, 2002b; and Lipp *et al.*, 2002). Some of the *Vibrio* species such as *V. harveyi* and *Vibrio parahaemolyticus* are also associated with bacterial infections in shrimp (Jiravanichpaisal and Miyazaki, 1995) and are generally considered to be opportunistic pathogens causing disease when shrimp are stressed. *Vibrios* also form a part of the normal microflora of the shrimp *Penaeus vannamei* (Vandenberghe *et al.*, 1999). Understanding the pathogenicity of certain strains of *Vibrios* is critical in aquaculture systems, where the fish and shellfish, viz., salmonids and penaeid shrimps are reared in intensive culture systems and in high densities under artificial and unstable conditions (Olafsen.,2001).A combination of these conditions automatically favors the proliferation of *Vibrios* and enhances their virulence and disease prevalence. This highly intensive aquaculture also has disastrous effect on the environment (Naylor *et al.*, 2000; Williams *et al.*, 2000).

Several cultivation dependent and independent studies have shown that *Vibrios* appear at particularly high densities in and/or on marine organisms, e.g., corals (Rosenberg and Ben-Haim, 2002), fish (Ringo and Birkbeck,1999), seagrass, sponges, shrimp (Gomez-Gil *et al.*, 1998; Vandenberghe *et al.*, 1998; 1999; 2003) and zooplankton (Johnson and Shunk,1936;Suantika *et al.*,2001).*Photobacterium leiognathi* and *P. phosphoreum* are found in symbiotic associations with fish, and *P. leiognathi*, *V. logei*, and *V. fischeri* are found in symbiotic associations with squid. These bacteria colonize the light organs of the host and play a role (via emission of light) in communication, prey attraction, and predator avoidance (Fidopiastis *et al.*,

1998; Fukasawa and Dunlap, 1986). In the light organs of the squid *Sepiolla* spp., the abundance of *Vibrios* can be as high as 10^{11} cells/organ (Fidopiastis *et al.*, 1998; Nishiguchi, 2000). Newly hatched squid excrete a mucus matrix from the pores of the light organs whereby *V. fischeri* cells present in seawater are found (Nyholm *et al.*, 2000 ; Nyholm and Mc Fall-Ngai, 2003). Subsequently, *V. fischeri* migrates into the organ and colonizes the crypt epithelium. Obviously, the flagella of *V. fischeri* play a crucial role in the colonization of the light organs, but hyper-flagellated *V. fischeri* cells containing up to 16 flagella are defective in normal colonization (Millikan and Ruby, 2002). *V. fischeri*, *V. logei*, and *P. leiognathi* are apparently the only three organisms colonizing the light organs of squid, but this seemingly specific partnership remains to be confirmed. *V. fischeri* cells entrapped in the light organs of squid can sense the density of nonspecific cells by signaling molecules or pheromones (e.g., N-acyl homoserine). Copepods may, in turn, feed on these bacteria. *V. cholerae* moves along and attaches to surfaces with the aid of the flagellum and pili, this may act as adhesions (Moorthy and Watnick, 2004). Because *V. cholerae* is closely associated with plankton, it is assumed that cholera outbreaks are linked with planktonic blooms and the sea surface temperature, and so such outbreaks may be predicted by monitoring the parameters like remote sensing. The wide ecological relationships and ability to cope with global climate changes may be a reflection of the high genome plasticity of *Vibrios* (Lipp *et al.*, 2002). Recently, a number of reports have highlighted the pathogenic potential of *Vibrios* toward humans and marine animals (e.g. corals, gorgonians, and shrimp), which may be coupled with rising of seawater temperature due to global warming (Martin *et al.*, 2002; Rosenberg and Ben Haim, 2002; Sechi *et al.*,2000).

Seafood-associated illnesses are mainly associated with the consumption of molluscan (viz., mussels, oysters, squids, cuttlefish, clams) owing to the filter feeding habit of these organisms that concentrates particulate matter and bacteria present in

surrounding waters. Two groups of pathogenic bacteria present in coastal seawater may be entrapped by bivalves: firstly the exotic bacterial pathogens like *Salmonella* and *Shigell*a that is shed into the water from infected animals and humans and the other is autochthonous members of the family Vibrionaceae (Potasman *et al.,* 2002). *Vibrio* resistance to depuration procedures of edible bivalves is a further reason for the worldwide incidence of *Vibrio* related seafood borne diseases. In order to decrease the number of unwanted microorganisms to acceptable levels for human consumption, bivalve depuration in controlled waters is used worldwide. (Perkins *et al.,* 1980; Richards, 1988; Prieur *et al.,* 1990; Olafsen *et al.,* 1993;Marino *et al.,* 1999). Interestingly, some *Vibrio* species have been reported to be resistant to depuration and are able to persist and multiply in bivalve tissues (Jones *et al.,* 1991; Shumway, 1992; Murphree and Tamplin., 1991), supporting the hypothesis that these bacteria represent a bivalve-specific community. Outbreaks of cholera have been associated with consumption of seafood including oysters, crabs and shrimp (Kaper, 1995). Given their abundance in water, 100-fold higher concentrations are found in filter-feeding shellfish such as edible bivalves than in the surrounding water (Wright *et al.,* 1996).During the warm summer months, virtually 100% of oysters can carry *V. vulnificus* and *V. parahaemolyticus (*Wright *et al.,* 1996; Cook *et al.,* 2002). Factors that favor active filter feeding by shellfish increase the probability that shellfish in a given area will take up the pathogen (Murphree and Tamplin, 1991).

Vibrio tapetis is the causative agent of brown ring disease (BRD), an epizootic disease that causes high mortalities in the introduced Manila clam, *Ruditapes philippinarum* cultured in Western Europe. *Vibrio tapetis* adheres to and disrupts the production of the periostracal lamina, causing anomalous deposition of periostracum around the inner shell (Prieur *et al.,* 1990; Novoa *et al.,* 1998). *Vibrio alginolyticus* infections have been related to production of heat labile and heat-stable toxins, with lethal and both lethal and ciliostatic activity respectively (Di Salvo *et al.,*

1978; Brown and Roland, 1984) *V. parahaemolyticus* causes gastroenteritis in which the hemolysins, thermostable direct hemolysin (TDH) and/or TDH-related hemolysin (TRH), have been considered to play a crucial role (Nishibuchi and Kaper,1995).Terrestrial and aquatic animals (including plankton, birds, fish, reptiles) may harbor virulent strains of *V. parahaemolyticus* and play a role as intermediate hosts and vehicles for spread (Sarkar *et al.*,1985). *V. parahaemolyticus* can be introduced into non-contaminated areas by relaying shellfish prior to commercial harvesting. Sewage discharge may indirectly influence the densities of *V. parahaemolyticus* present in shellfish growing areas (Watkins and Cabelli, 1985). *V. parahaemolyticus* favors the presence of particulates, zooplankton and other chitin sources (Kaneko and Colwell, 1973). *V. parahaemolyticus,* a chitinoclastic organism adsorbs onto the exoskeleton of copepods. This association has been reported to be the most important in dictating the annual cycle of *V. parahaemolyticus* in temperate and estuarine areas (Kaneko and Colwell, 1978). The isolation of *V. parahaemolyticus* from freshwater samples is by no means novel. There have been occasional reports on the recovery of this organism from freshwater areas (Sayler, 1976). Introduction of the pathogen into such closed water bodies through ambulatory cases or carriers can be presumed since these waters are constantly used for domestic and ablutionary purposes. Since *V. parahaemolyticus* is among the more salinity dependent *Vibrios*, one might expect the halophile to survive for a very short period in such alien environments. Adsorption onto plankton might, perhaps, prolong its survival conferring some kind of protection (Sarkar *et al.*, 1985). In Japan and eastern Asian countries, *V. parahaemolyticus* has been recognized as a major cause of food borne gastroenteritis. It has spread throughout Asia and to the United States elevating the status of *V. parahaemolyticus* to pandemic (Miyamoto *et al.*,2000).

The first account of Vibriosis in impounded lobsters was that of (Sanyal *et al.*, 1983), who reported the isolation of both *Vibrio parahaemolyticus* and *Vibrio*

alginolyticus from moribund aquarium-held lobsters. *Vibrio fluvialis* like organisms were isolated from diseased lobsters. Although the emergence of this pathogen poses economic threat that merits additional studies, the causative *V. fluvialis* like strains are probably not infectious for humans (Sanyal *et al.*,1983).

V. vulnificus is an important etiologic agent of wound infections and septicemia in humans (Finkelstein, 2002). *V. vulnificus* has been associated with primary septicemia in individuals following consumption of raw bivalves which is a serious, often fatal, disease. To date, *V. vulnificus* disease has almost exclusively been associated with oysters (Oliver and Kaper, 1997). *Vibrio mimicus* has also been established as a pathogenic member of the genus *Vibrio* (Davis *et al.,* 1981). Isolation of the pathogen from clinical samples has been made in different countries including the United States, Japan, Bangladesh, New Zealand, and Canada (Davis *et al.,* 1981). Association of toxigenic *V. mimicus* with freshwater prawns has been described in Bangladesh (Chowdhury, 1986).

Other *Vibrios*, e.g., *V. hollisae*, *V. damselae*, *V. alginolyticus*, *V. cincinnatiensis*, *V. fluvialis*, *V. furnissii*, *V. harveyi*, *V. metschnikovii*, and *V. mimicus*, have been sporadically found in human infections (Yamane *et al.*, 2004; Farmer and Brenner, 1992; Farmer, 1992; Carnahan *et al.,* 1994; Davis *et al.,* 1981; Brenner *et al.,* 1983; Abbott and Janda, 1994). Apparently, they are less important as human pathogens (Farmer, 1992; Farmer and Brenner, 1992).

A *Vibrio* surveillance system maintained by the Centre for Disease Control and Prevention reported 296 cases of infection caused by *Vibrios* in the United States in 2000 (CDC, 2001). Most strains were isolated from stool, wound, and blood samples and were identified as *V. parahaemolyticus*, *V. vulnificus*, and *V. cholerae*, respectively. Most patients who died were infected by *V. vulnificus*.

Some of the *Vibrio* species, viz., *Vibrio alginolyticus*, have been characterized as probionts (Gomez-Gil *et al.,* 2002) as well as pathogens (Lee *et al.,* 1996). Certain *Vibrio* strains have been reported to be potential probiotics for this shrimp (Gomez-Gil *et al.,* 1998,2002).Use of probiotics, i.e. live microorganisms that, when administered in adequate amounts, confer a health benefit on the host, has been reported to reduce the need for medication (e.g. antibiotics and pesticides) and water exchange, which are used massively in intensive shrimp-rearing (Verschuere *et al.,* 2000). In India, the occurrence of various *Vibrio* species in water, sediment and shrimp samples from multiple shrimp farm environments from the east and west coast of India was studied (Shubha *et al.,* 2005).

2.2. Aquaculture and *Vibrios*

Aquaculture emerged as one of the most promising food production in the later part of 20th century, with annual growth rate exceeding 11% per annum (Pillay, 1997). However, the industry is unfortunately encountering serious diseases during the culture period. Of the different diseases, Vibriosis is one of the most frequent disease affecting fishes, molluscans and crustaceans, which needs further attention.

It is a common practice in aquaculture systems to incorporate antibiotics to treat the bacteriological infections. The dispersion of antibiotics after treatment in shrimp ponds or hatcheries can lead to the development of resistance among the pathogens, and a changed microorganism composition in the aquatic environment (Molina Aja *et al.,* 2002).

2.3. Use of antibiotics

Bacteria have adapted defense mechanisms against the antibiotics and continue to develop new resistances, even as new antibiotics are being developed. Although penicillin was the first natural antibiotic to be discovered, the idea of using

microorganisms therapeutically was not new .A series of different antibiotics were quickly discovered after penicillin came into use. In 1943, one of Waksman's students discovered streptomycin (Schatz *et al.,* 1944), leading to a flood of researchers combing the world for new drugs. It was in this same period the gramicidin, the first antibiotic active against gram-positive bacteria was discovered (Hotchkiss and Dubos, 1941). Chlortetracycline, Chloramphenicol and others were discovered shortly there after (Garrod, and O'Grady. 1971). Many drugs discovered were too toxic for human use, or that had already been discovered. Nevertheless, this work lead to the discovery of many new drugs and within a decade, drugs comprising the major classes of antibiotics were found (Greenwood, 2000). Some antibiotic-producing bacteria were isolated from a wound infection and others from sewage, a chicken's throat, and a wet patch of wall in Paris (Garrod and Grady, 1971). In 1962, one of the later discoveries was a synthetic drug nalidixic acid, the first of the quinolones to be described, and although not therapeutically important by itself, modification of nalidixic acid led to the production of the highly effective fluoroquinolones. Members of this class, such as ciprofloxacin, norfloxacin, enrofloxacin, and ofloxacin, have become very important in the treatment of diseases in both humans and animals (Mitsuhashi, 1993.). Since 1960's, there have been few discoveries of new antibiotic drugs. The drugs developed since have mostly been chemical modifications of existing drugs. These modifications have been very useful in treating infectious diseases, leading to the enhanced killing of pathogens, increased spectrum of action, reduced toxicity, and reduced side effects.

In order to comprehend the problem of antibiotic resistance, as it exists today, it is useful to understand the history and development of both antibiotics and antibiotic resistance. Antimicrobial drugs have generally been classified into two categories- one includes the synthetic drugs, such as the sulfonamides and the quinolones; and the second includes the antibiotics synthesized by microorganisms. In recent years, increasing numbers of semi-synthetic drugs have been developed which are chemical

derivatives of antibiotics, thereby blurring the distinction between synthetic and natural antibiotics.

Unfortunately, since the 1970's, only one new class of antibiotics has been introduced (Lipsitch, 2002.) and a recent trend in antibiotic therapy has been to employ combinations of drugs with different mechanisms of action, in order to increase their effectiveness and to overcome the problem of drug resistance.

2.4. Antibiotics in veterinary and aquaculture: Drug use and antibiotic resistance

Antibiotics are added to various feeding mixtures used in poultry and animal farming as a preventive measure to keep the animals in good health. There are seven chemicals approved for sale when labeled for food fish use in Canada, including four antibiotic drugs (oxytetracycline, florfenicol, sulfadimethoxine plus ormetoprim, sulfadiazine plus trimethoprim), one anaesthetic (tricaine methanesulphonate) and two fungicides/disinfectants (formaldehyde and hydrogen peroxide) (Health Canada, 2001a). Oxolinic acid has been widely used in salmonid culture outside of Canada, including the United States, and off-label prescription potential exists where veterinarians can legally prescribe it. In addition, oxolinic acid provides a wide degree of information regarding fate and effect data, which could be relevant to other antibiotics. As with all intensive animal husbandry, aquaculture practices create an opportunity for the proliferation and spread of pathogens that can lead to significant mortality of stock and subsequent loss of revenue (Dixon, 1994). Antibiotics can be administered directly by injection or by releasing feed containing antibiotics directly into the aquatic ecosystem. Unconsumed medicated feed is available to wild animals. In addition, antibiotic containing feed can accumulate in the sediments or unabsorbed antibiotics can be released in fish faeces or urinary waste (Bjorklund *et al.,* 1990 and 1991). Subsequently influencing the natural bacterial flora, an important component of ecological food webs. It is estimated that 1.4 to 40.5% of fish feed passed uneaten

through an Atlantic salmon sea cage (Thorpe *et al.,* 1990). However, this may be a conservative estimate since diseased fish rarely feed (Bjorklund *et al.,*1990) and the majority of the active form antibiotic passes unabsorbed through the gastrointestinal tract of fish (Cravedi *et al.,* 1987; Bjorklund *et al.,* 1991).

The evolution of drug resistant strains of pathogenic bacteria is perhaps the most important implication of antibiotic use in aquaculture. Resistance to antibiotics is present in bacterial populations naturally (McPhearson *et al.,* 1991; Johnson and Adams 1992; Spanggaard *et al.,* 1993) and antibiotic use gives resistant strains the opportunity to proliferate and spread. Studies that examined antibiotic resistance following drug therapy at fish farms (Bjorklund *et al.,* 1990, 1991; McPhearson *et al.,* 1991; Nygaard *et al.,* 1992; Samuelsen *et al.,* 1992a; Spanggaard *et al.,* 1993; Ervik *et al.,* 1994; Kerry *et al.,* 1996a) and in microcosms (O'Reilley and Smith, 2001) shows an increased frequency of resistance to several drugs across a variety of bacterial species. However, Kapetanaki *et al.,* (1995) and Vaughan *et al.,* (1996) suggest that increased levels of bacterial drug resistance can arise independently of the presence of a drug (through sterile fish feed, sediments added to microcosm studies, uneaten fish food).

The emergence and spread of antimicrobial resistance poses a major challenge to the quality and cost of healthcare systems worldwide. Effective interventions are urgently needed to contain emerging resistance without these the problem will inevitably worsen, with dramatic human and financial consequences. The WHO Global Strategy for the Containment of Antimicrobial Resistance provides a practical framework and helps to prioritize those interventions that are likely to be most effective. The future containment of antimicrobial resistance requires a coordinated multidimensional approach in which effective change in antimicrobial usage; infection control and epidemiologically sound resistance surveillance are key concerns, which is to be addressed in future.

2.5. History of antibiotic resistance

There is evidence that although resistant microorganisms existed in nature before the use of antibiotics, such microorganisms were mostly absent from human flora (Hughes and Datta, 1983). However, in the intervening years, antibiotic resistant micro organisms have become frighteningly common. Almost as soon as antibiotics were discovered, researchers began to find microorganisms resistant to the new drugs. Even by 1909, when Ehrlich first began to study dyes and arsenicals, he found drug resistant trypanosomes. Resistant strains of *Staphylococcus aureus* in hospitals grew initially from less than 1% incidence, when penicillin first came into use, now resistance increases to 14% in 1946; to 38% in 1947, to more than 90% today (Greenwood, 2000). Worldwide, ampicillin and penicillin resistance can be found together in more than 80% of *S. aureus* strains. Within 30 years of their discovery, sulfonamides ceased to be an effective treatment for meningococcal disease (O'Brien, 1987). In the years since, reports of resistance have grown increasingly common and pathogens that are resistant to almost all antibiotics have been found. It has become painfully obvious that antibiotic resistance has reached a crisis stage and some clinicians have even forecasted that we are facing a return to the devastating diseases of the pre-antibiotic era (Hughes and Datta, 1983; Lipsitch *et al.*, 2002).

Antibiotics have been widely used in aquaculture worldwide to treat infections caused by a variety of bacterial pathogens of fish: *Aeromonas hydrophila, A. salmonicid*a, *Edwardsiella tarda, Pasteurella piscicida, Vibrio anguillarum,* and *Yersinia ruckeri.* Use of antimicrobial agents in aquaculture directly doses the environment, which results in selective pressures in the exposed ecosystem (Aarestrup, 1999 and 2000). The emergence of antimicrobial resistance following the use of antimicrobial agents in aquaculture has been identified in fish pathogens. In several countries *A. salmonicida* is frequently resistant to multiple drugs including sulphonamides, tetracycline, amoxycillin, trimethoprim, sulfadimethoxine and

quinolones (Aarestrup and McNicholas, 2002). Similar correlations between antimicrobial agents used in aquaculture and antimicrobial resistance are also reported among other fish pathogens (Akiba *et al.*, 1960)

During certain periods of the year, pathogenic *Vibrios* would endure adverse environmental conditions within aquaculture systems and when favorable environmental conditions are re-established, they are able to cause disease in wild animals (Ben-Haim *et al.*, 1997).The spread of antibiotic resistance from aquaculture settings to natural environments has recently been shown (Hameed and Balasubramanian, 2000; Liu, 1999). About 70% of the *Vibrios* isolated from aquaculture settings in Mexico were multiple-drug resistant. Several *Vibrio* isolates have also acquired resistance to the most commonly employed antibiotics (e.g., enrofloxacin, florfenicol, trimethoprim, and oxytetracycline) in shrimp rearing, suggesting that the recently initiated application of these antimicrobials has led to the generation of resistant strains of *Vibrios* (Molina Aja *et al.*,2002).

Use of antimicrobial agents in aquaculture also selects for antimicrobial resistance among bacteria that are not fish pathogens. Several studies have assessed the impact of use of antimicrobial agents in aquaculture on the bacteria in the sediment and within fish in the local environment. Bacteria resistant to antimicrobial agents used on specific fish farms have been isolated from sediment beneath the fish "net pens" on those fish farms (Allen *et al.*, 1977); in contrast, no resistance was present among bacteria from the intestinal contents of the fish from untreated areas (Allen *et al.*, 1977).

Many antibiotic resistance determinants in fish pathogens are frequently carried on transferable R plasmids. Horizontal spread of plasmids from fish pathogens may therefore transfer resistance genes to other bacteria including those that are pathogenic to humans. Horizontal transfer of resistance genes on plasmids has been

demonstrated between bacteria in the water of fish ponds(Anderson and Datta, 1965). and in marine sediments (Anderson and Sandaa, 1994). Plasmids carrying resistance determinants have also been transferred *in vitro* from fish pathogens to human pathogens including *Vibrio cholerae* (Angulo *et al.,* 2000). *Vibrio parahemolyticus (*Apgar *et al.,* 2005) and potential human pathogens including *Escherichia coli* (Armstrong *et al.,* 1990; Arvanitidou *et al.,* 2001).

Furthermore, plasmids carrying multiple antimicrobial resistance determinants have been transferred in simulated natural microenvironments between bacterial pathogens of fish, humans, and other animals, demonstrating that resistance determinants on plasmids can spread from fish pathogens to human pathogens (Arzese *et al.,* 2000). These studies indicate that the horizontal transfer of plasmids between related and diverse bacteria may facilitate dissemination of antimicrobial resistance determinants. Bacteria present in aquaculture systems may be transmitted to humans who come in contact with this ecosystem. For instance, *Vibrio* spp can cause wound infections in persons with open wounds or abrasions exposed to seawater or marine life (Ash, 2002). In 1991, an epidemic of *Vibrio cholerae* O1 infections affected Latin America; the epidemic strain in Latin America was susceptible to the 12 antimicrobial agents tested except in coastal Ecuador where the epidemic strain became multi drug-resistant (Ashkenazi *et al.,*2003).

The cholera epidemic in Ecuador began among persons working in shrimp farms. Multidrug-resistance was present in non-cholera *Vibrio* infections that were pathogenic to the shrimp. The resistance may have been transferred to *V. cholerae* 01 from other *Vibrios* (Ashkenazi, *et al.,* 2003). Bacteria from the aquaculture ecosystem may also been transmitted directly to humans through handling of fish. Recently, the fish pathogen *Streptococcus iniae* has caused invasive infections in persons who handled store-bought aquacultured tilapia and *S. iniae* was isolated from the aquaculture ecosystem and on fish in grocery stores (Bager *et al.,* 1999). Similarly a

new biotype of *Vibrio vulnificus* caused hundreds of serious infections among persons handling live tilapia produced by aquaculture in Israel (Baquero and Blazquez, 1997). Bacteria in fish also might have transmitted to humans when the cultured fish were eaten or when other foods, which got cross contaminated by bacteria from fish, were eaten. For instance, *V. parahaemolyticus* is a common food borne disease in Japan where infections have been linked to the consumption of aquacultured finfish (Baquero et al.,1991). Furthermore, *Salmonella spp.*, a common cause of food borne disease has been isolated from aquacultured fish and shrimp ponds(Bass, *et al.*, 1999). There were other reports, which indicate that bacteria present in aquaculture ecosystems can be transmitted to humans. Newly available molecular characterizations of antimicrobial resistance determinants provide further evidence of the transmission of antimicrobial resistance between aquaculture ecosystems and humans.

Some of the antimicrobial resistance determinants in *Salmonella* serotype *typhimurium* definitive type 104 may have originated in aquaculture. *S. typhimurium* DT 104 is typically resistant to ampicillin, chloramphenicol, florofenicol, streptomycin, sulfonamides and tetracycline. The strain was first isolated from a patient in 1985 and emerged during the 1990s as a leading cause of human *Salmonella* infections. Tetracycline resistance in *S. typhimurium* DT104 is due to a class G resistance gene (Baxter *et al.*,1998). Class G was first identified in 1981 in tetracycline-resistant isolates of *Vibrio anguillarum*, a pathogen of fish (Baya *et al.*, 1986). Furthermore, the recently described novel florofenicol resistance gene, *flo*R, in *S. typhimurium* DT 104, which also confers resistance to chloramphenicol, is almost identical, by molecular sequence, to the florofenicol resistance gene first described in *Photobacterium damsela*, bacteria found in fish. This resistance gene is rare and has not previously been reported from *Salmonella* isolates (Bayne *et al.*, 1983). Finally, all of the antimicrobial resistance determinants in *S. typhimurium* DT104 are grouped

on the chromosome within two distinct integrons and an intervening plasmid-derived sequence. The Class G and *floR* determinants are located within the intervening plasmid-derived sequence. By molecular sequence, the plasmid-derived sequence is closely related (94% identity) to a plasmid identified in *Pasteurella piscicid*a, a pathogen of fish (Bergh *et al.*, 1989). These and other reports indicate that antimicrobial resistance determinants selected for in aquaculture ecosystems can be transmitted to bacteria that cause illness in humans, perhaps at a greater frequency than, previously suggested (Blanch *et al.*, 2003).

With the expansion of fish culture in recent years, problems associated with bacterial fish pathogens have increasingly occurred. Concomitantly, a variety of important properties of microorganisms have been proven to be plasmid mediated. The use of antibiotics in the treatment of infectious diseases of fishes has resulted in the expansion of R plasmids in commercial aquaculture (Aoki *et al.*, 1977 and 1981; Chen, 1978; Watanabe, 1971), owing to the selective pressure exercised by chemotherapeutic agents when used over an extended period of time (Aoki, *et al.*, 1974;1975 and 1981). The presence of plasmids in bacterial fish pathogens may pose a potential public health hazard, since plasmids from animals may be transferred to humans either directly, by infection with pathogens such as *Aeromonas hydrophila* or *Edwardsiella tarda* (Jordan and Hadley, 1969; Joseph *et al.*, 1979), or indirectly, if they are transferred to human pathogens such as *Vibrio cholerae* or *Escherichia coli* by way of pathogenic fish bacteria (Aoki *et al.*, 1974 and 1977).

2.6. Plasmids

The term plasmid was originally used by Lederberg to describe extra chromosomal hereditary determinants and it is currently used to describe autonomously replicating extra chromosomal DNA of bacteria. They are found both in gram -negative and gram-positive bacteria as well as in some yeast and other fungi.

They range in size from 1 to more than 200 kb and are present in a wide variety of prokaryotic and eukaryotic organisms, but are mainly in bacteria. Plasmids are found in a variety of microorganisms and it is difficult to generalize about plasmids. Although most of them are covalently closed circular double stranded DNA molecules, some linear plasmids have also been recently isolated from bacteria. In general plasmids are not essential for the survival of bacteria, but they may nevertheless encode a wide variety of genetic determinants, which permit host bacteria to survive better in an adverse environment or to compete better with other organisms occupying the same ecological niche (Luis *et al.*, 1999).

The existence of plasmid was initially revealed as the "F factor" in *Escherichia coli* even before the double-helix structure of DNA was elucidated by Watson and Crick (Hayes, 1953; Lederberg, 1998). The occurrence of plasmids has been well documented among the majority of gram- negative and gram-positive isolates from the Eubacteria, and recently in a hyperthermophilic *Archaeon* (Erauso *et al.*, 1996).

The first linear plasmid was found in *Streptomyces rochei* in 1979 (Hayakawa *et al.*,1979) and now they have been detected in several bacterial genera such as *Agrobacterium*, *Borrelia*, *Nocardia*, *Rhodococcus*, *Thiobacillus*, and *Escherichia* (Hinnebusch and Tilly,1993). Most of the plasmid-encoded genes that have been characterized impart some growth advantages to the host. They are also responsible for nitrogen fixation in certain bacteria, resistance to heavy metals and radiation, production of certain endonucleases, metabolism of compounds such as toluene and camphor, plant tumor development, and also for the production of bacterial virulence determines. The genetic determinants encoded by plasmid enable the bacterial host to survive better in adverse environments and to compete better to occupy ecological niches. Examples of some typical plasmid-encoded traits include protection from UV light damage (Rochelle *et al.*, 1989), resistance to heavy metals

(Hansen *et al.,* 1984; Schutt, 1989), proliferation in the presence of antibiotics (Aviles *et al.,* 1993) and catabolism of xenobiotic compounds (Hada and Sizemore, 1981; Sayler *et al.,* 1990).

2.7. Role of plasmids

Plasmids display an amazing diversity of characteristics, such as size, modes of replication and transmission, host range, and the presence of variety of genes. They have adopted a variety of strategies to ensure their own faithful replication, maintenance, and transfer. They impart a wide assortment of phenotypes to the cells that harbor them. From a human perspective, some phenotypes may be problematic, for example, the expression of antibiotic resistance and expression of antibiotic resistance genes and pathogenicity genes that hinder human and animal health. Other properties may be beneficial, such as the ability to fix elemental nitrogen or features that can be exploited and used to improve soil fertility. It is the rich diversity of their form, function, and utility that can be explored in plasmid biology.

An obvious way of classifying plasmids was by function. There are five main types

- Fertility plasmids, which contain only *tra* genes. Their only function is to initiate conjugation
- Resistance plasmids, which contain genes that can build a resistance against antibiotics or poisons.
- Col plasmids, which contain genes that code for the production of colicins, proteins etc that can kill other bacteria.
- Degradative plasmids which enable digestion of unusual substance toluene or salicylic acid
- Virulence plasmids that turn the bacterium into a pathogen.

Plasmids may also be categorized into one or two major types: conjugative or non conjugative, depending on whether or not they carry a set of transfer genes, called the *tra* genes that promote bacterial conjugation. Generally conjugative plasmids are of high molecular weight and low copy number i.e. they are present in one to three copies per chromosome where as non conjugative plasmids are of low molecular weight and are high copy number i.e. present as multiple copies per chromosome (Willetts and Wilkins, 1984)

Plasmids can also be categorized on the basis of their being maintained as multiple copies (relaxed plasmids) or as limited copies (stringent plasmids) per cell. Frequently, plasmids contain some genes advantageous to the bacterial host. Plasmids which do not have any phenotypic traits ascribed to them are called cryptic plasmids. The number of copies also varies among plasmids, and bacterial cells can harbor two different types of plasmids, with hundreds of copies of one plasmid type and only one copy of other type. Unlike chromosomes, plasmids generally encode genes whose functions benefit the bacterium under certain specific circumstances (Tolmasky *et al.*, 1992).

2.8. Role of Plasmids in resistance

It took several decades to appreciate the existence of plasmid-mediated antibiotic resistance. The key to identifying plasmids as resistance factors was their property of providing simultaneous protection to multiple antibiotics of inherently different nature. But in hindsight, resistance based on plasmids as well as chromosomally based resistance, indubitably was an important mechanism early after the antibiotics were introduced and used commercially. Plasmids bearing antibiotic resistance markers can be found in organisms isolated before the antibiotic era (Falkow, 1975). Resistance based on transmissible plasmids affords the significant advantage of flexibility to the microorganism. Resistance to several antibiotics can be

brought together in single plasmids. Antibiotic resistance genes can be amplified when needed and deamplified when not needed. Plasmids can be stored in a minimum portion of the microbial population and regained as needed. Plasmids can serve as vectors to transfer genes. Plasmids serve an evolutionary role in the rearrangement of genetic parts both between and within organisms

However, gene transfer does little to account for the resistance characters themselves (Bennett and Richmond, 1978). But plasmid-borne resistance has the disadvantage to the bacterium that it cannot easily function to alter the target of the antibiotic within the cell, since the structural genes for the cell's essential proteins in almost all cases are carried on the chromosome. In gram-negative organisms, much less enzyme suffices to detoxify penicillin or cephalosporin because the enzyme is retained within the periplasm itself. *Escherichia coli* strain K-12 contains a gene that codes for a penicillinase, but this gene does not afford much protection, suggesting that the enzyme really has another unknown function, and therefore highly possible that it does function to protect against penicillin (Boman *et al.*, 1967; Burman *et al.*, 1973). One possibility is that at low levels of penicillin and high cell densities, it protects against penicillin in the amounts that may occur naturally. The second possibility is that this gene protects the strain against high levels of penicillin only after it has become tandemly duplicated. Still, gene amplification may be needed to protect the cell. Besides the gram-negative envelope structure, there are other aspects of bacterial biology that serve to increase the effectiveness of plasmid borne resistance mechanisms. For example, the derivatives of amino glycoside antibiotics produced by resistance mechanisms are such that they interfere with the transport of the unmodified drug (Demerec, 1948).

2.9. Role of mechanisms facilitating genetic rearrangements within an organism

In earlier paper (Koch, 1972), it was reported that the mobilization of genetic material would greatly slow the fundamental rate of evolution by supplying alternate but preexisting mechanisms to overcome a particular growth limitation rather than forcing a new solution to be sought. Some of these mechanisms lead to the mutation and therefore also faster *de novo* evolution provided that the genetic burden is not high. Early evolution could have taken place *via* point mutations and by chance through primitive replications, but mechanisms allowing duplication of more than several base pairs and rearrangement of segments of DNA may have involved biological evolved mechanisms, such as insertion sequences, at a very primitive stage. Numerous studies have demonstrated that genetic exchange by conjugation as well as transduction and transformation occurs between bacteria in the environment (O'Morchoe *et al.,* 1988; Ogunseitan *et al.,* 1990; Paul *et al.,* 1991; Kinkle *et al.,* 1993). All the three transfer mechanisms have been shown to occur in marine systems (Maruyama *et al.,* 1993; Barkay *et al.,* 1995).

2.10 Molecular properties of R plasmids

One of the features that keep plasmids at the forefront of microbiology is their ability to carry and transmit genes encoding resistance to antimicrobial compounds. This type of plasmids is widespread in bacteria and can be transferred between different microorganisms, a genetic property that represents a very serious medical problem in human and animal medicine. R plasmids can be isolated from host bacteria as circular DNA in both closed and nicked forms. There has been some debate as to the proportions of closed and nicked circles that arise during the isolation procedures. Nevertheless there is little doubt that both forms exist in the cell. The closed circular structure is probably adopted by R plasmids when not in replication. The R plasmid

sometimes may dissociate into its conjugative and resistance determinants (Franklin and Snow, 1989).

The numbers of R plasmids harbored by individual bacteria is influenced by the properties of the plasmid and host as well as by the culture conditions. As a general rule that larger plasmids are present only in a limited number of copies per chromosome in *E.coli,* whereas in *Proteus mirabilis* the number varies during the growth cycle (Franklin and Snow, 1989). In stationary phase, replication of the R plasmid continues for some times after chromosomal replication has ceased. Conditions that give rise to an increased number of R plasmid copies are sometimes associated with enhanced resistance. However the level of resistance does not always reflect the number of resistance gene copies in the cell (Franklin and Snow, 1989).

In general, plasmids are not essential for the survival of bacteria, but nevertheless encode a wide variety of genetic determinants, which may confer on their bacterial hosts, better prospects of survival in an adverse environment or to compete better with other microorganisms occupying the same ecological niche. Resistance plasmids (R-Plasmids) harbor a variety of genes encoding resistance to a wide spectrum of antimicrobial compounds, which include antibiotics, heavy metals, resistance to mutagenic agents like ethidium bromide, and even disinfectant agents such as formaldehyde (Foster, 1983). The medical importance of plasmids that encode for antibiotic resistance, as well as specific virulence traits has been well documented and demonstrated the important role that the bacterial genetic elements play in nature (Luis *et al.,* 1999). Although they encode specific molecules required for initiation of their replication, plasmids rely on host-encoded factors for their replication. Plasmid replication initiates in a predetermined *cis*-site called *ori* and can proceed either by a rolling circle or a theta replication mechanism. Chromosomal replication origin, (ori C) was isolated from plasmid of *V. harveyi.* The ori C was found to be functional in *E. coli (*Zyskind *et al.,* 1983).

Molecular and genetic work on plasmids resulted in extraordinary contributions to the modern fields of molecular genetics and molecular biology (Cohen, 1993). Molecular and genetic analysis of bacterial plasmids led to basic concepts such as "the operon" and "the replicon", and has provided essential information on DNA conjugation and fertility, control of gene expression, gene transfer and genetic recombination, and transposable elements. Studies of essential plasmid functions have resulted in important findings about basic aspects of initiation of DNA replication and its regulation, DNA partitioning, and plasmid copy number and incompatibility (Helsinki, 1996). In pathogenic microorganisms, plasmids that contribute directly to microbial pathogenicity in plants and animals, such as for instance iron transport in several pathogens or the presence of adhesins, invasins or antiphagocytic proteins, is well documented (Crossa *et al.*, 1989). In a more applied vein, plasmids played a central role in the initial development of recombinant DNA technology, gene cloning, and the constant evolution of molecular biology (Cohen, 1993). Furthermore, genetic and molecular analysis of plasmids proved to be essential in understanding the structure of transposons and integrons and the role these genetic elements play in the transmission of resistance to antimicrobial agents (Hall, 1995). The presence of these mobile genetic elements in transmissible plasmids, some of them capable of replicating in bacterial strains belonging to different species, makes matters quite serious since they contribute to the transmissibility of resistance genes from strains to strains as well as between different replicons within any given strain (Luis *et al.*, 1999).

2.11. Other roles of plasmids

Virulence factors of certain bacterial pathogens are encoded by the plasmids. The correlation between enhanced virulence and presence of a 50 M Da plasmid in *V.anguillarum* was reported (Crossa *et al.*, 1977). Plasmid plays an important role in adaptation of *Pseudomonas* spp to chronic petroleum pollution. 50% of the isolated oil

degrading bacteria from oil spills on industrial bay and off shore oil field and grown on liquid enriched media of crude oil and poly nuclear aromatic hydrocarbons had multiple plasmids (Deverix *et al.,* 1982).

Marine bacteria are able to survive in polluted environment due to the presence of self-transmissible plasmids as they transfer plasmid DNA coding for ecologically advantageous functions such as detoxification of heavy metals, oxidation of manganese, etc. Heterotrophic manganese oxidizing bacteria lost the capacity along with the loss of plasmids when maintained in laboratory (Gregory and Stanley, 1982).

Plasmids can be used for the transformation of bacteria as vectors (Hackett and Sarma, 1989). Studies on plasmids in wastewater bacteria showed that most of them had multiple plasmids 2 to 4 and have direct correlation between the number of plasmids and crude oil degradation by bacteria. (Flood gate, 1991). Plasmids in *V.parahaemolyticus* controls biodegradation, polymyxine resistance and low-level halophilism like characteristics (Chakraborty *et al.,* 1994). A plasmid measuring 70-100 kb was isolated from *Pseudomonas capcia* G4 (Shields *et al.,*1995). The plasmid may also affect the temperature tolerance in acido thermophilic *Archaebacterium thermoplasma* (Yasuda *et al..,*1995). But there is little effect of plasmids on production of siderophore by the *Aeromonas hydrophila* strains obtained from diarroheal samples of human patients and fresh water ponds (Naidu ,1997).

In the isolates of *Aeromonas hydrophila* collected from shellfish and water, it was shown that 60% of strains simultaneously possessed the plasmids and haemolytic activity (Borrego *et al.,* 1991). It was observed that *A. salmonicida* harbored 17 plasmids in size ranging from 12 –90 kD which encodes various proteins (Bell and Trust, 1989). Virulence plasmid of *V.anguillarum* is known to mediate a restriction system that prevents the conjugal transmission of plasmid DNA from *E.coli* donor to *V.anguillarum* (Singer *et al.,*1992). Plasmids of *V. fisheri* carry genes that are

important for the survival of these strains outside the squid symbiont (Boettcher and Ruby, 1994).

2.12. Plasmid profile

Various workers have studied the plasmid profiles of a number of bacterial species earlier. Many of studies have covered the different strains within species isolated from different locations. Plasmid profile of five fish pathogens- *A. salmonicida, A. hydrophila, Vibrio anguillarum, Pasteurella piscicida, Yersinia ruckeri, Edwardsiella tarda* and *Renibacterium salmoinarum*, showed that 75% of the strains were found to harbor one or more plasmids, with the majority of strains having multiple plasmids (Toranzo *et al.*,1983). The study also showed that some of the strains of *A.salmonicida* harbored six plasmids, *P. piscicida* were with three plasmids and *A. hydrophila* strains harbored a single plasmid having a molecular weight of 20 to 30 kb. In this study the highest molecular weight plasmids of 145 and 130 kb were detected in *V. anguillarum* (Toranzo *et al.*,1983). Plasmids from halophilic bacteria were shown to have varying molecular sizes ranging 6.4 kb to 8.75 kb (Hong, 1985). In *Edwardsiella ictaluri*, the causative agent of enteric septicemia of Channel catfish, two plasmids of 5.7 kb and 4.9 kb were reported (Lobb and Rhoades, 1987). Strains of *A. salmonicida* the agent of furunculosis also have fairly uniform plasmid pattern. The pattern consists of four small plasmids of 4.2, 3.6, 3.5 and 3.3 M Da and a larger plasmid. The larger plasmid was most often 50-56 M Da, but sometimes it was even larger. An additional plasmid was seen in a few species (Bast *et al.*, 1988). A similar plasmid profile was also revealed in the *A. salmonicida* (Toranzo *et al.*, 1991). However, Bell and Trust (1989) observed 2- 9 plasmids in strains of *A. salmonicida*. Thermophilic bacteria have been reported to harbour plasmids; 6 M Da and 47 M Da were isolated from *Thermus thermophilus* (Fee and Mathew,1988). A plasmid pTAI of 15.2kb was reported in isolates of an acidothermophilic bacterium archaebacterium, *Thermoplasma acidophilum* (Yasuda *et al.*, 1995). Zhao and Aoki (1992) reported

that *Pasteurella piscida* isolated from *S. quinqueradiata* had one large plasmid of 110 kb and two small plasmids of a 5 and 5.1 kb which were shared by all strains. However, Margarinos *et al.*, (1992) found common plasmid band of 20 and 7 M Da in all *Pasteurella piscicida* studied. But the European strains were found to have an additional 50 M Da plasmid. Jain and Tiwari (1993) screened the plasmid pattern of 32 isolates of *Shigella dysenteriae* from different parts of India and reported that all the strains had at least five plasmids of following sizes viz 120 M Da, 57 M Da, 10.5 M Da, 6.5 M Da and 2.5 M Da. Maximum numbers of plasmids seen were eight while minimum numbers of plasmids present were five. Studies carried out by the Pederson *et al.*, (1996) revealed that *Aeromonas salmonicida* strains have 2 to 3 plasmids and all of them share a common small sized plasmid. The strains of *A. salmonicida* from Atlantic coast was found to posses 4 to 6 plasmids within the size range of 4.3 to 8.1 kb and while the strains from Pacific coast possessed 6 plasmids in the range of 14.2 to 0.9 kb. In strains of Aeromonas, 36% had plasmids and the most plasmid containing strains with multiple were less than 12 kb in size (Pettibone *et al.*, 1996). But plasmids were observed only in two hundred ninety seven isolates out of the more than thousand aerobic hydrophilic bacteria isolated from coastal California marine sediments.

While majority of the isolates typically contained one large plasmid of 40 to 100 kb size, some contained multiple small plasmids, three to five numbers with 5 to 10 kb size (Sobecky *et al.*, 1997). *Vibrio salmonicida* isolated from Cod and Atlantic salmon have 61, 21, 3.4 and 2.8 M Da plasmids and a 61 M Da plasmid were found exclusively in *V. salmonicida* strains of Northern Norway (Amaru *et al.*,1998). Twenty-five *A. hydrophila* strains isolated from fresh water fish and water samples were screened for the presence of plasmids (Noterdaeme *et al.*,1991). Ten strains were found without plasmids while eleven harbored one plasmid of 20 kb and four strains contained two or three plasmids. The 20 kb plasmid was common to all plasmid

positive strains. Borrego *et al.*, (1991) studied the plasmid profile of sixty strains *A. hydrophila* isolated from shell fish and water and found that forty strains harbored one or more plasmids. The plasmid profile most frequently detected (15%) was the association of three small plasmids of 4.2, 3.2 and 2.8 M Da. Thirty four *A. hydrophila* strains isolated from various fish species and several geographical locations were examined (Ansari *et al.*,1992) for their plasmid carriage and reported that the plasmid occurrence rate was only 14.7 % with size range from 2.6 to 6 M Da. Multiple carriage of plasmid is more likely with strains having three or four plasmids.

2.13.Importance of plasmid profiling

Plasmid profiling can be used in the characterization and identification of bacteria. Plasmid profiles can be taken as a fingerprint in identifying the bacteria. Reud and Bayle (1989) used the plasmid profiling, since it is simple, easy to perform and useful to identify *Edwardsiella ictaluri* that causes enteric septicemia in channel catfish. According to Lobb and Rhoades (1987) restriction profiles of the separated plasmids reveal whether the plasmids are closely related or nor. Typing based on the plasmid profiling appears to be the most effective method for grouping strains with the same serotype obtained from clinical source and from environmental sources (Borrego *et al.*, 1991).

In recent years, a molecular-based approach, referred to as replicon typing, has been used to assign plasmids to incompatibility groups using specific DNA probes containing replication control genes from well characterized plasmids (Couturier *et al.*, 1988).The primary sources of the majority of these well-characterized plasmids have been bacteria from clinical and animal origins. This more direct and less time consuming method for classifying plasmids is possible due to the nature of the basic replicon of plasmids. The basic or minimal replicon of a plasmid consists of the genes and sites necessary to ensure and control autonomous replication. The genes essential

for plasmid replication and maintenance are typically clustered on a contiguous segment of DNA usually not more than 2–3 kb in size (Helinski *et al.,* 1996). It is this compact nature of plasmid replication origins that has facilitated the isolation and characterization of replicons from plasmids obtained from bacteria of clinical and animal origins. The bank of replicon probes developed by Couturier *et al.,* (1988) contain unique DNA sequences derived from 19 different basic replicons cloned in high copy number plasmid vectors. These collections of replicon (inc/rep) probes have been shown to be suitable for the molecular typing of plasmids from bacteria of medical importance. Interestingly, recent studies that have attempted to use these clinically based replicon probes to type plasmids from bacterial isolates obtained from terrestrial soils (Kobayashi and Bailey, 1994), as well as sediments (Sobecky *et al.,* 1997), bulk water, air water interfaces and biofilms of marine environments have been unsuccessful (Dahlberg *et al.,* 1997). None of the hundreds of plasmid containing isolates from these different environments shared homology to the *inc/rep* group-specific DNA probes currently available for plasmid typing. Such findings indicate that plasmids isolated from bacterial populations occurring in terrestrial soils and marine aquatic and sediment systems encode novel replication and incompatibility loci that lack homology to clinically- derived plasmid incompatibility groups. Moreover, the extent of plasmid diversity occurring in natural microbial communities, such as marine sediments, cannot be determined using the present molecular classification system based on plasmids of clinical and animal origins. Therefore, *inc/rep* probes specific for replicons isolated from the marine environment are necessary to characterize naturally occurring plasmid distribution and diversity (Sobecky *et al.,* 1997).

To better understand plasmid distribution, diversity and abundance in marine microbial communities, the isolation and characterization of replication sequences from naturally occurring plasmid populations is necessary. Ideally, such information could be used to develop a collection of environmentally based

incompatibility group specific replicon probes suitable for typing plasmids from non-clinical environments. An increasing body of literature, based largely on the analysis of plasmids from culturable bacteria from diverse environments, supports the existence of new plasmid groups which appear to have evolved along separate lines from plasmid groups occurring in clinical bacterial populations (Kobayashi and Bailey, 1994;Top *et al.*, 1994;Dahlberg *et al.*1997; Sobecky *et al.*, 1997;Van Elsas *et al.*, 1998). Therefore, studies designed to isolate and characterize plasmid replication and incompatibility sequences from environmental isolates will aid in the determination of plasmid diversity, as well as to provide more detailed insights into gene movement in microbial communities. The basic plasmid characterization study may further lead to the development of such replication probes that will help to identify the environmental isolates itself rather than from available clinical probes.

Wilk (1989) reported that plasmid profile along with serological and biological properties is helpful in isolating *V. anguillarum* strains from diseased fish. He classified the *V. salmonicida* using the same technique. Zhao and Aoki (1992) reported that plasmid profile could be used as a fingerprint of bacteria. They found that a plasmid of 5.1 kb size is specific to *Pasteurella piscida*, which causes influenza. Austin and Adams (1995) are of the opinion that plasmid profiling can be used as a rapid diagnostic technique for detection of furunculosis caused by Aeromonas salmonicida. However Dahlberg *et al.*,(1997) observed the plasmid types isolated from different habitats and from different sampling occasions showed little similarity indicating high variation.

2.14. Plasmids in *Vibrios*.

In *Vibrio*, plasmids have been found and their involvement in resistance to many antibiotics has been proven (Toranzo *et al.*, 1983).Antibiotic resistance

plasmids have been reported in strains of *Vibrio* isolated from cultured marine fish(Aoki *et al.,* 1973).Cryptic plasmids have been isolated from enteropathogenic strains of *Vibrio parahaemolyticus* (Guerry and Colwell, 1977*)*. Sizemore has observed a bacteriocinogenic plasmid in *Vibrio harveyi* (Mc Call and Sizemore, 1979). The plasmid presence in the Danish isolates of *Vibrio anguillarum* with phenotypic properties of haemagglutination, and biochemical activities was also reported (Larsen,1991). A 50 M Da plasmid pJM1 was present in avirulent strains encodes a very efficient iron sequestering mechanism that helps to compete with iron binding proteins in serum (Crossa *et al.,* 1980). It is to be observed that plasmid-carrying bacteria could grow under conditions of iron limitations. Non-virulent bacteria without plasmids were unable to produce siderophore activity, which is plasmid mediated nature (Tolmasky, 1985). A plasmid from *V cholerae* strain isolated in Malaysia encoding the CT toxin was detected. (Mohammad and Haque, 2002).

The complete sequence analysis of pPS41 from *Vibrio splendidus* showed that this plasmid could be mobilized by RK2 transfer system (Leigh *et al.*, 2000). The role of plasmids in the degradation of hydrocarbons was reported. *Vibrio parahaemolyticus* possessed a degradative plasmid, which helped in the break down many aromatic compounds. The halophilic characteristic of microbe has also been linked to a plasmid (Chakraborty, 1994). In the non-marine environments where there is low concentration of sodium chloride, the plasmids were acquired by the *Vibrios* and retained them by due to selection pressure. As soon as the *Vibrio parahaemolyticus* return to marine environment, the plasmid may be lost because of the lack of selection pressure of halophilism. But in some clinical isolates of *Vibrios*, a plasmid determined the growth of those isolates in less concentration of sodium chloride (Chakraborty, 1994). *Vibrio anguillarum* isolated from diseased trout carried a 67 kb plasmid similar to plasmid pJM, the virulence plasmid and also a 98 kb

cryptic plasmid. Larsen and John 1991 studied certain strains and observed that 42% isolates from healthy fish and from environment were without plasmids. Environmental authorities and fish farmers are naturally concerned about the increasing spread of pathogenic bacteria in the aquatic environment and hence the plasmid profiling should be used as an epidemiological trait in such cases

The antibiotic susceptibility differences, plasmid content and RAPD analysis of *Vibrio vulnificus* isolated from *Anandra granosa* (cockles) in Malaysia however yielded no plasmid profile predictive of particular antibiotic susceptibility. A non-conjugative plasmid of 2 M Da was observed during the characterization of *Vibrio cholerae* O139 Bengal isolated from sewage drainage water in Malaysia. Although strains contain plasmids, they were not genetically identical. The genetic diversity within the group demonstrated that the isolates were isolated from a single location, has almost genetic diversity among them and finally they found out that isolates appeared to be heterogeneous. They also studied the ability to transfer resistance is a potentially serious health hazard not only because of consequent problems regarding therapy but also because of risk of resistance spreading to other enter bacterial organisms including normal flora. Further the transmissibility of antimicrobial resistance may assist in establishment of persistence of organism in the host. (Son *et al.*,1997).

A plasmid encoding histidine decarboxylase gene *angH* that is essential for biosynthesis of siderophore Anguibactin was also reported from the fish pathogen *Vibrio anguillarum* (Courtney *et al.*, 1998). The fatal hemorrhagic septicemic disease in salmonids and other fish is caused by *Vibrio anguillarum*. The pathogenic strains survive within the host due to the possession of a 65 kbp virulence plasmid, which provides the bacteria with an iron sequestering system that is crucial. This confers upon them the synthesis of the siderophore Anguibactin, an iron scavenging compound and subsequent transport of ferric Anguibactin complex in the cell.

Anguibactin synthesis requires expression of gene from chromosome and the virulent plasmid p JM1.

The plasmid profiles of fish pathogenic isolates such as *Aeromonas* and *Vibrio ordalii* from Canada revealed that the resistance to oxytetracycline and streptomycin do not appear to be plasmid mediated. In this the antibiotic resistance was altered following plasmid curing and resistance was not transferable to *E.coli.* (Giles *et al.,* 1995). *Vibrio ordalii is* one of major causes of *Vibrios*is in wild and cultured marine salmonids in Japan and the USA (Actis *et al.,* 1985). The molecular characterization of different isolates of *Vibrio ordalii* showed that they contain a plasmid, designated as pMJ101. The pathogenic *Vibrios* isolated from moribund silver sea bream in fish farms in Hong Kong were screened for plasmids and they describe the antibiotic resistance associated plasmids. The different *Vibrio* strains studied had similar antibiotic resistant profiles (Liu *et al.,* 1999). It is reported that the antibiotic susceptibility profiles for the different *Vibrio* species in clinical and environmental setting and observed 29% bacteria gave large plasmid with molecular weights ranging from 9-123 kb. Bacterial resistance is usually associated with the presence of plasmids and the ability of plasmids for trans conjugation. But in general, plasmids, which can be trans conjugated usually, possess a high molecular weight (French *et al.,* 1989).

Vibrio ordalii is a major cause of *Vibrios*is in wild and cultured marine salmonids in Japan and carries pMJ101, a 30 kb cryptic plasmid that replicates in the absence of DNA polymerase I without producing single-stranded intermediates. *V. ordalii* is phenotypically and genetically distinct from *V. anguillarum* 775 (Schiewe *et al.,*1981), which also causes disseminated infections in salmonids (Schiewe *et al.,* 1981). The molecular characterization of different isolates of *V. ordalii* revealed the presence of a high-copy-number plasmid in all strains examined to date (Schiewe *et al.,* 1981). This plasmid, which was designated pMJ101, is a 30-kb extra

chromosomal element that has no DNA sequence homology with the pJM1 virulence plasmid present in *V. anguillarum* 775 (Crossa *et al.*, 1980). This plasmid is essential for the virulence of *V. anguillarum* and encodes a high-affinity siderophore-mediated iron acquisition system that allows this fish pathogen to acquire this essential micronutrient from the infected host (Crossa, 1989).

2.15. PCR based molecular characterization of microbes

PCR is a technique for the *in vitro* amplification of DNA, which lies between the regions of known sequence. This technology has proven to be a revolutionary method, which gives scientist the great advantage of generating a large number of target DNA sequences from trace amounts of DNA material. Since its introduction was first reported by Saiki (Saiki *et al.*, 1985). PCR has already become a wide spread technique in research laboratories.

2.16. Horizontal gene transfer

The horizontal transfer of genetic material within microbial communities has been instrumental in the emergence of novel functions and species (De la Cruz and Davies, 2000; Ochman *et al.*, 2000). The 'antibiotic resistance phenomenon' is perhaps the most striking recent example of the impact of horizontal transfer on microbial adaptation. This phenomenon refers to the rapid and widespread emergence of similar antibiotic resistance profiles among phylogenetically diverse gram negative clinical isolates over the last half-century (Davies, 1997).

Antibiotic resistance is the best-known example of rapid adaptation of bacteria to a new ecosystem. The ability of bacteria to expand their ecological niche, also in the presence of certain antibiotics, can be explained by the acquisition of resistance genes by horizontal gene transfer and/or by the accumulation of point mutations leading to the modification of existing genes. Several studies on bacterial pathogens of human and animal origin concluded that multiple antibiotic resistance is

a consequence of horizontal gene transfer (Sundstrom, 1998; Trieu and Courvalin, 1986). The principal mechanisms facilitating horizontal gene transfer among bacteria are transformation, transduction and conjugation.

Conjugation is the most frequently recognized mechanism for horizontal gene transfer. This mechanism exists in a wide variety of bacterial species and genera. In this process, mobilizable DNA molecules (plasmids, episomes, conjugative transposons) can be transferred from a donor to a recipient cell, via a contact-dependent transmission. The self-transmissible conjugative F-plasmid of *Escherichia coli* is the best-known example of an autonomously replicating molecule, which encodes all necessary factors required for conjugation. Some non-self-transmissible plasmids can also be mobilized *in trans* by an associated self-transmissible plasmid, which is not normally transferred at the same time. Conjugation can also mediate chromosomal exchange following the integration of a self-transmissible plasmid into the bacterial chromosome.

The conjugation involves physical contact between donor and recipient cells and can mediate the transfer of genetic material between domains (for example, between bacteria and plants, and between bacteria and yeast) (Buchanan *et al.*, 1987; Heinemann and Sparge, 1989). Typically, DNA is transferred from a donor to a recipient strain by either a self-transmissible or mobilizable plasmid.

The intercellular spread of the genetic determinants of resistance to antimicrobial agents is facilitated by mobile genetic elements, such as conjugative plasmids and conjugative transposons. Plasmids or transposons borne integrons are a key player in this process, being able to acquire, rearrange, and express genes, in this case, those conferring antibiotic resistance (Stokes and Hall, 1989). Whether integrons are located on a plasmid or chromosome, their structure and function are similar (Michael *et al.*, 2005).

Naturally occurring gene expression elements, called *integrons*, have been described as a very efficient genetic mechanism by which bacteria can acquire resistance genes (Hall and Collis, 1995; Martinez and De la Cruz, 1990; Stokes and Hall, 1989). Integrons promote the capture of one or more gene cassettes within the same attachment site, thereby forming composite clusters of antibiotic resistance genes. Over the past few years, the analysis of many antibiotic resistance genes identified in clinical and veterinary isolates of gram-negative organisms (particularly *Enterobacteriaceae*) established the importance of integrons in the dissemination of resistance among bacterial pathogens from different geographical origins (Falbo *et al.*, 1999).

Horizontal transfer of antibiotic resistance genes provides a potentially saving ecological impact on any bacterial population exposed to an antibiotic treatment. However, the transferred resistance gene must be expressed in a manner that benefits the recipient microorganism. Many recent investigations on the molecular basis for antibiotic resistance have highlighted the link between resistance determinants embedded in units of DNA called *integrons* and broad-host range plasmids. This novel class of specialized DNA elements was initially described mainly from comparisons of the DNA sequence surrounding different antibiotic resistance genes found in naturally occurring gram negative bacteria. Early attempts to describe integrons suggested that they consisted of two conserved regions flanking a variable region containing one resistance gene or more (Stokes and Hall, 1989).

Horizontal transfer of resistance genes is a successful mechanism for the transmission and dissemination of multiple drug resistance among bacterial pathogens. The impact of horizontally transmitted genetic determinants in the evolution of resistance is particularly evident when resistance genes are physically associated in clusters and transferred to the recipient cell. Recent advances in the

molecular characterization of antibiotic resistance mechanisms have highlighted the existence of genetic structures, called *integrons*, involved in the acquisition of resistance genes. These DNA elements have frequently been reported in multi-drug resistant strains isolated from animals and humans, and are located either on the bacterial chromosome or on broad-host-range plasmids. The role of integrons in the development of multiple resistances relies on their unique capacity to cluster and express drug resistance genes. Moreover, the spread of resistance genes among different replicons and their exchange between plasmid and bacterial chromosome are facilitated by the integration of integrons into transposable elements. The associations of a highly efficient gene capture and expression system, together with the capacity for vertical and horizontal transmission of resistance genes represents a powerful weapon used by bacteria to combat the assault of antibiotics (Falbo *et al.*, 1999).

The capture and spread of antibiotic resistance determinants by integrons underlies the rapid evolution of multiple antibiotic resistances among diverse Gram negative clinical isolates. The association of multiple resistance integrons (MRIs) with mobile DNA elements facilitates their transit across phylogenetic boundaries and augments the potential impact of integrons on bacterial evolution (Dean *et al.*, 2002).

2.17. Integrons and Sulfamethaxazole Trimethoprim element (SXT) as mobile elements for antibiotic resistance

After the introduction of antibiotics in the treatment of infectious diseases, antibiotic resistance has spread dramatically among microbes. The occurrence of drug resistant strains of *Vibrio cholerae* has been reported in India with increasing frequency (Bag *et al.*, 1998). Spread of antibiotic resistance in microbes has been attributed to the mobilization of drug-resistance markers by a variety of agents (e.g.,

plasmids, transposons, and integrons).

Integrons are DNA elements capable of mobilizing individual gene cassettes into bacterial chromosomes by site-specific recombination. Integrons consist of a central variable region that often harbors antibiotic-resistance gene cassettes, flanked by 5′ and 3′ conserved sequences(CS). Integrons have been categorized into four different classes on the basis of the distinctive integrase (*int*) genes they carry on their 5′-CS (Recchia and Hall,1995). Among the different integron families, class I integrons are found to be most prevalent in drug-resistant bacteria. Class I integrons have been detected in *V. cholerae* O1 strains isolated in Vietnam, Thailand, and Italy (Dalsgaard *et al.,* 2000; Falbo *et al.,* 1999). Amita *et al.,* 2003, however, have previously reported the presence of integrons in *V. cholerae* O1 strains isolated in India. The indiscriminate usage of antibiotics in human medicine and animal husbandry promotes the spread of multiple antibiotic resistance.

SXT is representative of a family of conjugative transposon like mobile genetic elements that encode multiple antibiotic resistance genes. The term conjugative transposon (CTn) encompasses a diverse and growing group of mobile genetic elements. Chromosomal integration by CTns is mediated by recombinases of either the integrase (Salyers *et al.*,1995; Scott and Churchwood,1995) or resolvase family (Wang and Mullani, 2000). In the laboratory, SXT is transmissible by conjugation to a variety of gram-negative organisms, and it can mediate the transfer of certain mobilizable plasmids, as well as chromosomal DNA, in an Hfr-like fashion. This integrase is required for SXT transfer but not for SXT-dependent transfer of mobilizable plasmids or chromosomal DNA (Hochut *et al.,* 2000).

Chapter 3

MATERIALS AND METHODS

3.1. Samples and their Source

Vibrios were isolated from water, crustacean, molluscans and plankton. Water and plankton samples were collected from different coastal environments of Kerala and from certain aquaculture farms. Crustacean, and molluscan samples were collected from market and used in this study.

3.2. Isolation and Identification of *Vibrios*

3.2.1. Collection of water samples

Water samples were collected from different coastal areas and two aquaculture farms. Surface water samples were collected in sterile polythene bags and transported aseptically to the laboratory within 2- 6 h.

3.2.2. Isolation of *Vibrio* strains from water

Water samples were serially diluted using physiological saline and 100µl was used as inoculum. The medium used for isolation and purification of isolates from all samples was Marine Zobells agar (Hi Media) unless otherwise specified. The plates were incubated overnight at 37^0C. Single cell colonies were isolated and purified in Zobells agar plates, sub cultured on Zobells agar slants and stocked.

3.2.3. Isolation of *Vibrios* from molluscans

Green Mussel (*Perna viridis*) and cuttle fish (Sepia spp.) samples collected from the market were used for the study. The tissue samples were homogenized with physiological saline. 10 g tissue was weighed in a beaker, transferred aseptically to a sterile mortar, ground well with a pestle, and mixed with 90 ml of physiological saline. After thorough mixing, the homogenate was centrifuged and 1 ml of the supernatant was used for serial dilution. Plating was done employing spread plate technique onto TCBS medium, using 0.1 ml of the prepared serial dilution, and the plates were incubated at 30^0C for 24 h. The single cell colonies from the plates were isolated, purified on Zobells agar, sub- cultured on Zobells slants, and stocked until further use.

3.2.4. Isolation of *Vibrios* from crustaceans

Penaeus indicus samples were used as source of *Vibrio*. 10 g tissue of the prawn was weighed and transferred aseptically to a sterile mortar, ground well with a pestle and mixed with 90 ml physiological saline. After thorough mixing, the homogenate was centrifuged and 1 ml of the supernatant was used for serial dilution. Plating was done employing spread plate technique onto TCBS agar, using 0.1 ml of the prepared serial dilution, and the plates were incubated at 30^0C for 24 h. The single cell colonies from the plates were isolated, purified on Zobells agar, sub-cultured on Zobells slants, and stocked until further use.

3.2.5. Collection of plankton samples.

Plankton were collected from Cochin estuary using a plankton collector (Bonko Net) and brought to the laboratory in an ice box and were further processed.

3.2.5.1. Isolation of *Vibrios*

Plankton was filtered from the water and the filtered plankton samples were used for *Vibrio* isolation. First, the plankton suspension in physiological saline was vortexed in order to dislodge the adhered bacteria present as biofilm on it. Plating was done employing spread plate technique onto TCBS agar, using 0.1 ml of the prepared serial dilution, and the plates were incubated at 30^0C for 24 h. The single cell colonies from the plates were isolated, purified on Zobells agar, sub- cultured on Zobells slants, and stocked until further use.

3.2.5.2. Sub culturing

From Zobells agar plates, pure single colonies were sub cultured onto Zobells agar slants.

3.2.6. Preliminary screening and identification of *Vibrios*

The purified isolates were used for screening *Vibrios*. All the isolates obtained from Zobells agar were repeatedly streaked on Zobells agar plates and confirmed their purity by gram staining and microscopic observations.

The isolates were then segregated as *Vibrio* genera based on their morphological and biochemical characteristics outlined in Bergey's Manual of Systematic Bacteriology (Baumann and Schubert, 1984).

3.2.6.1. Salt tolerance test.

Growth at varying concentrations of NaCl (0, 2, 6, 8 and 10 %) was tested by observing growth in 1% tryptone broth at pH 7 ± 0.4 containing varying concentration of NaCl. Growth was detected by observing turbidity at 600nm.

3.2.6.2. Swarming test

Swarming is a characteristic property of certain *Vibrio* species. This was tested on nutrient agar supplemented with 1.5 % NaCl. The overnight bacterial colonies were inoculated on to the nutrient agar plate, incubated overnight and were observed for swarming.

The information thus obtained from all the biochemical profile was used for identifying the *Vibrio* species, which is the available scheme for the identification of environmental *Vibrio* (Alsina and Blanch, 1994).

3.2.6.3. Identification of *Vibrio* strains to the species level

All the isolates recognized as *Vibrios* were further identified to the species level by using a commercially available '**Hi *Vibrio* identification system**' (HiMedia, Bombay). The results were interpreted and recorded using the identification table provided in the chart, supplied by the manufacturer.

3.2.7. Preservation of purified strains

The identified strains were maintained as stock cultures employing glycerol stocking, paraffin overlay method, and semisolid agar method.

3.3. Screening of strains for antibiotic resistance

3.3.1. Antibiotic resistance studies

All the strains of *Vibrios*, isolated from different sources i.e. water, molluscans, crustaceans and plankton were screened for their resistance against 22 different antibiotics available commercially as discs (Hi Media, Mumbai, India). The list of antibiotic disc, their abbreviations and the concentration per disc is as given in Table-1.

3.3.2. Antibiotics used

The twenty two antibiotic discs used in the study are listed below along with the abbreviation, potency of discs and interpretation of the diameters of the zones of inhibition. The interpretative chart was adapted from the Hi Media, Mumbai.

In the present study the Kirby-Bauer disk diffusion assay was used (Bauer *et al.*, 1966), although there are a number of standard methods for testing the susceptibility to antibiotics.

Table-1: List of antibiotics, potency and the interpretative zone was adapted from manufacturer, Hi Media Mumbai.

Sl.no.	Antibiotic used	Abbreviation	Potency (µg/disc)	Interpretation of the diameter of zone of inhibition		
				Resistant (mm or less)	Intermediate (mm)	Sensitive (mm or more)
1	Amoxycillin	Ac	30	13	14-17	18
2	Ampicillin	A	10	13	14-16	17
3	Amikacin	Ak	30	14	15-16	17
4	Carbenicillin	Cb	100	13	14-16	17
5	Cefuroxime	Cu	30	14	15-17	18
6	Chloramphenicol	C	30	12	13-17	18
7	Ciprofloxacin	Cf	5	15	16-20	21
8	Chlortetracycline	Ct	30	13	14-17	18
9	Co-trimoxazole	Co	25	10	11-15	16
10	Doxycycline hydrochloride	Do	30	12	13-15	16
11	Furazolidone	Fr	50	14	15-16	17
12	Gentamycin	G	10	12	13-14	15
13	Meropenem	Mr	10	13	14-15	16
14	Netilmicin	Nt	30	12	13-14	15
15	Nalidixic Acid	Na	30	13	14-18	19
16	Norfloxacin	Nx	10	12	13-16	17
17	Neomycin	Ne	30	12	13-16	17
18	Rifampicin	R	5	16	17-19	20
19	Streptomycin	S	10	11	12-14	15
20	Sulphafurazole	Sf	300	12	13-16	17
21	Trimethoprim	Tr	5	10	11-15	16
22	Tetracycline	T	30	14	15-18	19

(The abbreviations are as indicated by manufacturer, and according to NCCLS, 2001)

3.3.3. Preparation of inoculum

The strains were initially streak plated onto nutrient agar and incubated at 37°C for 18-24 h. Using two or more colonies, from the overnight grown plate, a cell suspension was prepared using sterile saline (0.89%NaCl). The turbidity of the bacterial suspension was then compared with a MacFarland's Barium sulfate standard solution corresponding to 1.5 and used (1.5 =10 cfu / ml). Any increase in turbidity was compared and adjusted with the same saline.

The standardized bacterial cell suspension was spread plated over the Mueller Hinton Agar plate using sterile cotton swabs such that a lawn of culture is obtained. The plates were air dried for 3-5 minutes at room temperature and the different antibiotic discs were placed over the surface of the agar aseptically, with the help of sterile forceps. A maximum of 5 discs were applied to one petri plate. The plates were then incubated over night at 37 ^{0}C. After incubation only zones showing complete inhibition were measured and the diameter of the zones measured.

3.3.4.Interpretation of Results

After incubation the diameter of the zones of inhibition was measured, and based on the size of the inhibition zone the result was interpreted as resistant /sensitive. The results were interpreted based on the recommendations of National Committee for Clinical Laboratory Standards for antimicrobial susceptibility tests (NCCLS, 2001). (Table-1). Strains showing intermediate zones of inhibition were interpreted as resistant to that drug. (Yamamoto *et al.*, 1995.)

3. 3. 5.Calculation of the MAR index

MAR index was calculated to determine the origin of isolates from samples of polluted or non-polluted. Multiple Antibiotic resistances indexing (MAR index) (Krumperman, 1983) of each *Vibrio* strain was calculated from the observed drug resistance pattern of the isolates from each sample using the following equation.

$$\text{MAR index} = \frac{\text{No. of antibiotics to which resistance is obtained}}{\text{Total no. of antibiotics tested}} = \frac{a}{b}$$

3. 4. Detection of plasmids in *Vibrios*

All the 100 *Vibrio* strains obtained from the four different sources were subjected to plasmid profile study

3.4.1. Plasmid DNA Isolation - Alkali lysis method

Plasmid DNA from the *Vibrio* strains was isolated following the alkali lysis method. This commonly used protocol for the bacterial plasmid isolation consists of lysis of bacterial cell wall, and denaturation of DNA and protein using alkali lysis which consists of 1% detergent SDS and 0.2 NaOH, followed by adding potassium acetate which neutralizes the media and precipitates the chromosomal DNA and most of the protein, leaving plasmid DNA and RNA in the solution. The phenol /chloroform iso-amyl alcohol extraction then removes any protein in the solution. Plasmid is then precipitated by addition of ice-cold absolute ethanol. This procedure effectively removes the contaminating proteins. (Birn Boim and Doly, 1979).

3.4.2. Cultivation of cells

The antibiotic resistant *Vibrio* cultures were streaked onto Luria Bertani (LB) agar plate supplemented with 2% sodium chloride to get isolated single cell colonies. The single cell colony from the overnight plate was inoculated into 3 ml LB containing 50 µg/ml ampicillin and incubated 37^{0}C for 12 –14 h on a shaker. From the pre-culture, 1% of the culture broth was transferred to 10 ml LB containing 50 µg/ml ampicillin and incubated 37^{0}C for 14 –18 h on a shaker. Later, the cells were harvested from the shake culture broth by centrifugation at 6000 rpm for 10 minutes at 4 ^{0}C. After centrifugation the supernatant was discarded and the cell pellet was used.

3.4.3. Plasmid isolation –Mini Prep

The bacterial pellet was resuspended in a 100 µl of Solution I, mixed well by vortexing and kept at room temperature for 5 minutes. This was followed by addition of freshly prepared Solution II (twice the volume of Solution I). The contents of the tube were mixed by inverting gently and the tube was kept on ice for 10 minutes. To this alkali treated suspension, the Solution III was added (160µl) and inverted gently for complete dispersion of solution III through the viscous bacterial isolate and stored on ice for 15 minutes. Further this was centrifuged at 10,000 rpm for 15 minutes at 4^0 C. The pellet was discarded and to the supernatant 1ml of phenol-chloroform (1:1) was added, mixed well and kept at room temperature for 10 minutes. Later the contents were centrifuged at 10,000 rpm for 10 minutes at 4^{0}C. The supernatant was transferred gently to a fresh eppendorf tube and added with 1ml of chloroform: isoamyl alcohol (25:1 parts). After keeping at room temperature

for 30 minutes, centrifuged at 10, 000 rpm for 15 minutes at 4^0 C and transferred the supernatant to a fresh 2 ml microfuge tubes. From this, DNA was precipitated by adding 2 volumes of ethanol at room temperature and mixed by gentle vortexing. This was allowed to stand at -20^0C for 2 minutes and centrifuged at 10, 000 rpm for 5 minutes at 4^0C. The supernatant was discarded and the tube was allowed to dry completely. The DNA pellet was rinsed with 1 ml of 70% ethyl alcohol and centrifuged at 10,000 rpm for 15 minutes at 4^0 C. The supernatant was removed and the pellet was air-dried. The DNA pellet was dissolved in 20µl TE buffer (pH-8.0). RNAse was added at a concentration of 20 µg /ml. The DNA suspension was stored at -20^0 C.

3.4.4.Agarose Gel Electrophoresis and Visualization of plasmid DNA

Gel electrophoresis is used to separate DNA molecules by size and to estimate the size of nucleic acid molecules of unknown length by comparison with the migration of molecules of known length. After electrophoresis, the gel is incubated in a solution containing the fluorescent dye ethidium bromide. This molecule binds to DNA by intercalating between the base pairs. Binding concentrates ethidium bromide in DNA and increases its intrinsic fluorescence. As a result, when the gel is illuminated with UV light, the regions of gel containing DNA bands fluorescees much brightly than the regions of gel without DNA.

The isolated plasmid DNA was mixed with 2µl of bromophenol loading dye and loaded onto 0.7% agarose gels. Electrophoresis was carried out in 1X Tris Acetate EDTA buffer in a horizontal gel apparatus at 80 volts using a power pack of 5v/cm DC current for 2-3 h or till the tracking dye reaches to ¾th of the gel length. After completion of electrophoresis, the agarose gel was removed gently from the

electrophoresis tank and the gels were stained in 0.5 mg/ml of ethidium bromide in water by keeping for 10 minutes. The stained gel was then viewed under gel documentation system. λ DNA Hind III digested fragment ladder was used as marker. The gel documentation was made under the documentation system of Amarsham Pharmacea, Biotech.

3.4.5. Retrieval of DNA fragments from Agarose Gel Electro Elution method

The plasmid DNA fragments were retrieved from the gel by electro-elution method. The plasmid DNA gel, while being visualized on UV transilluminator was cut using a sharp surgical blade. The gel piece containing DNA fragment was transferred into sterile eppendorf tubes. The gel piece was placed on a parafilm in platinum wire surrounded Electroeluter apparatus. 200 µl TE buffer was added to the platinum wire surrounded place where the gel bit was placed. The gel bit was so placed that the tip of the platinum was in the middle of the gel piece. A voltage of 30 V was applied for 3 minutes. After visualizing the suspended plasmid DNA with a UV lamp, the suspended plasmid DNA from the gel was collected in a sterile eppendorf tubes (Sambrook *et al.*, 1989).

Equal amount of ethanol was added to it and kept for 2 h in -20^0C and centrifuged at 10000 rpm for 2 minutes at 4°C. Supernatant was discarded and the pellet was washed with 70 % ethanol. The supernatant was discarded and dried the pellet in air and resuspended with in 20 µL DNA. This was stored at -20° C until further use (Sambrook *et al.*, 1989).

3.5. Molecular Characterization of plasmids

3.5.1. Plasmid Transformation

The transformation of plasmids was performed in order to confirm whether the plasmid encoded with resistance characters from the *Vibrio* strains can be transferred to other genera such as *E.coli*. All the plasmids isolated were suspended in TE buffer and were used to transform the strain, *E.coli* DH5α strain, which is sensitive to all antibiotics. After transformation, the *E.coli* transformants were checked for their antibiotic resistance pattern, before re isolation of the plasmid from the *E. coli* DH5α (Sam brook *et al.*, 1989).

3.5.1.1 Materials required (details in appendix I)

a. 0.1 M $CaCl_2$ freshly prepared from filter sterilized 1 M $CaCl_2$
b. LB agar
c. LB Broth
d. *E.coli* DH5α strain
e. Ampicillin
f. SOC Medium.
g. Antibiotic discs
h. Reagents for plasmid isolation
i Plasmid isolated from resistant *Vibrios*

3.5.1. 2 . Preparation of competent cells and transformation

Bacterial strain *E. coli* DH5α was used as recipient cells for the transformation experiment using the isolated plasmids. The strains were first prepared as competent cells for the said purpose using calcium chloride.

1 ml of overnight culture of *E. coli* DH5α cells was inoculated into 50 ml LB broth and incubated with shaking of 180 rpm for 2 h at 37 ^{0}C, till it reaches 0.8-1.0 O.D at 660 nm. The culture was centrifuged at 6000 rpm for 10 minutes at 4^{0}C. The supernatant was discarded completely; the pellet was resuspended in 0.1M ice- cold $CaCl_2$ and was incubated on ice for 45 minutes. After incubation it was centrifuged at 7000 rpm for 15 minutes at 4^{0}C. The pellet was again resuspended in 3 ml $CaCl_2$. Distributed the competent cells to sterile 1.5 ml tube and 20 µl sterile glycerol was added to it and kept at – 20^0 C.

To the competent cell suspension, 10µl of test plasmid DNA isolated from the *Vibrio* strains under study (0.01µg/ml) was added. Incubated for 30 minutes at 4 ^{0}C. A negative control without any DNA and a positive control with the plasmid pUC18 (0.6µg/ml) were also maintained. After this the samples were subjected to heat shock at 42^{0}C for 2 minutes followed by sudden chilling on ice. Later, 500µl of SOC medium was added, incubated at 37^0 C for 2 h, and then the cells were plated onto Luria Bertani agar medium containing the antibiotic ampicillin (at the rate of 50µg/ml). The transformants appeared on the plates, after overnight incubation at 37^{0}C, were further checked for their antibiotic resistance pattern. Then the plasmids were re-isolated from the transformants as well.

Along with this, the competent cells were plated after serial dilution onto LB plates without ampicillin.

3.5.1.3. Calculation of transformation efficiency

Transformation efficiency was calculated using the number of transformants (cfu /ml) grown in Luria Bertani agar containing ampicillin (at the rate of 50 μg/ml)divided by the number of recipient cells (cfu /ml) used.

Transformation efficiency

= No. of *E.coli* DH5 α transformants on Luria agar with ampicillin

No. of competent *E.coli* DH 5 α (recipient cells) on Luria agar without antibiotics.

= X cfu /ml

3.5.2. Plasmid Curing

Plasmid curing was carried out for those isolates containing plasmids. An attempt was made to cure or delete plasmids in these isolates by the following technique that would help to distinguish the location of the resistant characteristics shown by the *Vibrio.* i.e., the genetic location of the antibiotic resistance phenotype could be determined to be either on the chromosome or on the plasmid. After curing with ethidium bromide if the resistance was encoded in the plasmid, the plasmid-encoded property is lost and as a result the resistance pattern becomes sensitive with the subsequent loss of plasmids as well.

3.5.2.1. Materials required

1. Ethidium Bromide 1%
2. LB broth
3. Saline solution

4. LB agar
5. LB agar with ampicillin
6. Antibiotic discs
7. Reagents for plasmid isolation

3.5.2.2. Plasmid Curing using Ethidium Bromide

The protocol described of Molina Aja *et al.* (2002) to cure plasmids of *Vibrio* strains isolated from aquaculture water samples was used in this study.

Plasmid curing experiment was done only for those isolates of *Vibrio* that contained plasmids. Curing treatments were carried out using ethidium bromide as the curing agent. 200 µl of an overnight cultures of *Vibrio* strains with plasmid was added into 5 ml of LB broth containing 2% NaCl. Increasing concentrations ranging from 50 to 500 µg/ml of the curing agent was added to the culture tubes. The cultures were then incubated overnight at 37°C under constant vigorous agitation and observed for growth.

The bacterial cells from the culture tubes that contained the highest concentration of curing agent permitting visible growth (300 µg/ml) were serially diluted and plated onto LB agar plates containing 2% NaCl, incubated and grown up to single clones. These are the clones of the cured culture, were serially diluted in normal saline to get upto10^{9} dilutions. 100µl of these dilutions was plated on to LB agar medium without ampicillin. The plates were incubated overnight at 37^{0}C.

3.5.2.3. Replica plating to identify cured isolates

The cured clones, after making replica plating onto plates containing antibiotics, were tested for the antibiotic resistance pattern for all the antibiotics to which they were originally resistant. Bacterial isolates, that showed change in the resistance pattern to susceptible, were further submitted to plasmid extraction as explained in section 3.4.3.

Replica plating was done using the plates, obtained earlier by serial dilution as the master plate. Wooden blocks wrapped with velvet were sterilized and used to imprint the earlier plate onto the LB plates containing ampicillin. Those colonies present in the antibiotic containing plate were again checked for their antibiotic resistance pattern and their plasmid content to study the effect of curing.

3.5.3. Plasmid conjugation

3. 5. 3.1. Materials required (details in the appendix I)

1. 0.2 μm cellulose acetate filter paper(Sartorious corporation, Germany)
2. Mac Conkey agar plate containing ampicillin and streptomycin
3. Mac Conkey agar plate containing streptomycin
4. Saline solution
5. Antibiotic disc
6. Reagents for plasmid isolation
7. *E. coli* HB 101 cells Streptomycin ^{R}

3. 5. 3.2. Conjugation Method

Conjugations were done for all the *Vibrio* strains that contained the plasmid. Conjugation was done with *E.coli* HB 101 strains being the recipient and *Vibrio* containing the plasmid encoded resistance as the donor cells. The recipient *E.coli* HB 101 has a selectable streptomycin resistance marker (Liu *et al.*, 1999).

Donor and recipient cells were inoculated in LB broth and incubated overnight at 37^0C. After overnight incubation, donor and recipient cells were mixed in a 1: 3 proportion in a sterile bottle. The mixture was then taken by a sterile 5 ml syringe and filtered through 0.2 µm filter paper. The filter paper containing the bacteria was then placed onto the Mac Conkey agar containing the antibiotics ampicillin and streptomycin at the rate of 50 µg/ml and 25µg/ml respectively. The plates were incubated overnight at 37^0 C for 48 h.

After incubation, the filter paper containing bacteria were washed with normal saline. The conjugated bacterial suspensions were plated onto Mac Conkey agar containing ampicillin and streptomycin after serial dilution upto 10^{-8}. The inoculated plates were incubated after 48 h at 37^0 C. Only the exconjugants containing both antibiotic resistance markers were grown in the medium containing ampicillin and streptomycin. The conjugated bacteria present in the plate containing both the antibiotics were checked for their antibiogram pattern and for their plasmid content.

The recipient *E.coli* HB 101 cells were also plated after serial dilution onto Mac Conkey agar containing streptomycin and incubated at 24-48 h at 37^0 C.

3.5. 3. 3. Calculation of Conjugation efficiency

Conjugation efficiency was calculated using the following formula (Liu et al., 1999)

Conjugation efficiency = $\frac{\text{No. of transconjugants on Mac Conkey with ampicillin and streptomycin}}{\text{No. of recipient } \textit{E.coli} \text{ HB 101 cells on Mac Conkey with streptomycin.}}$

= X cfu/ml

3. 5. 4. Polymerase Chain Reaction (PCR)

3. 5. 4.1. PCR screening for the detection of the presence of Integrons and SXT conjugative element in the isolated plasmids.

PCR screening was done detecting the presence of Integrons in the isolated plasmids using the primers for the *int* genes .The presence of the SXT element was also screened among the isolated plasmids from *Vibrio* strains. The plasmids were examined for the presence of integron, which causes the horizontal gene transfer of antibiotic resistance genes. The examination of SXT was done to examine the presence of any conjugative self-transmissible element (CONSTIN such as SXT) among the isolated plasmids. *V.cholerae* El Tor strain (CO366) genomic DNA positive for integron and SXT was used as positive control.

3.5.4.2 Description of Primers and PCR conditions

PCR was done with the primer pairs.

3.5.4.2 a. PCR for *int* gene

Template used for PCR- Plasmid DNA

Primer sequence- primer used is *in* (5`- cs)

in (3`- cs)

Forward primer: 5' GGC ATC CAA GCA GCA AG 3'

Reverse primer: 5'AAG CAG ACT TTG ACC TGA 3'

3.5.4.2 a.1 PCR Mix composition.

10X PCR amplification buffer (containing 500 mM KCl, 100mM Tris-HCl (pH 9.0), 0.1% TritonX 100, 2 µl of 200mM $MgCl_2$)

3.5.4.2 a.2. PCR assay

PCR amplification was carried out in PCR tubes (Axygen) containing 2.5µl of 10X PCR amplification buffer, 2 µl of dNTP mixture (2.5 mM concentration each), 10 pmole of each of the primers, 1U of Taq polymerase (Genei, Banglore), 1µl of plasmid DNA template, 14.5 µl of sterile distilled water.

3.5.4.2 a.3. PCR conditions

Denatured at 94 ^{0}C for 5 minutes in PCR machine. Taken and plunged into the ice in icebox and kept for 5 minutes. Then 1.0 µl Taq DNA polymerase was added

Denaturation at 94^{0}C for 30 seconds.

Annealing at 62 ^{0}C for 1 minute 30 seconds

Extension at 68 ^{0}C for 1 minute.

Step 1-3= 1 cycle

Total 34 cycles.

3.5.4.2 b. PCR for SXT element .

Template used for PCR- Plasmid DNA

Primer sequence

Forward primer: 5' TTA TCG TTT CGA TGGC 3'

Reverse primer: 5'GCT CTT CTT GTC CGTTC 3'

Amplicon size - 946 bp.

PCR mix composition –same as in Section 3.5.4.2.a.1

PCR conditions -same as in section3.5.4.2 a.3.

3.5.4.3. Procedure for agarose gel of PCR products.

10 µl of the amplified PCR product were mixed with 2 µl of bromophenol blue loading dye and loaded onto 1.5 % agarose gels. Electrophoresis was carried out in 1X Tris Acetate EDTA buffer in a horizontal gel apparatus at 80 volts for using a power pack. To determine molecular weight, 500 bp DNA (Genei, Bangalore) was used as ladder. The gel was run for 2 h at 80 volts and stained in 0.5 mg/ml of ethidium bromide in water by keeping it for 10 minutes. The gel was visualized and documented using a video documentation system. (Amersham Pharmacia Biotech).

3. 6. Restriction digestion profile of isolated plasmids.

The plasmids isolated were subjected for the restriction digestion analysis using EcoR1, Bam H1, Hind III to determine the heterogeneity of the plasmids as well as to know the presence of the respective restriction enzyme sites. Only the plasmids, which were not digested with the above enzymes, were digested with Hae

III. These enzymes were selected based on their wide use in plasmid study particularly with unknown plasmids.

3.6.1.Restriction digestion of plasmid DNAs

The TE suspended plasmid DNAs isolated were first digested with restriction enzymes such as Eco R 1, Bam H1, Hind III and Hae III

For a reaction volume of 20µl, the following reactants were added in a sterile eppendorf tube.

Plasmid DNA	-----	10 µl
Restriction enzyme buffer (10X)	------	2µl
Restriction enzyme	------	2 µl
Sterile water	------	6 µl

The contents were mixed well and the mixture was then allowed to incubate overnight at 37^0C for enzyme digestion. The digestion was then stopped by incubated at 68^0C for 15 minutes in a dry bath (Banglore Genei). The digested DNA fragments in the restriction-digested mixture were analyzed by gel electrophoresis.

3. 6. 2 . Agarose gel electrophoresis of digested products.

Gel electrophoresis of digested products were performed in 1 % agarose gel.

10 µl of digested products were loaded onto gel and electrophoresis was done at 80 volts for 1 hour or until the dye had traversed the two third distance of the gel. λ DNA Eco R1/ Hind III double digest were used as a molecular marker.

The gel was stained in a freshly prepared ethidium bromide solution (1%) and viewed using the gel documentation system.

Chapter 4

RESULTS

4.1. Isolation and identification of *Vibrios*

4.1.1. Isolation of *Vibrios* from various samples

A total of 100 *Vibrio* like organisms were segregated from the 396 bacterial isolates obtained from water, molluscans, crustacean and planktons collected from different locations and aqua farms.(Table.1). More *Vibrios* could be isolated from water compared to other samples.

Table -1. *Vibrio* like isolates obtained from different sampling stations

Sl. no	Sample type	Isolates (n=100)	No. of *Vibrios*
1	Water	CBMW 01 to CBMW 55	55
2	Molluscan	CBMM 56 to CBMM 70	15
3	Crustacean	CBMC 71 to CBMC 85	15
4	Planktons	CBMP 86 to CBMP100	15

n= number of isolates

4. 2. Identification of *Vibrios* to Species level.

All the 100 isolates, which were Gram negative, oxidase positive, fermentative on MOF media that showed yellow/green coloured colonies on TCBS (Thiosulfate Citrate Bile salt Sucrose) agar were segregated as *Vibrios.*

From among them only those 90 *Vibrio* isolates which showed antibiotic resistance, were identified further to species level using Hi-*Vibrio* identification system (Hi Media, Bombay) (Alsina and Blanch, 1994). The results for the identification of these 100 isolates are presented in Table -2.

Table- 2. Identification of *Vibrios* (Hi -*Vibrio* identification system –HiMedia, Bombay)

Isolate no	V.P.Test	Arginine	1%.Salinetolerance	ONPG	Citrate	Ornithine	Mannitol	Arabinose	Sucrose	Glucose	Salicin	Cellobiose	Indole	TCBS	Swarming	Catalase test	0 % Na Cl	3 % NaCl	6% NaCl	8% NaCl	10% NaCl	Species
CBMW 01	-	+	+	+	+	+	+	+	-	+	+	+	+	G	-	+	+	+	+	-	-	*V.mimicus*
CBMW 02	-	-	-	-	-	-	-	-	-	+	-	+	-	G	-	+	-	+	+	-	-	*V.vulnificus*
CBMW 03		+	-	-	-	-	-	-	-	-	-	-	+	G/Y		+	+	-	+	-	-	*V.mimicus*
CBMW 04			-	-	-	-	-	-	-	+	-	-	-	G	-	+	-	+	-	-	-	*V.marinus*
CBMW 05			-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	+	+	-	-	*V.campbellii*
CBMW 06			+	-	-	-	-	-	-	-	-	-	-	Y	-	+	-	+	-	+	+	*V.proteolyticus*
CBMW 07	-		+	-	-	-	-		+	+	-	-	-	Y	-	+	-	-	+	+	+	*V.nereis*
CBMW08	-		-	-	-	-	-	-	-	-	-	-	+	Y	-	+	-	+	+	+	-	*V.carchariae*
CBMW09	+	-	-	-	-	-	-	-	+	+	-	-	-	Y	-	+	-	+	+	-	-	*V.metschnikovii*
CBMW11	-	-	-	-	-	-	+	-	+	+	+	-	+	Y	-	+	-	+	-	-	-	*V.mediterraneii*
CBMW12	-	-	-	-	-	+	-	-	+	+	-	-	+	Y	-	-	-	-	-	-	-	*V.cholerae*
CBMW13	+	-	+	-		-	-	-	-	+	-	-	+	Y	+	+	-	+	-	+	+	*V.proteolyticus*
CBMW14	-	-	+	+		-	-	-	-	+	-	-	-	G	-	+	-	+	+	-	-	*V. harveyi*
CBMW15	-	-	-	-	-	-	-		-	-		-	-	Y	-	+	-	+	-	-	-	*V.cholerae*
CBMW16	+	-		-		-	-	-	-	-	-	-	-	Y	-	+	-	+	-	-	-	*V.cholerae*
CBMW17	-	-	-	-		+	-	-	-	+	-	-	+	G/Y	-	+	+	+	+	-	-	*V.mimicus*
CBMW18	-	-	-	+	+	-	-	-	-	-	-	-	-	Y	-	+	-	+	-	+	-	*V. cincinnatiensis*
CBMW19	-	-	+	-	-	-	-	-	-	-	-	-	-	G	-	+	-	+	-	-	-	*V.marinus*

+ -positive; - negative ;Y- Yellow; G-Green

Table- 2. Identification of *Vibrios* (Hi -*Vibrio* identification system –Hi Media, Bombay) ...cont'd

Isolate no	V.P Test	Arginine	1%Salinetolerance	ONPG	Citrate	Ornithine	Mannitol	Arabinose	Sucrose	Glucose	Salicin	Cellobiose	Indole	TCBS	Swarming	Catalase test	0 % NaCl	3 % NaCl	6% NaCl	8% NaCl	10% NaCl	Species
CBMW20	-	-	+	-	-	-	-	-	+	+	-	-	+	Y	-	+	-	+	-	-	-	*V.splendidus*
CBMW21	+	+	+	-	-	-	+	-	+	+	-	-	-	Y	-	+	-	+	+	-	-	*V.damselae*
CBMW22	-	-	-	+	+	+	-	-	-	+	-	-	+	G/Y	-	+	+	+	+	-	-	*V.mimicus*
CBMW23	+	-	-	-	+	-	-	-	+	-	-	-	+	Y	+	+	-	+	+	-	-	*V.alginolyticus*
CBMW24	-	-	+	-	-	-	+	-	+	+	+	-	-	Y	-	+	-	+	+	+	+	*V. cincinnatiensis*
CBMW25	-	-	+	-	-	-	-	-	+	-	-	-	-	Y	-	+	-	-	+	-	-	*V.damselae*
CBMW26	+	-	-	-	-	-	-	+	-	+	+	-	-	Y	-	+	-	+	-	-	-	*V.anguillarum*
CBMW29	+	-	-	-	-	+	-	-	+	-	-	-	-	Y	-	+	-	-	-	-	-	*V.cholerae*
CBMW30	-	-	-	-	-	-	-	-	-	+	+	-	-	G	-	+	-	+	-	+	+	*V.hollisae*
CBMW31		-	+	-	+	-	-	-	-	-	-	-	+	Y	-	+	-	-	+	+	-	*V.mediterranei*
CBMW32	-	-	-	-	-	-	-	-	-	+	-	-	-	G	-	+	-	-	+	-	-	*V. vulnificus*
CBMW33	-	-	+	-	+	-	+	-	-	+	-	-	-	G	-	+	-	+	-	+	+	*V. furnissii*
CBMW34	-	-	-	-	-	+	-	-	-	-	+	-	+	Y	+	+	-	-	+	+	+	*V.alginolyticus*
CBMW35	+	-	-	-	+	-	-	+	-	+	+	-	-	Y	-	+	-	+	-	-	-	*V.anguillarum*
CBMW36	-	-	-	-	-	-	-	-	-	-	-	+	-	G	-	+	-	-	+	-	-	*V. vulnificus*
CBMW37	-	-	-	-	-	-	+	-	+	+	-	-	+	Y	-	+	-	+	+	-	-	*V. mediterranei*
CBMW38	-	-	+	-	+	-	-	-	-	+	-	-	-	Y	-	+	-	+	-	+	-	*V.pelagius*
CBMW39	-	-	-	+	-	+	-	-	+	-	-	-	-	Y	-	+	-	-	-	-	-	*V.cholerae*
CBMW41	-	-	-	-	-	-	-	-	-	+	-	-	-	G	-	+	-	+	-	-	-	*V.marinus*
CBMW42	-	-	-	-	-	-	-	+	-	-	-	-	-	Y	+	-	+	-	-	+	-	*V.costicola*

+- positive;- negative ;Y- Yellow; G- Green

Table- 2. Identification of *Vibrios* (Hi -*Vibrio* identification system –(Hi Media, Bombay) **...cont'd**

Isolate no	V.P.Test	Arginine	1% Salinetolerance	ONPG	Citrate	Ornithine	Mannitol	Arabinose	Sucrose	Glucose	Salicin	Cellobiose	Indole	TCBS	Swarming	Catalase test	0 % Na Cl	3 % NaCl	6% NaCl	8% NaCl	10% NaCl	Species
CBMW43	+	-	+	-	+	-	-	+	+	+	+	-	-	Y	-	+	-	+	+	+	+	*V. cincinnatiensis*
CBMW44	-	-	+	-	+	-	-	+	-	-	+	-	-	G	-	+	-	+	+	+	-	*V.parahaemolyticus*
CBMW47		-	+	-		-	-	-	-	+	-	-	+	G	-	+	-	+	+	+	+	*V. furnissii*
CBMW48	-	-		-	-	-	-	-	-	+	-	-	+	Y	+	-	-	-	-	+	-	*V.carchariae*
CBMW49	-	-	-	-	+	+	+	-	-	+	+	-	-	G	-	+	-	+	+	-	-	*V.parahaemolyticus*
CBMW50	-	-	-	-	+	-	+	-	-	+	-	-	-	G	-	+	-	+	+	+	-	*V.parahaemolyticus*
CBMW51	-	-	-	-	+	-	+	-	-	+	-	-	-	Y	-	-	-	+	-	+	-	*V.pelagius*
CBMW52	-	-	-	-	+	-	-	-	-	+	-	-	+	Y	-	+	-	+	+	-	-	*V.mediterranei*
CBMW53	-	-	+	-	-	-	-		-	+	-	-	+	Y	-	+	-	+	+	-	+	*V.proteolyticus*
CBMM56	-	-	+	-	-	-		-	-	+	-	-	+	Y	-	+	-	+	+	-	+	*Vcosticola*
CBMM57	-	-	+	-	+	-		-	-	+	-	-	-	Y	-	+	-	-	-	+	+	*V.alginolyticus*
CBMM58	-	-		-	+	-	-	-	-	-		-	-	G/Y	-	+	-	-	-	-	-	*V.mimicus*
CBMM59	-	-	-	-	-	-	-	-	-	-		-	-	G/Y	-	+	-	-	+	-	-	*V.mimicus*
CBMM60	-	-	-	-	-	-	-	-	-	-	-	-	-	Y	+	+	-	+	+	+	+	*V.proteolyticus*
CBMM61	-	-	-	-	-	-	-	-	+	-	-	-	+	Y	-	+	-	-	-	-	-	*V.splendidus*
CBMM62	-	+	-	-	-	-	-	-	+	-	-	-	-	Y	+	-	+	-	-	-	-	*V.marinus*
CBMM63	-	+	-	-	-	-	-	-	+	-	-	-	+	Y	-	+	-	+	+	-	+	*V.nereis*
CBMM64	-	-	-	-	-	-	-	-	+	-	-	-	-	Y	-	-	-	-	-	-	-	*V.orientalis*
CBMM65	-	-	-	-	-	+	-	-	+	-	-	-	-	Y	-	-	-	-	+	-	-	*V.carchariae*

+-positive; - -negative; Y- Yellow; G- Green

Table- 2. Identification of *Vibrios* (Hi -*Vibrio* identification system –Hi Media, Bombay)

....cont'd

Isolate no	V.P Test	Arginine	1%.Salinetolerance	ONPG	Citrate	Ornithine	Mannitol	Arabinose	Sucrose	Glucose	Salicin	Cellobiose	Indole test	TCBS	Swarming	Catalase test	0 % Na Cl	3 % NaCl	6% NaCl	8% NaCl	10% NaCl	Species
BMM 66	-	-	+	-	-	-	+	-	+	+	-	-	+	Y	-	+	-	+	-	-	-	*V.splendidus*
BMM 67	-	-	+	-	-	-	+	-	+	+	-	-	+	Y	-	+	-	+	-	-	-	*V.splendidus*
BMM 68	+	+	+	-	-	-	-	-	+	+	-		+	Y	+	+	-	+	+	+	+	*V.proteolyticus*
BMM 69	+	-	+	-		+	+	-	-	+	+	-	-	G	-	+	-	+	+	+	-	*V.parahaemolyticus*
BMM70	+	+	-	-	-	+	-	+	-	+	-	+	-	Y	+	-	+	+	-	-	-	*V.mediterranei*
BMC 71	-		+	-	-	-	-	-	-	+	+	-	+	G	-	+	-	+	+	+	+	*V.hollisae*
BMC 72	-	-		-	+	-	+	+	+		-	-	+	Y	-	+	-	+	+	+	-	*V.pelagius*
BMC 73	-	-	+	-	-	+	-	+	+	+	-	-	+	Y	+	+	-	+	+	+	-	*V.carchariae*
BMC 74	-	-	+	-	-	-	+	-	+	+	-	-	+	Y	-	+	-	+	-	-	-	*V.splendidus*
BMC 75	+	-	+	+	-	+	+	-	+	+	-	-	-	Y	-	+	-	+	-	-	-	*V.cholerae*
BMC 76	+	-	+	+	-	+	+	-	+	+	-	-	-	Y	-	+	-	+	-	-	-	*V.cholerae*
BMC 77	-	-	-	+	-	-	-	-	-	+	-	+	-	G	-	+	-	+	+	-	-	*V.vulnificus*
BMC 78	+	-	+	+	+	-	+	+	+	+	+	-	-	Y	-	+	-	+	+	+	+	*V. cincinnatiensis*
BMC 79	+	-	+	+	+	+	+	+	-	+	+	-	-	G	-	+	-	+	+	+	-	*V.parahaemolyticus*
BMC 80	+	-	+	+	+	+	+	+	-	+	+	-	-	G	-	+	-	+	+	+	-	*V.parahaemolyticus*
BMC 81	+	-	+	+	-	+	+	-	+	+	-	-	-	Y	-	+	-	+	-	-	-	*V.cholerae*
BMC 82	+	-	+	+	+	+	+	+	-	+	+	-	-	G	-	+	-	+	+	+	-	*V.parahaemolyticus*
BMC 83	-	-	-	+	-	-	-	-	-	+	-	+	-	G	-	+	-	+	+	-	-	*V.vulnificus*
BMC 84	-	+	+	-	-	-	-	-	+	+	-	-	-	Y	-	+	-	+	+	+	+	*V. costicola*
BMC 85	-	-	-	+	-	-	-	-	-	+	-	+	-	G	-	+	-	+	+	-	-	*V.vulnificus*

+ - positive; -- negative; Y-Yellow; G-Green

Table- 2. Identification of *Vibrios* (Hi -*Vibrio* identification system –Hi Media, Bombay)

....cont'd

Isolate no	V.P.Test	Arginine	1% Salinetolerance	ONPG	Citrate	Ornithine	Mannitol	Arabinose	Sucrose	Glucose	Salicin	Cellobiose	Indole	TCBS	Swarming	Catalase test	0 % Na Cl	3 % NaCl	6% NaCl	8% NaCl	10% NaCl	Species
CBM P86	-	-	+	-	-	-	-	-	-	+	-	-	-	G	-	+	-	+	-	-	-	*V.marinus*
CBMP87	+	+	+	+	+	-	+	+	+	+	+	-	+	Y	-	+	-	+	-	-	-	*V.anguillarum*
CBMP88	-	+	+	-		-	+	+	-	+	-	-	+	G	-	+	-	+	+	+	+	*V. furnissii*
CBMP89	+	-	+	+	-	+	+	-	+	+	-	-	-	Y	-	+	-	+	-	-	-	*V.cholerae*
CBMP90	+	-	+	+	-	+	+	-	+	+	-	-	-	Y	-	+	-	+	-	-	-	*V.cholerae*
CBMP91	+	-	-	+	-	+	+	-	+	+	-	-	-	Y	-	+	-	+	-	-	-	*V.cholerae*
CBMP92		-	+	+	+	+	+	+	-	+	+	-	-	G	-	+	-	+	+	+	-	*V.parahaemolyticus*
CBMP93	+		+	+	+	+	+	+	-	+	+	-	-	G	-	+	-	+	+	-	-	*V.parahaemolyticus*
CBMP96	-	-	-	+	-	-	-	-	-	+	-	+	-	G	-	+	-	+	+	-	-	*V.vulnificus*
CBMP97	+	+	+	-	-	-	-	-	+	+	-	-	+	Y	+	+	-	+	+	+	+	*V.proteolyticus*
CBMP98	-	-	+	-	-	-	-	-	+	+	-	-	+	Y	-	+	-	+	-	-	-	*V.splendidus*
CBMP99	+	-	+	+	+	+	+	+	-	+	+	-	-	G	-	+	-	+	+	+	-	*V.parahaemolyticus*
CBMP100	-	-	-	+	-	-	-	-	-	+	-	+	-	G	-	+	-	+	+	-	-	*V.vulnificus*

+- positive; - -negative ;Y- Yellow; G- Green

23 different *Vibrio* species could be identified from the selected four samples based on their biochemical characterizations (Table 2). Among the species observed *V. cholerae* was recorded to be a predominant species among all the samples studied viz. 10.64% from water, 20% from crustacean, and 23.08% from planktons.

A sample wise approach for assessing species composition indicated that *V. cholerae* demonstrated highest prevalence in water samples tested, while all other species were present in different levels: *V. mimicus* (8.51 %), *V. parahaemolyticus, V. cincinnatiensis, V. vulnificus, V. marinus, V. mediterranei, V. proteolyticus* (6.38% each), *V. furnissii, V alginolyticus, V. anguillarum, V. carchariae, V. damselae, V. pelagius* (4.26 % each),. *V. mediterranei, V. costicola, V. hollisae , V. campbellii, V. nereis, V. splendidus, V. metchnikovii, V. harveyi* (2.1 % each) (Table 3).

In molluscans, the prevalence of *V. mimicus, V. splendidus and V. proteolyticus* was highest over the other species obtained, i.e., *V. mediterranei, V. alginolyticus, V. carchariae, V. costicola, V. marinus, V.parahaemolyticus* and *V. nereis*.

In crustacean and plankton samples the occurrence of *V.cholerae, V. parahaemolyticus* and *V. vulnificus* was higher than that of the other species, like *V. cincinnatiensis, V. carchariae, V. costicola, V. hollisae, V.splendidus, V. vulnificus, V. furnissii, V. anguillarum, V. marinus, V. proteolyticus and V.pelagius,* which were only 6.67 % each.

Table-3. Per cent distribution of different species of *Vibrio* isolated from different samples.

Sl. no	*Vibrio* species	Per cent distribution in different samples (%)			
		Water	Molluscan	Crustacean	Plankton
1	*V. cincinnatiensis*	6.38	-	6.67	-
2	*V. furnissii*	4.26	-	-	7.69
3	*V. mediterranei*	2.13	6.67	-	-
4	*V. vulnificus*	6.38	-	20.00	15.38
5	*V.alginolyticus*	4.26	6.67	-	-
6	*V.anguillarum*	4.26	-	-	7.69
7	*V.carchariae*	4.26	6.67	6.67	-
8	*V.cholerae*	10.64	-	20.00	23.08
9	*V.costicola*	2.13	6.67	6.67	-
10	*V.damselae*	4.26	-	-	-
11	*V.hollisae*	2.13	-	6.67	-
12	*V.marinus*	6.38	6.67	-	7.69
13	*V.mediterranei*	6.38	-	-	-
14	*V. mimicus*	8.51	26.67	-	-
15	*V.parahaemolyticus*	6.38	6.67	20.00	23.08
16	*V.campbellii*	2.13	-	-	-
17	*V.nereis*	2.13	6.67	-	-
18	*V.proteolyticus*	6.38	13.33	-	7.69
19	*V.splendidus*	2.13	20.00	6.67	7.69
20	*V.metchnikovii*	2.13	-	-	-
21	*V. harveyi*	2.13	-	-	7.69
22	*V.pelagius*	4.26	-	6.67	-
23	*V.orientalis*	-	6.67	-	-

4. 3. Antibiotic resistance studies

Antibiotic resistance pattern can be of use in assessing the extent of drug resistance existing in nature. With this objective all the 100 *Vibrio* isolates were tested for their resistance patterns against 22 antibiotics using the disc diffusion method. The characterizations of strains as resistant (R) and sensitive (S), were interpreted based on the zone of inhibition around each antibiotic disc studied. The antibiogram profiles of the strains were prepared and the results are as represented in Table-4, 5, 6 and 7.

Table -4. Antibiotic resistance pattern of *Vibrios* isolated from water samples

Isolate	Identity of isolate	Resistance pattern	No of R
CBMW01	*V.mimicus*	Ac, A, Ak, Cb, Cu, Fr, Nt, Ne, S	9
CBMW02	*V.vulnificus*	Ac, A, Ak, Cb, Do, Fr, M, Nx, R, S, Sf, Tr	12
CBMW03	*V.mimicus*	Ac, A, Ak, Cb, Cu, Cf, Fr, G, Nt, Ne, Nx, S	12
CBMW04	*V.marinus*	Ac, A, Ak, Cb, Fr, G, M, Nt, Ne, Nx, R, S, Sf, Tr	14
CBMW05	*V.campbellii*	Ac, A, Cb, Cu, Cf, G, M, Ne, R, S, Sf, Tr	12
CBMW06	*V.proteolyticus*	Ac, A, Ak, Cb, Cu, Cf, Fr, G, M, Nt, Ne, Nx, R, S, Sf, Tr ,T	17
CBMW07	*V.nereis*	Ac, A, Ak,.Cb, Cu, Fr, G, M, Nt, Ne ,Nx, R, S, Sf, T, Tr	14
CBMW08	*V.carchariae*	Ac, A, Ak, Cb, Ne, S, Tr,	7
CBMW09	*V.metchnikovii*	Ac ,A, Cb, Cu, Ne, R,S,	7
CBMW11	*V.mediterranei*	Ac, A, Ak ,Cb, Cu, Ne, R, S, Sf, Tr	10
CBMW12	*V.cholerae*	Ac, A, Ak, Cb, Cu, Na, R, S, Tr	9
CBMW13	*V.proteolyticus*	Ac, A, Ak, Cb, Cu, Ne, R,S,	8
CBMW14	*V. harveyi*	Ac ,A, Ak, Cb,Cu, Cf, Ne, Nx, R	9
CBMW15	*V.cholerae*	Ac, A, Ak, Cb, Cu, Cf, Fr, Ne, Nx, S	10
CBMW16	*V.cholerae*	Ac, A, Ak, C, Do, Ct, M, Nx, Ne	9
CBMW17	*V.mimicus*	Ak, Ne, R, S	4
CBMW18	*V.cincinnatiensis*	Ac, A, Cb, Cu	4
CBMW19	*V.marinus*	Ac, A ,Cb, Cu, Fr, Ne, S	7
CBMW20	*V.splendidus*	Ac, A, Ak, Cb, R,Cu,Ne,	7
CBMW21	*V.damselae*	Ac, A, Ak, Cb, Cu, Cf ,Do, Na ,Nx, Nt, T, Tr, R, S, Sf, Tr, T	17
CBMW22	*V.mimicus*	Ac, A Cb, Cu, G, Ne, S	7
CBMW23	*V.alginolyticus*	Ac, A, Ak, Cb, Cu, Cf Co, Fr, G, M, Nt, Nx ,Ne, R, S, Sf, Tr, T	18
CBMW24	*V. cincinnatiensis*	Ac, A, Ak, Co, Cb, Cu, C, Cf, Fr, G, M, Na, Nt, R, Ne, S, Sf, Tr, T	19

No. of R= Number of antibiotics to which *Vibrio* isolates were resistant)

(The abbreviations are as indicated by manufacturer, and according to NCCLS, 2001)

Ac-Amoxycillin, A-Ampicillin, Ak-Amikacin, ,Cb-Carbenicillin, Co-Cotrimoxazole , Cu-Cefuroxime, C-Cholramphenicol, Cf-Ciprofloxacin, Ct-Chlortetracycline, Do-Doxycycline hydrochloride,Fr-Furazolidone, G-Gentamycin, M-Meropenem, Na-Nalidixic acid, Nt-Netilmycin, Nx-Norfloxacin, Ne-Neomycin, R-Rifampicin, S-Streptomycin, Sf-Sulfafur .azole, Tr-Trimethoprim, T-Tetracycline.

Table -4. Antibiotic resistance pattern of *Vibrios* isolated from water samples .....contd

Isolate	Identity of isolate	Resistance pattern	No of R
CBMW25	*V.damselae*	Ac, A, Ak, Cu, C, Co, Cf, Ct ,Do, Fr , G, M, Nt, Ne Na, Nx, R, S, Sf, Tr	21
CBMW26	*V.anguillarum*	Ac, A, Cb, R	4
CBMW29	*V.cholerae*	Ac, A, Ak, M, Cb, C, Ct, Na, S	9
CBMW30	*V.hollisae*	Ac, A, Cb, Na, M, R, S	7
CBMW31	*V.mediterranei*	Ac, A, Cb, C, Do, Sf	6
CBMW32	*V. vulnificus*	Ac, A, Cb, Cu	4
CBMW33	*V. furnissii*	Ac, A	2
CBMW34	*V.alginolyticus*	Ac, ,A, Cb, R	4
CBMW35	*V.anguillarum*	Ac, A	2
CBMW36	*V. vulnificus*	Ac, A, Cb, S, M, Cu, Fr, T	8
CBMW37	*V. mediterranei*	Ac, A, Cb, S, R	5
CBMW38	*V.pelagius*	Ac, A, Cb, Cu, G, R, S	7
CBMW39	*V.cholerae*	Ac, A, Ak, Cb, Cu, C, Cf, Ct, Do, Fr, M, Na, Nt, Nx, Ne, R, S, Sf, Tr, T	20
CBMW41	*V.marinus*	Ac, A, Cb, Cu, Do, Fr, G	7
CBMW42	*V.costicola*	Ac, A, Cb, Cu, Fr, R, S, Ne	8
CBMW43	*V. cincinnatiensis*	Cu, M ,Tr	3
CBMW44	*V.parahaemolyticus*	Ac, Ak, Cb, Cu, Fr, Ne, S	7
CBMW47	*V. furnissii*	Ac, A, Ak, Cb, Cu, Do, Fr, Na, Ne, R, S, T	12
CBMW48	*V.carchariae*	Ak, Cb, Fr, Na, Ne, Nt, R, S, Sf, T	11
CBMW49	*V.parahaemolyticus*	A, Ak, Cb, Cu, Fr, M, Nt, Ne, R	9
CBMW50	*V.parahaemolyticus*	Ac, A, Ak, Cb, Cu, Fr, M, Ne, R, S, Tr	11
CBMW51	*V.pelagius*	Ac, A, Ak, Cb, G ,M, Ne, R, S	9
CBMW52	*V.mediterranei*	Ac, A, Ak, Cb, Cu, Fr, Nt, Ne, S	9
CBMW53	*V.proteolyticus*	Ac, ,A, Cb, Cu, G	5

No. of R= Number of antibiotics to which *Vibrio* isolates were resistant)

(The abbreviations are as indicated by manufacturer, and according to NCCLS, 2001)

Ac-Amoxycillin, A-Ampicillin, Ak-Amikacin, Co-Cotrimaxazole, Cb-Carbenicillin, Cu-Cefuroxime, C-Chloramphenicol, Cf-Ciprofloxacin, Ct-Chlortetracycline, Do-Doxcycline hydrochloride, Fr-Furazolidone, G-Gentamycin, M-Meropenem, Na-Nalidixic acid, Nt-Netilmycin , Nx-Norfloxacin, Ne-Neomycin, R-Rifampicin, S-Streptomycin, Sf-Sulfafur izole, Tr-Trimethoprim, ,T-Tetracycline.

Table -5. Antibiotic resistance pattern of *Vibrios* isolated from molluscans

Isolate.	Identity of isolate	Resistance pattern	No. of R
CBMM56	*V. costicola*	Ac, A, Cb, Cf, Nx, R, Tr	7
CBMM57	*V. alginolyticus*	Ac, A, Cb, Cu, R	5
CBMM58	*V. mimicus*	Ac, A, Cb, Cu, Cf, R	6
CBMM59	*V. mimicus*	Ac, A, Cb, S, R	5
CBMM60	*V. proteolyticus*	Ac, A, Cb, Cu, Fr, S	6
CBMM61	*V. splendidus*	Ac, A, Cb, Cu, Do, Fr, ,G, Na, Nx, R, S,	11
CBMM62	*V. marinus*	Ac, A, Ak, Cb, Cu, Cf, Do, Fr, R, T, Ne,	11
CBMM63	*V. nereis*	Ac, A, Ak, Cb, Cu, Do, Fr, G, M, Na, Nx, R, S, Cf, Sf, Tr, T,	17
CBMM64	*V. orientalis*	Ac, A, Ak, Cb, Cu, Do, G, M, Nt, R,, S, Sf	12
CBMM65	*V. carchariae*	Ac, A, Ak, Cb, C, Cu, Ct, Cf, Fr, G, M, Na, Nx, Nt, Ne, R, S, Sf, Tr, T	20
CBMM66	*V. splendidus*	Ac, A, Ak, Cb, Cf, Fr, G, M, Na, Nx, Nt, Ne, R, S, Sf, Tr	16
CBMM67	*V. splendidus*	Ac, A, Ak, Cb, Cu, Cf, S, Nt, Na, Nx, R, S, T,	13
CBMM68	*V. proteolyticus*	Ac, A, Ak, Cb, Cu, C, R, S, Na, Nx, Nt, R, S, T	14
CBMM69	*V. parahaemolyticus*	Ac, A, Ak, Cb, Cu, C, Cf, S,T, Tr	10
CBMM70	*V. mediterranei*	Ac ,A, Ak, Cb, Cu, Fr, M, Nt, Na, Nx, R, Sf,Tr, T	14

(No. of R= Number of antibiotics to which *Vibrio* isolates were resistant)

(The abbreviations are as indicated by manufacturer, and according to NCCLS, 2001)

Ac-Amoxycillin, A-Ampicillin, Ak-Amikacin, Co-Cotrimaxazole, Cb-Carbenicillin, Cu-Cefuroxime, C-Chloramphenicol, Cf-Ciprofloxacin, Ct-Chlortetracycline, Do-Doxycycline hydrochloride, Fr-Furazolidone, G-Gentamycin, M-Meropenem, Na-Nalidixic acid, Nt-Netilmycin, Nx-Norfloxacin, Ne-Neomycin, R-Rifampicin, S-Streptomycin, Sf-Sulfafur. azole, Tr-Trimethoprim, T-Tetracycline.

Table–6. Antibiotic resistance pattern of *Vibrio* isolated from crustaceans

Isolate	Identity of isolate	Resistance pattern	No. of R
CBMC 71	*V. hollisae*	Ac, A, Cb, Cu	4
CBMC 72	*V. pelagius*	Ac, A, Cb, Cu, C, M, Nt, R, S	9
CBMC 73	*V. carchariae*	Ac, A, Cb, Cu, C, M, Nt, R, S	9
CBMC 74	*V. splendidus*	Ac, A, Cb, Cu, Cf, Ct, M, Na, Nt, R S,	11
CBMC 75	*V. cholerae*	Ac, A, Ak, Cb, Cu, C, Cf, Do, Na, Nt, Nx, R, S, Tr	14
CBMC 76	*V. cholerae*	Ac, A, Cb, Cu, M, Nt, R, T, Tr	9
CBMC 77	*V. vulnificus*	Ac, A, Cb, Cu, C, Cf, Ct, Na	8
CBMC 78	*V. cincinnatiensis*	Ac, A, Cb, Cu, C, Co, Cf, Ct, Na, R	10
CBMC 79	*V. parahaemolyticus*	Ac, A, Cb, Cu, C, Cf, M, Na, Nt, R, S,T	12
CBMC 80	*V. parahaemolyticus*	Ac, A, Cb, Na	4
CBMC 81	*V .cholerae*	Ac, A, Cb	3
CBMC 82	*V. parahaemolyticus*	Ac, A, Cb, Cu, C, R, S	7
CBMC 83	*V. vulnificus*	Ac, A, Cb, Cu, C, Cf, Ct	7
CBMC 84	*V. costicola*	Ac, A, Cb, Cu, Cf, M, Nt, R,S	9
CBMC 85	*V. vulnificus*	Ac, A, Cb, Cu, R, C, M, S	8

(No. of R= Number of antibiotics to which *Vibrio* isolates were resistant)

(The abbreviations are as indicated by manufacturer, and according to NCCLS, 2001)

Ac-Amoxycillin, A-Ampicillin, Ak-Amikacin, Co-Cotrimaxazole, Cb-Carbenicillin,Cu-Cefuroxime, C-Cholramphenicol, Cf-Ciprofloxacin, Ct-Chlortetracycline, Do-Doxycycline hydrochloride, Fr-Furazolidone, G-Gentamycin, M-Meropenem, Na-Nalidixic acid, Nt-Netilmycin, Nx-Norfloxacin, Ne-Neomycin, R-Rifampicin, S-Streptomycin, Sf-Sulfafur. azole, Tr-Trimethoprim, ,T-Tetracycline.

Table-7. Antibiotic resistance pattern of *Vibrio* isolated from plankton

Isolate	Identity of isolate	Resistance pattern	No. of R
CBMP86	*V. marinus*	Ac, A, Ak, Cb, Cu, Ne, R, S, Tr	**9**
CBMP87	*V. anguillarum*	Ac, A, Ak, Cb, Cu, Fr, G, R	**8**
CBMP88	*V. furnissii*	Ac, A, Ak, Cb, Cu, Ne R,S,	**8**
CBMP89	*V .cholerae*	Ac, A, Ak Cb,Cu, Cf, Fr, Nt, R, S	**10**
CBMP90	*V. cholerae*	Ac, A, Ak, C, Cb, Cu, Nt, Ne, R, S,	**10**
CBMP91	*V. cholerae*	Ac, A, Ak ,Cb, Cu, Ne, ,R	**7**
CBMP92	*V. parahaemolyticus*	Ac, A, Ak, Cb, R	**5**
CBMP93	*V. parahaemolyticus*	Ac, A, Ak, Cb, Cu, Fr, Nt, R, S, Tr	**10**
CBMP96	*V. vulnificus*	Ak, Fr, Ne, R, S	**5**
CBMP97	*V. proteolyticus*	Ac, A, Ak, Cb, Cu, Fr, G, M, Nx, R, S, Sf	**12**
CBMP98	*V. splendidus*	Ac, A, Ak, Cb, Cu, Cf, Fr, G, M, Nt, Ne, Nx, R, S	**14**
CBMP99	*V. parahaemolyticus*	Ac, A, Ak, Cb, Ne, R, S, Tr	**8**
CBMP100	*V. vulnificus*	Ac, A, Cb, Cu, Ne	**5**

(No. of R= Number of antibiotics to which *Vibrio* isolates were resistant)

(The abbreviations are as indicated by manufacturer, and according to NCCLS, 2001)

Ac-Amoxycillin, A-Ampicillin, Ak-Amikacin,Co-Cotrimaxazole, Cb-Carbenicillin,Cu-Cefuroxime, C-Chlramphenicol, Cf-Ciprofloxacin, Ct-Chlortetracycline, Do-Doxycyclinehydrochloride, Fr-Furazolidone, G-entamycin, M-Meropenem, Na-Nalidixicacid, Nt-Netilmycin,Nx-Norfloxacin, Ne-Neomycin, R-Rifampicin, S-Streptomycin, Sf-Sulfafur. azole, Tr-Trimethoprim, ,T-Tetracycline.

Fig . 1 Percentage of resistant and susceptible *Vibrios* isolated from the different samples

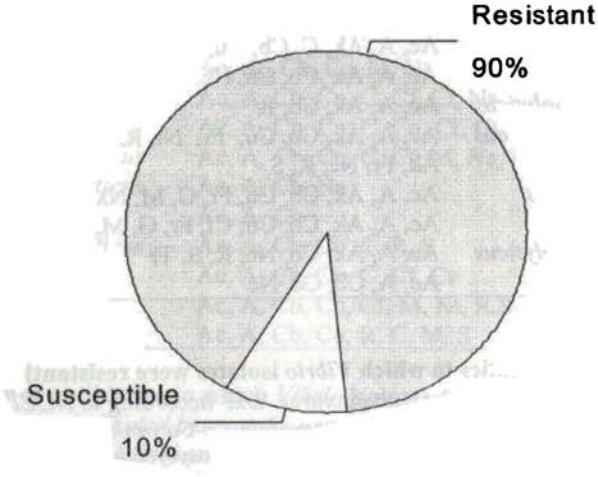
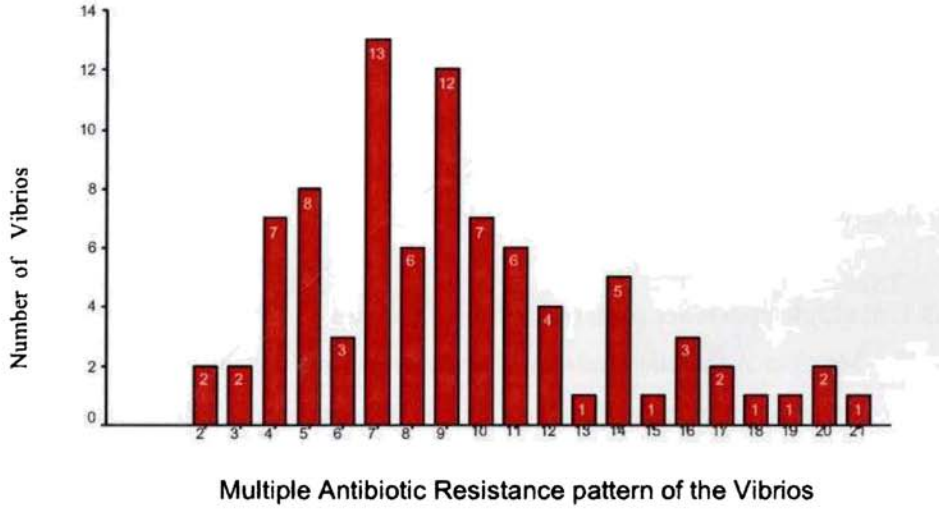


Fig . 2 Quantitative analysis showing multiple antibiotic resistance patterns from *Vibrio* isolates from coastal samples



Nos. on the X-axis indicate the number of antibiotic resistance

From the results presented in Tables 4 to7 & Fig .1 it is inferred that only 10 % of the *Vibrio* isolates studied were completely susceptible to all the 22 antibiotics tested in this study. 90% of the *Vibrios* in this study were resistant to all the 22 antibiotics.

Quantitative analysis of the observed pattern of resistance in the isolates is represented in Table 4, 5, 6, & 7 ; Fig. 2. The resistance pattern of antibiotics varied, with some of the isolates showing resistance to only 2 antibiotics, while some others were resistant to a maximum of 21 antibiotics. The occurrence of *Vibrio* isolates with the expression of resistance to 7 and 9 different antibiotics were the highest, followed by those with resistance to 5 different antibiotics.

4.3.1. Multiple resistance patterns in *Vibrio* Isolates

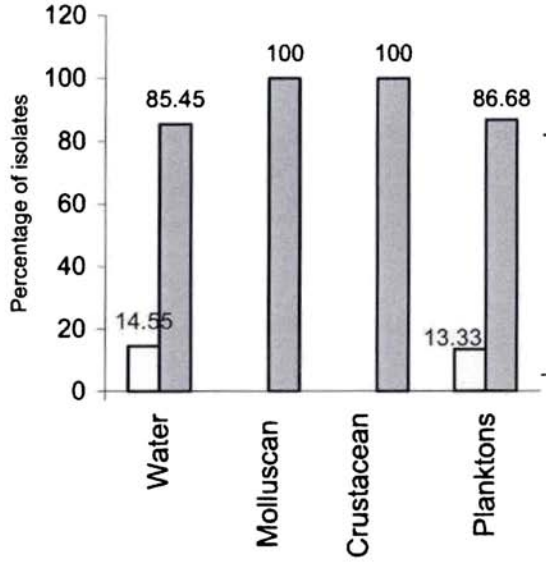
Multiple Antibiotic Resistant (MAR) organisms are those organisms, which are showing resistant to more than 3 antibiotics (Eleonor, *et al.*, 2001). The percentage of multiple antibiotic resistance in the *Vibrios* isolated from different samples were as represented in Table-8 and Fig. 3.

Table -8. Percentage of resistant, susceptible and MAR *Vibrios* isolated from different samples

Sample & Strains	n	(S)	(R)	Multiple Antibiotic Resistant (MAR) isolates		
				N	N/ R (%)	N / n (%)
Water (CBMW 01-CBM55)	55	8 (14.55%)	47 (85.45%)	44	93.62 %	80
Mollusc (CBMM56-CBMM70)	15	0	15 (100%)	15	100	100
Crustacean (CBMC71-CBMC85)	15	0	15 (100%)	15	100	100
Planktons (CBMP86- CBMP100)	15	2 (13.33%)	13 (86.67%)	13	100	86.67
TOTAL	100	10	90	87	96.67	87

n - total no. of Isolates; (S) –Susceptible, (R) -Resistant; N -Total no. of MAR isolates.
Multiple Antibiotic resistant (MAR) - Resistant to more than three antibiotics (Eleonor et al., 2001)

Fig. 3. Percentage of Resistance/ Susceptibility of *Vibrios* isolated from different samples.



Samples	Number of isolates (n)
Water	55
Molluscan	15
Crustacean	15
Plankton	15
Total	100

Values expressed as percentage of the individual samples collected (n)

The expression of resistance pattern (R-pattern) of the *Vibrios* varied from sample to sample. 85.45 % (n=47) of the *Vibrios* isolated from the water sample and 86.67 % (n=13) of the *Vibrios* isolated from the plankton were resistant to one or more than one antibiotic used in this study. All the *Vibrios* isolated from molluscan (n=15) and crustacean(n=15) samples were shown as resistance to the studied antibiotics (Table-8 and Fig.3). Out of the 100 *Vibrio,* only a small portion of isolates, ie, eight (14.55%) isolates from water and two (13.33%) isolates from plankton ,were susceptible to all the 22 antibiotics used in this study.

While 100% *Vibrios* isolated from molluscans (N=15) and crustacean (N=15) samples showed Multiple Antibiotic Resistance (MAR) (Table-8); only 85.45% (N=44) of *Vibrio* isolates from water and 86.67% (N=13) isolates from plankton were MAR. There were significant differences in the R-pattern of the isolates from the different samples.

4.3.2. The activity profile of the antibiotics used in this study.

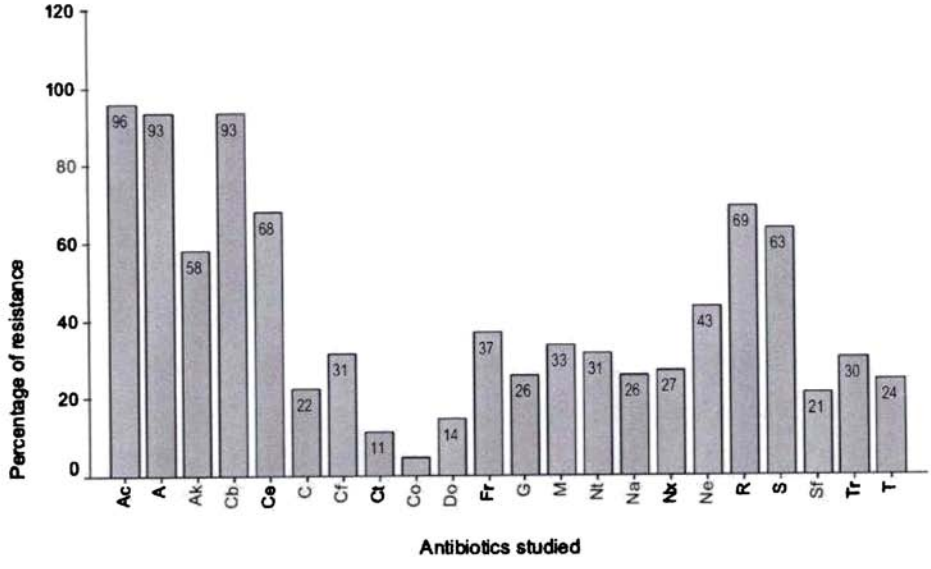
Based on the results obtained from the antibiotic resistance study conducted, the activity profile of the antibiotics was computed and the same is presented in Table-9 and Fig.4.

Table - 9. The activity profile of the antibiotics in terms of the percentage of *Vibrios* resistant to the antibiotics tested

Sl. No	Antibiotics tested	n=100	
		R	%
1	Amoxycillin	86	95.56
2	Ampicillin	84	93.33
3	Amikacin	52	57.78
4	Carbenicillin	84	93.33
5	Cefuroxime	61	67.78
6	Chloramphenicol	20	22.22
7	Ciprofloxacin	28	31.11
8	Chlortetracycline	10	11.11
9	Co-trimoxazole	4	4.44
10	Doxycycline hydrochloride	13	14.44
11	Furazolidone	33	36.67
12	Gentamycin	23	25.56
13	Meropenem	30	33.33
14	Netilmicin	28	31.11
15	Nalidixic acid	23	25.56
16	Norfloxacin	24	26.67
17	Neomycin	39	43.33
18	Rifampicin	62	68.89
19	Streptomycin	57	63.33
20	Sulphafurazole	19	21.11
21	Trimethoprim	27	30.00
22	Tetracycline	22	24.44

R=resistant isolates; n=total no of isolates screened

Fig –4. The activity profile of the antibiotics tested



Numbers indicate the percentage of isolates resistant to the particular antibiotic

(The abbreviations are as indicated by manufacturer, and according to NCCLS, 2001)

Ac-Amoxycillin, A-Ampicillin, Ak-Amikacin, Co-Cotrimaxazole, Cb-Carbenicillin, Cu-Cefuroxime, C-Chlramphenicol, Cf-Ciprofloxacin, Ct-Chlortetracycline, Do-Doxycycline hydrochloride, Fr-Furazolidone, G-Gentamycin, M-Meropenem, Na-Nalidixic acid, Nt-Netilmycin , Nx-Norfloxacin, Ne-Neomycin, R-Rifampicin, S-Streptomycin, Sf-Sulfafur azole, Tr-Trimethoprim, T-Tetracycline.

It was observed that among the 90 *Vibrios* resistant to any of the antibiotics tested, 95.56 % (n=86) were resistant to amoxycillin, 93.33% (n=84) to ampicillin and carbencillin, 68.89% (n=62) to rifampicin, 67.78% (n=61) to cefuroxime, 63.33% (n=57) to streptomycin and 57.78% (n= 52) to amikacin; 43.33%(n=39) to neomycin, 36.67% (n=33) to furazolidone, 33.33%(n=30) to meropenem, 31.1% (n=28) to netilmycin and ciprofloxacin, 30% (n=27) to trimethoprim and 26.67% (n=24) to norfloxacin, 25.56% (n=23) to gentamycin and nalidixic acid, 24.44% (n=22) to tetracycline. 22.22% (n=20) to chloramphenicol and 21.11% (n=19) to sulfafurazole. Resistance to doxycycline hydrochloride was exhibited only by 14.44% (n=13) isolates while 11.1% (n=10) were resistant to chlortetracycline. But cotrimoxazole resistance was observed in only 4.4% (n=4) of the isolates tested.

4.3.3 The Multiple Antibiotic Resistance index (MAR-Index) of *Vibrio* isolates

MAR index of all the *Vibrio* isolates is presented in Table -10, 11, 12, 13, 14 and Fig.5. A significant number of strains i.e. 82.98%, 100%, 80% and 100% from water, molluscans, crustacean and plankton respectively showed a MAR index value of >0. 2.

Table-10. MAR index of *Vibrios* isolated from water samples

Culture no.	No. of antibiotics against resistance shown (a)	No of antibiotics used (b)	MAR index (a/b)
CBMW01	09	22	0.41
CBMW02	12	22	0.55
CBMW03	12	22	0.55
CBMW04	14	22	0.64
CBMW05	12	22	0.55
CBMW06	17	22	0.77
CBMW07	16	22	0.73
CBMW08	07	22	0.32
CBMW09	07	22	0.32
CBMW11	10	22	0.45
CBMW12	09	22	0.41
CBMW13	08	22	0.36
CBMW14	09	22	0.41
CBMW15	10	22	0.45
CBMW16	09	22	0.41
CBMW17	04	22	0.18
CBMW18	04	22	0.18
CBMW19	07	22	0.32
CBMW20	07	22	0.32
CBMW21	17	22	0.77
CBMW22	07	22	0.32
CBMW23	18	22	0.82
CBMW24	19	22	0.86

Table-10. MAR index of *Vibrios* isolated from water samples . contd.

Culture no.	No. of antibiotics to which resistance was shown (a)	No of antibiotics used (b)	MAR index (a/b)
CBMW25	21	22	0.95
CBMW26	04	22	0.18
CBMW29	09	22	0.41
CBMW30	07	22	0.32
CBMW31	06	22	0.27
CBMW32	04	22	0.18
CBMW33	02	22	0.09
CBMW34	04	22	0.18
CBMW35	02	22	0.09
CBMW36	09	22	0.41
CBMW37	05	22	0.23
CBMW38	07	22	0.32
CBMW39	20	22	0.91
CBMW41	07	22	0.32
CBMW42	08	22	0.36
CBMW43	03	22	0.14
CBMW44	07	22	0,32
CBMW47	12	22	0.55
CBMW48	11	22	0.50
CBMW49	09	22	0.41
CBMW50	11	22	0.50
CBMW51	09	22	0.41
CBMW52	05	22	0.23
CBMW53	05	22	0.23

Table-11. MAR index of *Vibrios* isolated from molluscan samples.

Culture no.	No. of antibiotics to which resistance was shown (a)	No of antibiotics used (b)	MAR index (a/b)
CBMM 56	07	22	0.32
CBMM 57	05	22	0.23
CBMM 58	06	22	0.27
CBMM 59	05	22	0.23
CBMM 60	06	22	0.27
CBMM 61	11	22	0.50
CBMM 62	11	22	0.50
CBMM 63	17	22	0.77
CBMM 64	12	22	0.55
CBMM 65	20	22	0.91
CBMM 66	16	22	0.73
CBMM 67	13	22	0.59
CBMM 68	14	22	0.64
CBMM 69	10	22	0.45
CBMM70	14	22	0.64

Table –12. MAR index of *Vibrios* isolated from crustacean samples

Culture no.	No. of antibiotics to which resistance as shown (a)	No of antibiotics used (b)	MAR index (a/b)
CBMC 71	04	22	0.18
CBMC 72	09	22	0.41
CBMC 73	11	22	0.50
CBMC 74	11	22	0.50
CBMC 75	14	22	0.64
CBMC 76	09	22	0.41
CBMC 77	08	22	0.36
CBMC 78	10	22	0.45
CBMC 79	12	22	0.55
CBMC 80	04	22	0.18
CBMC 81	03	22	0.14
CBMC 82	07	22	0.32
CBMC 83	07	22	0.32
CBMC 84	09	22	0.41
CBMC 85	08	22	0.36

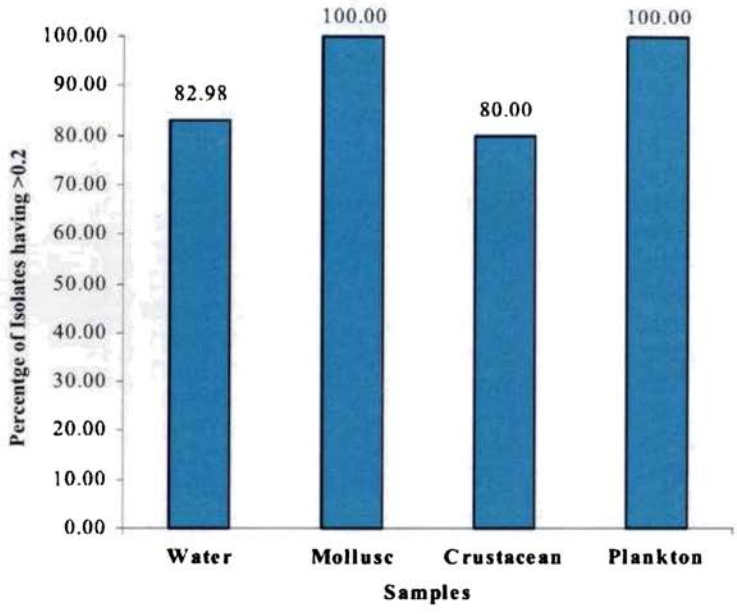
Table -13. MAR index of *Vibrios* isolated from plankton samples.

Culture no.	No. of antibiotics to which resistance was shown (a)	No of antibiotics used (b)	MAR index (a/b)
CBM P86	09	22	0.41
CBMP87	08	22	0.36
CBMP88	08	22	0.36
CBMP89	10	22	0.45
CBMP90	10	22	0.45
CBMP91	07	22	0.32
CBMP92	05	22	0.23
CBMP93	10	22	0.45
CBMP96	05	22	0.23
CBMP97	12	22	0.55
CBMP98	14	22	0.64
CBMP99	08	22	0.36
CBMP100	05	22	0.23

Table -14. MAR index of the *Vibrio* isolates under study

Samples	No. of R Isolates	% of MAR Index	
		≤0.2	>0.2
Water	47	17.02	82.98
Molluscans	15	0	100.00
Crustaccan	15	20.00	80.00
Plankton	13	0	100.00
Total	90	12.22	87.78

Fig. 5. Percentage of *Vibrio* isolates having MAR index values >0.2



4.3.4. Segregation of *Vibrios* to different Clusters based on their Resistance pattern

Based on the differential pattern of antibiotic resistance showed by the isolates, they were classified into 4 clusters and the results are presented in Table-15 and Fig.6.

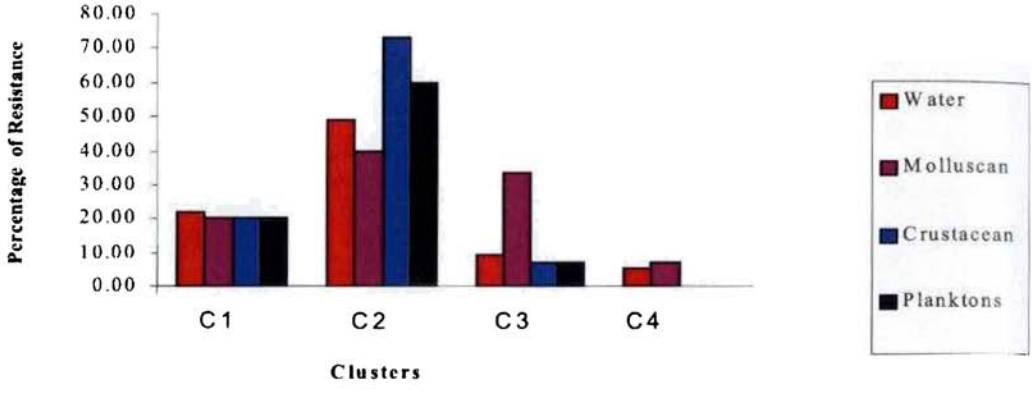
- **Cluster -1** - resistance to $\leq$ **6** antibiotics
- **Cluster -2** - resistance to **7-12** antibiotics
- **Cluster -3** - resistance to **13-17** antibiotics
- **Cluster -4** - resistance to **18-22** antibiotics

Table-15. Segregation of *Vibrios* into different clusters based on their resistance pattern

Samples	n	Resistance to antibiotics				Total R
		Cluster-1	Cluster-2	Cluster-3	Cluster-4	
		$\leq$ 6	7 -12	13-17	18-22	
Water (CBMW01-CBM55)	55	**12** (21.82%)	**27** (49.09%)	**5** (9.09%)	**3** (5.45%)	**47** (85.45%)
Molluscan (CBMM56-CBMM70)	15	**3** (20.00%)	**6** (40.00 %)	**5** (33.33%)	**1** (6.67 %)	**15** (100%)
Crustaccan (CBMC71-CBMC85)	15	**3** (20.00%)	**11** (73.33%)	**1** (6.67%)	0	**15** (100%)
Planktons (CBMP86- CBMP100)	15	**3** (20.00 %)	9 (60.00%)	**1** (6.67%)	0	**13** (86.67%)

Numbers in bold indicate the number of isolates resistant to antibiotics

Fig. 6. Segregation of *Vibrios* into different clusters based on their resistance pattern



It was observed that 21.82% (n=12) of the *Vibrios* from the water samples and 20% (n=3) each from molluscans, crustacean and plankton, with resistances to more than 3 but up to six antibiotics were grouped as Cluster-1. While 73.33% (n=11) of the *Vibrios* from crustacean and 60% (n=9) from the plankton sample were grouped as cluster -2; 49.09% (n=27) strains from water and 40% (n= 6) from molluscs were also grouped as cluster-2, all exhibiting resistance to more than 6 antibiotics.

4.4. Plasmid isolation from *Vibrios*

All the 90 isolates which showed resistance to one or more than one antibiotic were further screened for the presence of R-plasmids.

Attempts to isolate plasmids from *Vibrio* that showed antibiotic resistance, yielded fruitful results. Of the 90 resistant *Vibrio* isolates tested, only 28.89% (n=26) isolates were observed to harbor plasmids. The rest i.e. 71.11% (n=64) isolates of the resistant strains were without plasmids.

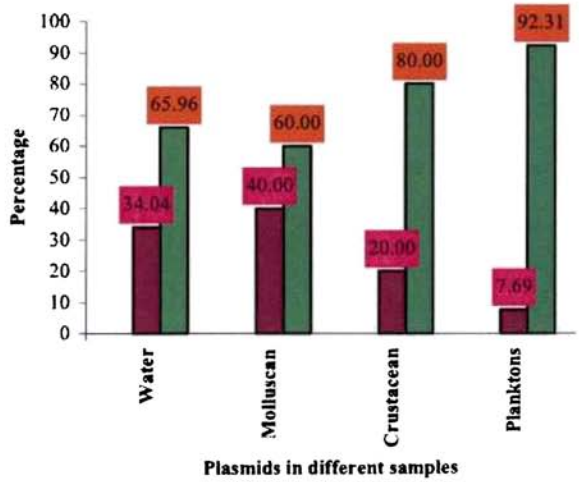
Of the various samples evaluated, the *Vibrio* strains isolated from molluscan samples were found to harbor more R- plasmids (40%) followed by water (34.04%) and crustacean (20%). The results of the plasmid isolation are summarized in Table-16 and Fig. 7.

Table 16. Percentage of plasmids isolated from MAR-*Vibrios*

ample	*Vibrio* isolates tested (n)	No. isolates with antibiotic resistance	No. of - *Vibrios* with plasmid	No. of *Vibrios* without plasmid
Water CBMW01- CBMW55	55	47	16 (34.04%)	31 (65.96%)
Molluscan CBMM56-CBMM70	15	15	6 (40%)	9 (60%)
Crustacean CBMC71-CBMC85	15	15	3 (20%)	12 (80%)
Planktons CBMP86- CBMP100	15	13	1 (7.69%)	12 (92.31%)
Total	100	90	26 (28.89 %)	64 (71.11)

Values in parenthesis indicates percentage

Fig .7. Percentage of plasmids isolated from MAR *Vibrios*



4.5. Plasmid profiles of MAR *Vibrio* isolates

The plasmids isolated were named as given in the corresponding tables. The plasmid profile of the MAR-*Vibrios* isolated from different samples is presented in Table-17, 18 and 19 and Fig.8, 9, & 10. The size of the plasmid isolated from different strains ranged from 1.4 kb to 27 kb.

The plasmid profile of the *Vibrios* isolated from water sample is presented in Table-18 and 19 and Fig.8 and 9. Only Sixteen MAR-*Vibrio* strains out of 47 showed the presence of plasmids. Strains CBMW26 (*V.anguillarum;* CBMW37, CBMW52 (*V. mediterranei;)* CBMW47 (*V. furnissii*); CBMW32 & CBMW33 (*V. vulnificus*) showed the presence of more than one plasmid.

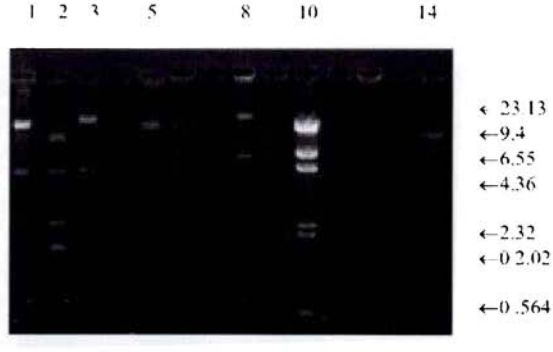
The plasmid profiles of isolates showed diversity and the variations were observed in plasmid number, molecular size, antibiotic resistance genes present, etc. A few isolates (6 strains) from water samples were found to contain more than one plasmid (1.4 to 27.7 kb in size).

It was observed from Fig.10 and Table -19 that among the six MAR-*Vibrios* isolated from molluscan , four contained a single plasmid, and the remaining 2 strains were with multiple plasmids

Table-17. Profile of multiple plasmids in *Vibrios* isolated from water sample

sl	Culture no	Identity	Plasmid	Approximate size	No. of plasmids
2	CBMW26	*V. anguillarum*	**pVEK1**	22.1, 6.2	**2**
3	CBMW37	*V. mediterranei*	**pVN36**	14.4, 6.6, 2.1, 1.4	**4**
4	CBMW47	*V. furnissii*	**pVB9**	27.7, 15.0, 6.7	**3**
5	CBMW13	*V. proteolyticus*	**pVP10**	25.1	**1**
6	CBMW32	*V. vulnificus*	**pVMM1**	12.3, 4.16	**2**
7	CBMW42	*V. costicola*	**pVPD3**	23	**1**

Fig.8. Profile of multiple plasmids in MAR *Vibrios* isolated from water samples

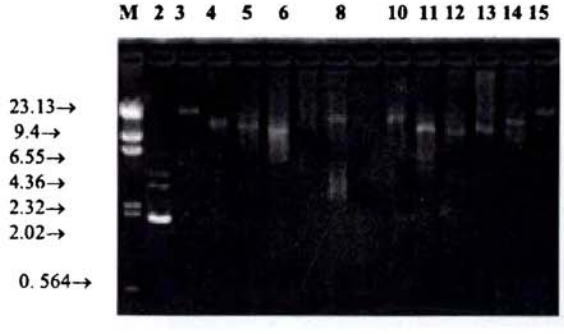


Plasmids isolated from different *Vibrio* species- Lane- 1, 2, 3, 5, 8, 14 have plasmids pVEK1, pVN36, pVB9, pVP10; pVMM1& pVPD3 respectively; lane 10 has λ DNA Hind III digest as marker

Table-18. Profile of single/ double plasmids in *Vibrios* isolated from water samples

	Culture no	Identity	Plasmid	Approximate size in Kb	No. of plasmids
1	CBMW01	*V. mimicus*	pVCL5	18.31	1
2	CBMW25	*V. damselae*	pVCVA8	16.2	1
3	CBMW08	*V .carchariae*	pVP5	13.5	1
4	CBMW09	*V. metschnikovii*	pVP17	16.9	1
5	CBMW52	*V .mediterranei*	pVKG1	16.6, 2.9	2
6	CBMW31	*V .mediterranei*	pVO14	19.2	1
7	CBMW32	*V. vulnificus*	pVMM1	12.3, 4.16	2
8	CBMW33	*V. furnissii*	pVMM2	13.16	1
9	CBMW34	*V. alginolyticus*	pVMM3	13.58	1
10	CBMW35	*V. anguillarum*	pVMM4	16.11	1
11	CBMW36	*V. vulnificus*	pVMM5	25.4	1

Fig.9. Profile of single/double plasmids in MAR *Vibrios* isolated from water sample

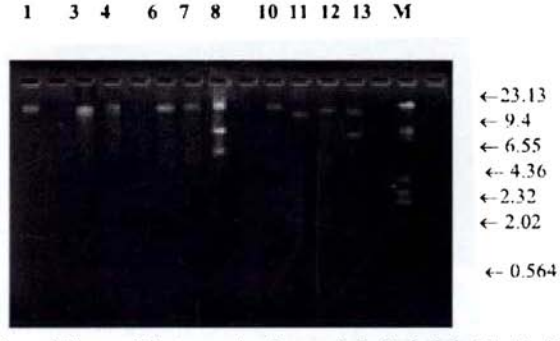


Plasmids isolated from different MAR *Vibrio* species- Lanes 2, 3, 4, 5, 6 ,8, 10, 11, 12, 13, 14, 15 has pUC 18 from *E.coli*; pVCL5; pVCVA8; pVP5; pVP17; pVKG1; pVO14; pVMM1;p VMM2; pVMM3; pVMM4; pVMM5 respectively; and Lane M has. λ DNA Hind III Digest as marker

Table-19. Profile of single/ double plasmids in MAR-*Vibrios* isolated from molluscan, crustacean and plankton

	Culture no	Identity	Plasmid	Approximate plasmid size in Kb	No. of plasmids
1	CBMP87	*V. anguillarum*	pVPV9	25	1
2	CBMM58	*V mimicus*	pVMUS10	14.93	1
3	CBMM62	*V. mediterranei*	pVMUS15	14.38	1
4	CBMM57	*V. alginolyticus*	pVMUS1	19.36	1
5	CBMM56	*V. costicola*	pVMUS7	16.69	1
6	CBMM70	*V. mediterranei*	pVSPF4	16.08,8.27,5.98	3
7	CBMC84	*V. costicola*	pVSY3	13.38	1
8	CBMC73	*V. carchariae*	pVSY1	15.36	1
9	CBMC78	*V. cincinnatiensis*	pVSY6	14.3	1
10	CBMM64	*V. orientalis*	pVMUS11	14.3,6.44	2

Fig.10. Profile of single/ double plasmids in MAR-*Vibrios* isolated from molluscan, crustacean and plankton



Plasmids isolated from different *Vibrio* species-Lanes 1, 3, 4, 6, 7, 8, 10, 11, 12, 13 have pVPV9, pVMUS10, pVMUS15, pVMUS1, pVMUS7, pVSPF4, pVSY3, pVSY1,p VSY6, pVMUS 11respectively; and Lane M has λ DNA Hind III Digest as marker

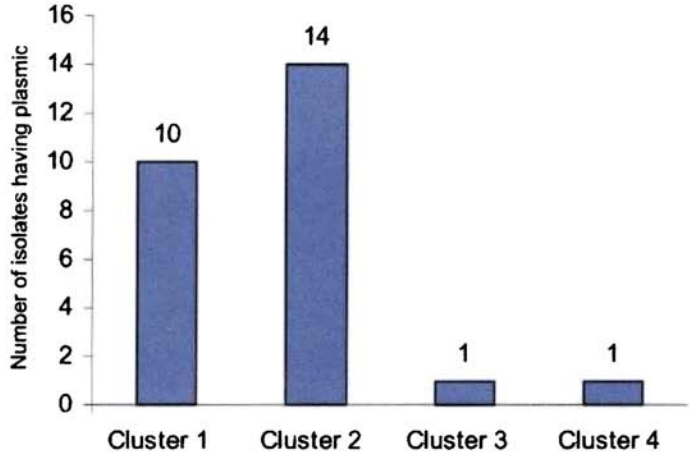
4. 6. Classification of plasmid containing *Vibrio* isolates from different samples in various clusters.

In an attempt to correlate the presence of plasmids to the MAR-pattern, the plasmid containing isolates were clustered (as per section-4.3.5) and the results are depicted in Table-20 and Fig.11. Of the 26 *Vibrio* isolates with plasmids, fourteen belonged to cluster-2 (showing resistance to 7 -12 antibiotics), 10 belonged to cluster - 1, and only one each in cluster-3 (showing resistance to 13-18antibiotics) and cluster 4 group (with resistance to more than 18 antibiotics).

Table .20. Clustering of *Vibrios* with plasmids

	Resistance to number of Antibiotics	**Water**	**Molluscan**	**Crustacean**	**Plankton**	**Total**
Cluster 1	(< 6)	8	2	0	0	10
Cluster 2	(7 -12)	7	3	3	1	14
Cluster 3	(13 - 18)	0	1	0	0	1
Cluster 4	(18 -22)	1	0	0	0	1
	Total (P)	16	6	3	1	26

Fig.11. Clustering of *Vibrios* with plasmids



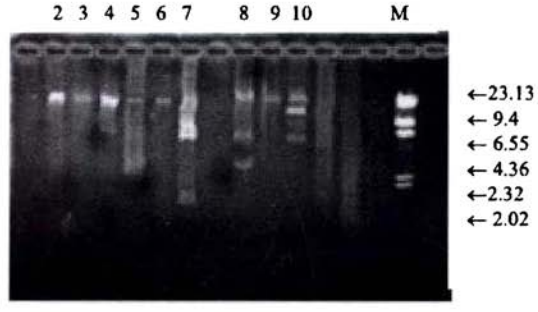
4.7. Characterization of isolated plasmids.

4.7.1. Transformation

Transformation of the plasmids isolated from the *Vibrios* strains were carried out with *E. coli* DH 5α as the recipient. The transformation efficiency, resistance pattern of the transformant *E. coli* DH 5α and re-isolation of the plasmids were also done. It was observed that only 12 out of 26 plasmids could successfully transform *E. coli* DH5α, which was sensitive to all tested antibiotics .The transformants were recovered in ampicillin containing LB agar plate. Results of the transformation, the R-pattern of the transformants, and the transformation efficiency analysis are as presented in Table-21.The plasmids from the transformant *E. coli* DH5α were recovered by re-isolation after the transformation to the recipient strain (Fig.12 and 13)

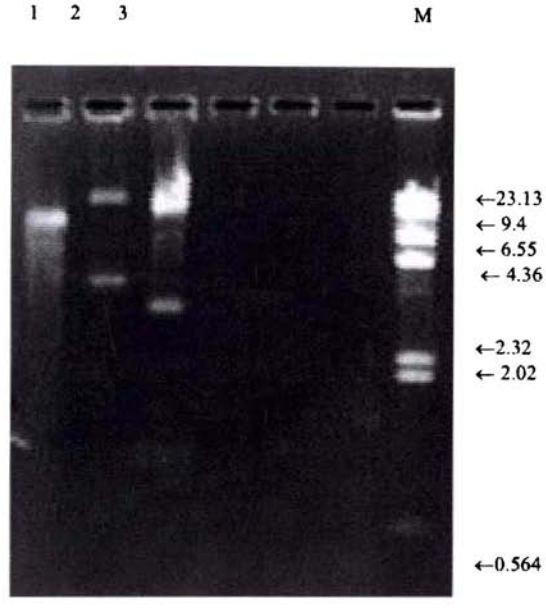
The R-pattern of the transformed *E. coli* DH5α indicated that the resistance pattern was indeed transferred from the corresponding donor *Vibrio* isolates to the recipient strain, each R-pattern showing a variation.(Table-22) This study helped to indicate that these R-genes were plasmid mediated.

Fig.12. Plasmids isolated from transformed *E.coli* DH 5α



Plasmids isolated from the transformed *E.coli* DH5α – Lanes 2 , 3, 4, 5, 6, 7, 8, 9, 10 have pVP10T, pVMUS10T, pVMUS11T, pVKG1T, pVMUS1T, pVN36T, pVSPF4T, pVO14, & pVB9T and Lane M has λ DNA Hind III Digest.as marker

Fig.13 Plasmids isolated from transformed *E.coli* DH 5α



Plasmids Isolated from the transformed *E.coli* DH5α - Lanes1, 2 ,3 has pVP5T, pVMM1T, pVEK1T and Lane M has Marker λ DNA

Table-21. R-pattern of the plasmids and their transformation efficiency

Donor *Vibrio* culture no.	Plasmid name	R- pattern associated with donor *Vibrio* isolate	R- pattern of transformant *E.coli DH5 α*	R-pattern of plasmid	Transformation efficiency
CBMW08	**pVP5**	Ac, A, Ak, Cb, Ne, S, Tr	Ac, A, Ak, Cb, S (5)	Ac, A, Ak, Cb, S (5)	3.13×10^{-8}
CBMW13	**pVP10**	Ac, A, Ak, Cb, Cu, Ne, R, S	Ac, A, Cb ,Cu, R, S (6)	Ac, A, Cb ,Cu,,R, S (6)	7.15×10^{-7}
CBMW32	**pVMM 1**	Ac, A, Cb, Cu	Ac, A, Cu ,Cb (4)	Ac, A, Cu, Cb (4)	5×10^{-8}
CBMW31	**pVOMM 14**	Ac, A, Cb, C, Do, Sf	Ac, A, Cb, Sf (4)	Ac, A, Cb, Sf (4)	43.75×10^{-5}
CBMW37	**pVN 36**	Ac, A, Cb, S, R	Ac, A, Cb, R, S (5)	Ac, A, Cb, R, S (5)	5×10^{-7}
CBMW47	**pVB 9**	Ac, A, Ak, Cb, Cu, Do, Fr, Na, Ne, R, S, T,	Ac, A ,Ak, Na, R (5)	Ac, A ,Ak, Na, R (5)	3.13×10^{-7}
CBMW52	**pVKG 1**	Ac, A, Ak, Cb, Cu, Fr, Nt, Ne, S	Ac, A, Cu ,Cb, (4)	Ac, A, Cu ,Cb, (4)	34.1×10^{-5}
CBMW26	**pVEK1**	Ac, A Cb,R	Ac, A Cb (3)	Ac, A, Cb (3)	5×10^{-5}
CBMW58	**pVMUS 10**	Ac, A, Cb, Cu, Cf, R	Ac, A, Cb, R (4)	Ac, A, Cb, R (4)	2.38×10^{-9}
CBMM64	**pVMUS 11**	Ac, A, Ak, Cb, Cu, Do, G, M, Nt, R, S, Sf	Ac, A, Do ,G, M, R, S (6)	Ac, A, Do ,G, M, R, S (6)	5×10^{-9}
CBMM57	**pVMUS1**	Ac, A, Cb, Cu, R	Ac, A,R (3)	Ac, A,R (3)	4.17×10^{-9}
CBMM70	**pVSPF4**	Ac ,A, Ak Cb,Cu, Fr M, Nt, Na, Nx, R, Sf, Tr, T	Ac, A, Ak, Cb, Cu, Fr, Tr, Sf (8)	Ac, A, Ak, Cb, Cu, Fr, Tr, Sf (8)	3.58×10^{-9}

The numbers in parenthesis indicate the number of antibiotic resistance genes on the plasmid.
Ac-Amoxycillin, A-Ampicillin,Ak-Amikacin,Co-Cotrimaxazole,Cb-Carbenicillin,Cu-Cefuroxime,C-Chlramphenicol, Cf-Ciprofloxacin, Ct-Chlortetracycline, Do-Doxy cyclinehydrochloride,Fr-Furazolidone,G-Gentamycin,M-Meropenem,Na-Nalidixic acid, Nt-Netilmycin , Nx-Norfloxacin, Ne-Neomycin, R-Rifampicin, S-Streptomycin, Sf-Sulfafurxazole, Tr-Trimethoprim, T-Tetracycline
(The abbreviations are as indicated by manufacturer, and according to NCCLS, 2001)

The plasmids harbored 4-8 resistance determinants on them. All 12 plasmids harbored ampicillin and amoxicillin resistance determinants on them. Nalidixic acid resistance was seen only on plasmid pB9 isolated from CBMW47, which was identified as *V. furnissii.* Chloramphenicol resistance was not found to be plasmid mediated in this study, although it was observed in some of the isolates. It is presumed that this is chromosome-mediated resistance.

4.7. 2. Curing of plasmids.

In the present investigation, all the *Vibrio* isolates with plasmids (n=26)were subjected to curing with ethidium bromide in order to understand the association of plasmids with drug resistance phenotype. Curing procedure yielded reproducible results and in the cured isolates, resistances that were plasmid encoded, were found sensitive to the corresponding antibiotics .The results suggest confirming the plasmid mediated nature of the drug resistance in these *Vibrio* isolates (Table –22).

Curing protocols revealed that the plasmid encoded resistance phenotype of isolates from water becomes sensitive after curing. However, certain drug resistance markers were expressed, even after curing, indicating that these R-markers are chromosomal in nature.

Efforts to isolate plasmids after curing and the absence of plasmid bands on the agarose gels corroborated that the plasmids- encoded resistances are indeed lost. And no plasmids were obtained after curing. (Fig.14).

Table -22. Studies on plasmid curing in *Vibrio* isolates

Culture no	Plasmid	Identity of *Vibrio* spp	R Pattern before curing (Plasmid borne)	R Pattern after curing (Chromosomal borne)	Plasmid content (before curing)	Plas cont (afte curi
CBMW01	pVPCL5	*V. mimicus*	Ac, A, Ak, Cb, Cu, Fr, Nt, Ne, S	Ac, Fr, Ne, Cu	18.31	
CBMW25	pVCVA8	*V. .damsela*	Ac, A, Ak, Cu, C, Co, Cf, Ct ,Do, Fr, G, M, Nt, Ne Na, Nx, R, S, Sf, Tr	Ac ,A, Ak, Cu, C, Ct ,Cf Co, Do ,G, Fr , M, Nt, Na, Ne, Nx	16.2	
CBMW08	pVP5	*V. carchariae*	Ac, A, Ak, Cb, Ne, S, Tr,	Ac, A, Tr, Ne	13.5	
CBMW09	pVP17	*V. metschnikovii*	Ac ,A, Cb, Cu, Ne, R,S,	Ac, A, Cb, Ne	9.9	
CBMW13	pVP10	*V. proteolyticus*	Ac, A, Ak, Cb, Cu, Ne, R,S,	Ac , A, Ak, Ne,	25.1	
CBMW26	pVEK1	*V. anguillarum*	Ac, A Cb, R	R	22.1, 6.2	
CBMW31	pVOMM14	*V. mediterranei*	Ac, A, Cb, C, Do, Sf	Ac, A ,Cb. C, Do,	19.2	
CBMW32	pVOMM1	*V. vulnificus*	Ac, A, Cb, Cu	Ac	12.3,4.16	
CBMW33	pVOMM2	*V. furnissii*	Ac, A	Ac	13.16	
CBMW34	pVOMM3	*V. alginolyticus*	Ac, ,A, Cb, R	Ac, A	13.58	
CBMW35	pVOMM4	*V. anguillarum*	Ac, ,A	Ac ,A	12.11	
CBMW36	pVOMM5	*V. vulnificus*	Ac, A, Cb, S, M, Cu, Fr, T	Ac, A, Cb, S, Fr, T	22.7	
CBMW37	pVN36	*V. mediterranei*	Ac, A, Cb, R, S	Ac	14.4, 6.6, 2.1, 1.4	

Ac-Amoxycillin, A-Ampicillin,Ak-Amikacin,Co-Cotrimaxazole,Cb-Carbenicillin,Cu-Cefuroxime,C-Chlramphenicol, Cf-Ciprofloxacin, Ct-Chlortetracycline, Do-Doxy cyclinehydrochloride,Fr-Furazolidone,G-Gentamycin,M-Meropenem,Na-Nalidixicacid, Nt-Netilmycin , Nx-Norfloxacin, Ne-Neomycin, R-Rifampicin, S-Streptomycin, Sf-Sulfafurxazole, Tr-Trimethoprim, T-Tetracycline
(The abbreviations are as indicated by manufacturer, and according to NCCLS, 2001)

Table -22. Studies on plasmid curing in *Vibrio* isolates ***cont'd***

Vibrio culture no	Plasmid	Identity	R Pattern before curing (Plasmid borne)	R Pattern before curing (Plasmid borne)	Plasmid content (before curing)
CBMW42	pVPD3	*V. costicola*	Ac, A, Cb, Cu, Fr, R, S, Ne	Ac, A, Cb, Cu,Ne	23
CBMW47	pVB9	*V. furnissii*	Ac, A, Ak, Cb, Cu, Do, Fr, Na, Ne, R ,S, T,	Ac,A,Cb,Cu,Do, Fr ,Ne,S, T	27.7, 15.0, 6.7
CBMW52	pVKG1	*V. mediterranei*	Ac, A, Ak, Cb, Cu, Fr, Nt, Ne, S	Ak, Fr ,Nt ,Ne, S	16.6,2.9
CBMM56	pVMUS 7	*V. costicola*	Ac, A, Cb, Cf, Nx, R, Tr	Ac, Cb, Nx, Tr	16.69
CBMM57	pVMUS1	*V. alginolyticus*	Ac, A, Cb, Cu, R	Ac, A, Cu, Cb	19.36
CBMM58	pVMUS10	*V. mimicus*	Ac, A, Cb, Cu, Cf, R	Ac, A , Cu,Cf	14.93
CBMM62	pVMUS 15	*V. marinus*	Ac ,A, Ak Cb, Cu, Cf, Do, Fr ,R ,S,T, Ne	Ac, A, Cb, Do, Fr, T, Ne	14.38
CBMM64	pVMUS11	*V. orientalis*	Ac, A, Ak , Cb, Cu, Do, G, M, Nt, R, S, Sf	Ac,A,Ak,Cb,Cu, ,Nt,	14.3,6.44
CBMM70	pVSPF4	*V mediterranei*	Ac ,A, Ak, Cb, Cu, Fr M, Nt, Na, Nx, R, Sf, T, Tr	Ac, M ,Nt,Na, ,Nx, Na, R,	16.08,8.27,5.98
CBMC73	pVSY1	*V. mediterranei*	Ac, A, Cb, Cu, C, M, Nt, R, S	Cu, C, Nt,S	15.36
CBMC84	pVSY3	*V. costicola*	Ac, A, Cb, Cu, Cf, M, Nt, R, S	A Cu, ,Cf, Nt, R,S	14.38
CBMC78	pVSY6	*V .cincinnatiensis*	Ac, A, Cb, Cu, C, Co, Cf, Ct, Na, R	A, Cu C, Co, Cf, Ct	14.3
CBMP87	pVPV9	*V .anguillarum*	Ac, A, Ak, Cb, Cu, Fr ,G,R	All are sensitive	16.2

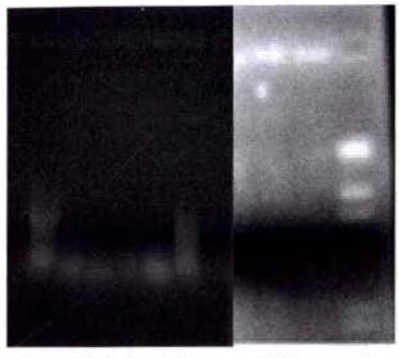
Ampicillin,Ak-Amikacin,Co-Cotrimaxazole,Cb-Carbenicillin,Cu-Cefuroxime,C-Chlramphenicol,Cf-Ciprofloxacin,Ct-Chlortetracycline,Do-Doxy cyclinehydrochloride,Fr-Furazolidone,G-Gentamycin,M-Meropenem,Na-Nalidixicacid, Nt-Netilmycin , Nx-Norfloxacin, Ne-Neomycin, R-Rifampicin, S-Streptomycin, Sf-Sulfafurxazole, Tr-Trimethoprim, T-Tetracycline
(The abbreviations are as indicated by manufacturer, and according to NCCLS, 2001)

Fig.14. Curing of plasmids in *Vibrios* isolated from different samples.

M 2 3 4 5 6 7 8

Plasmids isolated from cured *Vibrio* species -Lanes 2,3,4,5,6,7 & 8 and has cured plasmids pVP5,pV P10 ,pVP17, pVPD3, pVP10, pVP17, pVB9,pVCL5,pVCVA8 M as Marker

1 2 3 4 5 6 7 8 9 10 11 M



Plasmids isolated from cured *Vibrio* species - Lanes 1,2,3,4,,5,6,7,8,9,10 ,& 11 has cured plasmids such as pVPV9, pVO14, pVKG1, p,VOMM1,pVOMM3,pVOMM4, pVOMM5, pVSY1,pV SY 6, pVSY3 , pVSPF4 and lane M has Marker λ DNA Hind III Digest

M 2 3 4 5 6 7 8

Plasmids Isolated from cured *Vibrio* species -Lanes 2,3,4,5,6,7 & 8 has cured plasmids such as pVMUS10, pVMUS7, pVMUS15, pVMUS11,pVMUS1, pVEK1, pVN 36 and M is λ DNA Hind III Digest as marker

From the results presented in Table-23 and Fig.14 it is inferred that the strain CBMW01 when subjected to antibiotic sensitivity tests gave the following resistance pattern Ac, A, Ak, Cb, Cu, Fr, Nt, Nc, S .The same strain after curing has retained only the following resistance pattern Ac, Fr ,Ne, Cu and became sensitive to the other 5 antibiotics. The plasmid curing experiments and attempts were made to re-isolate plasmids from the cured strains implied that the lost antibiotic resistance determinants may be residing on the cured pVPCL5 plasmid.

Similarly antibiotic resistance pattern for the strain CBMW25 was found to be Ac, A, Ak, Cu, C, Co, Cf, Ct ,Do, Fr , G, M, Nt, Nc Na, Nx, R, S, Sf &Tr The same strain after curing produced a R-pattern .Ac ,A, Ak, Cu, C, Ct ,Cf Co, Do ,G, Fr , M, Nt, Na, Ne & Nx. These results indicated that the pVCVA8 encoded R-genes , R, S, Sf &Tr were lost.

The strain CBMW08 when subjected to antibiotic sensitivity tests gave the following resistance pattern -Ac, A, Ak, Cb, Nc, S, Tr. The same strain after curing retained only the following resistance pattern -Ac, A,Tr, Nc and became sensitive to the rest of antibiotics. The plasmid loss was revealed by their absence, when tried to isolate from the cured strains. The results indicate that the lost antibiotic resistant genes may be encoded in the cured pVP5 plasmid.

Similarly antibiotic resistance pattern for the strain CBMW09 was found to be Ac ,A, Cb, Cu, Ne, R & S. The same strain after curing produced showed a pattern Ac, A, Cb, Nc. The results indicate that pVP17 plasmid encoded genes Cu, R & S were lost.

The strain CBMW13 when subjected to antibiotic sensitivity tests gave the following resistance pattern Ac, A, Ak, Cb, Cu, Nc, R,S. The same strain after curing retained only the following resistance pattern Ac , A, Ak, Cb and became sensitive to

the rest of antibiotics. These results indicated that the lost antibiotic resistant genes may be encoded on pVP10 plasmid

Similarly, the antibiotic resistance pattern of the strain CBMW26 was found to be Ac, A, Cb, R. The same strain after curing showed resistance only to R, which indicated that the pVEK1 plasmid encoded genes, was lost.

The strain CBMW31 was resistant to Ac, A, Cb, C, Do & Sf. The same strain after curing retained resistance only to Ac, A, Cb, C and Do, but became sensitive to the other antibiotics. Similarly antibiotic resistance pattern for the strain CBMW 32 was found to be Ac, A, Cb, Cu, which were lost after curing indicating that plasmid pVOMM1 encoded R-genes were lost.

The strain CBMW33 when subjected to antibiotic sensitivity tests gave the following resistance pattern Ac, A The same strain after curing has retained only the following resistance pattern Ac, and became sensitive to the rest of antibiotics. The plasmid loss was revealed by their absence, when tried to reisolate from the cured strains, which indicate that the lost antibiotic resistant genes may be encoded in the cured pVOMM2 plasmids.

The antibiotic resistance pattern for the strain CBMW34 was Ac, A, Cb, R. The R-pattern of the same strain after curing was only Ac, A , which indicate that plasmid pVOMM3 encoded genes for Cb and R were lost.

The strain CBMW35 gave the following resistance pattern-Ac, A. The same strain after curing has retained the same resistance pattern Ac, A, which was indicative that both these R-genes were present on the plasmid pVOMM4.

Similarly antibiotic resistance pattern for the strain CBMW 36 was found to be Ac, A, Cb, S, M, Cu, Fr, T. The same strain after curing produced Ac, A, Cb, S, Fr, T. indicating that plasmid pVOMM5 encoded genes were lost.

The strain CBMW 37 when subjected to antibiotic sensitivity tests gave the following resistance pattern Ac, A, Cb, R, S The same strain after curing has retained resistance to only Ac and became sensitive to the rest of antibiotics. The plasmid loss was revealed by their absence, when tried to reisolate from the cured strains. which indicate that the lost antibiotic resistant genes may be encoded in the cured pVN36 isolate plasmids.

Similarly antibiotic resistance pattern for the strain CBMW 42 was found to be Ac, A, Cb, Cu, Fr, R, S, Ne. The same strain after curing produced Ac, A, Cb, Cu, Ne, which indicate that plasmid pVOMM5 encoded genes were lost.

The strain CBMW 47 when subjected to antibiotic sensitivity tests gave the following resistance pattern Ac, A, Ak, Cb, Cu, Do, Fr, Na, Ne, R, S, T. The same strain after curing has retained only the following resistance pattern Ac, A, Cb, Cu, Do, Fr, Ne, S, T and became sensitive to the rest of antibiotics. The plasmid loss was revealed by their absence, when tried to reisolate from the cured strains, implying that the lost antibiotic resistant genes may be encoded in the cured pVB9 plasmids.

Similarly antibiotic resistance pattern for the strain CBMW 52, was found to be Ac, A, Ak, Cb, Cu, Fr, Nt, Ne, S The same strain after curing showed resistance to Ak, Fr ,Nt ,Ne, S, which indicate that plasmid pVKG1 encoded genes were lost.

The strain CBMM 56 when subjected to antibiotic sensitivity tests gave the following resistance pattern Ac, A, Cb, Cf, Nx, R, Tr. The same strain after curing has retained only the following resistance pattern Ac, Cb, Nx, Tr and became sensitive

to the rest of antibiotics. The plasmid loss was revealed by their absence, when tried to reisolate from the cured strains, which indicate that the lost antibiotic resistant genes may be encoded in the cured pVMUS 7 plasmids.

Similarly antibiotic resistance pattern for the strain CBMW 57 was found to be Ac, A, Cb, Cu, R The same strain after curing produced Ac, A, Cu, Cb which indicate that that plasmid pVMUS1 encoded genes were lost.

The strain CBMM 58 when subjected to antibiotic sensitivity tests gave the following resistance pattern Ac, A, Cb, Cu, Cf, R The same strain after curing has retained only the following resistance pattern Ac, A , Cu, Cf and became sensitive to the rest of antibiotics. The plasmid loss was revealed by their absence, when tried to reisolate from the cured strains, which indicate that the lost antibiotic resistant genes may be encoded in the cured pVMUS10 plasmids.

Similarly antibiotic resistance pattern for the strain CBMM 62 was found to be Ac ,A, Ak Cb, Cu, Cf, Do, Fr ,R ,S,T, Ne .The same strain after curing produced Ac, A, Cb, Do, Fr, T, Ne, which indicate that plasmid pVMUS 15 encoded genes were lost.

The strain CBMM 64when subjected to antibiotic sensitivity tests gave the following resistance pattern Ac, A, Ak , Cb, Cu, Do, G, M, Nt, R, S, Sf The same strain after curing has retained only the following resistance pattern Ac, A, Ak, Cb, Cu, ,Nt and became sensitive to the rest of antibiotics. The plasmid loss was revealed by their absence, when tried to reisolate from the cured strains, which indicate that the lost antibiotic resistant genes may be encoded in the cured pVMUS11 plasmid.

Similarly antibiotic resistance pattern for the strain CBMM70 was found to be Ac ,A, Ak Cb, Cu, Fr M, Nt, Na, Nx, R, Sf, T, Tr .The same strain after curing

produced Ac, M ,Nt,Na, ,Nx, Na, R which indicate that pVSPF4 plasmid encoded genes were lost.

The strain CBMC 73 when subjected to antibiotic sensitivity tests gave the following resistance pattern Ac, A, Cb, Cu, C, M, Nt, R, S The same strain after curing has retained only the following resistance pattern Cu, C, Nt, S and became sensitive to the rest of antibiotics. The plasmid loss was revealed by their absence, when tried to reisolate from the cured strains, which implies that the lost antibiotic resistant genes may be encoded in the cured pVSY1 plasmid.

Similarly antibiotic resistance pattern for the strain CBMC 84 was found to be Ac, A, Cb, Cu, Cf, M, Nt, R, S same strain after curing produced A Cu, ,Cf, Nt, R,S indicates that pVSY3 plasmid encoded genes were lost.

The antibiogram of strain CBMC78 was - Ac, A, Cb, Cu, C, Co, Cf, Ct, Na, R.The same strain after curing has retained only the following resistance pattern- A, Cu C, Co, Cf, Ct and became sensitive to the rest of antibiotics. The plasmid loss was revealed by their absence, when tried to reisolate from the cured strains which indicate that the lost antibiotic resistant genes may be encoded in the cured pVSY6 plasmid.

Similarly antibiotic resistance pattern for the strain CBMP87 was found to be Ac, A Ak Cb, Cu, Fr, G, R same strain after curing produced that all are sensitive indicates that pVPV9 plasmid encoded genes becoming lost (Table 24 & figure 14).

4.7. 3. Conjugation experiments

The conjugation efficiency, resistance pattern of the ex-conjugants and the plasmid extraction from the transconjugants *E. coli* HB101 were carried out. All the plasmids studied, except two, were found to be conjugative plasmids. After conjugation, the exconjugants possessing the characteristic resistance pattern and the ex-conjugants were recovered from Mac Conkey agar plates incorporated with Ampicillin and Streptomycin.

Conjugation efficiency analysis showed that the *Vibrio* isolates from water sample conjugated with an efficiency varying from 10^{-2} to 10^{-9}(Table –23). The *Vibrio* isolates from Mollusk and crustacean samples also conjugated with a varying efficiency ranging from 10^{-3} to10^{-9}. However, *Vibrio* isolates, one each from water and plankton sample failed to conjugate, as evidenced by the unchanging R-pattern of the recipient.

The studies on the drug resistance patterns of the recovered transconjugants revealed that the resistance markers were transferred to the recipient strains of *E. coli* HB101.

Table -23. Conjugation efficiency and resistance pattern of exconjugant (*E. coli* HB 101)

Vibrio culture no.	Plasmid name	Antibiotic resistance pattern of Donor	R-resistance pattern of exconjugant *E.coli HB 101*	Conjugation efficiency
CBMW25	**pVCVA8**	Ac, A, Ak, Cu, C, Co, Cf, Ct ,Do, Fr , G, M, Nt, Ne Na, Nx, R, S, Sf, Tr	Ac, A, R, S, Sf, Tr,	0.215×10^{-8}
CBMW08	**pVP5**	Ac, A, Ak, Cb, Ne, S, Tr,	Ac, Ak, Cb ,S, Tr	0.444×10^{-4}
CBMW09	**pVP17**	Ac ,A, Cb, Cu, Nc, R,S,	Ac, A,Cu, R,S	0.046×10^{-2}
CBMW13	**pVP10**	Ac, A, Ak, Cb, Cu, Ne, R,S,	Ac, A, Cb ,Cu, R ,S	2.357×10^{-4}
CBMW26	**pVEK 1**	Ac, A Cb, R	Ac, A, Cb,S	4.25×10^{-3}
CBMW31	**pVO14**	Ac, A, Cb, C, Do, Sf	Ac ,A, Sf, S	15.333×10^{-5}
CBMM32	**pVOMM1**	Ac, A, Cb, Cu	Ac, A, Cb, S	0.222×10^{-4}
CBMW33	**pVOMM2**	Ac, A	Ac, A,S	0.333×10^{-4}
CBMW34	**pVOMM3**	Ac, ,A, Cb, R	Cb, R, S	6×10^{-5}
CBMW35	**pVOMM4**	Ac, ,A	Ac, S	10.42×10^{-5}
CBMW36	**pVOMM5**	Ac, A, Cb, S, M, Cu, Fr, T	M, Cu, S	5.5×10^{-4}
CBMW37	**pVN36**	Ac, A, Cb, R, S	Ac, A, Cb, S, R	1.071×10^{-6}

Ac-Amoxycillin,A-Ampicillin,Ak-Amikacin,Co-Cotrimaxazole,Cb-Carbenicillin,Cu-Cefuroxime,C-Chlramphenicol, Cf-Ciprofloxacin, Ct-Chlortetracycline, Do-Doxycycline hydrochloride, Fr-Furazolidone, G-Gentamycin, M-Meropenem, Na-Nalidixic acid, Nt-Netilmycin , Nx-Norfloxacin, Ne-Neomycin, R-Rifampicin, S-Streptomycin, Sf-Sulfafur azole, Tr-Trimethoprim, T-Tetracycline
(The abbreviations are as indicated by manufacturer, and according to NCCLS, 2001)

Table -23. Conjugation efficiency and resistance pattern of exconjugant (*E. coli* HB 101) **.....contd.**

Vibrio culture no.	Plasmid name	Antibiotic resistance pattern of Donor	R- resistance pattern of Exconjugant *E.coli HB 101*	Conjugation efficiency
CBMW42	pVPD3	Ac, A, Cb, Cu, Fr, R, S, Ne	Ac, A, Fr, R, S	8*10-5
CBMW47	pVB 9	Ac, A, Ak, Cb, Cu, Do, Fr, Na, Ne, R ,S, T,	Ac, A, Ak ,Na, R,S , T	0.3*10-5
CBMW52	pVKG 1	Ac, A, Ak, Cb, Cu, Fr, Nt, Ne, S	Ac ,A, Cb, Cu, S	1.78*10-5
CBMM56	pVMUS 7	Ac, A, Cb, Cf, Nx, R, Tr	Ac, A, Cf, Cb, R,S	0.18*10-9
CBMM57	pVMUS 1	Ac, A, Cb, Cu, R	Ac, A, R ,S	2.14*10-3
CBMM58	pVMUS10	Ac, A, Cb, Cu, Cf, R	Ac, A, Cb, R,S	1.07*10-6
CBMM62	pVMUS 15	Ac ,A, Ak Cb, Cu, Cf, Do, Fr ,R ,S,T, Ne	Ac, Ak, A, Cu, Cf, R, S	0.02*10-3
CBMM64	pVMUS 11	Ac, A, Ak , Cb, Cu, Do, G, M, Nt, R, S, Sf	Do,G,M,R,S, Sf, ,	2.28*10-3
CBMM70	pVSPF4	Ac ,A, Ak Cb, Cu, Fr M, Nt, Na, Nx, R, Sf, T, Tr	Ac, A, Ak, Cb, Cu, Fr,S, Sf ,Tr, T	1.5*10-6
CBMC72	pVSY1	Ac, A, Cb, Cu, C, M, Nt, R, S	Ac, A, Cb, R,M,S	11.66*10-7
CBMC 84	pVSY3	Ac, A, Cb, Cu, Cf, M, Nt, R, S	Ac, A, Cb,M,S	5.83*10-10
CBMC 78	pVSY6	Ac, A, Cb, Cu, C, Co, Cf, Ct, Na, R	Ac, Cb, Na, R,S	6.83*10-9

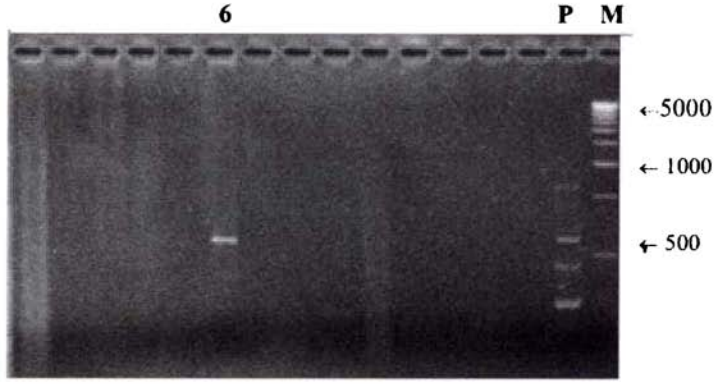
Ac-Amoxycillin, A-Ampicillin,Ak-Amikacin,Co-Cotrimaxazole,Cb-Carbenicillin,Cu-Cefuroxime,C-Chlramphenicol, Cf-Ciprofloxacin, Ct-Chlortetracycline, Do-Doxy cyclinehydrochloride,Fr-Furazolidone,G-Gentamycin,M-Meropenem,Na-Nalidixicacid, Nt-Netilmycin , Nx-Norfloxacin, Ne-Neomycin, R-Rifampicin, S-Streptomycin, Sf-Sulfafur azole, Tr-Trimethoprim, T-Tetracycline

(The abbreviations are as indicated by manufacturer, and according to NCCLS, 2001)

4.7.4. PCR Analysis

PCR based detection method was used for studying the presence of integrons and sxt elements from the plasmids isolated from *Vibrio* isolates. It was observed that only one single plasmid pP17 from strain CBW09 identified as *Vibrio metschnikovii* was positive for the presence of *int* genes of integrons, giving a PCR product of 800 bp size.(Fig.15). All other plasmids were negative for the presence of integrons and *sxt*.

Fig.15. Screening for integron *int* genes in isolated R-plasmids of *Vibrios* using primers for *inF* and *inR* using PCR.



Lane 6-pVP17 positive for the integron *int* genes
Lane P-*V.cholerae* El Tor CO366 genomic DNA positive for integron (positive control)
Lane M -Marker

4. 8 . Restriction digestion of plasmids.

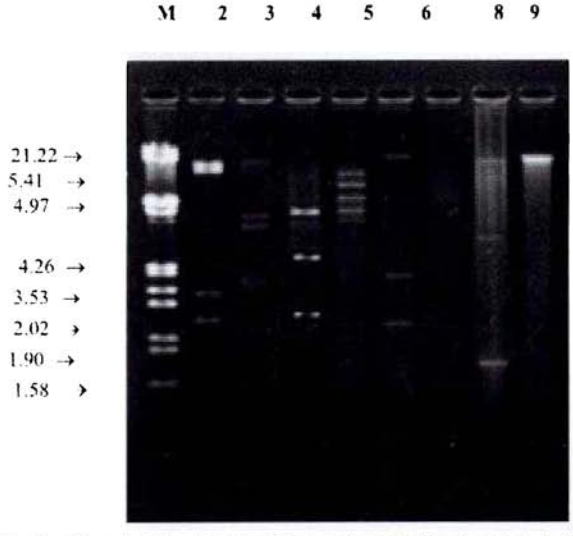
The results of the restriction digestion of plasmids are shown in Table- 24, 25, 26, 27, 28, & 29 and Fig. 16, 17, 18, 19, 20 & 21.

Restriction digestion using four different enzymes i.e. EcoR1, HindIII and Bam H1 and HaeIII, revealed different restriction profiles for the various plasmids isolated. Over all results indicated that the fragments obtained after digestion were of different size and they are heterogeneous in nature.

Table- 24. Restriction digestion of plasmids with Bam H1

Plasmid	Identity	No of fragments obtained	Size of fragments digested (kb)
pVOMM2	*V. furnissii*	3	11, 3.4, 1.5
pVOMM4	*V. anguillarum*	3	12, 3.5, 2.1
pVSY3	*V. costicola*	3	5.4, 4.6, 3.5
pVOMM5	*V.vulnificus*	4	10, 6.1, 4.9, 4.4
pVPV9	*V. anguillarum*	3	18, 4.2, 3.1
pVMUS10	*V.mimicus*	3	10 ,4.9, 1.6

Fig.16 Restriction digestion of plasmids with Bam H1

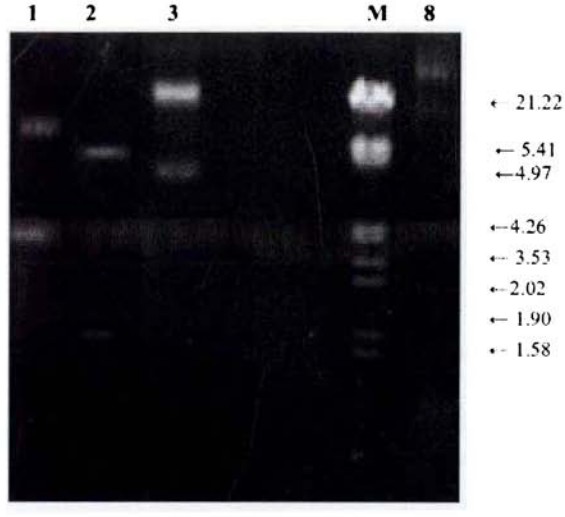


Restriction digested fragments of isolated plasmids- Lanes 2, 3, 4, 5, 6, 8,& 9 has pVOMM2; pVOMM4; pVSY3; pVOMM5; pVPV9; pvMUS10; pVO14 undigested respectively and Lane M has λ DNA Hind III /E.coR1 double digest as marker

Table- 25. Restriction digestion of the plasmids with EcoR1 and BamHI

Plasmid	Identity	No of fragments obtained	Size of fragments digested (kb)
pVMUS15 (BamH1digest)	*V. mediterranei*	2	10,4.6
pVPD3(E.coR1 digest)	*V. costicola*	3	5,1
pVMUS15 (E.coR1 digest)	*V. mediterranei*	2	11, 3.6
pVMUS1 E.coR1 digest	*V. alginolyticus*	2	23,18.

Fig. 17. Restriction digestion of plasmids with Eco R1 and Bam H1

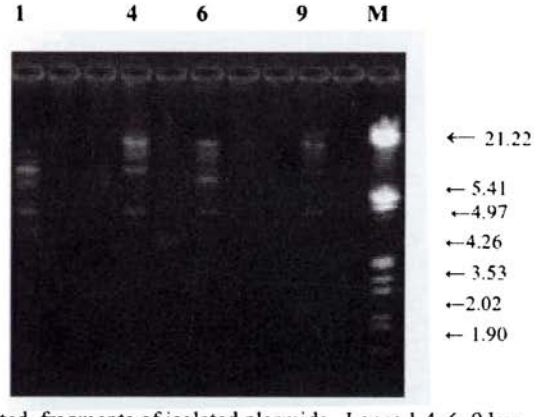


Restriction digested fragments of isolated plasmids - Lanes1 has pVMUS15 Bam HIDigest; Lane 2 has pVPD3 digested with EcoR1; lane 3 has pVMUS 15 digested with E.coR1 and lane M & 8 has pVMUS1digested with E.coR1; and Lane M has λ DNA Hind III /E.coR1 double digest as marker

Table- 26. Restriction digestion of the plasmids by EcoRI

Plasmid	Identity	No of fragments obtained	Size of fragments digested (kb)
pVEK1	*V. anguillarum*	5	10, 9, 4.6 ,4.2, 3
pVOMM5	*V. vulnificus*	3	13,8,4.4
pVO14	*V. mediterranei*	3	11,6,4.4
pVOMM4	*V. anguillarum*	3	11,6,4.4

Fig.18. Restriction digestion of plasmids with EcoR1

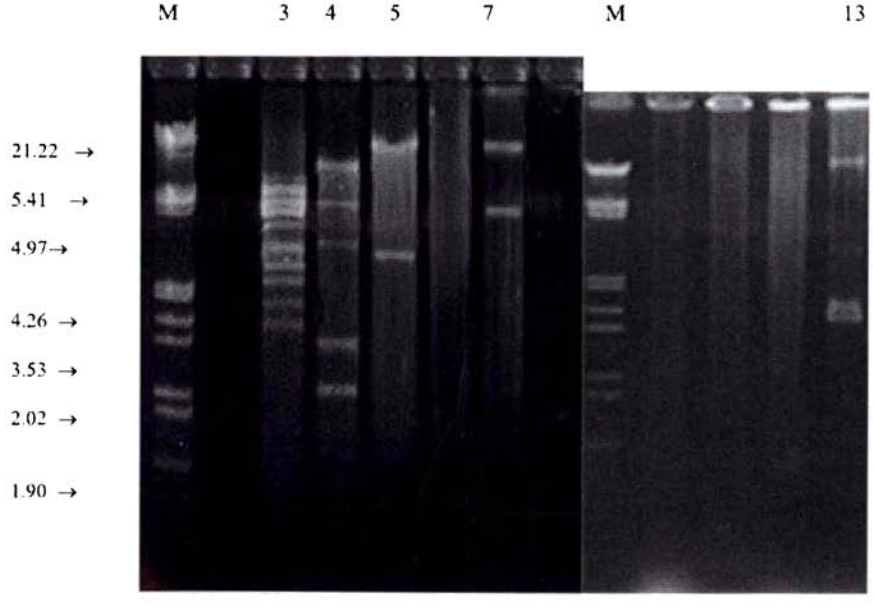


Restriction digested fragments of isolated plasmids Lanes 1,4, 6, 9 has pVEK1; pVOMM5; pVO14 ; pVOMM4 and M has Marker λ DNA Hind III /E.coR1 /Double Digest

Table -27. Restriction digestion of plasmids with EcoRI

Plasmid	Identity	No of fragments obtained	Size of fragments digested (kb)
pVP 17	*V .metschnikovii*	9	5.4, 4.9, 4.8, 4.7, 4.6, 4.5, 4.2, 3.5, 2.2
pVP 5	*V. carchariae*	5	7, 5.4, 4.6, 2, 1.9
pVCL5	*V. mimicus*	2	16, 4.3
pVMUS7	*V. costicola*	2	16, 4.9
pVOMM3	*V. alginolyticus*	3	21, 3.3, 3.2

Fig.19. Restriction digestion of plasmids with EcoR1

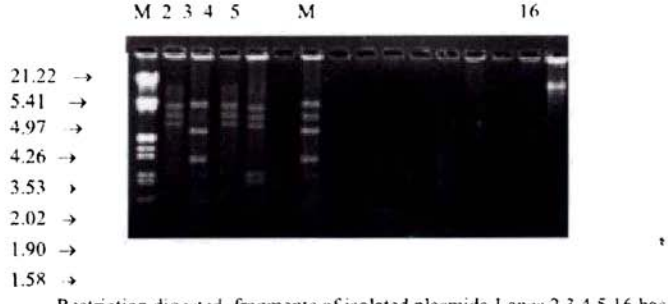


Restriction digested fragments of isolated plasmids - Lanes-3, 4, 5, 7, & 13 have pVP17; pVP5; pCL5; pVMUS7; pVOMM3 digested with EcoRI and lane M has λ DNA Hind III /E.coR1 double digest as marker

Table -28. Restriction digestion of plasmids with HindIII

Plasmid	Identity	No of fragments obtained	Size of fragments digested (kb)
pVP10	*V. proteolyticus*	4	11, 5.5, 5.4, 4.9,
pVSY1	*V. carchariae*	3	5.5, 4.3, 2,
pVSY6	*V. cincinnatiensis*	4	18, 5, 4.9, 4.7
pVOMM1	*V. vulnificus*	5	5, 4.9, 4.7, 3.5, 3.5, 2
pVKG1	*V. mediterranei*	4	7, 5.4, 4.2, 2.0

Fig. 20 Restriction digestion of plasmids with Hind III

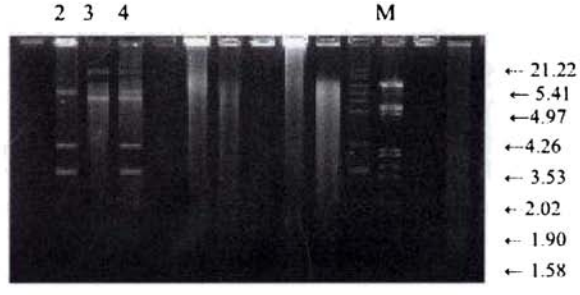


Restriction digested fragments of isolated plasmids-Lanes 2,3,4,5,16 has pVP10 ; pVSY1, pVSY6; pVOMM1; pVKG1 digested withHindIII ; pVO14 undigested, respectively and lane M has M- Marker λ DNA Hind III :F coR1 double digest

Table –29. Restriction digestion of plasmids with Hind III and Hae III

Plasmid	Identity	No of fragments obtained	Size of fragments digested (kb)
pVCVA 8 (Hind III)	*V .damselae*	3	9, 4.9, 3.5
pVCVA 8(HaeIII)	*V. damselae*	2	11, 5.5

Fig. .21. Restriction digestion of plasmids with HindIII and HaeIII



Restriction digested fragments of isolated plasmid- Lanes 2 ,3,4 has pVCVA 8 Hind III; CVA 8 HaeIII; .pVCVA 8 Hind III/ HaeIII digest and Lane M has Marker λ DNA Hind III /E.coR1 Double Digest

Chapter 5

DISCUSSION

Increase in antibiotic resistance has become more serious because of the dissemination by the various mobile elements by horizontal gene transfer. Different methods of horizontal gene transfer such as transformation, conjugation, transduction occurs among bacterial populations in the environment. Since the acquired resistance by plasmids is the most dangerous, antibiotic therapy in future may turn ineffective. Further, transfer of these acquired resistance to other organisms in the environment, may create havoc in the ecosystem. This will be a serious threat to aquaculture organisms as well as to the human health. Realizing the need to understand the rise of antibiotic resistance, the experiment was designed. The objective of the study was to assess the antibiotic resistance in the *Vibrios* from coastal water samples, aqua farm water samples, certain seafood samples (molluscans and crustacean) and characterize the isolated plasmids in relation to their drug resistance. Since plasmids can easily transfer their resistance to other microorganisms, rapid dissemination of resistance to a variety of microorganisms occurs in the aquatic environment. Consumption of raw or improperly cooked sea foods or fishes thus becomes a great threat to human health. The findings of the present investigation may help to understand the gravity of the problem and devise a nation wide antibiotic policy. Strict regulations for the use of antibiotics in animal husbandry and aquaculture could be necessary to curtail any further increase in antibiotic resistance in the aquatic ecosystem.

5.1. Incidence of *Vibrios* from different samples

Coastal environment, by its nature, presents a theatre of ecological diversity and evolutionary adaptation (Carla *et al.*,2005). *Vibrio* species occur widely in aquatic environments and are a part of normal flora of coastal seawater. They also exists as normal flora in fish and shellfish but have also been recognized as opportunistic pathogens in many marine animals (Austin and Austin, 1999). *Vibrios* are one of the most important pathogens for reared aquatic organisms such as penaeid shrimps (Lightner, 1993), several fish species and molluscans (Austin, 1988), and also for corals (Ben-Haim *et al.*, 2003). *Vibrios* constitute a major portion of the microbiota in brackishwater pond ecosystem.

From the 396 isolates obtained from different sources, a total of 100 bacterial isolates were segregated as *Vibrios*, i.e approximately 25.25% of the sample size. This was based on the morphological and biochemical characteristics of the genus *Vibrios* ie, gram negative, rod/cocci shaped, oxidase positive and MOF fermentative tests (Holt *et al.*, 1994.). The attempt to identify the different species of *Vibrio* isolates from different samples also indicated that 100 isolates exhibited the characteristic biochemical reactions of *Vibrio* spp.

Distribution of different species of *Vibrio* in different samples was analyzed in the present investigation. Of the different *Vibrio* isolates obtained from the different samples (water, molluscans, crustacean and plankton), *V. cholerae* was predominant by its presence. It was observed that as many as 23 different *Vibrio* species were identified based on their biochemical characterizations. Among the species observed *V. cholerae* was recorded to be a predominant species among all the samples studied viz. 10.64% from water, 20% from crustacean, and 23.08% from planktons. While all other species were also present at different levels: *V. mimicus* (8.51 %), *V.*

parahaemolyticus, V. cincinnatiensis, V. vulnificus, V. marinus, V. mediterranei, V. proteolyticus (6.38% each), *V. furnissii, V alginolyticus, V. anguillarum, V. carchariae, V. damselae, V. pelagius* (4.26 % each). *V. mediterranei, V. costicola, V. hollisae , V. campbellii, V. neries, V. splendidus, V. metchnikovii, V. harveyi* (2.1 % each). Looking at the diversity exhibited, the presence of confirmed pathogenic *Vibrios* like *V. cholerae, V. parahaemolyticus, Vibrio mimicus, and Vibrio vulnificus, Vibrio anguillarum and Vibrio alginolyticus* are made obvious.

In shrimp farms from India, Otta *et al.*, (1999) and Vaseeharan and Ramasamy (2003) observed earlier that *Vibrio* species accounted for 38–81% of the bacterial biota. . Presence of *V. cholerae* isolates in crustacean and plankton samples, may pose a serious human health hazard. *Vibrio cholerae* is reported to be associated with a variety of aquatic organisms including crustaceans, zooplankton, phytoplankton and algae (Colwell, 1996). *Vibrio cholerae* is unusual among pathogens in its ability to cause epidemic and pandemic disease. Further, in our study, it was evident that *V. mimicus* demonstrated the highest prevalence in molluscan samples. *Vibrio mimicus* is a species closely related to *V. cholerae* (Vieira *et al.*, 2001). In addition, *V. mimicus* isolates have been identified that encode the two main virulence factors of *V. cholerae*, CT and TCP, highlighting their potential as reservoirs of these elements and their possible emergence as agents of cholera (Boyd *et al.*, 2000). Majority of seafood-associated diseases are resulting from consumption of molluscan (such as mussels, oysters & clams), which may be due to the filter feeding habit of these organisms that concentrate particles present in surrounding waters. (Allam *et al.*, 2000). Many potential pathogenic *Vibrios* are naturally active in coastal waters and may be entrapped by bivalves and transmitted to humans. Microbiological studies have shown that plankton play a significant role as a reservoir of *V. cholerae* in the environment and are also significant in biological productivity of the oceans (Islam *et*

al., 1990). Abundance of the *Vibrio* species in the water and their ability to persist within bivalve tissues are responsible for their high concentration within this host that can act as an environmental reservoir of human pathogens. *Vibrio* resistance to depuration procedures of edible bivalves is a further reason for the worldwide incidence of *Vibrio*-related seafood borne diseases. Only a few *Vibrio* strains are considered true pathogens and a majority of *Vibrio* species are opportunistic pathogens. The pathogenic effects of certain strains of *Vibrios* are dependent on environmental conditions of aquaculture systems.

V .anguillarum was the first species to be identified as a full fledged fish pathogen, and has now been reported in more than 42 species in widely distributed regions (Colwell and Grimes, 2000). Although *V. anguillarum* is regarded as the dominant species causing *Vibrio*sis, several other *Vibrio* species are also pathogenic. *V. alginolyticus* has been found in finfish, shellfish, and marine sediments and has been associated with acute septicemia in sea bream *(Sparus aurata); V. damsela* were found to infect damselfish *(Chromis punctipinnis),* a tropical aquarium species, which also reported to infect some species of sharks and human beings (Grimes *et al.*, 1986). *V. vulnificus* is usually encountered as a highly virulent but opportunistic human pathogen, though bio group 2 infects eels and causes development of a red patch on the trunk or tail (Tison *et al.,* 1982).

Two other species of importance, *V. parahaemolyticus* and *V. vulnificus* can cause gastrointestinal illness or non-intestinal illness such as septicemia. In our study, the abundance of these species in isolates obtained from crustacean and molluscans show the potential risk of these seafoods causing food borne illness (Lipp *et al.,* 1997). These species were also isolated from estuarine and brackish waters. (Joseph *et al.,* 1983). *Vibrio* infections are not limited to humans, as recent reports of *Vibrio* species capable of killing coral tissue (Benhaim *et al.,* 2003 and Kushmaro *et al.,*

2001) and *Vibrios* represent a major concern in aquaculture systems and marine aquaria (Tison *et al.*, 1982).

V. carchariae is an agent of disease causing organism in sharks and human wound infections (Farmer *et al.*, 1986). *V. vulnificus* is found as free-living inhabitant of estuarine and marine environments throughout the world (Veenstra *et al.*, 1994). When shrimps and fishes are reared in high densities under very artificial and unstable conditions, the opportunistic pathogen may turn pathogenic. Therefore, other species in the present investigation also may become opportunistically pathogenic (Bergh *et al,* 2001; Olafsen, 2001). The species composition of *Vibrios* isolated from the coastal waters, in this study, revealed that many of these opportunistic pathogens are prevailing in the aquatic system and may turn to be occasionally pathogenic.

Occurrence of halophilic *Vibrios* in aquatic environments has been reported from India (Sarkar *et al.*, 1985.) Epidemiological studies have established *V. parahaemolyticus* as the worldwide agent of gastroenteritis and results of ecological studies demonstrate that it can be isolated from seafood, as well as estuarine, and brackish waters. (Joseph *et al.*, 1983.)

5.2. Antibiotic resistance

Antibiotic resistance analysis (ARA) is a method that is based on the patterns of antibiotic resistance of bacteria from human and animal sources. The premise behind this method is that human fecal bacteria will have greater resistance to specific antibiotics followed by those in the livestock and in the wildlife, and that the livestock bacteria will have greater resistance to other antibiotics (Hager, 2001a). These differences occur because human beings are exposed to different antibiotics than cattle, pigs and poultry. Isolates of faecal streptococci and *E. coli* are taken from

various sources (human, livestock, and wildlife) and these isolates are grown on a variety of antibiotics. Following incubation, isolates are scored as 'growth/no growth' for each concentration of an antibiotic (Hager, 2001a). The resistance pattern of an organism is used to identify its source. Either sample-level analysis or isolate-level analysis can be used with this method. If it is assumed that the sample was taken from a single major source, then sample level analysis could be used. On the other hand, if the sample is contaminated by more than one source, isolate-level analysis should be used (Wiggins *et al.*, 1999). ARA is considered a low cost method, which requires basic microbiology training to perform (Hager, 2001a). Several studies with average rates of correct classification ranging from 62 to 84% cite the ARA method as a useful tool in assessing sources of fecal contamination (Wiggins, 1996; Parveen *et al.*, 1997; Wiggins *et al.*, 1999; Harwood *et al.*, 2000).

Antibiotic resistance is another marker often used for differentiating the strains of bacterial species. Antibiotic sensitivity pattern and multiple drug resistance are efficient methods of characterizing different *Vibrio* isolates. The drug resistance profiles changes over a period of time in different bacterial species because the organisms under a variety of conditions can acquire drug resistance. Of these, exposure to antibacterial agents is one of the important conditions. Our study indicated that all the isolates from crustacean and molluscan samples were showing antibiotic resistance (Table 5and 6). Filter feeding nature of molluscan and planktonic feeding nature of shrimps may be one of the reasons for the accumulation of antibiotic resistance by the process of biomagnification. However, more research needs to be conducted on bacteria from other sources to further strengthen this hypothesis.

Multiple antibiotic resistance (MAR) patterns of *Vibrio* isolates indicate that there was high incidence of MAR in crustacean and molluscan samples. In our study, considering the bioaccumulation and biomagnifications of bacteria in filer feeding and

planktivorous shrimps and molluscans sample size were suitably controlled, which also might have influenced the difference in MAR.

A number of instances of resistances in organisms were explained as their natural resistance to particular antibiotics (Davies, 1997). The *Vibrio* strains isolated from clinical samples were studied for sensitivity of antimicrobial activity against clinical isolates. Many researchers have studied the sensitivity of only the clinically significant species in detail (Lee *et al.,* 1981). With rare exception, they found the bacteria sensitive to tetracycline, which is usually the antibiotic of choice for the treatment of infections.

The drug resistance profiles of 100 *Vibrio* isolates from water, molluscan (mussel and sepia), crustacean and plankton were studied using 22 different antibiotics (Table 4-7). *Vibrio* strains are infamous for drug resistance due to their property to acquire and transfer resistance by genetic transfer. Most of the resistance strains tested was resistant to multiple drugs. An interesting observation is that molluscans isolates exhibited high MAR other categories (Table 8), which supports our earlier hypothesis that molluscans accumulates the *Vibrio* load in large amounts and association fairly large population of *Vibrio* accumulated in the mussels may increase a chance of horizontal gene transfer.

Resistance to individual drug was different from different sources (Table 9). Strains isolated from samples were showing highest resistance against amoxycillin, ampicillin and carbenicillin. Similar reports were shown in the studies conducted in shrimp farming systems and unfarmed farms in Asia (Rouque *et al.,* 2001) in Phillipines (Baticodos *et al.,* 1990, Leano *et al.,* 1999) India (Abraham *et al.,* 1997).

The high influx of sewage, the practice of use of antibiotics in terrestrial systems and indiscriminate use of coastal areas as open latrines could be the probable reasons for the high incidence of MAR *Vibrio* encountered in the present study (Table 11). Bacteria with antibiotic resistance factors have a selective advantage over antibiotic sensitive forms in the natural environment and when sufficient numbers of resistant bacteria are ingested they can transfer their resistance factors to the normal intestinal flora in human beings (Hatha *et al.,* 1993). The increasing number of drug resistance bacteria in sewage, and food products poses a health hazard to human being and animals. Indiscriminate use of antibiotics in the treatment of diseases and in culture systems has to be therefore controlled and need to be checked intermittently.

5.3. Plasmid profiles

Plasmids are circular DNA, which are separate from the chromosome. They often carry genes that provide antibiotic resistance, the ability to use alternate substrates, produce toxins, or other genes that can offer a survival advantage. Since plasmids are easily transferable from bacterium to bacterium the environmental strains can undergo sudden changes in their plasmid carriage causing diversity in plasmid profile and the resulting antibiotic resistance pattern. Plasmids that carry the genes necessary for conjugation are called conjugative plasmids, while non-conjugative plasmids can only be spread during conjugation brought on by a conjugative plasmid. Plasmid mediated gene exchange between bacteria plays an important role in bacterial adaptation and flexibility. In a study of a large collection of enteric bacteria isolated before the discovery and use of antibiotics, Hughes and Datta (1983) found that, although there was little antibiotic resistance among these strains, 24% contained plasmids, suggesting that, although plasmids are useful in spreading resistance, their presence does not necessarily mean an organism is resistant. However, over the year an increase in the use of antibiotics for the treatment of infectious diseases in fishes

has resulted in gaining antibiotic resistance and the expansion of R plasmids in commercial aquaculture (Aoki *et al.,* 1977) owing to the selective pressure exercised by the chemotherapeutic agents when used over an extended period of time (Aoki *et al,* 1971;1981). It is reported that 34% of environmental *Vibrio*, *Aeromonas*, *E. coli*, and *Pseudomonas* isolates from Chesapeake Bay and Bangladesh were found to contain plasmids (McNicol *et al.,* 1982).In our study, (28.89 %)of the *Vibrio* isolates harbored plasmids, with the sizes ranging from 1.6 to 27 kb. There were eleven *Vibrio* strains with a single plasmid, three strains were with two plasmids, and one each with three and four plasmids respectively. However, plasmids of smaller molecular weight were also observed in some of the isolates. Similar plasmid profiles in *Vibrio* spp were reported from earlier studies: *Vibrio* spp from cultured silver sea bream, *Sparus sarba* in China (Liu *et al.,* 1999), *V. ordalii* (Tiainen *et al.,*1995), *V. vulnificus* (Radu *et al.,* 1998), and most extensively in *V. anguillarum* (Pederson *et al.,* 1996). In our study, a proportionate comparison on different samples indicated highest incidence of plasmids in *Vibrios* isolated from molluscan than in other isolates. A part of our study had also revealed that highest MAR was recorded in molluscan samples. Therefore, it is evident that the antibiotic resistance is plasmid mediated.

5.4. Gene transfer mechanism in *Vibrio* isolates

Over the years, the research on horizontal transfer revealed that this phenomenon in bacteria spreads genes farther than was previously thought possible. A bacterium can gain antimicrobial resistance in at least one of two ways, either via mutation or horizontal gene transfer. Mutations in chromosomal genes leading to antibiotic resistance occur at different rates, depending on the organism and the mechanism of resistance. Resistance due to the inactivation of a gene will occur at a higher rate than resistance arising from mutation of a protein to a specific binding site.

Once resistance has developed, the genes can spread widely through horizontal gene transfer. Horizontal gene transfer can occur through one of three ways: transduction, transformation, and conjugation. Transduction is the transfer of genetic material by incorporation of bacterial DNA by a bacteriophage during packaging, and the subsequent infection of another bacterium. Transfer of resistance by transduction was first shown with penicillin resistance in staphylococci in 1958 (Garrod and Grady, 1971). Although phages generally have a restricted host range, they are common in many environments and may therefore play an important role in transfer of resistance genes.

Several studies have shown gene transfer between marine organisms or between marine organisms and enteric bacteria *in vitro*. Adams *et al.*, (1998) used the fish pathogen *Aeromonas salmonicida* to transfer a plasmid encoding tetracycline resistance to *E. coli* in offshore seawater and sediment samples.

5.4.1. Transformation of *Vibrio* plasmids

Transformation involves the uptake of naked DNA from the environment and has the potential to transmit DNA between very distantly related organisms. Naked DNA is made available through secretion or after cell lysis. Although DNA may get easily be destroyed in the environment, it can also be stabilized by binding to particles (Davison,1999). In order to be transformed, a bacterium must be competent. There are many bacterial species that are either naturally competent or in which competency can be induced by environmental conditions (Davison, 1999). As with studies of other environments, the evidence for horizontal gene transfer in marine and aquatic environments is largely circumstantial, relying on microcosm studies, *in vitro* transfer between aquatic organisms, and the presence of plasmids. Transformation and transduction are theorized to occur in the marine environment, but few studies have been done explicitly to demonstrate the phenomenon. Certain bacterial species, such

as *Neisseria gonorrhoeae* and *Haemophilus infuenzae*, are perpetually competent to accept DNA, whereas others, such as *Bacillus subtilis* and *Streptococcus pneumoniae,* become competent upon reaching a certain physiological stage in their life cycle (Heidelberg, 2000). Results of our transformation experiment of plasmids in *Vibrio* spp indicate that the plasmid mediated bacterial resistance in *Vibrio* spp is transferable to other bacterial genera (*E. coli*). Similar previous studies on transformation experiments were reported in plasmids of *Vibrio* isolates from *Sparus sarba* (Liu *et al.*, 1999) and penaeid shrimp (Molina *et al.*, 2002). Sizemore and Colwell (1977) found antibiotic resistant bacteria in most samples, including those collected 100 miles offshore and from depths of 8200 meters. Isolates considered autochthonous to the marine environment were examined for plasmids and used in mating experiments. Of the 16 isolates studied, 50% contained plasmids. Several of these were able to transfer plasmids to *E. coli* (Sizemore and Colwell, 1977), which is concurrent to our findings. Since these plasmids mobilize into *E. coli* DH5α suggest that the plasmids are of broad host range. Similar findings were reported in plasmids isolated from *Pseudomonas* spp (Shahid, 2004).

5.4.2. Conjugation of *Vibrio* plasmids

Conjugation refers to the transfer of genetic material through direct cell-to-cell contact accomplished by a pilus. Originally thought to be a highly specific interaction that could only occur between closely related bacterial species, now know that conjugation occurs between diverse species and even between gram positive and gram-negative bacteria (Davison, 1999). Conjugation involves physical contact between donor and recipient cells and can mediate the transfer of genetic material between domains (for example, between bacteria and plants, and between bacteria and yeast) (Buchanan *et al.*, 1987; Heinemann and Sprague, 1989). Typically, DNA is transferred from a donor to a recipient strain by either a self-transmissible or mobilizable plasmid. *V. parahaemolyticus* strains isolated from Chesapeake Bay were

found to be antibiotic resistant and to contain plasmids. Conjugation experiments showed that resistance plasmids could be transferred from *E. coli* to *V. parahaemolyticus in vitro* (Guerry, 1975). Results of the conjugation experiment in this study involving the *Vibrio* containing resistant plasmid, as the donor and *E. coli* HB 101 as the recipient indicated that the majority of the plasmid associated resistant markers were transferred to the *E. coli* strain. However, a few of the strains (from water and plankton samples) failed to transfer the resistance by conjugation into the recipient, which may be due to its nonmobilizable nature. After conjugation, plasmids were screened for, in the transconjugants. Although there was a failure to recover the plasmids from the transconjugants, but the resistance pattern of the plasmid was observed in them. This may be due to the integrative property of plasmids into the chromosomes in the form of episomes.

5.4.3 Curing procedure to assess genetic location of antibiotic resistance.

Curing procedure is performed to ascertain the genetic location of the resistance in the bacteria (plasmid mediated or chromosome mediated) or to demonstrate the plasmid borne nature of antibiotic resistance. Curing is performed using ethidium bromide, which intercalate with the R factors' by selectively complexing with the plasmid DNA and inhibiting their replication. Results of the curing experiment indicated loss of plasmids in all of the *Vibrio* strains and demonstrated a change in their resistance pattern. The *Vibrio* strains that were cured of their plasmids were susceptible to these antibiotics. This result indicated that some of these resistance may be encoded on plasmids in some strains, while in some others they may chromosome mediated,as reported in earlier studies (Aoki *et al.*, 1984). In our study, a large population of *Vibrio* stains (75.37%), was devoid of plasmids but showed an antibiotic resistance pattern, which indicated that in these bacteria, resistance might be mediated *via* chromosome.

5.4.4. PCR for testing the presence of integron and SXT element in plasmids

Plasmid borne integrons are a key player in being able to acquire, rearrange, and express genes conferring antibiotic resistance (Stokes and Hall, 1989). Irrespective of integrons, if located on a plasmid or chromosome, their structure and function are similar. They contain a gene for a DNA integrase (*intI*), which catalyzes the site-specific recombination of gene cassettes at the integron-associated recombination site (*attI*). Acquisition of multiple cassettes results in a contiguous array of genes. Each gene cassette typically consists of a single ORF and a further recombination site known as a 59-base element (be). Transcription of integrated gene cassettes is driven by a promoter, Pc (Levesque, 1994; Stokes and Hall, 1989). Integrons are not restricted to clinical settings, because they can be amplified from soil DNA and are found in many genome sequences (Nield, 2001; Holmes, 2003) Integrons and gene cassette arrays have been found in the chromosomes of *Vibrio, Pseudomonas*, *Xanthomonas*, *Treponema*, *Geobacter*, *Dechloromonas*, *Methylobacillus*, and *Shewanella* species (Heidelberg, 2000; Holmes *et al.*, 2003). In this study, in the experiment to assess the presence of integron, one of the plasmids isolate was positive for *int* gene, which indicated the presence of an integrons on this plasmid Their activity might have facilitated a community level response to intensive antibiotic use, which in turn helped in the emergence of integron-encoded, and multiple antibiotic resistances in disparate bacterial species. However, PCR screening for SXT element were found to be negative. From the results, it is evident that there are integron mediated horizontal gene transfer may occur in rare cases, to augment the horizontal gene transfer responsible for antibiotic resistance from *Vibrio* spp to other genus.

5.4.5. Restriction profile of plasmid DNA

Restriction profile of plasmid DNA in our experiment was conducted to assess the heterogeneity of plasmids in *Vibrio* isolates. Treatment with four different

enzymes yielded different fragments, which is suggestive of multiple restriction sites in the plasmids It was also reported that considerable variation based on the endonucleases digestion patterns after studying about the conjugative plasmids, there exists a diversity (Cecilia *et al.*,1997). Also suggest that the plasmids are actively transferred under natural conditions.. Above all, the formation of restriction fragments is indicative of the genetic variation in plasmids in *Vibrios*. Similar results were reported by earlier studies in plasmids isolated from *Bacillus* and *Pseudomonas* spp (Gowri, 2001). Further, information on the restriction sites of the tested plasmid may pave way for the selection of a suitable vector system that can be employed in the transfer of genes to other organisms.

Chapter 6

SUMMARY AND CONCLUSION

Since the discovery and introduction of antibiotics, the microbial resistance to antibiotics has steadily increased. Human activities have significant influence on bacterial populations in the natural environment, in antibiotic resistance due to a large-scale selective pressure through agricultural waste management and other practices. Over the years, the development of resistance to antibiotics was not considered a serious problem because of the notion that there would be new antibiotics discovered. The reality is, whether for economic, political, or scientific reasons, no novel antibiotics have been produced for several years, and few are on the horizon. While humans have been idle in this biological arms race, bacteria continue to develop resistance against each new drug we create. Infections once easily curable are now regarded as a growing threat from the drug-resistant microbial agents of these diseases. Antimicrobial use in animal husbandry, beef and pork farming, poultry and aquaculture has been blamed for the increase in microbial resistance to antibiotics. Pathogens that are autochthonous to the environment can acquire resistance genes from animal fecal bacteria. Aquatic pathogens, such as *Vibrio* spp would be very dangerous if they did not respond to antibiotic treatment. But can this use lead to detrimental effects in humans and other aquatic organisms in future?

To address this problem, a study was designed to screen for *Vibrios* from four different sources (water, plankton, crustacean and two molluscan samples-sepia and mussel). Further, the *Vibrio* isolates were tested for antibiotic resistance pattern. In order to establish the genetic mechanism of antibiotic resistance, the resistant *Vibrios* were further assessed for the presence of plasmids. After the detection of the plasmids in *Vibrio* isolates, plasmid encoded antibiotic resistance were confirmed by

further molecular characterization such as transformation and conjugation. Antibiotic resistance marker and its location were confirmed from the resistant plasmids from *Vibrio* isolates by using curing protocols. It was found that while some of the antibiotic resistances were plasmid encoded, many others were chromosome mediated.

In order to elucidate the role of integrons and SXT elements, the isolated plasmids were tested for the presence of integrons and SXT elements using PCR. Plasmid borne integrons play a key role in sequestering antibiotic resistance genes by being able to acquire, rearrange, and express these R-genes. The heterogeneity of plasmids in *Vibrio* isolates were tested by treating the plasmids with restriction digestion using EcoRI, BamH1 and HindIII and HaeIII.

To summarize

- A total of 100 *Vibrio* like organisms were segregated from the 396 bacterial isolates obtained from water, molluscan, crustacean and planktons collected from different locations and aqua farms. More *Vibrios* could be isolated from water compared to other samples.
- Among the *Vibrio* isolates, the predominant group was *V. cholerae*.
- Sample wise analysis indicated that all the isolates from crustacean and molluscan samples were showing the highest antibiotic resistance. Further, high incidence of MAR was recorded in crustacean and molluscan samples over plankton and water samples.
- Antibiotic wise cluster comparison indicated that molluscan isolates exhibited the highest MAR (more than 7 and less than 12 drugs) over other categories. A significant portion of strains from the all the four samples was showing MAR index value greater than 0. 2.
- In all the isolates, resistance exhibited to the beta lactum group of antibiotics was the highest.

- Plasmid profile studies indicated that of the 90 resistant isolates tested, only 28.89 % (n=26) isolates were with plasmids. 71.11% isolates of the resistant strains were without plasmids.
- It was observed that 12 plasmids out of 26 plasmids were transformed to *E. coli* DH5α, indicating the risk of horizontal gene flow.
- Further, conjugation studies revealed that majority of the plasmids were successfully conjugated to the recipient *E. coli* HB101.
- Curing protocols revealed that the plasmid encoded resistance phenotype of isolates becomes sensitive after curing. However, certain drug resistance markers were expressed, even after curing. The plasmid extraction reveals that although the resistance-encoded plasmids were lost and no plasmids obtained, indicative of the chromosome mediated R-genes.
- The study also revealed the presence of the ***int*** of the integrons on the plasmid pP17 isolated from *V. metschnikovii,* however, PCR screening for SXT element were found to be negative.
- Restriction digestion with four different enzymes; EcoR1, Hind III, HaeIII and Bam H1 yielded different fragments indicative of the genetic variation in plasmids in *Vibrios.* Further, information on the restriction fragments of the tested plasmid may pave way for the selecting suitable vector system that can be employed in the transfer of genes to other organisms.

Overall results indicated that *Vibrio* spp present in aquatic system, acquire antibiotic resistance by means of plasmids and they are capable of transferring the resistance by means of transformation, conjugation and by other mobile elements like integrons.

CONCLUSION

While the seriousness of the problem of antibiotic resistance is now recognized, the complex web of resistance linking humans, animals, and the environment is getting realized. More often, antibiotics are used as a preventive measure against diseases. Antibiotic use for agriculture leads to the increased resistance in the environment since antibiotics are inevitable element during agriculture/aquaculture and antibiotic residues are excreted as waste that is frequently spread onto farmland as organic fertilizer. Fecal bacteria survive long periods in the environment and spread through runoff into groundwater, rivers, and marine ecosystems.

However, horizontal gene transfer occurs in the animals and guts of humans and in a variety of ecosystems, creating a pool of resistance in the rice fields and open waters. Even if people are not in direct contact with resistant disease through food animals, there are chances of contact with resistant fecal pathogens from the environment. Additionally, pathogens that are autochthonous to the environment can acquire resistance genes from the environment. Our study revealed that autochthonous bacteria *Vibrio* spp gained antibiotic resistance in the environment. Further, it was evident that horizontal gene transfer occurs in *Vibrio* by means of plasmids, which further augments the gravity of the problem. Non-pathogenic bacteria may also acquire resistance genes and serve as a continuing source of resistance for other bacteria, both in the environment, and in the human gut. As the effectiveness of antibiotics for medical applications decline, the indiscriminate use of in aquaculture and in humans can have disastrous conditions in future due to horizontal gene transfer and the spread of resistant organisms: We must recognize and deal with the threat posed by overuse of antibiotics.

Chapter 7

REFERENCES

Aarestrup, F. M.(1999):Association between the consumption of antimicrobial agents in animal husbandry and the occurrence of resistant bacteria among food animals. *Int. J. Antimicrob. Agents* .,**12**, 279-285.

Aarestrup, F. M.(2000):Characterization of glycopeptide-resistant *Enterococcus faecium* (GRE) from broilers and pigs in Denmark: genetic evidence that persistence of GRE in pig herds is associated with coselection by resistance to macrolides. *J. Clin. Microbiol.*, **38**, 2774-2777.

Aarestrup, F. M., and P. M. McNicholas.(2002):Incidence of high-level evernimicin resistance in *Enterococcus faecium* among food animals and humans. *Antimicrob. Agents Chemother.*, **46**, 3088-3090.

Abbott, S. L., and J. M. Janda. (1994):Severe gastroenteritis associated with *Vibrio hollisae* infection: report of 2 cases and review. *Clin. Infect. Dis.*, 18.

Abraham, T. J., R. Manley, R. Palaniappan and K .Dhevendaran.(1997):Pathogenicity and antibiotic sensitivity of luminous *Vibrio harveyi* isolated from diseased penaeid shrimp. *J. Aquat. Trop.*, **12**, 1-8.

Actis, L. A., S. Potter and J .H. Crosa .(1985):Iron regulated outer membrane protein OM 2 of *Vibrio anguillarum* is encoded by virulence plasmid PJM1. *J. of Bact.*,**161**, 736-742.

Adams, C. A., B. Austin, P. G. Meaden and D. McIntosh.(1998):Molecular characterization of plasmid-mediated oxytetracycline resistance in *Aeromonas salmonicida*. *Appl. Environ. Microbiol.*, **64**, 4194-4201.

Akiba, T., T. Koyama, Y. Isshiki, S. Kimura and T. Fukushima.(1960):Studies on the mechanism and development of multiple drug-resistant *Shigella* strains. *Nippon Iji Shimpo.*,**1866**, 45-50.

Allam, B., C. Paillard and M.Auffret. (2000):Alterations in hemolymph and extrapallial fluid parameters in the Manila clam, *Ruditapes philippinarum*, challenged with the pathogen *Vibrio tapetis*. *J Invertebr Pathol.*, **76**, 63-69.

Allen, D.A., B. Austin and R. R.Colwell.(1977):Antibiotic resistance patterns of metal-tolerant bacteria isolated from an estuary. *Antimicrob.Agents*

Chemother.,**12**, 545-547.

Alsina,M., and A.R.Blanch.(1994):A set of keys for biochemical identification of environmental *Vibrio spp. J. of Applied Bacteriol.* **76**, 79-85.

Amaru, C. E., and G.Biosca. (1998):*Vibrio vulnificus* biotype 2, pathogenic to cells also an oppurtunistic pathogen for human. *Appl. Environ. Microbiol.*,**62**, 1454-1457.

Amita, S., M.R.Chowdhury, G.Thungapathra, T. Ramamurthy,G. Balakrish Nair and A.Ghosh. (2003):Class I Integrons and SXT Elements in El Tor Strains Isolated before and after 1992 *Vibrio cholerae* O139 Outbreak, Calcutta, India. *Emerging Infectious Diseases.*, **9**, 500-502.

Anderson, E., and N.Datta.(1965):Resistance to penicillins and its transfer in Enterobacteriaceae. *Lancet*, 407-409.

Anderson, S. R., and R.A .Sandaa. (1994):Distribution of tetracycline resistance determinants among gram-negative bacteria isolated from polluted and unpolluted marine sediments. *Appl. Environ. Microbiol.* **60**, 908-912.

Angulo, F. J., K. R. Johnson and R. V. Tauxe. (2000):Origins and consequences of antimicrobial resistant nontyphoidal *Salmonella*: Implications for the use of fluoroquinolones in food animals. *Microb.Drug Resis* .,**6**, 77-83.

Ansari, A., R. M. Haneef, J.L.Torres and Yadav. (1992):Plasmids and antibiotic resistance in *Aeromonas hydrophila* strains isolated in Malayasia from healthy and diseased fish. *J .of Fish Disea.*, **12**, 191-196.

Aoki,T., S.Egusa,T.Kimura and T.Watanabe.(1971):Detection of R factors in naturally occurring *Aeromonas salmonicida* strains. *Appl. Microbiol.* ,**22**, 716-717.

Aoki,T., S.Egusa, and T.Watanabe. (1973):Detection of R bacteria in cultured marine fish, Yellowtail (*Seriola quinqueradiata*). *Jpn. J. Microbiol.*, **17**, 7-12.

Aoki, T., S.Egusa and T.Arai .(1974):Detection of R factors in naturally occurring *Vibrio anguillarum* strains. *Antimicrob Agents Chemother.* ,**6**, 534-538.

Aoki, T., S. Egusa and T.Arai. (1975):Reduced nitrofuran sensitivity conferred by R-factor. *Jpn. J. Microbiol.*,**19**, 327-329.

Aoki, T., T.Arai and S. Egusa. (1977):Detection of R plasmids in naturally occurring fish-pathogenic bacteria, *Edwardsiella tarda. Microbiol. Immunol.* ,**21**, 77-83.

Aoki, T., T. Kitao, and K.Kawano.(1981):Changes in drug resistance of *Vibrio anguillarum* in cultured ayu(*Plecoglossus altivelis*). *J. Fish Dis.* ,**4**, 223-230.

Aoki, T., T. Kitao, S.Watanabe and S.Takeshita.(1984):Drug resistance and R

plasmids in *Vibrio anguillarum* isolated in cultured ayu (*Plecoglossus altivelis. Microbiol. Immunol.*, **28**, 1-9.

Apgar, B.,G.Greenberg and G. Yen. (2005):Prevention of group B streptococcal disease in the newborn. *Am. Fam. Physician* **71**, 903-910.

Armstrong, J. L., N. D. Wood, and L. A. Porteous. (1990):Transconjugation between bacteria in the digestive tract of the cutworm *Peridroma saucia. Appl. Environ. Microbiol.*, **56**, 1492-1493.

Arvanitidou, M., V. Katsouyannopoulos and A. Tsakris. (2001):Antibiotic resistance patterns of *Enterococci* isolated from coastal bathing waters. *J. Med. Microbiol.* **50**, 1001-1005.

Arzese, A. R., L.Tomasetig and G. A. Botta. (2000):Detection of tetQ and erm F antibiotic resistance genes in *Prevotella* and *Porphyromonas* isolates from clinical specimens and resident microbiota of humans. *J.Antimicrob. Chemother.*, **45**, 577-582.

Ash, R. J., B. Mauck and M. Morgan. (2002):Antibiotic resistance of gram negative bacteria in rivers of United States. *Emerg. Infect. Dis.*, 8, 713- 716.

Ashkenazi, S., I. Levy, V. Kazaronovski and Z. Samra. (2003.):Growing antimicrobial resistance of *Shigella* isolates. *J. Antimicrob. Chemother.*, **51**, 427-429.

Austin, B. (1988): *Marine Microbiology*. Cambridge UK ,Cambridge University Press.

Austin, B., and D.Austin. (1999): Bacterial Fish Pathogens: Disease of farmed and wild fish ,3rd ed,. Springer-Praxis publishing, Chichester.pp 457.

Austin, B, and C.Adams. (1995): Biotechnology and Fish pathogen *Aeromonas salmonicida. J.of Mar.Biotechnolo.*, **2**, 67-72.

Aviles, M., J. C. Codina, A. Perez-Garcia, F. Cazorla, P. Romero and A. De Vicente. (1993):Occurrence of resistance to antibiotics and metals and of plasmids in bacterial strains isolated from marine environments. *Wat. Sci. Tech.*, **27**, 475-478.

Bag, P.K., M. S. C. Sharma, A. Ghosh, A. Basu and R. Mitra.(1998):Rapid spread of the new clone of *Vibrio cholerae* O1 biotype El Tor in cholera endemic areas in India. *Epidemiol Infect* .,**121**, 245-51.

Bager, F., F. M. Aarestrup, M. Madsen and H. Wegener. (1999):Glycopeptide resistance in *Enterococcus faecium* from broilers and pigs following discontinued use of avoparcin. *Microb. Drug Resis.*, **5**, 53-56.

Baquero, F., J. Martinez-Beltran and E. Loza.(1991):A review of antibiotic resistance patterns of *Streptococcus pneumoniae* in Europe. *J. Antimicrob. Chemother.*,

28, 31-83.

Baquero, F., and J. Blazquez. (1997):Evolution of antibiotic resistance. *Trends Ecol. Evol.*, **12**, 482-487.

Barkay, T., N. Kroer, L. D. Rasmussen and S. J. Sorensen.(1995): Conjugal transfer at natural population densities in a microcosm simulating an estuarine environment. *FEMS Microbiol. Ecol.*, **16**, 43-54.

Bass, L., C. A. Liebert, M. D. Lee, A. O. Summers, D. G. White and Thayer, J. M. (1999):Incidence and characterization of integrons, genetic elements mediating multiple-drug resistance, in avian *Escherichia coli*. *Antimicrob. Agents Chemother.*, **43**, 2925-2929.

Bast, L., J.G.Daly , S.A. Degrandis and R.M.W.Sterenson.(1988):Evaluation of plasmid profiles of *Aeromonas salmonicida* as epidemiological makers of furunculosis infections in fish. *J. of Fish Dis.*, **11**, 133-145s.

Baticados, M. C. L., C.R.Lavilla-Pitogo, E.R.Cruz-Lacierda, L.D. De la Pena, N.A.Sunaz.(1990): Studies on the chemical control of luminous bacteria *Vibrio harveyi* and *V. splendidus* isolated from diseased *Penaeus monodon* larvae and rearing water. *Dis. Aquat. Org .*, 133-139.

Bauer, A. W., M. Kirby, J. C. Sherris, and M. Turck .(1966): Antibiotic susceptibility testing by a standardized single disc method. *Am. J. Clin. Pathol.*, **45**, 493-496.

Baumann, P., A. L. Furniss, and J. V. Lee. (1984):Genus I. *Vibrio pacini* 1854, 411AL, In I. N. R. Krieg and J. G. Holt(ed.). *Bergey's manual of systematic bacteriology*, Vol. 1., The Williams & Wilkins Co., Baltimore. pp. 518-538.

Baumann, P., and R. H. W. Schubert.(1984): *Vibrionaceae*,. In N. R. Krieg and J. G. Holt (ed.) Bergey's manual of systematic bacteriology, Vol. 1., The Williams and Wilkins Co., Baltimore, pp. 516–550.

Baxter P,W. R., and M. W. Gilliland.(1998):Bacterial pollution in runoff from agricultural lands. *J. Environ. Qual.*, **17**, 27-34.

Baya, A. M., P. R. Brayton, V. L. Brown, D. J. Grimes, E. Russek ,Cohen, and R. R. Colwell.(1986):Coincident plasmids and antimicrobial resistance in marine bacteria isolated from polluted and unpolluted Atlantic Ocean samples. *Appl. Environ. Microbiol.*, **51**, 1285-1292.

Bayne, S., M. Blankson and D. Thirkell.(1983):Enumeration and speciation of group D *Streptococci* from above and below a sewer outfall their susceptibilities to six antibiotics and a comparison with clinical isolates. *Antonie Van Leeuwen.*, **49**, 299-310.

Bell, R. J., and Trust, T.J. (1989):*Aeromonas salmonicida* plasmids: Plasmid directed synthesis of protein *in vitro* and in *E.coli* minicells. *J.Gen.Micro.*,**135**,513-524.

Ben Haim, Y., F. L.Thompson, C. C.Thompson, M. C. Cnockaert, B. Hoste, J.Swings and Rosenberg, E. (2003):*Vibrio coralliilyticus* sp. nov., a temperature-dependent pathogen of the coral *Pocillopora damicornis. Int. J. Syst. Evol. Microbiol.*, **53**, 309-315.

Ben Haim, Y., E. Banim, A. Kushmaro, Y. Loya and E. Rosenberg. (1997): Inhibition of photosynthesis and bleaching of zooxanthellae by the coral pathogen *Vibrio shiloi. Appl. Environ. Microbiol.*, 223-229.

Bennett, P. M., and M. H. Richmond.(1978): *Plasmids and their possible influence on bacterial evolution.*, Academic Press, Inc. New York.

Bergh, O., K. Y. Borsheim, G. Bratbak, and M. Heldal.(1989): High abundance of viruses found in aquatic environments. *Nature.*, **340**, 467-468.

Bergh, O., F. Nilsen and O. B. Samuelsen. (2001):Diseases, prophylaxis and treatment of the Atlantic halibut *Hippoglossus*: a review. *Dis. Aquat. Org.*, **48**, 57-74.

BirnBoim and Doly.(1979):A rapid alkaline extraction procedure for recombinant plasmid DNA. *Nucleic Acid Research.*, **7**, 1513-1523.

Bjorklund, H., J. Bondestam and G. Bylund. (1990):Residues of oxytetracycline in wild fish and sediments from fish farms. *Aquaculture.*, **86**, 359-367.

Bjorklund, H.V.,C.M.I. Rabergh and G. Bylund.(1991):Residues of oxolinic acid and oxytetracycline in fish and sediments from fish farms. *Aquaculture.*, **97**, 85-96.

Blanch, A. R., J. L. Caplin, A. Iversen, I.Kuhn, A.Manero, H. D.Taylor and X.Vilanova.(2003): Comparison of Enterococcal populations related to urban and hospital wastewater in various climatic and geographic European regions. *J. Appl. Microbiol.* **94**, 994-1002.

Boettcher, K. J.,and E. J. Ruby.(1994): Occurence of plasmid DNA in the *Sepiolid* squid symbiont *Vibrio fischeri. Current microbiology.*, **29**, 279-286.

Boman, H. G., K. G. Eriksson, J. Foldes and E. B. Lindstrom (1967): The regulation and possible evolution of a penicillinase like enzyme in *Escherichia coli.* Elsevier Publishing Co. Amsterdam.

Borrego,J.J., M.A. Morinigo, E.M.Manzanares, M.Bosca, D.Castro, J.L.Barja, A. E.Toranzo.(1991):Plasmid associated virulence properties of environmental isolates of *Aeromonas hydrophila*. The *J. of Med. Microbiol.*, **35**, 5 ,264-269.

Boyd, E. F., K. E. Moyer, L. Shi, and M. K. Waldor. (2000): Infectious CTX and the

Vibrio pathogenicity island prophage in *Vibrio mimicus*: Evidence for recent horizontal transfer between *V. mimicus* and *V. cholerae*. *Infect. Immun.*, 1507-1513.

Brenner, D. J., F. W. Hickman-Brenner, J. V. Lee, A. G. Steigerwalt, G. R. Fanning, D. G. Hollis, J. J. Farmer III, R. E. Weaver, S. W. Joseph and and R. J. Seidler.(1983): *Vibrio furnissii* (formerly aerogenic biogroup of *Vibrio fluvialis*) a new species isolated from human feces and the environment. *J. Clin. Microbiol.*, **18**, 816-824.

Brown, C., and Roland, G.(1984):Characterization of exotoxin produced by a shellfish pathogenic *Vibrio* sp. *J Fish Dis.*,**7**,1-10.

Buchanan,W., V. Passiatore, J. E and F.Canon.(1987):The mob and oriT mobilization functions of a bacterial plasmid promote its transfer to plants. *Nature.*, 170-175.

Bullock, G. L. (1987): *Vibriosis* in fish. *Fish Dis. Leaf and U.S. Fish Wildl. Serv.*, 77.

Burman, L. G., J. T. Park, E. B. Lindstrom and H. G.Boman.(1973): Resistance of *Escherichia coli* to penicillins: identification of the structural gene for the chromosomal penicillinase. *J. Bacteriol.* **116**, 123-130.

Carla, P., G.Gabriella and C.A.Laura.(2005): Persistence of *Vibrios* in marine bivalves: the role of interactions with haemolymph components. *Environmental Microbiology.*,**7**, 761-772.

Carnahan, A. M., J. Harding, D. Watsky, and S. Hansman.(1994.):Identification of *Vibrio hollisae* associated with severe gastroenteritis after consumption of raw oysters. *J. Clin. Microbiol.*, **32**, 1805-1806.

CDC (2001):Outbreak of *Vibrio parahaemolyticus* infection associated with eating raw oysters and clams harvested from Long Island Sound - Connecticut, New Jersey and New York, **48**. pp48-51.

Cecilia,D., C.Linberg, V.D.Torsvik and M. Hermansson.(1997):Conjugative plasmids isolated from bacteria in marine environments show various degree of homology to each other and are not closely related to well characterized plasmids. *Appli. and Environ. Microbiol.*, **63**,4962-4697.

Chakraborty, T., A. N. Chakraborty and S.G.Dastidar.(1994):Plasmid determined degradative metabolism and halophilism of *Vibrio parahaemolyticus*. *Indian Jnl. of Exp.Biol.* **32**, 619-622.

Chakraborty, S., G.B. Nair and S. Shinoda.(1997): Pathogenic *Vibrios* in the natural aquatic environment. *Rev Environ Health.*, **12**, 63-80.

Chen, H.Y., and G. H. Kou. (1978):Studies on bacterial drug-resistance in

aquaculture. In. Drug resistance of bacteria in pond-reared eels (*Anguilla japonica*). *ICRR Fish. Ser.* 34, 1-14.

Chowdhury, M. A. R., K. M. S. Aziz, Z. Rahim, and B. A. Kay.(1986):Antibiotic resistance patterns of *Vibrio mimicus* isolated from human and environmental sources in Bangladesh. *Antimicrob. Agents Chemother.* **30**, 622-623.

Cohen, S. N. (1993):Bacterial plasmids: their extraordinary contribution to molecular genetics. *Genetics.*,**135**, 67-76.

Colwell, R. R. and A. Huq. (1994):*Vibrios* in the environment: viable but nonculturable *Vibrio cholerae.* Washington, DC: American Society for Microbiology.

Colwell , R. R. (1996): Global climate and infectious disease: the cholera paradigm. *Science.,* **274**, 2025-2031.

Colwell, R. R., and D. J. Grimes.(2000):*Nonculturable microorganisms.* Washington,D.C., ASM Press.

Colwell, R. R. H., A.Islam, M. S. Aziz, K. M. A. Yunus, M. Khan, N. H. Mahmud, A. Sack, G. B.Nair, J.Chakraborty, A.S.David., and E. R Cohen. (2003): Reduction of cholera in Bangladeshi villages by simple filtration. *PNAS.,* **100**, 1051-1055.

Cook, D. W.,O. P Leary, J.C.Hunsucker,E.M.Sloan, J.C. Bowers, R.J.Blodgett and A.J. DePaola.(2002):*Vibrio vulnificus* and *Vibrio parahaemolyticus* in U.S.retail shell oysters: a national survey from June 1998 to July 1999. *Food Pro .*,**65**, 79-87.

Courtney, E. B., C. S. James, H. F. Robert and L. Actis.(1998):Plasmid mediated histamine biosynthesis in the fish pathogen *Vibrio anguillarum. Plasmid .*,**39**, 235-244.

Couturier, M. F., F. Bex, P.L. Bergquist and W. K. Maas.(1988):Identification and classification of bacterial plasmids. *Microbiol. Rev.* ,**52**.375-395.

Cravedi, J.P., G. Choubert and G. Delous.(1987):Digestibility of chloramphenicol, oxolinic acid and oxytetracycline in rainbow trout and influence of these antibiotics on lipid digestibility. *Aquaculture.,* **60**, 133-141.

Crossa, J. H., M.H.Scheiwe and S. Falkow. (1977): Evidence for plasmid contribution to the fish pathogen *V.anguillarum. Infec. and immun.*,**18**, 509-513.

Crossa, J. H. (1980): A plasmid associated with virulence in the marine fish pathogen *Vibrio anguillarum* specifies an iron sequestering system. *Nature.*,**283**, 566-568.

Crossa, J. H., L. L. Hodges and M. Schiewe. (1980): Curing of a plasmid is correlated with an attenuation of virulence in the marine fish pathogen *Vibrio anguillarum. Infect. Immun.*, 897-902.

Crossa, J. H. (1989): Genetics and molecular biology of siderophore-mediated iron transport in bacteria. *Microbiol Rev. Infect. Dis.*, 517-530.

Dahlberg, C., C. Linberg, V. L. Torsvik and M. Hermansson.(1997):Conjugative plasmids isolated from bacteria in marine environments show various degrees of homology to each other and are not closely related to well-characterized plasmids. *Appl. Environ. Microbiol.* **63**, 4692-4697.

Dalsgaard A, F. A., Serichantalergs O, Sandvang D. (2000): Distribution and content of class I integrons in different *Vibrio cholerae* O-serotype strains isolated in Thailand. *Antimicrob. A. Chemother.* **44**, 1315-21.

Davis, B. R., G. R. Fanning, J. M. Madden, A. G. Steigerwalt, H. B. Bradford, Jr., H. L. Smith, Jr. and D. J. Brenner.(1981):Characterization of biochemically atypical *Vibrio cholerae* strains and designation of a new pathogenic species, *Vibrio mimicus. J. Clin. Microbiol.* ,**14**, 631-639.

Davies, J. E. (1994):Inactivation of antibiotics and the dissemination of resistance genes. *Science.,* **264**, 375-382.

Davies, J. E. (1997):Origins, acquisition and dissemination of antibiotic resistance determinants. *Ciba Foundation Symp.,* **207**, 15-27.

Davison, J. (1999):Genetic exchange between bacteria in the environment. *Plasmid* .,**42**, 73-91.

De la Cruz, F., and Davies, J. (2000):Horizontal gene transfer and the origin of species: lessons from bacteria. *Trends in Microbiol.,* **8**, 128-133.

Dean, A., Magnus,R., Anne, M. G., and Didier ,M., (2002):Bacterial resistance evolution by recruitment of super integron gene cassettes . *Mole. Microbiol.*,**43**, 1657-1669.

Demerec, M. (1948): Origin of bacterial resistance to antibiotics. *J. Bact.*,**56**, 63-74.

Di Salvo, L.H., J. Blecka and R. Zebal, (1978) *Vibrio anguillarum* and larval mortality in a Californian coastal shellfish hatchery. *Appl. Environ. Microbiol.* **35**, 219-221.

Dixon, B. ,(1994): Antibiotic resistance of bacterial fish pathogens. J. *World Aquacult. Soc.*, **25**, 60-63.

Deveriex, R. and R. K. S. (1982):Plasmid incidence in marine bacteria isolated from petroleum polluted sites on different petroleum hydrocarbons.

Mar.Pollution.Bull. ,**13**,198-202.

Eleonor, A., and Leobert, D.(2001).Antibiotic Resistance of Bacteria from Shrimp Ponds .*Aquaculture.*,**195**, 193-204.

Erauso, G., S. Marsin, R.N. Benbouzid, M.F. Baucher, T.Barbeyron, Y.P.Zivanovic, and P. Forterre.(1996):Sequence of plasmid pGT5 from the Archaeon *Pyrococcus abyssi* evidence for rolling circle replication in a hyperthermophile. *J. Bact* .,**178**, 3232-3237.

Ervik, A., B. Thorsen, V. Eriksen, B.T. Lunestad and O.B. Samuelsen. (1994): Impact of administering antibacterial agents on wild fish and blue mussels *Mytilus edulis* in the vicinity of fish farms. *Dis. Aquat. Org.*, **18**, 45-51.

Falbo,V,C.A., F.Tosini, C.Pezzella, A.M.Dionisi, I.Luzzi.(1999): Antibiotic resistance conferred by a conjugative plasmid and a class I integron in *Vibrio cholerae* O1 El Tor strains isolated in Albania and Italy. *Antimicrob. Agents. Chemother.* **43**, 693-6.

Falkow, S. (1975): Infectious multiple drug resistance. Pion Limited. London.

Farmer, J. J. 1986. International commitee on systematic bacteriology. Subcommitee on the taxonomy of *Vibrionaceae.* Minutes of the meeting. *Int. J. Syst. Bacteriol.* **39:**210-212.

Farmer, J. J., III. and F. W. Hickman-Brenner.(1992):The genera *Vibrio* and *Photobacterium*. In H. G. T. p. A. Balows, M. Dworkin, W. Harder, and K. H. Schleifer (ed.) (Ed.): The Prokaryotes. A handbook on the biology of bacteria: ecophysiology, isolation, identification, and applications. Springer-Verlag KG, Berlin, Germany, pp. 2952-3011.

Farmer, J. J., III.(1992): The Family *Vibrionaceae.* In H. G. T. p. A. Balows, M. Dworkin, W. Harder, and K. H. Schleifer (Ed.): The prokaryotes. A handbook on the biology of bacteria: ecophysiology, isolation, identification, and applications, Springer-Verlag, KG,, Berlin, Germany, pp. 2938-2951.

Fee, J. A., and M.W. Mathew.(1988):Excursion into the molecular biology of *Thermus* Evidence that adhesion is a plasmid borne trait. In First *Int.Nat.Sympo.Mol.Biolo.*, Baltimore.

Fidopiastis, P. M., S. Von Boletzky, and E. G. Ruby. (1998):A new niche for *Vibrio logei*, the predominant light organ symbiont of squids in the genus *Sepiola. J. Bact.* **180**, 59-64.

Finkelstein, R., S. Edelstein, and G. Mahamid.(2002):Fulminant wound infections due to *Vibrio vulnificus. Isr. Med. Assoc. J.*,**4**, 654-655.

Floodgate, G. D. (1991):Oil degradation by marine bacteria. *In Second Inter Nati.*

Marine Biotech. Conf. and Abtracts. pp. 69.

Foster, T. J. (1983): Plasmid-determined resistance to antimicrobial drugs and toxic metal ions in bacteria. *Microbiol. Rev.*, **47**, 361-409.

Franklin, T. J., and G. A. Snow.(1989):*Biochemistry of Antimicrobial Action.* Chapman and Hall,New York.

French, G. L., M. L. Woo, Y. W. Hui and K. Y. Chan.(1989):Antimicrobial susceptibilities of halophilic *Vibrios. Jnal. of Antimicrob. Chemotherapy.* **24**, 183-194.

Fukasawa, S., and P. Dunlap. (1986): Identification of luminous bacteria isolated from the light organ of the squid *Doryteuths kensaki. Agric. Biol. Chem.*, 1645-1646.

Garrod, L. P., and F. O.Grady. (1971):*Antibiotic and Chemotherapy.* Livingstone. Edinburgh.

Giles, J.S.H., and Heany. (1995):The plasmid profile of fish pathogenic isolates of *Aeromonas salmonicida*, *Vibrio anguillarum*, and *Vibrio ordalii* from the Atlantic and pacific coasts of Canada. *Canadian J. of Microbiol.*,**41**, 209-216.

Gomez-Gil, B., A. Roque, J. F. Turnbull, and L.M.Tron.(1998):Species of *Vibrio* isolated from hepatopancreas, haemolymph and digestive tract of a population of healthy juvenile *Penaeus vannamei. Aquaculture.*, **163**, 1-9.

Gomez-Gil, B., A. Roque and G. Velasco-Blanco. (2002):Culture of *Vibrio alginolyticus* C7b, a potential probiotic bacterium, with the microalga *Chaetoceros muelleri. Aquaculture.*, **211**, 43-48.

Gowry,S.(2001):Atrazine degradation potentials and catabolic plasmid profile of autochtonous bacteria, Manonmaniam Sundaranar University, Ph.D.Thesis,Tirunelveli,Tamilnadu.

Greenwood, D. (2000): *Antimicrobial Chemotherapy.* Oxford University Press. New York.

Gregory , E., and J.J.Stanley. (1982):Widespread distribution of ability to oxidise manganese among fresh water bacteria. *Appli.Env. Microbiol.***44**, 509-511.

Grimes, D. J., R.W. Atwell, P.R. Brayton, L.M.Palmer, D.M. Rollis, D.B. Roszak, F.L. Singleton, M.L. Tampling and R.R. Colwell.(1986):The fate of enteric pathogenic bacteria in estuarine and marine environments. *Microbiol. Sci.*, **3**, 324-329.

Guerry, P. (1975):The ecology of bacterial plasmids in Chesapeake Bay Ph.D, University of Maryland,College Park.

Guerry P and R.R. Colwell. (1977):Isolation of Cryptic plasmid deoxyribonucleic acid from kanagawa positive strains of *Vibrio parahaemolyticus*. *Infection and immunity.*, **16**, 328-334.

Guidolin, A. and P. A. Manning.(1987):Genetics of *Vibrio cholerae* and its bacteriophages. *Microbiol. Rev.*, **51**, 285-298.

Hada, H. S. and R. K. Sizemore. (1981): Incidence of plasmids in marine *Vibrio* spp. isolated from an oil field in the northwestern Gulf of Mexico. *Appl. envir. Microbiol.*, **41**, 199-202.

Hager, M. C., (2001a): Detecting bacteria in coastal waters. Part 1. *The J. for Surface Water Quality Profession.*, **2**, 16-25.

Hall, R. M. and Stokes, H.W. (1993):Integrons: novel DNA elements which capture genes by site-specific recombination. *Genetics.*,**90**, 115-132.

Hall, R. M. and Collis, C. M. (1995):Mobile gene cassettes and integrons: capture and spread of genes by site-specific recombination. *Mol Microbiol.*, **15**, 593–600.

Hameed, A. S. S., and G. Balasubramanian. (2000):Antibiotic resistance in bacteria isolated from *Artemia nauplii* and efficacy of formaldehyde. *Aquaculture.*, **183**, 195-205.

Hansen, C. L., G. Zwolinsk, D. Martin and J. W. Williams.(1984):Bacterial removal of mercury from sewage. *Biotechnol. Bioeng.*, **26**, 1330-1303.

Harwood,V.J., J.Whitlock and V.Withington. (2000):Classification of antibiotic resistance patterns of indicator bacteria by discriminant analysis: use in predicting the source of fecal contamination in subtropical waters. *Appl. and Environ. Microbio.* **66**, 3698-3704.

Hackett, N.R. and D. S. Sarma. (1989):Characterization of the small endogenous plasmid of *Halobacterium* strain SB3 and its use in transformation of *H. halobiun. Can. J. Microbiol.* **35**, 86-91.

Hatha ,A.A.M., P.Gomathinayagam and P.Lekshmanaperumalasamy.(1993):Incidence of multiple antibiotic resistant *Escherichia coli* in the Bhavani River. *World journal of Microbiol. and Biotechnol.*, **9**, 609-610.

Hayakawa, T., T. Tanaka, K. Sakaguchi, N. Otake and H. Yonehara.(1979):A linear plasmid-like DNA in *Streptomyces sp.* producing lankacidin group antibiotics. *J. Gen. Appl. Microbiol.*, **25**, 255-260.

Hayes, W.J.G.(1953):Observations on a transmissible agent determining sexual differentiation in *Bacterium coli*. *Microbiol.*, **8**, 72-88.

Health Canada.(2001a):Use of drugs in aquaculture. http://www.hc-sc.gc.ca/vetdrugs-medsvet/aquaculture.

Heidelberg,J.F., J.A. Eisen, W.C., Nelson, R.A. Clayton, M.L. Gwinn, R.J. Dodson, D.H. Haft, E.K. Hickey, J.D. Peterson, L. A.Umayam, S.R..Gill, K.E.Nelson, T.D.Read, H.Tettelin,D.Richardson, M.D.Ermolaeva, J. Vamathevan, S. Bass, H. Qin, I.Dragoi, P.Sellers, L.McDonald, T.Utterback, R.D. Fleishmann, W.C. Niernan, O .White, S.L, Salzberg, H.O. Smith, R.R. Colwell, J.J.Mekalanos, J.C.Venter and C.M Fraser. (2000): DNA sequence of both chromosomes of the cholera pathogen *Vibrio cholerae. Nature* **406**, 477-483

Heidelberg, J. F., K. B. Heidelberg and R. R. Colwell. (2002a): Seasonality of Chesapeake Bay bacterioplankton species. *Appl. Environ. Microbiol.*, 5488-5497.

Heidelberg, J. F., K. B. Heidelberg and R. R. Colwell.(2002b): Bacteria of the gamma-subclass *Proteobacteria* associated with zooplankton in Chesapeake Bay. *Appl. Environ. Microbiol.*, **68**, 5498-5507.

Heinemann, J. and F. J. Sparge. (1989): Bacterial conjugative plasmids mobilize DNA transfer between bacteria and yeast. *Nature*, 205-209.

Helinski, D., A and R. Novick. (1996):Replication control and other stable maintenance mechanisms of plasmids. In: *Escherichia coli* and *Salmonella.* Society for Microbiology Press.Washington, DC.

Hinnebusch, J., and H. Tilly. (1993):Linear plasmids and chromosomes in bacteria. *Mol Microbiol.*, **10**, 917-922.

Hochut, B., J. Marrero, and M. K. Waldor.(2000):Mobilization of plasmids and chromosomal DNA mediated by the SXT element, a constin found in *Vibrio cholerae* O139. *J. Bact.*, **182**, 2043-2047.

Hochut, B., Y. Lotfi, D. Mazel, S. M. Faruque, R. Woodgate, and M. K. Waldor. (2001):Molecular analysis of the antibiotic resistance gene clusters in the Vibrio cholerae O139 and O1 SXT constins. *Antimicrob. Agents Chemother.*,**45**, 2991-3000.

Holmes, A. J., M.R. Gillings, B.S.Nield, B.C.Mabbutt, K.M.H.Nevalainen, H.W.Stokes. (2003):The gene cassette metagenome is a basic resource for bacterial genome evolution. *Environ. Microbiol.*, **5**, 383- 394.

Holt, J. G., N. R. Krieg, P. H. A. Sneath, T.Staley and S. T.Williams.(1994) : *Bergey's Manual of Determinative Bacteriology 9th edn.* MD: Williams and Wilkins. Baltimore.

Hong, Y. K. (1985): Isolation of plasmids from the moderately halophilic bacteria.

Bull.Fish.Scien.Cong., **18**, 557-562.

Hotchkiss, R. D., and R. J. Dubos.(1941):The isolation of bactericidal substances from cultures of *Bacillus brevis*. *J. Biol. Chem.*,**141**,155-162.

Hughes, V. M., and N. Datta. (1983):Conjugative plasmids in bacteria of the 'pre-antibiotic' era. *Nature.*, **302**, 725-726.

Islam, M. S., B. S. Drasar and D. J. Bradley.(1990):Long-term persistence of toxigenic *Vibrio cholerae* O1 in the mucilaginous sheath of a blue-green alga, *Anabaena variabilis*. *J. Trop. Med. Hyg.*,**93**:133–139.

Jiravanichpaisal, P., and T.Miyazaki.(1995): Comparative histopathology of Vibriosis in black tiger shrimp, *Penaeus monodon*. Asian Fisheries Society, Manila, Philippines.

Jain ,A. and V.Tiwari.(1993): Plasmid pattern of *Shigella dysentriae* Type I and its correlation to antibiotic sensitivity pattern. *J. Micro.biotech.*, **8**, 1-6.

Johnson, F. H. and I.V. Shunk. (1936):An interesting new species of luminous bacteria. *J. Bacteriol.*, **31**, 585-593.

Johnson, R. and J. Adams. (1992).The ecology and evolution of tetracycline resistance. *Trends in Ecology and Evolution.*,**7**, 295-299.

Jones, S.H., T.L. Howell and K.O.Neil.(1991):Differential elimination of indicator bacteria and pathogenic *Vibrio sp.* from eastern oyster (*Crossostrea virginica* Gmelin, 1791) in a commercial controlled purification facility in Maine. *J. Shellfish. Res.*, **10**, 105-112.

Jones. N. H. (1984): The cholera *Vibrio*: a centenary. *Briti. Medl. J.* ,**288**,379-381.

Jordan, G. W., and W. K. Hadley. (1969):Human infection with *Edwardsiella tarda*. *Ann. Intern. Med.*,**70**, 283-288.

Joseph, S. W., R. R. Colwell and J. B. Kaper.(1983):*Vibrio parahaemolyticus* and related halophilic *Vibrios*. *CRC Crit. Rev. Microbiol.*, 77-124.

Joseph, S. W., P. Dailey, W. S. Hunt, R. J. Seidler, D. A. Alien, and R. R. Colwell. (1979):*Aeromonas* primary wound infection of a diver in polluted water. *J. Clin. Microbiol,*. **10**, 46-49.

Kaneko, T., and R. R. Colwell.(1973):Ecology of *Vibrio parahaemolyticus* in Chesapeake Bay. *J. Bacteriol.*, **113**, 24-32.

Kaneko, T., and R. R. Colwell. (1978):The annual cycle of *Vibrio parahaemolyticus* in Chesapeake bay. *Microb. Ecol.*, **4**, 135-155.

Kaper, J. B., J. G.Morris and M. M.Levine.(1995):Cholera. *Clin Microbiol Reviews.*, **8**, 48-86.

Kapetanaki, M., J. Kerry, M. Hiney, C. O .Brian, R. Coyne and P. Smith.(1995): Emergence, in oxytetracycline-free marine mesocosms, of microorganisms capable of colony formation on oxytetracycline containing media. *Aquaculture* .,**134**, 227-231.

Kerry, J., M. Slattery, S. Vaughan and P. Smith. (1996a): The importance of bacterial multiplication in the selection, by oxytetracycline-HCl, of oxytetracycline-resistant bacteria in marine sediment microcosms. *Aquaculture.*, **144**, 103-119.

Kinkle, B. K., M. J. Sadowsky, E. L. Schmidt and W. C. Koskinen. (1993): Plasmids pJP4 and r68.45 can be transferred between populations of bradyrhizobia in soil. *Appl. envir. Microbiol.*, **59**, 1762-1766.

Kobayashi, N. and M. J. Bailey.(1994):Plasmids isolated from the sugar beet phyllosphere show little or no homology to molecular probes currently available for plasmid typing. *Microbiology.*, **140**, 289-296.

Koch, A. L.(1972):Enzyme evolution: I.The importance of untranslatable intermediates. *Genetics.*, **72**, 297-316.

Krumperman, P.H. (1983): Multiple antibiotic resistance indexing of *Escherichia coli* to identify high risk sources of fecal contamination of foods. *Appl Environ Microbiol.*, **46**, 165-170.

Kushmaro, A., E. Banin, Y. Loya, E. Stackebrandt and E. Rosenberg.(2001):*Vibrio shiloi spp.* nov., the causative agent of bleaching of the coral *Oculina patagonica. Int. J. Syst. Evol. Microbiol.*, **51**, 1-6.

Larsen, J. L. and E. O. John.(1991):Occurrence of plasmids in Danish isolates of *Vibrio anguillarum* serovars O1 and O2 and association of plasmids with phenotypic characteristics. *Appl. Environ. Microbiol.*, **57**,2158-2163.

Leano, E. M., V.B.M. Inglis, I.H. MacRae.(1999) :Antibiotic resistance of *Vibrio spp.* and *Aeromonas spp.* isolated from fish and shrimp tissues and rearing water in Panay Island, Philippines. *UPV J. Nat. Sci.*, **3.**

Lederberg, J. (1998): Personal perspective ; Plasmid (1952-1997). *Plasmid* .,**39**, 1-9.

Lee, J. V., P. Shread, A. L. Furniss and T. N. Bryant. (1981):Taxonomy and description of *Vibrio fluvialis spp.* nov (synonym group F vibrios, group EF6). *J. Appl. Bacteriol.*, 73-94.

Lee, K. K., S. R. Yu, F. R. Chen, T. I. Yang and P. C. Liu. (1996): Virulence of *Vibrio alginolyticus* isolated from diseased tiger prawn, *Penaeus monodon. Curr. Microbiol.*, **32**, 229-231.

Leigh G, P., T. M. John and A. S.Patricia. (2000):Complete nucleotide sequence of a cryptic plasmid from the marine bacterium *Vibrio splendidus* and

identification of open reading frames. *Plasmid.*, **43**, 99-102.

Levesque, C., S.Brassard and J. Lapointe and P.H. Roy. (1994):Diversity and relative strength of tandem promoters for the antibiotic resistance genes of several integrons. *Gene.*, **142**, 49-54.

Liu, Y., W. T. Yie-Jun, M. L. Foo-Rita, L. Julia, H. Xu, and Y. S. Woo, and Norman. (1999):Antibiotic resistance and plasmid profiles of *Vibrio* isolates from cultured Sparus sarba. *Mar. Poll. Bull.*, **39**, 245-249.

Lightner, D. V. (1993):Diseases of cultured penaeid shrimp. In CRC Handbook of Mariculture, Crustacean Aquaculture., pp 393-486.

Lipp, E. K., A. Huq, and R. R. Colwell. (2002): Effects of global climate on infectious disease: the cholera model. *Clin. Microbiol. Rev.*, **15**, 757-770.

Lipp E.K and J.B.Rose.(1997):The role of seafood in food-borne diseases in the United States of America. *Rev Sci Tech.*,**16**, 620-40.

Lipsitch, M., R. S. Singer and B. R. Levin. (2002): Antibiotics in agriculture: When is it time to close the barn door? *PNAS.*, **99**, 5752-5754.

Lobb, C. J., and M. Rhoades. (1987):Rapid plasmid analysis for identification of *Edwardsiella ictaluri* from infected catfish. *Appl. Environ. Microbiol.*, **55**, 1267-1272.

Luis A., T.M.E. Actis and J. H. Crossa. (1999):Bacterial Plasmids: Replication of extrachromosomal genetic elements encoding resistance to antimicrobial compounds. *Front. in Biosci.*, **3**, 43-62.

Margorinos, B., J.L.Romalde, I.Bandin, B. Fouz, and E. Toranzo.(1992): Phenotypic,antigenic and molecular characterization of *Pasteuerella piscicida* strains from Fish. *Appli.Env.Micro.*, **58**, 3316-3352.

Marino, A., G .Crisafi, L.Maugeri, A. Nostro and V.Alonzo. (1999):Uptake and retention of *Vibrio cholerae* non O1, *Salmonella typhi*, *Escherichia coli* and *Vibrio harveyi* by mussels in sea water. *Microbiologica.*, **22**, 129-138.

Martin, Y., J. L. Bonnefont, and L. Chancerelle. (2002):*Gorgonian*s mass mortality during the 1999 late summer in French Mediterranean coastal waters: the bacterial hypothesis. *Water Res.*, **36**, 779-782.

Martinez, E., and F.De la Cruz.(1990):Genetic elements involved in Tn21 site-specific integration, a novel mechanism for the dissemination of antibiotic resistance genes. *E.M.B.O. J.* **9**, 1275-1281.

Maruyama, A., M. Oda and T. Higashihara. (1993):Abundance of virus-sized non-DNase-digestible DNA (coated DNA) in eutrophic seawater. *Appl. Environ. Microbiol.*,**3**, 712-718.

Mc Call, O. J. and K.R. Sizemore.(1979): Description of a bacteriocinogenic plasmid in *Benecka harveyi*. *Appli. Environ. Microbial.*, **38**, 974-979.

McNicol, L. A., T. Barkay, M. J. Voll and R. R. Colwell. (1982):Plasmid carriage in *Vibrionaceae* and other bacteria isolated from the aquatic environment. *J. Wash. Acad. Sci.*, **72**, 60-66.

McPhearson, R M., A. DePaola, S.R. Zwyno, M.L. Motes and A.M. Guarino.(1991): Antibiotic resistance in Gram-negative bacteria from cultured catfish and aquaculture ponds. *Aquaculture.*, **99**, 203-211.

Mead, P. S., L. Slutsker, V. Dietz, L.F. McCaig, J.S. Bresee, C. Shapiro, P.M.Griffin, and R.V. Tauxe.(1999): Food-Related Illness and Death in the United States. *Emerg. Infecti. Diseas.* **5**, 607-625.

Michael, R. G., H. W. Stokes and J. H. Andrew. (2005): Integrons in *Xanthomonas*: A source of species genome diversity. *P. N. A. S.*, **102**, 4419- 4424.

Millikan, D. S and E. G. Ruby. (2002): Alterations in *Vibrio fischeri* motility correlate with a delay in symbiosis initiation and are associated with additional symbiotic colonization defects. *Appl. Environ. Microbiol.* **68**, 2519-2528.

Mitsuhashi, S. (1993):Drug resistance in bacteria: history, genetics and biochemistry. *J. Int. Med. Res.*, **21**, 1-14.

Miyamoto, Y., T.Kato, Y.Obara, S.Akiyama, K.Takizawa and S,Yamai.(2000): *In vitro* haemolytic characteristic of *Vibrio parahaemolyticus*: it's close correlation to human pathogenicity. *J. of Bact.*, **100**, 1147- 1149.

Mohammad, N.F and Q.M. Haque.(2002):Detection of plasmids from *Vibrio cholerae* strains isolated in Malaysia using the perfect prep plasmid MiniKit. *Letters in Applied Microbiol.*,**69**, 36-40.

Molina, A.A., A. Garcia Gasca, A. Abreu Grobois, C. Bolan Mejia , A. Roque and B. G. Gill. (2002):Plasmid profiling and antibiotic resistance of *Vibrio* strains isolated from cultured *Penaeid* shrimp. *F.E.M.S. Microbiol. Lett.*, **213**, 7-12.

Moorthy, S., and P. I. Watnick .(2004):Genetic evidence that the *Vibrio cholerae* monolayer is a distinct stage in biofilm development. *Mol. Microbiol.*, **52**, 573-587.

Murphree, R. L. and M. L. Tamplin .(1991):Uptake and retention of *Vibrio cholerae* O1 in the Eastern oyster, *Crassostrea virginica*. *Appl. Environ. Microbiol.*, **61**, 3656-3660.

National Committee for Clinical Laboratory Standards.(2001) : Performance standards for antimicrobial susceptibilities testing—9th informational supplement.National Committee for Clinical Laboratory Standards, Wayne,

Pa.

Naidu, A. J. and Yadav,M.(1997):Influence of Iron, growth temparature and plasmids on siderophore production in *Aeromonas hydrophila. J. of. Medical micro.*, **46**, 833-838.

Naylor, R. L., R. J. Goldburg, J. H. Primavera, N. Kautsky, M. C. Beveridge, J. Clay, C. Folke, J. Lubchenco, H. Mooney and M. Troell. (2000):Effect of aquaculture on world fish supplies. *Nature.*, **405**, 1017-1024.

Nield, B.S., A.J. Holmes, M.R.Gillings, G.D. Recchia, B.C. Mabbutt, K.M.H. Nevalainen, and H.W. Stokes .(2001):Recovery of new integron classes from environmental DNA. *FEMS Microbiol. Lett.*, **195**: 59-65.

Nishibuchi, M. and J. B. Kaper.(1995): Thermostable direct hemolysin gene of *Vibrio parahaemolyticus:* a virulence gene acquired by a marine bacterium. Infect. Immun. **63**:2093-2099

Nishiguchi, M. K. (2000):Temperature affects species distribution in symbiotic populations of *Vibrio spp. Appl. Environ. Microbiol.*, **66**, 3550-3555.

Noterdaeme, L., S.Bigawa, K.A.Willems and F.Olliever.(1991):Biochemical and physiological characteristics and plasmid profiles of *Aeromonas hydrophila* strains isolated from fresh water fish and from water. *J .of Fish. Disea.*, **14**, 312-321.

Novoa, B., A. Luque, D.Castro, J.J. Borrego and A. J. Figueras. (1998): Characterization and infectivity of four bacterial strains isolated from brown ring disease-affected clams. *Inverteb. Pathol.*, **71**, 34-41.

Nunes-Duby, S. E., H.J.Kwon, R.S.Tirumalai, T.Ellenberger and A. Landy.(1998): Similarities and differences among 105 members of the *Int* family of site-specific recombinases. *Nucleic. Acid. Res.* **26**, 391-406.

Nygaard, K., B.T. Lunestad, H. Hektoen, J.A. Berge and V. Hormazabal.(1992): Resistance to oxytetracycline, oxolinic acid and furazolidone in bacteria from marine sediments. *Aquaculture.*, **104**: 31-36.

Nyholm, S. V., E. V. Stabb, E. G. Ruby, and M. J. McFall-Ngai.(2000): Establishment of an animal-bacterial association: recruiting symbiotic *Vibrios* from the environment. *PNAS.*, **97**, 10231-10235.

Nyholm, S. V., and M. J. McFall-Ngai.(2003):Dominance of *Vibrio fischeri* in secreted mucus outside the light organ of *Euprymna scolopes*: the first site of symbiont specificity. *Appl. Environ. Microbiol.*, **69**,3932-3937.

O Brien,T. F. (1987): Resistance of bacteria to antibacterial agents: Report of task force 2. *Rev. Infect. Dis.*, **9**, S 224-S 260.

O Morchoe, S., O. Ogunseitan, G. S. Sayler and R. V. Miller.(1988):Conjugal transfer of R68.45 and FP5 between *Pseudomonas aeruginosa* strains in a freshwater environment. *Appl. Envir.Microbiol.*, **54**, 1923-1929.

Ochman, H., G.J. Lawrence and E. A. Groisman. (2000): Lateral gene transfer and the nature of bacterial innovation. *Nature.*, **405**, 299 - 304.

Ogunseitan, O. A., G. S. Sayler and R. V.Miller.(1990):Dynamic interactions between *Pseudomonas aeruginosa* and bacteriophages in lakewater. *Microb. Ecol.*, **19**, 171-185.

Olafsen, J., H.V.Mikkelsen, H.M.Giaever and G.H.Hansen.(1993):Indigenous bacteria in hemolymph and tissues of marine bivalves at low temperatures. *Appl Environ Microbiol.*, **59**, 1848-1854.

Olafsen, J. (2001):Interactions between fish larvae and bacteria in marine aquaculture. *Aquaculture.*, **200**, 223-247.

O'Reilly, A. and P. Smith.(2001): Use of indirect conductimetry to establish predictive no effect concentrations of oxytetracycline and oxolinic acid in aquatic sediments. *Aquaculture.*, **196,**13-26.

Oliver, J. D., and J.B.Kaper.(1997): Vibrio Species Food Microbiology: Fundamentals and Frontiers. ASM Press. Washington, D.C.

Otta, S. K., Indrani Karunasagar and Iddya Karunasagar. (1999): Bacterial flora associated with shrimp culture ponds growing *Penaeus mondon* in India. *J. of Aqua. in Trop.* **,14,** 309- 318.

Pacini, F. (1854): *Gaz. Med. Italiana.*, **6**, 405-412.

Parveen, S., R.L. Murphree, L.Edmiston, C.W.Kaspar, K.M. Portier and M.L.Tamplin.(1997):Association of multiple antibiotic resistance profiles with point and nonpoint sources of *Escherichia coli* in Apalachicola Bay. *Appl. Environ.Microbiol.*, **7**, 2607-2612.

Paul, J. H., M. E. Frisher and J. M. Thurmond. (1991): Gene transfer in marine water column and sediment microcosms by natural plasmid transformation. *Appl. Envir. Microbiol.* **57,** 1509-1515.

Pedersen, K.,J. Dalsdaard and J.L. Larsen. (1996):Characterization of a typical *A.salmonicida* isolates by ribotyping and plasmid profiling. *Appl .Bacter.*, **80**, 37-44.

Pena, E. A. and L. D.Taylor.(2001): Antibiotic resistance bacteria from shrimp ponds. *Aquaculture.*, **195**, 193-204.

Perkins, F. O., D.S.Haven, R.Morales-Alamo, and M.W. Rhodes.(1980):Uptake and

elimination of bacteria in shellfish. *J. Food Prot.*,**43,** 124-126.

Pettibone , G. W., J.P.Mear and B.M.Sampsell. (1996): Incidence of Antibiotic and metal resistance and plasmid carriage in *Aeromonas* strains isolated from brown bullhead. *Lett.Appl.Micro.,* **23**, 234-240.

Pillay T.V.R., 1997.Freshwater aquaculture and research priorities in the next millennium. In Proceedings of National seminar on "Freshwater aquaculture"-Immediate research priorities,May 1997,CIFA,Bhuvaneswar.

Potasman, I., A. Paz and M. Odeh. (2002):Infectious outbreaks associated with bivalve shellfish consumption: a worldwide perspective. *Clin. Infect. Dis.,* **35**, 921-928.

Prieur, G., G. Mevel, J.L.Nicolas, A.Plusquellec and M.Vigneulle.(1990): Interactions between bivalve molluscan and bacteria in the marine environment. *Oceanogr. Mar. Biol. Annu. Rev.,* **28**, 277-352.

Radu, S., N. Elhadi, Z. Hassan, G. Rusul, S. Lihan, N. Fifadara , Yuherman and E.Purwati. (1998): Characterization of *Vibrio vulnificus* isolated from cockles (*Anadara granosa)*:Antimicrobial resistance, plasmid profiles and random amplification of polymorphic DNA analysis. *F.E.M.S. Microbiol. Lett,.* **165**, 139-143.

Recchia, G. D. and R.M.Hall. (1995): Gene cassettes: a new class of mobile element. *Microbiology* .,**141**, 3015-3027.

Reud, W. S. and J. A. Bayle. (1989): Plasmid homologies in *Edwardsiella ictaluri. Appli. Environ. Microbiol.,* **55**, 3253-3255.

Rheinheimer, G. (1992): *Aquatic Microbiology*. Wiley. London, UK.

Richards, G. P. (1988): Microbial purification of shellfish: a review of depuration and relaying. *J.Food Prot.,* **51**, 218- 251.

Ringo, E. and T. H. Birkbeck. (1999):Intestinal microflora of fish larvae and fry. *Aquaculture. Res.,* **30**, 73-93.

Rochelle, P. A., J. C. Fry and M. J. Day. (1989):Factors affecting conjugal transfer of plasmids encoding mercury resistance from pure cultures and mixed bacterial suspensions of epilithic bacteria. *J. Gen. Microbiol.,* **135**, 409-424.

Roque, A., A. A. Molina, M.C. Bolan and B.G.Gill. (2001):*In vitro* susceptibility to 15 antibiotics of *Vibrios* isolated from penaeid shrimps in Northwestern Mexico. *Int. J. Antimicrob. Agents.*,383-387.

Rosenberg, E. and Y. Ben-Haim.(2002):Microbial diseases of corals and global warming. *Environ. Microbiol.,* **4**, 318-326.

Saiki, R. K., S. Scharf, F. Faloona, K.B.Mullis , G.T.Horn and H.A. Ehrlich. (1985): Enzymatic amplification of beta globulin genomic sequence and restriction analysis for diagnosis of sickle cell anaemia. *Science.*,**230**,1350-1354.

Sakazaki, R., and T. A. Balows. (1981): The genera *Vibrio, Plesiomonas*, and *Aeromonas*, pp.. In H. S. In M. P. Starr, H. G. Truper, A. Balows, and H. G. Schlegel (Ed): *The prokaryotes: a handbook on habitats, isolation, and identification of bacteria.*, Springer Verlag KG, Berlin. pp 1271-1301.

Salyers, A. A., N. B.Shoemaker, A. M. Stevens and L. Y. Li. (1995):Conjugative transposons: an unusual and diverse set of integrated gene transfer elements. *Microbiol. Rev.*, 579-590.

Sambrook, J., E.F. Fritsch, T. Maniatis. (1989):Molecular Cloning: A Laboratory Manual, 2nd Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Samuelsen, O., V. Torsvik and A. Ervik.(1992a):Long-range changes in oxytetracycline concentration and bacterial resistance towards oxytetracycline in a fish farm sediment after medication. *Sci. Total Environ.*, **114**: 25-36.

Sanyal, S. C., M. I. Huq, P. K. B. Neogi, K. Alam, M. I. Kabir, and A.S.M.H. Rahman. (1983): *Vibrio mimicus* as an aetiological agent of diarrohea and its pathogenesis. *Indian. J. Med. Microbiol.*, **1**, 1-12.

Sarkar, B. L., G. B. Nair, A. K. Banerjee and S. C. Pal. (1985):Seasonal distribution of *Vibrio parahaemolyticus* in freshwater environs and in association with freshwater fishes in Calcutta. *Appl. Environ. Microbiol.*, **49**, 132-136.

Sayler, G. S., J. D. Nelson, Jr., A. Justice and R. R.Colwell.(1976):Incidence of *Salmonella spp., Clostridium botulinum* and *Vibrio parahaemolyticus* in an estuary. *Appl. Environ. Microbiol.*, **31**, 723-730.

Sayler, G. S., S. W. Hooper, A. C. Layton and J. H. King.(1990): Catabolic plasmids of environmental and ecological significance. *Microb. Ecol.*, **19**, 1-20.

Schatz, A., E. Bugie, and S. A. Waksman.(1944):Streptomycin, a substance exhibiting antibiotic activity against gram positive and gram negative bacteria. *Proc. Soc. Exp. Biol. Med.*, **55**, 66-69.

Schiewe, M. H., T. J.Trust and Crossa, J. H. (1981):*Vibrio ordalii* spp. nov. a causative agent of *Vibriosis* in fish. *Current microbio.*, **6**, 343-348.

Schiewe, M. H. and J. H. Crossa. (1981):Molecular characterisation of *Vibrio anguillarum* biotype 2. *Canad.J. of Microbiol.*, **27**, 1011-1018.

Schutt, C. (1989): Plasmids in the bacterial assemblage of a dystrophic lake: Evidence for plasmid encoded nickel resistance. *Microb. Ecol.*, **17**, 49-62.

Scott, J. R. and G. G. Churchward. (1995):Conjugative transposition. *Annu. Rev. Microbiol.*, **49**, 367-397.

Sechi, L. A., I. Dupre, A. Deriu, G. Fadda, and S. Zanetti. (2000):Distribution of *Vibrio cholerae* virulence genes among different *Vibrio* species isolated in Sardinia, Italy. *J. Appl. Microbio.*,. **88**, 475-481.

Shahid ,M. A.(2004):Plasmid mediated amikacin resistance in clinical isolates of Pseudomona aeroginosa. *Indian J. of Medi. Microbiol* ., **22**, 182-184.

Shields, M. S., M.J.Reagin, R.R.Gregor, R.Campbell and C.Somerville.(1995): TOM,a new aromatic degradative plasmid from *Pseudomonas capacia* G4 strain. *Appl.Env.Micro.*, **61**, 1352-1356.

Shubha G.S.K., H.S.Kumar, I.Karunasagar,Matsuaki Nishibuchi, Iddya Karunasagar. (2005):The occurrence of *Vibrio* species in tropical shrimp culture environments; Implications for food safety. *Internati. J. of Food Microbiol.*, **30**, 356-365.

Shumway,S.G.(1992):Mussels and public health. In The Mussel Mytilus: Ecology, Physiology, Genetics and Culture. Elsevier. Amsterdam, the Netherlands.

Simidu, U., E. Kaneko, and N. Taga. (1977):Microbial studies of Tokyo Bay. *Microb. Ecol.*, **3**, 173-191.

Singer, J., T.W.Choe, K.A. Schmdit and R.A.Makula.(1992):Virulence plasmid pJM1 prevents the conjugal entry of plasmid DNA into the marne fish pathogen *V.anguillarum* 775. *J.Gen.Microbiolo.*,**138**, 2485-2490.

Sizemore, R. K. and R. R. Colwell.(1977):Plasmids carried by antibiotic resistant marine bacteria. *Antimicrob. Agents Chemo.*, **12**, 373-382.

Sobecky , P. A., T.J. Mincer, M.C Chang and D.R. Helinsky.(1997):Plasmids isolated from marine sediment microbial communities contain replication and incompatibility regions unrelated to those group of known plasmid groups. *Appl. Env. Micro.* **63**, 888-895.

Son, R., G. Rusul, A. M. Sahilah, A. Zainuri, A.R.Raha and I.Salmah.(1997): Antibiotic resistance and plasmid profile of *Aeromonas hydrophila* isolates from cultured fish, Tilapia (*Tilapia mossambica*). *Letters in Appl. Microbiol.* **24**, 479-482.

Spanggaard, B., F.G.L. Jorgensen and H.H. Huss.(1993):Antibiotic resistance in bacteria isolated from three freshwater farms and an unpolluted stream in Denmark. *Aquaculture.*, **115**,195-207.

Stokes, H.W., and Hall, R. M. (1989):A novel family of potentially mobile DNA elements encoding site specific gene integration functions: integrons. *Mol.*

Microbiol. **3**, 1669-1683.

Suantika, G., P. Dhert, G. Rombaut, J. Vandenberghe, T. De Wolf, and P. Sorgeloos. (2001):The use of ozone in a high density recirculation system for rotifers. *Aquaculture,* **201**, 35-49.

Sundstrom, L. (1998):The potential of integrons and connected programmed rearrangements for mediating horizontal gene transfer. *APMIS* .,**84**, 37-42.

Thompson, F. L., B. Hoste, K. Vandemeulebroecke and J. Swings.(2001):Genomic diversity amongst *Vibrio* isolates from different sources determined by fluorescent amplified fragment length polymorphism. *Syst. Appl. Microbiol,*. **24**, 520-538.

Thompson, F. L., Tetsuya Iida, and Jean Swings.(2005a):Biodiversity of *Vibrios. Microbiol. and Mol. biolol. Rev.*, **68**, 403-431.

Thompson, F. L., C. C.Thompson, S. Naser, B.Hoste, K.Vandemeulebroecke, C.Munn, D. Bourne and J.Swings. (2005b): *Photobacterium rosenbergii* sp. nov. and *Enterovibrio coralii* sp. nov., *Vibrios* associated with coral bleaching. *Int. J. Syst. Evol. Microbiol.* **55**, 913-917.

Thorpe, J.E., C. Talbot, M.S. Miles, C. Rawlins and D.S. Keay. (1990): Food consumption in 24 hours by Atlantic salmon (*Salmo salar*) in a sea cage. *Aquaculture.,* **90**: 41-47.

Tiainen, T., K. Pedersen and J.L. Larsen. (1995): Ribotyping and plasmid profiling of *Vibrio anguillarum* serovar O2 and *Vibrio ordalii. J. Appl. Bacteriol.*,**79**, 384-392.

Tison, D. L., M. Nishibuchi, J. D. Greenwood and R. J. Seidler.(1982):*Vibrio vulnificus* biogroup 2; new biogroup pathogenic for eels. *Appl. Environ. Microbiol.*, **44**, 640-646.

Tolmasky, M. E., L.A. Actis, A.E.Torranzo, J.L.,Barja and J.H. Crossa.(1985): Plasmids mediating iron uptake in *Vibrio anguillarum* strains isolated from turbot in Spain. *J. of Gen. Microbiol.*,**131**, 1989-1997.

Tolmasky, M. E., L.A. Actis and J.H. Crossa.(1992): *Plasmids.* Academic Press. San Diego.

Top, E., I. D. Smet, W. Verstraete, R. Dijkmans and M. Mergeay.(1994):Exogenous isolation of mobilizing plasmids from polluted soils and sludges. *Appl. Environ. Microbiol.* **60**, 831-839.

Toranzo, A. E., J.L. Barja, R.R. Colwell and F.M.Hetrick .(1983): Characterization of plasmids in bacterial fish pathogens. *Infecti. and Immun.*, **39**, 184-192.

Toranzo, A. E., Y.Santos.,S. Nunez., and J.L. Bajra.(1991):Biochemical and serological characteristics, drug resistance and plasmid profiles of spanish isolates of *Aeromonas hydrophila. Fish path.*, **26**, 55-60.

Trieu, C. P. and P.Courvalin.(1986):Evolution and transfer of aminoglycoside resistance genes under natural conditions. *J. Antimicrob. Chemother.*, **18**, 93-102.

Vandenberghe, J., Y. Li, L. Verdonck, J. Li, H. S. Xu and J. Swings.(1998).*Vibrios* associated with *Penaeus chinensis* (Crustacea: Decapoda) larvae and post-larvae in Chinese shrimp hatcheries. Aquaculture., **169**,121-132.

Vandenberghe, J., L. Verdonck, R. R.Arozarena, G. Rivera, A. Bolland, M. Balladares, B. G.Gil, J. Calderon, P. Sorgeloos, and J. Swings. (1999): *Vibrios* associated with *Litopenaeus vannamei* larvae, postlarvae, broodstock, and hatchery probionts. *Appl. Environ. Microbiol.*, **65**, 2592-2597.

Vandenberghe, J., F. L. Thompson, B. G.Gil, and J. Swings.(2003):Phenotypic diversity amongst *Vibrio* isolates from marine aquaculture systems. *Aquaculture.*, **219**, 9-20.

Vaughan, S., R. Coyne and P. Smith.(1996):The critical importance of sample site in the determination of the frequency of oxytetracycline resistance in the effluent microflora of a freshwater fish farm. *Aquaculture.*, **139**, 47-54.

Van Elsas, J. D., B. B. M.Gardener, A.C.Wolters and E.Smit.(1998):Isolation, characterization, and transfer of cryptic gene mobilizing plasmids in the wheat rhizosphere. *Appl. Envir. Microbiol.*, **64**, 880-889.

Vanderzant, C., R.Nickelson and P.W.Judkins. (1971): Microbial Flora of Pond-Reared Brown Shrimp *Penaeus aztecus* . *Appl. Microbiol.*, **21**, 916-921.

Vaseeharan, B., and P.Ramasamy.(2003):Abundance of potentially pathogenic microorganisms in *Penaeus monodon* larvae rearing systems in India. *Microbiol. Res.*, **158**, 299- 308.

Veenstra, J., P. J. Rietra, J. M. Coster, E. Slaats and S. D. Go.(1994): Seasonal variations in the occurrence of *Vibrio vulnificus* along the Dutch coast. *Epidemiol. Infect.*, **112**, 285-290.

Verschuere, L., G. Rombaut, P. Sorgeloos, and W. Verstraete(2000):Probiotic bacteria as biological control agents in aquaculture. *Microbiol. Mol. Biol. Rev.*,**64**, 655-671.

Vieira, V. V., L. F. Teixeira, A. C. Vicente, H. Momen and C. A. Salles. (2001): Differentiation of environmental and clinical isolates of *Vibrio mimicus* from *Vibrio cholerae* by multilocus enzyme electrophoresis. *Appl. Environ.*

Microbiol., **67**, 2360-2364.

Wang, H., and Mullany, P.(2000):The Large Resolvase TndX Is Required and Sufficient for Integration and Excision of Derivatives of the Novel Conjugative Transposon Tn5397. *J. Bact.*, **182**, 6577-6583.

Watanabe, T., T. Aoki, Y. Ogata and S. Egusa.(1971):R factors related to fish culturing. *Ann. N.Y. Acad. Sci.*, **182**, 383-410.

Watkins, W. D. and V. J. Cabelli.(1985):Effect of fecal pollution on *Vibrio parahaemolyticus* densities in an estuarine environment. *Appl. Environ. Microbiol.*, **49**, 1307-1313.

West, P. A., G. C. Okpokwasili, P. R. Brayton, D. J. Grimes and R. R. Colwell. (1984):Numerical taxonomy of phenanthrene degrading bacteria isolated from the Chesapeake Bay. *Appl.Environ. Microbiol.*, **48**, 1988-1993.

West, P. A., P. R. Brayton, R. R. Twilley, T. N. Bryant and R. R. Colwell.(1985): Numerical taxonomy of nitrogen-fixing "decarboxylase-negative" *Vibrio* species isolated from aquatic environments. *Int. J. Syst. Bacteriol.*, **35**, 198-205.

White, P. A., C.J. McIver and W.D. Rawlinson. (2001): Integrons and gene cassettes in the *Enterobacteriaceae*. *Antimicrob. Agents Chemother.*, **45**, 2658- 2661.

WHO (2001): Cholera, 2000. *Wkly. Epidemiol. Rec.*, **76**, 233-240.

Wiggins, B.A.(1996):Discriminant analysis of antibiotic resistance patterns in fecal streptococci, a method to differentiate human and animal sources of fecal pollution in natural waters. *Appl.Environ. Microbiol.*, **62**, 3997-4002.

Wiggins, B. A., R. W. Andrews, R. A. Conway, C. L. Corr, E. J. Dobratz, D. P. Dougherty, J. R. Eppard, S. R. Knupp, M. C. Limjoco, J. M. Mettenburg, J. M. Rinehardt, J. Sonsino, R. L. Torrijos, and M. E. Zimmerman. (1999): Use of antibiotic resistance analysis to identify nonpoint sources of fecal pollution. *Appl. Environ. Microbiol.*, **65**, 3483-3486.

Wilk, R. K., F.L. Anderson, Daae and K.A. Hoff.(1989): Virulence studies based on plasmids of fish pathogen *Vibrio salmonicida*. *Appl . Environ .Microbiol.*, **55**, 819-825.

Willetts, N. and B. Wilkins. (1984):Processing of plasmid DNA during bacterial conjugation. *Microbiol. Rev.*, **48**, 24-41.

Williams, M. J., J. D. Bell, M. V. Gupta, M. Dey, M. Ahmed, M. Prein, S. Child, P. R. Gardiner, R. Brummett and D. Jamu .(2000): Responsible aquaculture can aid food problems. *Nature.*, **406**, 673.

Wright, A. C., R.T. Hill, J.A.Johnson, M.C.Roghman, R.R. Colwell and J.G. Jr.Morris. (1996): Distribution of *Vibrio vulnificus* in the Chesapeake Bay. *Appl. Environ. Microbiol.*, **62**, 717-724.

Wu, H.B. and J.P.Pan.(1997):Studies on the pathogenic bacteria of the Vibriosis of *Seriola dumerili* in marine cage culture. *J. Fish. China.*, **21**, 40-46.

Yamamoto, T., G.B.Nair, C.C.Parodi, and Takeda.(1995): *In vitro* susceptibilities to antimicrobial agents of *V.cholerae* Oland O139.*Antimicrob. Agents. Chemotherapy.* **39**, 241-244.

Yamane, K., J. Asato, N. Kawade, H. Takahashi, B. Kimura, and Y. Arakawa.(2004): Two cases of fatal necrotizing fasciitis caused by *Photobacterium damsela* in Japan. *J. Clin. Microbiol.*, **42**, 1370-1372.

Yasuda, M., Yamagishi, and T. Oshima. (1995): The plasmids found in isolates of the acidothermophilic archaebacterium *Thermoplasma acidophilum. F.E.M.S. Microbiol. Letters.*, **128**, 157-162.

Zhao, J. and T,Aoki. (1992): Plasmid profile analysis of *Pasteurella piscicda* and use of a plasmid DNA probe to identify the species. *J. of.Aquatic. Animal health.*, **4** , 198-202.

Zyskind, J. W., J.M. Cleary, W.S.A. Brusilaw, N.E. Harding and D.W. Smith. Chromosomal replication origin from the marine bacterium *Vibrio harveyi* functions in *Escherichia coli*: oriC consensus sequence.(1983):*P. N.A.S.*, **80**, 1164-1168.

APPENDIX I

Preparation of Media and reagents

Luria Bertani Broth

Bactotryptone	10g
Bacto yeast extract	5g
NaCl	10g

Dissolved the ingredients in 1000ml distilled water . pH adjusted to 7.5 and dispensed into test tubes. Sterilize at 121 ^{0}C for 15 minutes.

Luria Bertani agar

Bactotryptone	10g
Bacto yeast extract	5g
NaCl	10g
Agar	20g

Dissolved the ingredients in 1000 ml distilled water. pH was adjusted to 7.5 Mixed well and sterilize at 121 ^{0}C for 15 minutes. Cooled to 50-55 ^{0}C and the medium was dispensed to sterile plates. The plates was stored at 4°C and used within 2 weeks.

Mueller Hinton agar

Beef infusion	300g
Casein hydrolysate	17.5g
Starch	1.5g
Agar	17g

Dissolved the ingredients in 1000 ml distilled water. pH was adjusted to 7.5 Mixed well and sterilize at 121 ^{0}C for 15 minutes. Cooled to 50-55 ^{0}C and the medium was dispensed to sterile plates. The plates was stored at 4°C and used within 2 weeks.

Nutrient agar

Peptic digest of animal tissue	5g
Sodium chloride	5g
Beef extract	1.5g
Yeast extract	1.5g

Dissolved the ingredients in 1000 ml distilled water. pH was adjusted to 7.5 Mixed well and sterilize at 121 ^{0}C for 15 minutes. Cooled to 50-55 ^{0}C and the medium was dispensed to sterile plates. The plates was stored at 4°C and used within 2 weeks.

Marine Zobells Agar

Peptic digest of animal tissue	5 g
Yeast extract	1 g
Ferric citrate	0.1 g
Sodium chloride	19.45 g
Magnesium chloride	8.8g
Sodium sulfate	3.24g
Calcium Chloride	1.8g
Potassium chloride	0.55g
Sodium bicarbonate	0.16g
Potassium bromide	0.08g
Strontium chloride	0 .034g
Boric acid	0.022g
Sodium silicate	0.004g

Sodium flourate	0.0024g
Ammonium nitrate	0.0016g
Disodium phosphate	0.008g

Dissolved the ingredients in 1000 ml distilled water. pH was adjusted to 7.5 Mixed well and sterilize at 121 ^{0}C for 15 minutes. Cooled to 50-55 ^{0}C and the medium was dispensed to sterile plates. The plates was stored at 4°C and used within 2 weeks.

Mac Conkey Agar medium

Peptic digest of animal tissue	20.0
Lactose	10g
Bile salts	5g
Sodium chloride	5.0g
Neutral red	0.07g
Agar	15g

Dissolved the ingredients in 1000 ml distilled water. pH was adjusted to 7.5 Mixed well and sterilize at 121 ^{0}C for 15 minutes. Cooled to 50-55 ^{0}C and the medium was dispensed to sterile plates. The plates was stored at 4°C and used within 2 weeks.

TCBS agar

Proteose peptone	10g
Yeast extract	5g
Sodium thiosulphate	10g
Sodium citrate	10g
Ox Gall	8.0g
Sucrose	20g

Sodium chloride	10.0g
Ferric citrate	1.0g
Bromothymol blue	0.04g
Thymol blue	0.04g
Agar	15g

Dissolved the ingredients in 1000 ml distilled water. pH was adjusted to 7.5 Mixed well and sterilize at 121 ^{0}C for 15 minutes. Cooled to 50-55 ^{0}C and the medium was dispensed to sterile plates. The plates was stored at 4^{o}C and used within 2 weeks.

MOF medium

Casein enzyme hydrolysate	1g
Yeast extract	0.1g
Tris hydroxy methyl aminomethane	0.5g
Boric acid	0.011g
Ammonium sulfate	0.5g
Disodium phosphate	0.004g
Ammonium nitrate	0.008g
Sodium chloride	9.7g
Magnesium chloride	4.4g
Sodium sulfate	1.6g
Calcium chloride	0.9g
Potassium chloride	0.275g
Sodium bicarbonate	0.08g
Potassium bromide	0.04g

Strontium chloride	0.017g
Sodium silicate	0.002g
Sodium fluoride	0.0012g
Phenol red	0.01g
Agar	3g

Dissolved the ingredients in 1000 ml distilled water. pH was adjusted to 7.5 Mixed well and sterilize at 121 ^{0}C for 15 minutes. Cooled to 50-55 ^{0}C and the medium was dispensed to sterile plates. The plates was stored at 4°C and used within 2 weeks.

SOB Medium

Bactotryptone	20.0 g
Bactoyeast extract	5 g
Sodium chloride	0.5 g

Dissolved in 900 ml water. Added 10 ml of 250 mM KCl. Adjusted the pH to 7.0 . Adjusted the volume of solution to 1000 ml with deionised water. Autoclaved and kept in refrigerator.

SOC Medium

SOB medium containing 20 mM glucose. After the SOB medium has been autoclaved , allowed to cool and 20 ml of sterile 1M solution of glucose and 5 ml of $MgCl_2$ was added to it. Mixed well and used .

Normal saline

89 g Sodium chloride was dissolved in distilled water and autoclaved .

Ampicillin

A 50-mg/ml stock solution of the sodium salt of ampicillin in water was made, sterilized by filtration through 0.22µm filter cellulose acetate (Sartorious corporation, Germany) and stored at 4^0 C.

For plates : The autolaved Luria Bertani agar medium (Hi Media) was allowed to cool to 40-55^{0}C before adding ampicillin to a final working concentration of 50 µg/ml of antibiotic ampicillin. Plates containing ampicillin was stored at 4^0C and used within 2 weeks.

Solution I

Glucose	50 mM
Tris (pH 8.0)	25mM
EDTA (pH 8.0)	0.5mM

Autoclaved at 15 lbs pressure for 15 minutes and stored at 4^0 C

Solution II (Freshly prepared)

Sodium hydroxide (NaOH)	0.2 N
Sodium dodecyl sulphate (SDS)	1%

Stock solutions of each one prepared , autoclaved and kept at room temparature.

Solution III

Potassium acetate	5 M

pH adjusted to 5.2 with glacial acetic acid. Autoclaved and stored at - 20^0C.

Tris –EDTA (TE) Buffer

EDTA, pH 8.	10mM
Tris-HCl, pH 8.0	1mM

Autoclaved and kept at room temperature.

SDS solution (10%)

SDS	10 g

Dissolved in 100 ml sterile water and kept at room temperature.

RNAse A 10 mg/ml

RNAse	10 mg
Tris HCL-pH 7.5	1mM
$MgCl_2$	10mM

Boiled for 2 minutes and cooled and kept in –20° C.

Tris (pH 8.0) saturated with Phenol.

Tris Acetate EDTA (TAE, 10X)

Tris base	.....2M
Ethylene diamine tetra acetate disodium salt	.... 0 .05 M

pH adjusted to 8 with glacial acetic acid (Qualigens).

Buffer used for loading DNA samples onto gels

Bromophenolblue (SRL)	0.05g
Glycerol (SRL)	5 ml

The volume was adjusted to 10 ml with 1X TAE .

Ethidium Bromide

Ethidium bromide	10 mg
Sterile distilled water	100ml

Ethidium bromide was completely dissolved and the container was wrapped with aluminum foil and stored at 4^0 C.

250 mM KCl

Dissolved 1.86 g of potassium chloride in 100 ml of deionised water.

1 M $CaCl_2$

1 M $CaCl_2$ was prepared by adding 147.02 g of Calcium Chloride crystals in distilled water. Dissolved the crystals well and filter sterilized through 0.2 μ m cellulose acetate filter paper (Sartorious corporation, Germany) filter paper and dispense in bottles and stored at -20^0C.

0.1 M $CaCl_2$

0.1 M $CaCl_2$ was freshly prepared from 1 M $CaCl_2$.

McFarland Turbidity standard

0.5 McFarland turbidity standard was prepared by adding 0.5 ml of a 1.175% (w/v) barium chloride dihydrate solution to 99.5 ml of 1% v/v sulfuric acid. Sealed the tubes with paraffin wax to prevent evaporation. It was stored in dark at room temparature.

APPENDIX II

Research Papers published :

1.**Manjusha, S., Sarita G.Bhat, Elyas K.K, and Chandrasekaran, M., 2005.** Multiple Antibiotic Resistances of *Vibrio* isolates from Coastal and Brackish Water Areas. *American Journal of Biochemistry and Biotechnology* 1(4):201-206.

Presentation at International Conference.

1.Manjusha,S.,SaritaG.Bhat,ElyasK.K,BernardRajeevS.W.,and Chandrasekaran, M. Occurrence of integron containing plasmid pP17CBMW09 in *Vibrio metchnikovii* isolated from brackish water of Kerala.

A Poster presentation was done at an **International Conference "BIOVISION 2007"** October 22 –10-2007 to 24 –10-2007, at Sahradaya college of Engineering and Technology, Thrissur, Kerala.