3.1 Introduction

During the past, research has been mainly focused on antibiotic resistant bacteria in clinical environments. But, recently the rapid increase in community-acquired infections due to resistant bacteria has driven the interest to antibiotic resistance in natural environments (Martínez, 2008; Forsberg et al., 2012). Natural environments are reservoirs of antibiotic resistance genes. So far, the occurrence and emergence of antibiotic resistance in marine environment has been given little attention. There are three different ways through which antibiotic resistant bacteria occurs in marine environment such as surface runoff of antibiotic resistant bacteria from terrestrial sources, selection of antibiotic resistant strains due to anthropogenic antibiotic discharge and selection for antibiotic resistance as a result of antibiotic production in marine environments (Hatosy and Martiny, 2015).
The current trend of increasing antibiotic resistance is a crisis in global scale and it is a major health and economic issue. During the past few decades, there is rapid emergence in antimicrobial resistance in many bacterial genera due to the excessive use of antibiotics in humans, agriculture and aquaculture systems (Cabello, 2006). Release of sewage also results in entry of large number of drug resistant bacteria from various sources into the environment. Resistant genes are further transferred from non-pathogens to pathogens through horizontal gene transfer via conjugation, transduction and transformation. This could lead to transfer of drug resistance features to autochthonous microorganisms such as Vibrio. Thus, the search for genetic elements such as plasmids, transposons and integrons associated with antibiotic resistance in microorganisms has also become very important. Multiple drug resistance among Vibrio spp. in estuarine/marine environments may have further implications for those who consume seafood contaminated with these pathogenic vibrios and also for the recreational and commercial users of these environments (Shaw et al., 2014).

3.2 Review of literature

3.2.1 Antibiotics and their mode of action

The first antibiotic, penicillin, was discovered by Alexander Fleming in 1928. Later, streptomycin was discovered by Selman Waksman in 1943 from Streptomyces griseus and in 1944 it was introduced for treatment of tuberculosis. Further, many new antibiotics were discovered. The discovery of antibiotics has revolutionized healthcare in many aspects, and since then numerous lives have been saved (Davies and Davies, 2010).
The modes of action of the antibiotics are not similar. Different antibiotics have difference mechanisms by which it inhibits the growth or kills the bacteria. Based on the mode of action, antibiotics are mainly grouped into four, which includes those that:

1) Inhibit cell wall biosynthesis
2) Inhibit protein synthesis
3) Alter nucleic acid metabolism
4) Inhibit folate metabolism

Table 3.1 Mode of action of commonly used antibiotics (adapted from Davies and Davies, 2010)

<table>
<thead>
<tr>
<th>Antibiotic class</th>
<th>Mode of action</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beta lactams</td>
<td>Inhibits peptidoglycan biosynthesis</td>
<td>Penicillins (ampicillin), cephalosporins (cephamycin), penems (meropenem), monobactams (aztreonam)</td>
</tr>
<tr>
<td>Aminoglycosides</td>
<td>Inhibits translation</td>
<td>Gentamicin, amikacin, streptomycin, spectinomycin</td>
</tr>
<tr>
<td>Glycopeptides</td>
<td>Inhibits peptidoglycan biosynthesis</td>
<td>Vancomycin, teicoplanin</td>
</tr>
<tr>
<td>Macrolides</td>
<td>Inhibits translation</td>
<td>Erythromycin, azithromycin</td>
</tr>
<tr>
<td>Tetracyclines</td>
<td>Inhibits translation</td>
<td>Minocycline, tigecycline, oxytetracycline</td>
</tr>
<tr>
<td>Phenicols</td>
<td>Inhibits translation</td>
<td>Chloramphenicol</td>
</tr>
<tr>
<td>Quinolones</td>
<td>Affects nucleic acid metabolism</td>
<td>Ciprofloxacin, nalidixic acid</td>
</tr>
<tr>
<td>Pyrimidines</td>
<td>Inhibition of C1 metabolic pathways</td>
<td>Trimethoprim</td>
</tr>
<tr>
<td>Sulfonamides</td>
<td>Inhibition of C1 metabolic pathways</td>
<td>Sulfamethoxazole</td>
</tr>
<tr>
<td>Cationic peptides</td>
<td>Disrupts cell membrane</td>
<td>Colistin</td>
</tr>
</tbody>
</table>
All beta-lactam antibiotics are bactericidal and disrupt the bacterial cell wall synthesis by inhibiting the peptidoglycan synthesis. Sulphonamides competitively bind to dihydropteroate synthase (DHPS), an enzyme in the folic acid biosynthesis pathway, and inhibit the formation of dihydrofolic acid (Sköld, 2000). Trimethoprim competitively inhibits the dihydrofolate reductase (DHFR) enzyme and interrupts the folic acid pathway thereby disrupting the nucleic acid synthesis (Davies and Davies, 2010). The drug binds to the dihydrofolate reductase enzyme which is involved in the synthesis of folic acid. Aminoglycosides and tetracyclines bind to the aminoacyl site of 16S ribosomal RNA (rRNA) within the 30S ribosomal subunit and inhibits protein synthesis pathway. Macrolides on the other hand binds to the 50S ribosomal subunit to inhibit protein synthesis (Davies and Davies, 2010). Quinolones act by inhibiting the activities of DNA gyrase and topoisomerase IV. The initial target site of action for colistin antibiotic is the bacterial cell membrane. Colistin binds to lipopolysaccharides and phospholipids in the outer cell membrane of Gram-negative bacteria (Falagas and Kasiakou, 2005). It displaces the divalent cations (Ca$^{2+}$ and Mg$^{2+}$) from the phosphate groups of membrane lipids and ultimately disrupts the bacterial outer cell membrane and leads to bacterial death (Landman et al., 2008).

3.2.2 Antibiotic resistance mechanism in bacteria

Antimicrobial resistance is the ability of a microbe to survive and multiply in the presence of an antimicrobial agent that normally inhibits or kill the particular microorganism. Any use of antimicrobials, contributes to the emergence of antibiotic resistance. Its widespread unnecessary and
indiscriminate use makes the situation worse. Bacteria and other microbes evolve rapidly to resist to newly developed drugs. The pace at which novel antibiotics are being discovered has slowed drastically, whereas the antibiotic use is rising rapidly.

**Figure 3.1** Mechanism of antibiotic resistance (adapted from Verma and Rawat, 2014)

There are four main mechanism of bacterial resistance to antibiotics: (1) enzymatic degradation of drugs, (2) alteration of bacterial proteins that are targets for the antimicrobial agent, (3) changes in cell membrane permeability to antibiotics and (4) by expulsion of the antimicrobial agents from the cell through bacterial efflux pumps (Dever and Dermody, 1991). A bacterium attains antibiotic resistance through intrinsic or acquired mechanisms. Intrinsic resistance is the innate ability of bacteria to resist antibiotics and is attained by naturally occurring genes.
found on the bacterial genome. Acquired resistance is through mutations in genes targeted by the antibiotic and by transfer of resistance determinants through transformation, transduction or conjugation (Levy and Marshall, 2004; Alekshun and Levy, 2007).

Bacteria resist the action of β-lactam antibiotics by producing β-lactamases (penicillinases), which hydrolyze the β-lactam ring and inactivates the antibiotic (Davies and Davies, 2010). Bacterial resistance to aminoglycosides is accomplished by acetyltransferases, phosphotransferases and nucleotidyltransferases which modifies the aminoglycosides (Alekshun and Levy, 2007; Zhang et al., 2009). Quinolones are resisted by bacteria by expelling the antibiotic through the efflux proteins, by mutation in the quinolone target molecule (DNA gyrase) and also by blocking the entry of quinolones (Fonseca et al., 2008). Mutations in the trimethoprim target dihydrofolate reductase enzyme are the main mechanism through which bacteria acquire very high-level trimethoprim resistance (Skold, 2001). Other mode of resistance to pyrimidine antibiotics is through efflux proteins and development of permeability barriers. Resistance to sulphonamides is primarily attributed to possession of mutated chromosomal dihydropteroate synthase (DHPS) gene (Enne et al., 2001; Davies and Davies, 2010). Tetracycline resistance occurs by monooxygenation, efflux pumps and modifying target molecule (Chopra and Roberts, 2001). The expulsion of chloramphenicol by chloramphenicol specific efflux proteins (Davies and Davies, 2010) and production of chloramphenicol acetyltransferases (Alekshun and Levy, 2007; Dang et al., 2008) are the major resistance mechanisms adopted by chloramphenicol resistant bacteria. Glycopeptide resistance is achieved through reprogramming the
peptidoglycan synthesis and production of a modified peptidoglycan (Courvalin, 2006). Macrolide resistant bacteria have enzymes that modifies the RNA and protects the ribosome by preventing the entry of the antibiotic (Alekshun and Levy, 2007; Aminov and Mackie, 2007).

Above figure shows a recent report published by the UK government. It reveals an alarming prediction. They report that by 2050, antimicrobial resistant infections could kill 10 million people across the world which is more than the current death toll from cancer.

**Figure 3.2** Deaths caused due to antimicrobial resistance infections every year compared to other major causes of death *(Adapted from ‘The Review on Antimicrobial Resistance’ by UK government, December 2014)*
3.2.3 Spread of antibiotic resistance genes among bacteria

The spread of antibiotic resistance genes from environment to pathogenic bacteria is a global issue. Natural environment is known to be a major reservoir of potential antibiotic resistant genes (ARGs) and plays a pivotal role in the dissemination of these genes (Hatosy and Martiny, 2015). The presence of antibiotics in the environment due to misuse and overuse of antibiotics creates a selective pressure for the emergence and transmission of these antibiotic resistance genes in mutants (Canton, 2009). The pathogenic bacteria acquire the ARGs from environmental gene pool through horizontal gene transfer processes such as transduction, transformation and conjugation. Natural water bodies are the hotspots for horizontal gene transfer (HGT) of ARGs between environmental bacteria and the human and animal pathogens. This can lead to serious public health issue complicating the disease treatment (Igbinosa and Odjadjar, 2015).

Conjugative transfer of ARGs was initially discovered in 1950s (Davies and Davies, 2010). It was then the first plasmids were isolated and plasmid-mediated antibiotic resistance was discovered. Plasmids and mobile DNA elements belonging to the class integrative conjugative elements are the transferable genetic elements. For example *V. cholerae* acquires ARGs from intrinsically resistant environmental bacteria through these mobile genetic elements (Martinez, 2008). It further transfers these ARGs with other commensals or other pathogens in the human gut, thereby making the treatment of many infections complicated (Sedas, 2007). ARG transfer is possible even between phylogenetically distant
organisms like species of Gram-positive and Gram-negative bacteria (Kruse and Sørum, 1994). Among various bacteria, Escherichia coli have been recognized as a major carrier of antibiotic resistance genes (Zhao and Dang, 2012). Larger plasmids usually harbour a number of mobile genetic elements (MGEs) like IS elements, transposons, integrons, gene cassettes and conjugative transposons (Osborn et al., 2000; Toussaint and Merlin, 2002). These accessory elements give plasmids a selective advantage in transferring ARGs.

Plasmid-mediated antibiotic resistance is causing great trouble in the treatment of infectious diseases. Curing or elimination of plasmids from bacterial strains is a way to determine the antibiotic resistance mediation. Curing occurs either naturally through cell division or through any physical or chemical agent (Elias et al., 2013). Chemical agents such as acridine orange (AO) and ethidium bromide (intercalating agents) and sodium dodecyl sulfate (anionic detergent) are widely used for plasmid curing (Molina-Aja et al., 2002; Manjusha and Sarita, 2011; Costa et al., 2014; Yano et al., 2014; Letchumanan et al., 2015a). Intercalating agents are known to eliminate plasmids by inhibition of plasmid replication. In a study by Reboucas et al. (2011), 0.2 mg/ml AO was used to cure plasmid from Vibrio spp. from shrimps. Oxytetracycline resistance was lost in many isolates after curing, indicating plasmid mediated resistance to oxytetracycline. All the isolates exhibited resistance to ampicillin even after curing, indicating it to be chromosomal mediated. In another study, penicillin G, ampicillin and aztreonam resistant Vibrio isolates were subjected to plasmid curing with 0.1 mg/ml of AO. After the curing treatment, 11 resistant isolates became susceptible to the antibiotics
In a study on antibiotic resistant *Vibrio* spp. from shrimps in Thailand, ethidium bromide (0.2 mg/ml) was used to eliminate plasmids. Results suggested that oxytetracycline resistance in the isolates was lost after the plasmid curing (Yano et al., 2014). In a study from India, sodium dodecyl sulfate was used to cure plasmids from *V. parahaemolyticus* strains. The results concluded all strains were resistant to the antibiotics even after curing, thus revealing the resistance of *V. parahaemolyticus* isolates to be chromosomal borne (Devi et al., 2009). Among the physical agents, elevated growth temperature is most commonly used in *Vibrio* plasmid curing (Letchumanan et al., 2015b). Incubation of bacteria at higher temperature leads to complete or partial deletion of plasmid DNA (Letchumanan et al., 2015 b).

Uptake of naked extracellular DNA from environment is achieved through transformation. In a study from China it was found that extracellular DNA carrying ARGs was more abundant than intracellular DNA. This revealed that extracellular DNA is also an important environmental reservoir for ARGs that can be transferred through transformation (Mao et al., 2014). Transduction or the DNA transfer between bacteria via bacteriophages also plays an important role in the spread of antibiotic resistance genes in the environment (Muniesa et al., 2013). Bacteriophages have higher survival in water bodies than inside the host body, which makes them suitable for dissemination of ARGs among bacteria (Duran et al., 2002). Through metagenomic studies, bacteriophages carrying beta-lactamase genes and methicillin-resistance gene (*mecA*) have been detected in activated sludge, urban sewage and wastewater treatment plants (Colomer-Lluch et al., 2011; Rolain et al.,
2012). Recently, high levels of beta-lactam antibiotic and fluoroquinolone resistance genes were detected in phage DNA from hospital and urban treated effluents using qPCR assays (Marti et al., 2014).

There are about 38 different tetracycline resistance (tet) genes and three oxytetracycline resistance (otr) genes which are characterised (Roberts, 2005; Thompson et al., 2007). Many of the tetracycline resistance genes are found either on non-mobile plasmids or incomplete transposons in the chromosome (Roberts, 2005). The aac, aph and ant genes encoding acetyltransferases, phosphotransferases and nucleotidylytransferases respectively are the major genes involved in aminoglycoside resistance (Chandrakanth et al., 2008). Sulfonamide and trimethoprim resistance are usually encoded by sul and dfr genes (Skold 2000, 2001). The environmental reservoir of these genes include various wastewater treatment plants (da...
Silva et al., 2007; Moura et al., 2007), river water (Mohapatra et al., 2008) and the aquaculture ponds (Jacobs and Chenia, 2007). The $bla$ gene encoding the beta-lactamase confers beta-lactam resistance in bacteria. Currently, nearly 400 different $\beta$-lactamases encoded by different $bla$ genes have been characterised (Li et al., 2007). The $bla$ gene is often reported in environment from animal derived pathogens such as Vibrio, Aeromonas, Enterobacter, Salmonella etc. (Volkmann et al., 2004; Taviani et al., 2008; Zhang et al., 2009). Reports show that different types of genetic elements are involved in the transfer of $bla_{CTX-M}$ genes (Bou et al., 2002; Chanawong et al., 2002; Saladin et al., 2002). Among them ISEcp1-like insertion sequences are most common (Lartigue et al., 2004).

The $erm$ gene confers resistance to macrolide, lincosamide and streptogramin antibiotics (Roberts, 2008). The $erm$ gene can be easily spread from one host to another as they are usually located on plasmids (Liu et al., 2007) and transposons (Okitsu et al., 2005).

### 3.2.4 Antibiotic use in India

Van Boeckel et al. (2014) conducted a survey on the global antibiotic consumption from 2000-2010. Their study revealed an alarming report that, India is the largest consumer of antibiotics in the world in 2010 followed by China. Figure 3.4 gives a picture about the consumption of antibiotics in India from 2000 to 2010. Overall the consumption of cephalosporins, quinolones and broad spectrum penicillins is very high and has been increasing considerably over the years. The carbapenem resistance in $E. \text{coli}$ isolates increased from 10% to 13% and in the Klebsiella pneumoniae isolates fluoroquinolone resistance increased from
Prevalence of Antibiotic Resistance and Plasmid Profiles of Vibrio From Food and...

57% to 73% (CDDEP, 2015). High population density and lack of proper public health measures are the main reasons for rapid spread of antibiotic resistant pathogens in India (Laxminarayan and Chaudhury, 2016).

![Figure 3.4 Trends in antibiotic consumption in India, 2000–2010 (Retrieved from Laxminarayan and Chaudhury, 2016)](image)

In order to prevent or regulate the over-the-counter sale of antibiotics in India, the Central Drugs Standard Control Organization (CDSCO) included 24 important antibiotics under Schedule H1 in 2014 (Laxminarayan and Chaudhury, 2016). These antibiotics could be sold only with a valid prescription from a registered medical practitioner.

In India, antibiotics are not only overused/misused in clinical sectors. It is also exploited indiscriminately in various animal production sectors also. Recent study revealed presence of high load of antibiotic residues in poultry meat and milk samples (Kakkar and Rogawski, 2013).
3.2.5 Antibiotic resistance in *Vibrio*

Vibrios are generally known to be highly susceptible to most of the clinically used antibiotics (Shaw *et al.*, 2014). Commonly used antibiotics for treatment for *Vibrio* infections include cephalexin, cefuroxime, cefotaxime, ceftazidime, tetracycline, doxycycline, fluoroquinolone, amikacin, gentamicin and trimethoprim-sulfamethoxazole (Al-Othrubi *et al.*, 2014; Letchumanan *et al.*, 2015a).

The first report on antimicrobial resistant *V. cholerae* was from Tanzania (Mhalu *et al.*, 1979) and later from Bangladesh (Glass *et al.*, 1980). French *et al.* (1989) studied antibiotic susceptibility of 244 halophilic vibrios isolated from Hong Kong and reported most of the strains to be resistant to sulphamethoxazole, trimethoprim, penicillins and older cephalosporins. In Africa, the *V. cholerae O1* strain that caused 1996-97 epidemic, carried a conjugative multiple-resistance plasmid with class 1 integrons that encoded resistance to trimethoprim and aminoglycosides (Dalsgaard *et al.*, 2000).

Integrating conjugative elements (ICE) carry many ARGs and the first *V. cholerae* ICE was described in 1992 in a *V. cholerae* O139 isolate from Madras, India (Waldor *et al.*, 1996). Presence of class 1 integrons carrying antibiotic resistance genes was also reported in *V. cholerae* non-O1 and O139 strains from India (Thungapathra *et al.*, 2002).

There are numerous studies on antibiotic susceptibility of *Vibrio* from India. Devi *et al.* (2009) tested the antibiotic susceptibility of *V. parahaemolyticus* isolated from shrimp farms along the southwest
Prevalence of Antibiotic Resistance and Plasmid Profiles of *Vibrio* From Food and Environmental Sources Along the South West Coast of India

Lesley et al. (2011) studied the antibiotic resistance of *V. parahaemolyticus* isolated from cockles in Malaysia and found that all the tested strains showed resistance to streptomycin, tobramycin, carbenicillin, teicoplanin, cephalothin, clindamycin, rifampicin, sulfamethoxazole and ofloxacin. A study on the antibiotic resistance in *Vibrio* sp. isolated from seafoods collected from Persian gulf, revealed all the strains to be multiple drug resistant (Raissy et al., 2012a). Ottaviani et al. (2013) reported high antibiotic resistance in *V. parahaemolyticus* isolated from wild shrimps in Italy. The strains exhibited resistance to amoxicillin, ampicillin, cefalexin, colistin, erythromycin, cefalothin and streptomycin. Costa et al. (2014) reported that *V. parahaemolyticus* isolates from shrimps were resistant to penicillin, tetracycline, ampicillin and cephalothin.
V. parahaemolyticus strains isolated from shrimps in Louisiana Gulf in Mexico were resistant to ampicillin, oxytetracycline and tetracycline (Han et al., 2007). A recent study on antibiotic susceptibility profile of Vibrio cholerae isolated from catfish in Malaysia showed that all isolates were multiple antibiotic resistant (Norshafawati et al., 2017).

3.3 Objectives of the study

1) To determine the prevalence of antibiotic resistance in vibrios isolated from the Cochin estuary, pokkali cum shrimp farm and seafood collected from retail markets.

2) To evaluate the extent of multiple antibiotic resistance (MAR) and to delineate the resistance profiles.

3) To screen the vibrios for the presence of antibiotic resistance genes.

4) To screen the antibiotic resistant vibrios for presence of plasmids.

5) To carry out plasmid curing experiments to ascertain any possible plasmid-mediated antibiotic resistance.

3.4 Materials and Methods

A total of 280 Vibrio strains were subjected to antibiotic sensitivity screening. This included 180 isolates from Cochin estuary, 70 from pokkali cum shrimp farm and 30 from seafood collected from retail markets.

3.4.1 Antibiotic sensitivity test

The antibiotic sensitivity of the strains were analysed and compared using the disc diffusion method (Bauer et al., 1966). Twenty-five different
antibiotic discs (Himedia, India) of 8-mm diameter were used for the test. The antibiotics belonged to eleven different classes according to their chemical structure. The concentration of each antibiotic used, their class and abbreviations are given in Table 3.2.

**Table 3.2** Details of the antibiotics used in the study

<table>
<thead>
<tr>
<th>Antibiotic class</th>
<th>Antibiotics used</th>
<th>Abbreviations</th>
<th>Concentration (mcg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aminoglycosides</td>
<td>Amikacin</td>
<td>Ak</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>Streptomycin</td>
<td>S</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Gentamicin</td>
<td>Gen</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Netillin</td>
<td>Net</td>
<td>10</td>
</tr>
<tr>
<td>Beta-lactams</td>
<td>Amoxycillin</td>
<td>Amx</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Ampicillin</td>
<td>Amp</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Carbenicillin</td>
<td>Cb</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Cephalothin</td>
<td>Cep</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>Ceftriaxone</td>
<td>Ctr</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>Ceftazidime</td>
<td>Caz</td>
<td>30</td>
</tr>
<tr>
<td>Fluoroquinolones</td>
<td>Ciprofloxacin</td>
<td>Cip</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Enrofloxacin</td>
<td>Ex</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Norfloxacin</td>
<td>Nx</td>
<td>10</td>
</tr>
<tr>
<td>Folate pathway inhibitors</td>
<td>Trimethoprim</td>
<td>Tr</td>
<td>5</td>
</tr>
<tr>
<td>Macrolides</td>
<td>Erythromycin</td>
<td>E</td>
<td>15</td>
</tr>
<tr>
<td>Nitrofurans</td>
<td>Furazolidone</td>
<td>Fr</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>Nitrofurantoin</td>
<td>Nit</td>
<td>100</td>
</tr>
<tr>
<td>Polymyxins</td>
<td>Colistin</td>
<td>Cl</td>
<td>10</td>
</tr>
<tr>
<td>Quinolones</td>
<td>Nalidixic acid</td>
<td>Na</td>
<td>30</td>
</tr>
<tr>
<td>Sulfonamides</td>
<td>Cotrimoxasole</td>
<td>Cot</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>Sulphamethoxazole</td>
<td>Sm</td>
<td>100</td>
</tr>
<tr>
<td>Tetracyclines</td>
<td>Doxycycline</td>
<td>Do</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>hydrochloride</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Oxytetracycline</td>
<td>O</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>Tetracycline</td>
<td>Te</td>
<td>30</td>
</tr>
<tr>
<td>Miscellaneous class</td>
<td>Chloramphenicol</td>
<td>C</td>
<td>30</td>
</tr>
</tbody>
</table>
Discs containing the following antibacterial agents were placed on the plates swabbed with enriched bacterial culture and incubated over-night at 37 °C. After incubation, the diameter of the zone of inhibition was measured and the results were interpreted based on recommendations of Clinical Laboratory Standards Institute (CLSI, 2012).

3.4.2 MAR indexing

Isolates that are resistant to 3 or more antibiotics were grouped as multiple antibiotic resistant. Multiple antibiotic resistance (MAR) indexing of the isolates was determined by calculating the ratio between the number of antibiotics to which an isolate is resistant and the total number of antibiotics to which the isolate was exposed (Krumperman, 1983).

3.4.3 Detection of beta-lactam antibiotic resistance genes

3.4.3.1 DNA isolation

Refer section 2.4.7.1

3.4.3.2 Detection of blaTEM gene

Primers used were forward 5’ GAGTATTCAACATTTTCGT 3’ and reverse 5’ ACCAATGCTTAATCAGTGA 3’ (Marynard et al., 2003). PCR amplification was optimized in a total reaction volume of 25 µl consisting of sterile Milli Q water (15.5 µl), 10X PCR buffer (2 µl), primer (1 µl each), dntpmix (1 µl, 200 mM), template (4 µl), and Taq DNA polymerase (0.5 µl). PCR condition included 1 cycle of initial denaturation for 5 min at 94 °C followed by 30 cycles of denaturation at 94 °C for 30 sec, annealing at 50 °C for 30 sec, extension at 72 °C for 1.5 min; 1 cycle of final extension for 5 min at 72 °C.
3.4.3.3 Detection of \textit{bla}_{CTX-M} gene

The primers used were CTX-M F (5′ CGATGTGCGATACCAGTAA 3′) and CTX-M R (5′ TTAGTGACCAGACAGGGC 3′) (Batchelor et al., 2005). PCR amplification was optimized in a total reaction volume of 25 µl consisting of sterile Milli Q water (15.5 µl), 10X PCR buffer (2 µl), primer (1 µl each), dntp mix (1 µl, 200 mM), template (4 µl), and Taq DNA polymerase (0.5 µl). PCR conditions included an initial denaturation at 94 °C for 5 min; 30 cycles of denaturation at 94 °C for 30 sec, annealing at 60 °C for 30 sec, extension at 72 °C for 1.5 min; final extension at 72 °C for 5 min.

3.4.3.4 Detection of \textit{bla}_{NDM-1} gene

The primers used in this study were NDM-Fm (5′-GGTGGCGTGCTGTCG-3′) and NDM-Rm (5′-CGGAATGGCCTCATCACGATC-3′, positions), which amplified an internal fragment of 621 bp of the \textit{bla}_{NDM-1} gene (Nordmann et al., 2011). PCR amplification was optimized in a total reaction volume of 25 µl consisting of sterile Milli Q water (15.5 µl), 10X PCR buffer (2 µl), primer (1 µl each), dntp mix (1 µl, 200 mM), template (4 µl), and Taq DNA polymerase (0.5 µl). PCR conditions included an initial denaturation for 10 min at 94 °C; 36 cycles of amplification consisting of 30 sec at 94 °C, 40 sec at 52 °C, and 50 sec at 72 °C; and 5 min at 72 °C for the final extension.

3.4.3.5 Gel documentation and image analysis

The PCR amplified products were separated by electrophoresis on agarose (1.5% w/v) gel in 1X TBE Buffer (Himedia, India) containing 0.5 µg/ml of ethidium bromide. The amplicon size was compared with a 100 bp DNA ladder. The gels were then visualized under UV
Chapter 3

transilluminator and recorded as tiff file by using Gel Documentation System (GelDoc EZ imager, Bio-Rad, USA).

3.4.4 Plasmid profiling of the drug resistant strains

The drug resistant strains were screened for the presence of plasmids. Bacterial strains were grown in 10 ml Luria Bertani broth (Himedia, India) containing 1% sodium chloride and ampicillin and incubated overnight at 37 °C in a shaker incubator (200 rpm) (Scigenics Biotech, India) for 16–18 h. About 1.5 ml of this culture was used for plasmid extraction following the alkaline lysis method (BirnBoim and Doly, 1979). Briefly, 1.5 ml of the culture was transferred into a microfuge tube and centrifuged at 6000 rpm for 5 min. Supernatant was removed and the pellet was re-suspended in 100 µl distilled water followed by 100 µl lysis buffer (10% SDS, 0.5 M EDTA, 10 N NAOH). The tubes were kept in boiling water bath for 10 min, then 50 µl of 1 mM MgCl₂ was added into it in the hot condition itself. Tubes were kept in ice for 2 min, and then centrifuged at 12000 rpm for 2 min. Then 3 mM potassium acetate was added and kept for 2 min in ice and then centrifuged at 12000 rpm for 2 min. The supernatant was transferred to a new tube and 600 µl of isopropanol was added into it and kept in ice for 10 min. Tubes were then centrifuged at 10000 rpm for 10 min. Supernatant was discarded and the pellet was rinsed in ice-cold 70% ethanol at 5000 rpm for 5 min and air-dried for about 10 min to allow the ethanol to evaporate. The pellet was re-suspended in 30 µl distilled water and kept at 4 °C overnight for dissolving.

The extracted plasmids were subjected to electrophoresis in 0.8% agarose gel (Agarose, Himedia, Mumbai, India) 1% (w/v) in 1X TBE
Buffer (HiMedia, India) containing 0.5 μg/ml of ethidium bromide. Electrophoretic separation was carried out at 75 V for 1 hour and a molecular weight marker (Supercoiled DNA ladder, HiMedia, India) was included. The gels were then visualized under UV transilluminator and recorded as tiff file by using Gel Documentation System, (GelDoc EZ imager, Bio-Rad). The obtained plasmid profiles were noted.

### 3.4.5 Plasmid curing experiment

Plasmid curing treatments were carried out using acridine orange (Molina-Aja et al., 2002). An overnight culture of plasmid containing resistant *Vibrio* strain (200 μl) was added into five different 5 ml cultures of LB broth supplemented with 0.1 mg mL$^{-1}$ of acridine orange and incubated at 35 °C for 24 h under constant agitation. Subsequently, the plasmid cured strains were tested for the antibiogram pattern, for the antibiotics to which they were originally resistant. The resistance was considered chromosomal DNA mediated when observed after the curing procedure; otherwise, it was characterized as mediated by plasmid.

### 3.5 Results

#### 3.5.1 Antibiotic resistance among *Vibrio*

##### 3.5.1.1 Antibiotic resistance among *Vibrio* from Cochin estuary

All the *Vibrio* strains from Cochin estuary were found to be multiple drug resistant. All the strains (100%) were resistant to amoxicillin, ampicillin, cephalothin (beta-lactam) and colistin (polymyxin). A high percentage of resistance was also observed towards furazolidone and nitrofurantoin-91.6% (nitrofurans), carbenicillin-70% (beta-lactam),

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*Genotyping, virulence characterization and survival kinetics of Vibrio spp. from food and environmental sources along the South west coast of India*
sulphamethoxazole-82.8% (sulphonamide) and enrofloxacin-84.2% (fluoroquinolone). A medium-to-low level resistance was exhibited towards ceftazidime (beta-lactam), erythromycin (macrolide), cotrimoxasole (sulphonamides), trimethoprim (folate pathway inhibitors), streptomycin, gentamicin and amikacin (aminoglycoside), doxycycline hydrochloride, oxytetracycline (tetracycline), nalidixic acid (quinolone) and norfloxacin (fluoroquinolone). All the strains were sensitive to netillin (aminoglycoside), ciprofloxacin (quinolone) and ceftriaxone (beta-lactam) (Figure 3.5).

Figure 3.5 Percentage of antibiotic resistance among Vibrio from Cochin estuary

3.5.1.2 Antibiotic resistance among Vibrio from shrimp farm

All the Vibrio strains from shrimp farm exhibited multiple drug resistance. All the strains were resistant to cephalothin (beta-lactam).
High level of resistance was observed towards amoxicillin (88%), ampicillin (78%) and colistin (72%) (Figure 3.6). All were sensitive to ciprofloxacin, cotrimoxasole, netillin and tetracycline. Medium to low level resistance was observed towards all other tested antibiotics.

Figure 3.6 Percentage of antibiotic resistance among *Vibrio* from shrimp farm

### 3.5.1.3 Antibiotic resistance among *Vibrio* from seafood

All the *Vibrio* strains isolated from seafood were multiple drug resistant. All the strains were resistant to enrofloxacin, furazolidone and trimethoprim. A majority also exhibited resistance to amoxicillin (86%), ampicillin (80%), cephalothin (90%) and colistin (93%). All were sensitive to cotrimoxasole, ceftriaxone, doxycycline hydrochloride, gentamycin, nalidixic acid, netillin, norfloxacin and sulphamethoxazole (Figure 3.7).
3.5.1.4 Relative antibiotic resistance among *Vibrio* isolated from Cochin estuary, shrimp farm and seafood

All the *Vibrio* strains from Cochin estuary, shrimp farm and seafood were multiple drug resistant. Figure 3.8 shows the relative antibiotic resistance among *Vibrio* from the three sources. Prevalence of antibiotic resistance was relatively high among the *Vibrio* isolates from Cochin estuary. Among the 25 different antibiotics tested, strains from Cochin estuary showed resistance towards 22 antibiotics. All the strains were sensitive towards netillin, ciprofloxacin and ceftriaxone. *Vibrio* strains from shrimp farm exhibited resistance towards 21 out of the 25 antibiotics tested and all exhibited sensitivity to ciprofloxacin, cotrimoxasole, netillin and tetracycline. Among the *Vibrio* from seafood, resistance was observed towards 17 out of the 25 antibiotics tested. All were sensitive to
cotrimoxasole, ceftriaxone, doxycycline hydrochloride, gentamycin, nalidixic acid, netilin, norfloxacin and sulphamethoxazole. Ciprofloxacin resistance was observed only among the *Vibrio* from seafood.

Figure 3.8 Relative antibiotic resistance among *Vibrio* isolated from Cochin estuary, shrimp farm and seafood

Resistance towards cotrimoxasole and tetracycline was exhibited by *Vibrio* from Cochin estuary alone. Similarly, ceftriaxone resistance was
observed only among *Vibrio* from seafood. Overall, *Vibrio* strains from the 3 sources showed significant difference in their resistance towards Amp (p<0.001), Amx (p<0.001), C (p<0.001), Caz (p<0.001), Cb (p<0.01), Cep (p<0.01), Cip (p<0.01), Cl (p<0.001), Ctr (p<0.001), Do (p<0.001), E (p<0.001), Ex (p<0.001), Fr (p<0.001), Na (p<0.001), Nit (p<0.001), O (p<0.01), Sm (p<0.001), S (p<0.001), Te (p<0.05) and Tr (p<0.001) (Appendix 3.1).

### 3.5.2 MAR indexing and antibiotic resistance pattern among *Vibrio*

The MAR index of *Vibrio* isolated from the three sources is shown in Tables 3.3 a, b, c. MAR index among *Vibrio* from Cochin estuary ranged from 0.24 to 0.6 and among the shrimp farm isolates it ranged from 0.16 to 0.48. The MAR index among *Vibrio* from seafood ranged from 0.16 to 0.44. The highest MAR index of 0.6 was observed among the *Vibrio* strains from Cochin estuary.

There was variation in the antibiotic resistance pattern among the *Vibrio* from estuary, shrimp farm and seafoods. The different antibiotic resistant patterns observed among the *Vibrio* are shown in Tables 3.3 a, b, c. A total of 34 different antibiotic resistance patterns were observed among the *Vibrio* strains from estuary. The most frequently observed pattern was Amp, Amx, Cb, Cep, Cl, Ex, Fr, Nit, Sm. A total of 35 different antibiotic resistance patterns were observed among the strains from shrimp farm. The most repeated pattern was Amp, Amx, Caz, Cb, Cep, E, Sm, Tr. Among the *Vibrio* from shrimp farm a total of 19 different antibiotic resistance patterns were observed and the pattern Amp, Amx, C, Cb, Cep, Cl, Ex, Fr, Nit, Tr was the most frequently observed.
Table 3.3(a)  MAR indexing and antibiotic resistant patterns of *Vibrio* strains from Cochin estuary

<table>
<thead>
<tr>
<th>MAR index</th>
<th>Antibiotic resistance pattern</th>
<th>No. of strains showing the pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.24</td>
<td>Amp, Amx, Cep, Cl, Fr, Sm</td>
<td>2</td>
</tr>
<tr>
<td>0.24</td>
<td>Amp, Amx, Cep, Cl, Nit, Sm</td>
<td>2</td>
</tr>
<tr>
<td>0.28</td>
<td>Amp, Amx, Caz, Cep, Cl, S, Sm</td>
<td>2</td>
</tr>
<tr>
<td>0.32</td>
<td>Amp, Amx, Cep, Cl, E, Ex, Fr, Nit</td>
<td>7</td>
</tr>
<tr>
<td>0.32</td>
<td>Amp, Amx, Cep, Cl, E, S, Sm</td>
<td>7</td>
</tr>
<tr>
<td>0.32</td>
<td>Amp, Amx, Cep, Cl, Ex, Fr, Nit, Sm</td>
<td>11</td>
</tr>
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<td>2</td>
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<td>Amp, Amx, Cep, Cl, Ex, Fr, Nit, O, Sm</td>
<td>7</td>
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Table 3.3 (b)  MAR indexing and antibiotic resistant patterns of *Vibrio* strains from shrimp farm

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<th>MAR index</th>
<th>Antibiotic resistance pattern</th>
<th>No. of strains showing the pattern</th>
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</thead>
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</tr>
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</tr>
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<td>Amp, Ak, Amx, Cep, E, Ex, Fr, Sm</td>
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Table 3.3(c) MAR indexing and antibiotic resistant patterns of *Vibrio* strains from seafood

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<th>MAR index</th>
<th>Antibiotic resistance pattern</th>
<th>No. of strains showing the pattern</th>
</tr>
</thead>
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</tr>
<tr>
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<td>Amp, Amx, Cb, Cep, Cl</td>
<td>1</td>
</tr>
<tr>
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<td>Amp, Amx, Ex, Cl, Cep, Nit</td>
<td>1</td>
</tr>
<tr>
<td>0.28</td>
<td>Amp, Amx, Cb, Cep, Cl, Ex, Nit</td>
<td>2</td>
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<tr>
<td>0.32</td>
<td>Amp, Amx, Cep, Cl, Ex, Fr, Nit, Tr</td>
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<td>Amp, Amx, Ak, Cep, Cl, Ex, Fr, Tr</td>
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<td>Amp, Amx, C, Caz, Cb, Cep, Cl, Ex, Fr, Tr, S</td>
<td>1</td>
</tr>
</tbody>
</table>

3.5.3 Distribution of antibiotic resistance genes in *Vibrio* from Cochin estuary, shrimp farm and seafood

All the *Vibrio* strains from Cochin estuary, shrimp pond and seafoods were further screened for the presence of antibiotic resistance genes. Since majority of the strains exhibited resistance towards beta-lactam antibiotics they were further screened for the presence of beta-lactam antibiotic resistance genes such as *bla*<sub>TEM</sub>, *bla*<sub>CTX</sub> and *bla*<sub>NDM-1</sub> genes.
Figure 3.9 shows the percentage distribution of antibiotic resistance genes in *Vibrio* from Cochin estuary, shrimp farm and seafood.

Figure 3.9 shows the percentage distribution of antibiotic resistance genes in *Vibrio* from three sources. All the *Vibrio* isolated from the three sources harboured the *bla*TEM gene. The *bla*CTX-M gene was present in 1.1% of strains from Cochin estuary. None of the strains from seafood and shrimp farm harboured *bla*CTX-M gene. New Delhi metallo-beta lactamase (*bla*NDM-1) gene was present in 13.3% of strains from Cochin estuary, 6.6% from seafood and 14.2% strains from shrimp farm. Figure 3.10 displays the agarose gel images of PCR amplified *bla*TEM, *bla*CTX-M and *bla*NDM-1 genes.
Figure 3.10  Agarose gel image showing PCR amplified (a) $ \text{bla}_{\text{TEM}} $ Lane M: 100 bp DNA ladder; lane 1: negative control; lanes 2-6 Vibrio strains (b) $ \text{bla}_{\text{CTX-M}} $ lane M: 100 bp DNA ladder; lane 1 negative control; lane 2 Vibrio strain (c) $ \text{bla}_{\text{NDM-1}} $ gene Lane M: 200 bp to 10 kb ladder; lane 1: negative control; lanes 2-8: Vibrio strains.

3.5.4 Plasmid profiles among Vibrio from Cochin estuary, shrimp farms and seafood

Plasmid profiling revealed the presence of plasmids in 58 Vibrio strains. The size of plasmids ranged from 0.5 to 33 kb. Plasmids were present in 30 strains (16.6%) from Cochin estuary, 23 strains (32.8%) from shrimp farm and 5 strains (16.6%) from seafoods. Plasmid of size 33 kb was the most frequently encountered.

Among the 30 Vibrio strains from Cochin estuary that carried plasmids, 23 of them harboured single plasmid of 33 kb in size, 6 strains had 2 plasmids, 4 strains had 3 plasmids and 3 strains revealed the presence of 4 plasmids. Among the 23 strains from shrimp farm, 21
harboured a single plasmid of 33 kb and 2 had 2 plasmids each. Among the 5 strains from seafood all harboured a single plasmid of 33 kb. The plasmid profiles of the strains are given in Table 3.4.

Table 3.4 Table showing the plasmid profiles observed among the *Vibrio* strains from Cochin estuary, shrimp farms and seafood

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<tr>
<th>Isolates from Cochin estuary</th>
<th>Plasmid size in kb</th>
<th>Isolates from Shrimp farm</th>
<th>Plasmid size in kb</th>
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<tbody>
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<td>FSV5</td>
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<td>33, 2, 1.5</td>
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<td>DWA218</td>
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<td>WV151</td>
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<td>MWV3</td>
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<tr>
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<td>33</td>
<td>MSA3</td>
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<td>ASA17</td>
<td>33, 0.75</td>
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<td>33</td>
<td>Isolates from seafood</td>
<td>Plasmid size in kb</td>
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<td>M37</td>
<td>33</td>
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<td>M10W1</td>
<td>33</td>
<td>M91</td>
<td>33</td>
</tr>
<tr>
<td>PM 1S5</td>
<td>5, 1, 2, 3</td>
<td>M10</td>
<td>33</td>
</tr>
<tr>
<td>1W3</td>
<td>7, 8.9</td>
<td>M4</td>
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<td>33, 15</td>
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<td></td>
</tr>
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</table>
3.5.5 Plasmid curing of Vibrio from Cochin estuary, shrimp farms and seafood

All the plasmid harbouring strains were further subjected to plasmid curing experiments for detection of plasmid mediated antibiotic resistance. Among the 30 Vibrio strains from Cochin estuary 24 revealed the presence of plasmid mediated antibiotic resistance (Table 3.5). Among the Vibrio from shrimp farm 13 and all the 5 isolates from seafood exhibited plasmid mediated antibiotic resistance (Table 3.6, 3.7). Plasmid mediated resistance was shown towards 13 antibiotics (Ak, Amp, Amx, Caz, Cb, Cl, E, Ex, Fr, Nit, S, Sm, Tr). Resistance to carbenicillin was the most frequently lost phenotype after plasmid curing. Eight Vibrio isolates from Cochin estuary, 6 from shrimp farm and 1 from seafood lost their carbenicillin resistance after curing treatment. Resistance towards ceftazidime was plasmid mediated in 4 isolates from Cochin estuary, 3 from shrimp pond and 1 from seafood. Plasmid mediated nitrofurantoin resistance was observed in 3 isolates from Cochin estuary, 5 from shrimp farm and 1 from seafood. Sulphamethoxasole resistance was plasmid mediated in 9 isolates from Cochin estuary and 3 from shrimp farm. Amoxycillin resistance was plasmid borne in 1 isolate from Cochin estuary, 6 from shrimp farm and 3 from seafood. Plasmid mediated ampicillin resistance was observed among 2 isolates from Cochin estuary and one from shrimp pond. Two isolates from Cochin estuary exhibited plasmid mediated erythromycin resistance. One isolate each from Cochin estuary showed plasmid borne resistance to enrofloxacin, streptomycin, amikacin, furazolidone and trimethoprim. Similarly one isolate from shrimp pond had plasmid mediated colistin resistance.
Table 3.5 Changes in antibiotic resistance pattern (ARP) after plasmid curing in *Vibrio* from Cochin estuary

<table>
<thead>
<tr>
<th>Strain</th>
<th>ARP before curing</th>
<th>ARP after curing</th>
<th>Plasmid size</th>
<th>Plasmid mediated resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>M10S2 (CONTROL)</td>
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<td>Amp, Amx, Cep, Cl, E, Ex, Sm, Nit</td>
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<td>Cb</td>
</tr>
<tr>
<td>PM1S2</td>
<td>Amp, Amx, Caz, Cb, Cep, Cl, Ex, Nit, Sm</td>
<td>Amp, Amx, Cep, Cl, Ex, Nit, Sm</td>
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<tr>
<td>1W5</td>
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<td>Amp, Amx, Cep, Cl, Ex, Nit, O, Tr</td>
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<td>33, 8.9</td>
<td>Cb</td>
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<tr>
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<td>Amx, Cep, Cl, Ex, Nit, Sm</td>
<td>33, 2, 1.5</td>
<td>Cb</td>
</tr>
<tr>
<td>2S5</td>
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<td>Amx, Amp, C, Cep, Cl, Fr, Sm</td>
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<td>Cb</td>
</tr>
<tr>
<td>M9W1</td>
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<td>Amp, Cb, Cep, Cl, Ex, Fr, Nit, Sm</td>
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<tr>
<td>PM 1S1</td>
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Table 3.5 Continued ….
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<th>Common Resistance Profile</th>
<th>Size (kb)</th>
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<td>E, Nit, Sm</td>
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<tr>
<td>PM1W3</td>
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<td>Amx, Amx, Ak, Cep, Cl, Ex, Fr, Nit, S, Tr</td>
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<td>Sm</td>
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<tr>
<td>1S3</td>
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<td>Amx, Amp, Cb, Cep, Cl, Ex, Fr, Nit, Sm</td>
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<td>Ak</td>
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<td>Nit, Sm</td>
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<td>1W3</td>
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Table 3.6 Changes in antibiotic resistance pattern (ARP) after plasmid curing in *Vibrio* from shrimp farm

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<th>Strain</th>
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<th>ARP after curing</th>
<th>Plasmid size</th>
<th>Plasmid mediated resistance</th>
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<td>Amp, Caz, Cep, Sm</td>
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Table 3.7 Changes in antibiotic resistance pattern (ARP) after plasmid curing in Vibrio from seafood

<table>
<thead>
<tr>
<th>Strain</th>
<th>ARP before curing</th>
<th>ARP after curing</th>
<th>Plasmid size</th>
<th>Plasmid mediated resistance</th>
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</thead>
<tbody>
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3.6 Discussion

3.6.1 Antibiotic resistance of Vibrio from Cochin estuary, shrimp farm and seafood

Vibrios are generally considered to be highly susceptible to most clinically used antimicrobials (Oliver, 2006). However, in the past few decades, antimicrobial resistance has emerged and evolved in many bacterial genera due to the excessive use of antimicrobials in human, agriculture and aquaculture systems (Mazel and Davies, 1999; Cabello, 2006). This emerging issue has gained great concern due to increased resistance of pathogenic V. parahaemolyticus towards clinically used antibiotics. Recently, higher frequency of drug-resistant Vibrio has been reported (Okoh and Igbinosa, 2010; Hua and Apun, 2013).

The presence of multiple antibiotic resistance among environmental microorganisms may be due to long-term exposure to antibiotic containing
effluents discharged from hospitals, agriculture and aquaculture farms or through horizontal transfer of antibiotic resistant genes from human pathogens (Martinez, 2012). It was alarming to note that in our study all the *Vibrio* strains from Cochin estuary, shrimp farm and seafood have acquired multiple antibiotic resistance. However, strains from the three sources varied widely in their resistance pattern. This could be due to the difference in the type and amount of antibiotic residues to which the isolates were exposed. As pointed out by Hsu *et al.* (1992) differences in percentage of bacterial resistance to various antibiotics may reflect the history of antibiotic application and hence there is a possibility of using bacterial drug resistance as an indicator of antibiotic application.

High percentage of β-lactam resistance was exhibited by our isolates. Resistance towards β- lactam antibiotics have been previously reported in *V. parahaemolyticus* and other vibrios from different sources (Molina-Aja *et al.*, 2002; Manjusha *et al.*, 2005; Devi *et al.*, 2009). In a previous study sensitivity of *V. parahaemolyticus* from the south west coast towards nitrofurantoin and trimethoprim has been reported (Devi *et al.*, 2009). Similarly, in another study from Tunisia, more than 70% of isolates of *Vibrio* showed susceptibility to trimethoprim-sulfamethoxazole (Lajnef *et al.*, 2012). In the present study, isolates from Cochin estuary and shrimp farm demonstrated resistance towards sulphamethoxazole and low level resistance towards trimethoprim. While all the *Vibrio* strains isolated from seafoods were resistant towards trimethoprim and none of them were resistant to sulphamethoxazole. Resistance towards enrofloxacin was shown by all the strains from seafood and 85% strains from Cochin estuary, while only 14% of strains from shrimp farm had acquired
Prevalence of Antibiotic Resistance and Plasmid Profiles of Vibrio From Food and...

resistance towards it. All the strains from shrimp farm were sensitive towards gentamicin whereas strains from other sources exhibited low level resistance towards both the drugs.

A recent study on antibiotic resistance of Vibrio spp. isolated from Palk Bay revealed widespread distribution of multidrug resistant Vibrio across the Palk Bay (Sneha et al., 2016). In their study, the Vibrio isolates exhibited resistance towards beta-lactams, vancomycin, nitrofurantoin, gentamicin, azithromycin, oxytetracycline, tetracycline and chloramphenicol. Antibiotics like chloramphenicol, ampicillin, tetracycline, chlortetracycline, nalidixic acid, gentamycin, sulfafurazole and trimethoprim are commonly used in aquaculture farms to ensure continuous production of seafood (Roque et al., 2001; Manjusha and Sarita, 2011; Yano et al., 2014). In addition, treatment recommendations for Vibrio infections include cephalothin, cefuroxime, cefotaxime, ceftazidime, tetracycline, doxycycline, fluoroquinolone, amikacin, gentamicin and trimethoprim-sulfamethoxazole (Daniels and Shafaie, 2000; CDC, 2013; Letchumanan et al., 2015a). In the present study, most of the Vibrio strains have attained resistance towards life saving drugs such as cephalothin, ceftazidime, doxycycline, enrofloxacin, nitrofurantoin, trimethoprim, sulphamethoxasole, streptomycin, amikacin and nalidixic acid. Treatment of infections caused by such Vibrio has major clinical implications as this may lead to therapeutic failure and finally death of the patient. Even though our findings are of local in nature, it may cause global consequences, if these antibiotic resistant strains are transported through ballast water, seafoods and ocean water currents to other parts of the world (Ruiz et al., 2000; Ge et al., 2010, 2012).
All the isolates from Cochin estuary and majority from other two sources showed a MAR index higher than 0.2, and it ranged up to 0.6. MAR indices higher than 0.2 are often considered to have originated from higher-risk sources (Krumperman, 1983) of contamination, such as those from hospital sewage, commercial poultry farm waste etc., that somehow find their way to the open sea via illegal dumping of waste or transferred by infected humans. The study area is a famous tourist hot spot and shrimps grown in this area are exported to various countries, making the findings of our study all the more important. Therefore, continued monitoring of both the prevalence and the antimicrobial susceptibility profile of Vibrio is important to better ensure seafood and public health safety from our study area.

### 3.6.2 Distribution of antibiotic resistance genes in Vibrio from Cochin estuary, shrimp farm and seafood

The $bla_{TEM}$ gene was present in all the Vibrio strains from Cochin estuary, shrimp farm and seafood. This gene encodes resistance to penicillins. The $bla_{CTX-M}$ gene was found only in a few Vibrio strains isolated from Cochin estuary. The gene encodes for extended spectrum β-lactamases conferring resistance to extended spectrum beta-lactam antibiotics (cephalosporins, penicillin and monobactams). Reports suggest antibiotic resistance genes are very diverse (Letchumanan et al., 2015a). Currently, there are forty β-lactamases that encodes for plasmid-mediated CTX-M enzymes alone (Tzouvelekis et al., 2000). Therefore, the absence of $bla_{CTX-M}$ in beta-lactam resistant Vibrio strains could be due to presence of other encoding genes in isolates in the present study. Their resistance may possibly be mediated by the efflux systems also.
In a previous study it was found that the resistance of *V. parahaemolyticus* towards ampicillin was not mediated by the *bla* gene instead it was conferred by an efflux system (Pazhani *et al.*, 2014). In a recent study on prevalence of β-lactamase genes in clinical *E. coli* and *K. pneumoniae* isolates from Northeast India, *bla*$_{CTX-M}$ was the most prevalent gene in *E. coli* and *bla*$_{TEM}$ in *K. pneumonia* (Bora *et al.*, 2014). In a previous report, Enterobacterial strains were isolated during 2000-2003 from different countries like France, India, Poland and Turkey (Lartigue *et al.*, 2004). The strains produced emerging CTX-M-type extended-spectrum-β-lactamases and among the genes *bla*$_{CTX-M-15}$ was the most frequently observed gene. The New Delhi metallo-β-lactamase (NDM-1) gene, *bla*$_{NDM-1}$ was detected in 13.3% of strains from Cochin estuary, 14.2% of strains from shrimp farm and 6.6% of strains from seafoods. This is indeed an alarming situation since strains harbouring *bla*$_{NDM-1}$ is resistant to carbapenems, extended spectrum cephalosporins, penicillin and monobactams. Carbapenems are the last drug of choice for infections caused by extended spectrum β-lactamase producing bacteria.

In a previous study on antibiotic resistance in *Vibrio* isolated from Palk Bay, *bla*$_{NDM-1}$ gene was not detected in any of the isolates studied (Sneha *et al.*, 2015). *Vibrio fluvialis* harbouring *bla*$_{NDM-1}$ gene was isolated from acute diarrhoea patients in Kolkata, India (Chowdhury *et al.*, 2016).

The *Vibrio* strains from our study areas may act as potential reservoirs of drug resistance genes in the environment. Resistance genes can be further transferred from non-pathogens to pathogens through horizontal gene transfer *via* conjugation, transduction and transformation.
3.6.3 Plasmid profiling and plasmid curing of antibiotic resistant vibrios from Cochin estuary, shrimp farm and seafood

The plasmid is known to be one of the most important mediators facilitating the fast spread of antibiotic resistance among bacteria (Dale and Park, 2004). Plasmids were present in 30 strains (16.6%) from Cochin estuary, 23 strains (32.8%) from shrimp farm and 5 strains (16.6%) from seafoods. Plasmid of size 33 kb was the most frequently observed profile from all the environments; this is similar to the finding by Zhang et al. (2006), stating the presence of >30 kb plasmids in environmental Vibrio isolates. Bacterial antibiotic resistance patterns are known to sometimes be associated with the presence of large plasmids, as well as the abilities of plasmids in conjugation. Transferable R plasmids are usually as big as 30 kb, and the indispensable components of a conjugative plasmid make it big in size compared to other plasmids (Guiney and Landa, 1989).

In our study, when we compared the antibiotic resistance patterns and the plasmid profiles, we could not find any correlation. Even among the strains with same resistance pattern, the plasmid profiles were different and some strains even lacked plasmids, which was similar to findings by Lajnef et al. (2012). So, in some strains resistance may be plasmid coded, and in some it may be chromosomally borne. Plasmid profiles have been previously studied in Vibrio species such as V. parahaemolyticus (Devi et al., 2009), V. ordalii (Tiainen et al., 1995), V. vulnificus (Radu et al., 1998) and V. salmonicida (Sorum et al., 1990), and most extensively in V. anguillarum (Pedersen et al., 1996, 1999), where a high diversity of profiles have been observed. In a study on
Vibrio spp. isolated from tropical waters of Malaysia 32 different plasmid profiles with size ranging from 2.2 to 24.8 kb were detected among the resistant isolates (You et al., 2016). In the present study, the size of plasmids ranged from 0.5 to 33 kb. A high incidence of plasmids in Vibrio spp. of both polluted and pristine environments may be ecologically important to the survival of these bacteria in the environment (Zhang et al., 2006).

Acquired antibiotic resistance in bacteria is generally plasmid mediated and is easily transferred to other bacteria in the environment through horizontal/vertical gene transfer mechanisms (Manjusha and Sarita, 2011). The extrachromosomal DNA may be responsible for the rapid emergence of multiple antibiotic resistance in bacteria (Schelz et al., 2006). In order to ascertain the antibiotic resistance mediation, plasmid curing experiments are performed. In the present study, acridine orange was used as curing agent to eliminate plasmids from our Vibrio strains. Plasmid mediated resistance was observed towards 13 antibiotics (Ak, Amp, Amx, Caz, Cb, Cl, E, Ex, Fr, Nit, S, Sm, Tr). Plasmid mediated carbenicillin resistance was the most frequently observed phenotype in our study. In a previous study by Reboucas et al. (2011), plasmid mediated oxytetracycline resistance was the frequently observed profile in Vibrio species isolated from marine shrimp. Similarly, in a study on Vibrio from shrimp farm in Thailand, oxytetracycline resistance was eliminated through plasmid curing using ethidium bromide (Yano et al., 2014). In another study on vibrios isolated from coastal waters of Kerala, chromosomal borne resistance was observed towards amoxicillin, ampicillin, furazolidone and tetracycline after plasmid curing with 0.05 to
0.5 mg/ml of ethidium bromide (Manjusha and Sarita, 2011). The presence of antibiotic resistance genes in the bacterial plasmid may lead to rapid dissemination of drug resistance among pathogenic strains in our environment.