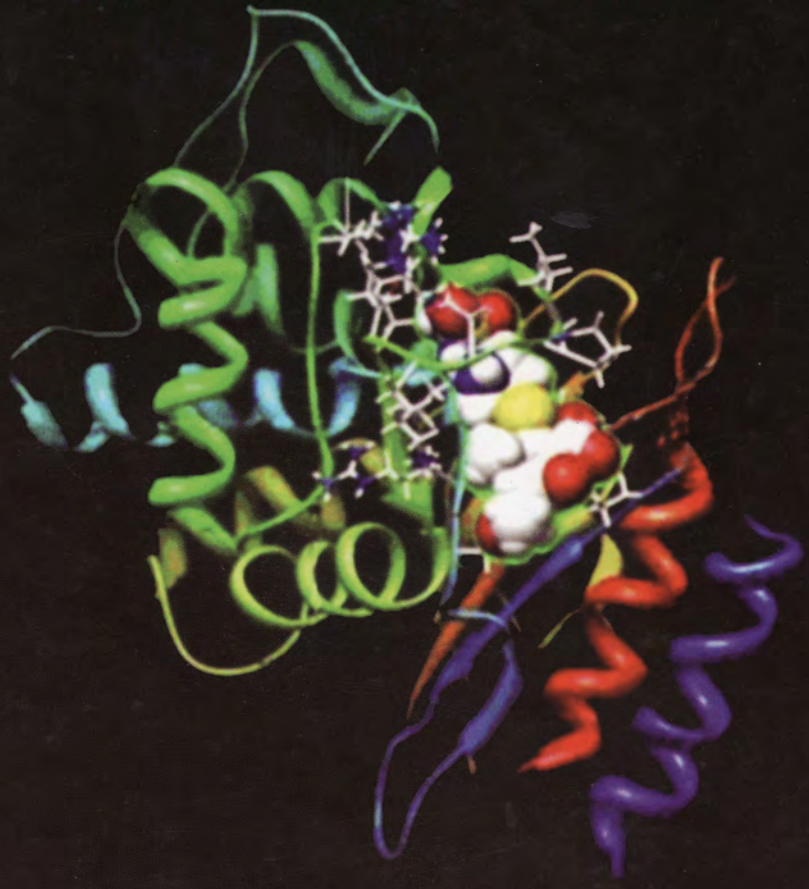

Current Trends in Antibiotic Resistance in Infectious Diseases

Editor
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CONTENTS

Chapter 5

Drug Resistance in Marine Bacteria

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ABSTRACT

The emergence of antibiotic resistance among marine bacteria has both a direct and an indirect impact on human and animal health. An important part of the dispersal and evolution of antibiotic resistant microorganisms depends on and occurs in the water environments. In marine water, bacteria from different origins (human, animal and environmental) are able to mix and resistance consequently evolves as a result of unwanted yet spontaneous exchange and shuffling of genes, genetic platforms and genetic vectors. At the same time antibiotics, disinfectants, pesticides and heavy metals are released in marine environment and may ultimately exert selection pressures, as well as ecological damage in water communities, eventually resulting in increased antibiotic resistance. Preventative and management methods aimed at reducing resistant bacterial load in wastewaters, and the sheer amounts of antimicrobial agents originating, in most cases from hospitals and farms, include optimization of disinfection procedures and management of wastewater and manure. A policy for preventing mixing of bacteria originating from human and animal sources with environmental organisms seems advisable. This chapter focuses on mechanisms of antibiotic resistance, research on antibiotic resistance in marine bacteria and routs of transmission of marine antibiotic resistant pathogens to human.

1. INTRODUCTION

Marine environment occupies 71% of the earth surface with rich biodiversity, starting from the largest whales to microscopic life forms. Among the biota in marine environment, bacteria play an important role in the dynamic functions of the ecosystem.

Bacteria are the huddled masses of the microbial world, performing tasks that include everything from causing disease to fixing nitrogen. A recent estimate suggests that the sea may support 2 million different bacterial species (Curtis *et al.*, 2002) among the estimated number only 0.01 to 0.1% of marine bacteria are culturable. The relationship between humans and the marine environment is intractable and well established since the distant past through dependence on transportation, exploration of living and non-living resources and waste disposal. The impact of the anthropogenic activity on the marine environment causes many adverse effects on marine life such as loss of biodiversity, pollution and impairment of water quality. One such impact is the development of antibiotic resistance among marine bacteria. The incidence of antibiotic-resistant bacteria in aquatic environments has increased dramatically as a consequence of the widespread use of antibiotics by humans. This increase is a direct result of a variety of factors, perhaps the most important of which is the evolutionary selection for resistant strains due to the continued exposure to antibiotics and the ability of such strains to exchange plasmids encoding resistance. The high incidence of antibiotic resistant bacteria has been reported and documented particularly in chronically polluted waters around the world (Smith *et al.*, 1974; Husevag *et al.*, 1991; Sandaa 1993 and Thavasi *et al.*, 2007).

2. MECHANISM OF ANTIBIOTIC RESISTANCE

There are four main mechanisms by which microbes exhibit resistance to antimicrobials as follows:

1. Inactivation or modification of drug: e.g. enzymatic deactivation of Penicillin G in some penicillin-resistant bacteria through the production of β-lactamases.
2. Alteration of target site: e.g. alteration of penicillin-binding proteins (PBP) the binding target site of penicillin's in Methicillin resistant *Staphylococcus aureus* (MRSA) and other penicillin resistant bacteria.
3. Alteration of metabolic pathway: e.g. some sulphonamide-resistant bacteria do not require para-aminobenzoic acid (PABA), an important precursor for the synthesis of folic acid and nucleic acids in bacteria inhibited by sulphonamides. Instead, like mammalian cells, they turn to utilizing preformed folic acid.
4. Reduced drug accumulation: by decreasing drug permeability and/or increasing active efflux (pumping out) of the drugs across the cell surface.

3. THE ORIGIN OF ANTIBIOTIC RESISTANCE AMONG MARINE BACTERIA

When penicillin became widely available after the Second World War, it was considered as a medical miracle, rapidly vanquishing the biggest wartime killer, infected wounds. Discovered initially by a French medical student, Ernest Duchesne, in 1896, and then

rediscovered by Scottish physician Alexander Fleming in 1928, the product of the soil mould *Penicillium* inhibited many types of disease-causing bacteria. But just four years after drug companies began mass production of penicillin in 1943; microbes that could resist it began appearing. *Staphylococcus aureus* was the first bacteria to resist penicillin. This bacterium is often a harmless passenger in the human body, but it can cause illness, such as pneumonia or toxic shock syndrome, when it overgrows or reproduce unchecked. The antibiotic resistance mechanism in marine bacteria may evolve through the transformation of genetic material from resistant strains to wild strains. In addition, influence of anthropogenic activity in the coastal region like, sewage input from urban, hospitals and pharmaceutical industries are the main source for antibiotics. Along with antibiotic residues the sewage and land runoff also bring terrestrial resistant bacteria into the coastal waters. It has been reported that 90% of the bacterial population in the coastal waters exist in the terrigenous zone, which contribute the major part in transformation of genetic materials to the marine bacteria.

It has been demonstrated that antibiotic-resistant bacteria from effluents and land runoff eventually enters marine receiving waters (Feary *et al.*, 1972; Smith 1970, 1971; Smith *et al.*, 1974). Intensive fish farming has resulted in massive use of antibacterial agents for treatment and control of fish diseases. Therefore, increased resistance problems have arisen as a consequence of the often uncritical use of antibacterial agents (Husevag *et al.*, 1991; Sandaa, 1993). The fish do not utilize all the antibiotics provided; some are released into the water and sediments as uneaten fish feed or excreted in fish faeces. In addition, a large number of bacteria and actinomycetes occurring in aquatic ecosystems are capable of synthesizing compounds of antibiotic nature (Lemos *et al.*, 1985) which can also contribute to the development of antibiotic resistance. These inhibitory substances are 2-15 kDa large molecules and their concentration in water could be as high as $1\mu g/cm^3$. Bacteria slowly but steadily synthesise and secrete into water a number of antibiotic substances, such as phenazines, pyrrolnitrin, bacteriocins, glycolipids and bromopyrrolic compounds (Lemos *et al.*, 1991 and Dakhama *et al.*, 1993). All of these substances have antimicrobial activity and can inhibit bacterial respiration and biosynthesis of cellular structures (Jensen, 1984 and Baron *et al.*, 1989). A lot of algae, mainly *Chlorophycae, Rhodophycae* and *Phyophyceae* also produce such substances with antimicrobial antibiotic activities, which inhibit the growth of bacteria (Klein and Alexander, 1986). All these sources lead to the development of antibiotic resistance in the natural environment.

Although specific evidence for the origin of multiple drug resistance R plasmids is not available, a number of lines of circumstantial evidence suggest that R plasmids existed before the antibiotic era. The widespread use of antibiotics provided selective conditions for the spread of the R plasmids with one or more antibiotic resistance genes. For example, a strain of *E. coli* that was freeze dried in 1946 contained a plasmid with genes conferring resistance to tetracycline and streptomycin, when these antibiotics were not discovered. Later, widespread medical and agricultural use of antibiotics provided selective conditions for the rapid spread of these R plasmids. R plasmids are thus a predictable outcome of natural selection. They pose significant limits for the long-term use of any single antibiotic as an effective chemotherapeutic agent.

In some cases strains isolated for heavy metal and pesticide resistance have demonstrated antibiotic resistance against 12 antibiotics (unpublished data by Thavasi *et al.*). This evidence has supported the assertion regarding the existence of antibiotic resistance before the discovery of antibiotics, but when the pesticides and heavy metals were in use. This point clearly suggests that heavy metal and pesticide pollution may contribute to increased antibiotic resistance through indirect selection.

Thus, the plasmid mediated resistance in heterotrophic populations suggests a free exchange (or) transfer of resistance genes between the sewage fed pathogens and the normal microflora in the ecosystem. Apart from this rout, antibiotic producing microorganisms are naturally present in marine environment and many indigenous bacteria may evolve becoming resistant strains as a protection mechanism against these antibiotics. The antibiotic producing organism themselves also use antibiotic-resistance strategies, including inactivation of antibiotics, alteration or replacement of target as a protection mechanisms from their own or similar antimicrobial compounds (Cundiffe, 1989). These reports revealed that, plasmid mediated multiple antibiotic resistance is common in the normal microflora in marine environments since a long time.

4. MECHANISM OF ANTIBIOTIC RESISTANCE GENE MOBILITY AMONG MARINE BACTERIA

There are three main antibiotic resistance gene mobility mechanisms proposed as follows:

4.1. R Plasmids

Since the first R plasmid was detected in the 1950s, plasmids carrying drug resistance genes have been detected in most bacterial groups, indicating a large pool of R plasmids among antibiotic-resistant bacteria. However, as the number of characterised R plasmids increases, it seems that often the new ones are variants of previously described plasmids. It actually may be that the numbers of different R plasmids are relatively limited, but the number of variants of each R plasmid may increase concurrent with the use of antibiotics in various ecological niches. One example in support of this hypothesis is the occurrence of IncU plasmids with varying resistance region components but with an identical plasmid backbone structure. They have been detected in *Aeromonas bacteria* causing fish disease, in environmental aeromonads and in enterobacteria from human clinical cases (Rhodes *et al.*, 2000; L'Abe´e-Lund and Sørum, 2001). R plasmids have also been isolated from normal flora bacteria of healthy food-producing animals (Sunde and Sørum, 2001).

4.2. Transposons

Transposons are genetic elements that are able to move between various genetic structures intracellularly or between bacterial cells (conjugative transposons) (Bennett,

2000). Transposons can simply be made up of two identical insertion elements flanking a functional gene or genes, for instance drug resistance genes. They move by the action of the transposase encoded by the two flanking IS-elements that move the whole structure from one DNA site to another. Other complex transposons, consists of elements where the transposase/resolvase is encoded together with other genes as drug resistance genes within a structure flanked by short inverted repeats. Among the most well-known transposons are the staphylococcal transposons with terminal copies of IS257. IS257 is related to several DNA reorganisations that are found associated with various antibiotic resistance genes (Rouch and Skurray, 1989; Yazdankhah *et al.*, 2000). These transposons show a high degree of sequence homology and may be responsible for transfer of the strA–strB genes between environments as diverse as orchard soil and marine water, and may be the source of strA–strB in the intestinal flora of man and animals (Sundin and Bender, 1996).

4.3. Integrons

An integron is the genetic structure consisting of a gene encoding a site-specific integrase and a recombination site for insertion of gene cassettes (Hall, 1997; Hansson *et al.*, 1997). Class 1 integrons are important in relation to mobilization of antibiotic resistance. More than 70 versions of class 1 integrons have been found in bacteria from all environments where antibiotics are used or where antibiotics are found. In environments with no known exposure to antibiotics, there seem to be other classes of integrons that theoretically may be ready to take part in the development of more variants of resistance integrons as soon as antibiotics reach those ecological niches (Nield *et al.*, 2001).

5. RESEARCH ON ANTIBIOTIC RESISTANCE IN MARINE BACTERIA

Multiple antibiotic resistant bacteria have been isolated from marine air-water interfaces (Feary *et al.*, 1972; Smith, 1970, 1971; Smith *et al.*, 1974; Hermannson *et al.*, 1987 and Thavasi *et al.*, 2007). Antibiotic resistance mediated by plasmids has also been reported to occur in marine environments (Thavasi *et al.*, 2007). Although many marine bacteria are capable of receiving plasmids (Genthner *et al.*, 1988), most studies on conjugal gene transfer in aquatic environments have been carried out in freshwater rivers and lakes (O'Morchoe *et al.*, 1988). The potential for conjugal gene transfer in wastewaters has been especially thoroughly investigated (Mancini *et al.*, 1987). For methodological reasons, most studies of conjugal plasmid transfer in marine environments have been performed by adding donors and recipients to sterilized sea water (Goodman *et al.*, 1993) or sediments (Breittmyer *et al.*, 1990). The organism most frequently used as a donor, recipient or both is *Escherichia coli*. To our knowledge, all previous investigators have used constructed plasmids or plasmids isolated from non marine environments. Such deviations from natural marine conditions make it difficult to predict to what extent such processes take place in nature.

Antibiotic resistance in microorganisms may be associated with reduced penetration of the antibiotic into the cell or can be a result of active processes such as changes in the transport of those compounds into or from the microbial cells (Hermansson *et al.*, 1987). Bacterial resistance to antibiotics is located in plasmids of 1-30 megadaltons molecular weight (Kobori *et al.*, 1984). Genes assembled in plasmids protect bacterial populations against antibiotics. It is the R plasmid that plays a substantial role in bacterial resistance to antibiotics (Silva and Hofer, 1995). The R plasmid can be transferred between various strains of bacteria through the processes of conjugation and transformation (Herwig *et al.*, 1997). There are four classic mechanisms of resistance specified by plasmids: inactivation, impermeability, bypasses and altered target site; all of which occur in aquatic environments. Also, intracellular binding seems to be a valid mechanism for immobilising an inhibitor (Foster, 1983). Resistance can also be associated with the production of enzymes that modify and inactivate antibiotics (Koch, 1981). According to Hermansson *et al.* (1987) some strains of antibiotics resistant bacteria do not contain any plasmids. In such a case resistance to antibiotics depends on the mobile genetic elements, called transposons (Herwig *et al.*, 1997).

A study conducted by Thavasi *et al.* (2007) in an estuarine environment indicated that, the total heterotrophic bacterial population contains 60-80% and 66-83% of multiple antibiotic resistant strains in water and sediment samples respectively (Table 1). Among the strains isolated, *Nesseria mucosa* showed resistance against 13 out of 20 antibiotics used with an MAR index of 0.65 (Table 2). Many *Nesseria* sp. are known for their pathogenic tendencies. Incidence of this pathogenic bacterium in the coastal water confirms the entry of terrigeneous multiple antibiotic resistance (MRA) strains into the marine system and their contribution to the spread of antibiotic resistance among other marine bacteria. To confirm the transformation, we carried out transformation of plasmids to both plasmid cured and wild strains, and we detected the expression of MAR in the transformed strains. These results revealed that MAR genes can be transported through plasmids.

Table 1: THB and multiple antibiotic resistant strains

Month	Sample	THB* CFU/ml/g	MAR strains CFU/ml/g
October	Water	6.5×10^5	4.3×10^3
	Sediment	7.9×10^6	3.2×10^4
November	Water	8.3×10^5	5.6×10^4
	Sediment	8.8×10^6	6.3×10^5
December	Water	9.4×10^5	8.0×10^4
	Sediment	9.1×10^6	7.2×10^5

*CFU-Colony Forming Units, CFU/ml-in water, CFU/g-in sediment,
THB-Total Heterotrophic Bacteria,

Table 2: Multiple antibiotic resistance index

Strain name	*MAR index
Bacillus megaterium	0.3
B. subtilis	0.25
Branhamella catarrhalis	0.6
Citrobacter intermedius	0.3
Enterobacter aerogenes	0.25
Klebsilla ozaenae	0.35
Micrococcus luteus	0.25
Neisseria mucosa	0.65
N. sicca	0.5
Pseudomonas fluorescencea	0.5

*Multiple Antibiotic Resistant

Formula for calculating MAR index (Krumperman, 1983)

$$\text{MAR index} = \frac{\text{Number of antibiotics to which the isolate is resistant}}{\text{Total number of antibiotics tested}}$$

6. TRANSMISSION OF PATHOGENIC ANTIBIOTIC RESISTANT MARINE BACTERIA TO HUMANS

Transmission of pathogens to humans through marine environments most frequently occurs though the following activities:

6.1. Eating of contaminated seafood
6.2. Direct contact with sea water
6.3. Exposure to marine aerosols
6.4. Exposure to zoonoses

6.1. Seafood

The most important route of infection by marine pathogens is by consumption of contaminated seafood resulting in symptoms from self-limiting gastroenteritis (typical seafood poisoning) to invasive infections that are potentially fatal. The frequency of antibiotic resistance in potentially pathogenic halophilic vibrios isolated from Italian seafood was found to be relatively low (Ottaviani *et al.*, 2001). In Taiwan and several other Asian countries, *Vibrio parahaemolyticus* is an important food-borne pathogen. In a study of 371 isolates from patients who suffered food-borne illness, about 10% of the isolates were resistant to seven or more antibiotics (Wong *et al.*, 2000). These studies indicate that seafood may be a source of food-acquired antibiotic resistant bacteria in the human consumer.

Filter-feeding shellfish such as scallops and oysters tend to concentrate bacteria from overlying waters. Experimentally contaminated oysters have been shown to retain *Salmonella typhimurium* for up to 49 days (Janusen, 1974). As shellfish are often consumed

raw or after minimal cooking, they may serve as a vehicle for transmission of antibiotic-resistant bacteria carrying R factors to humans. In many countries, the bacteriological quality of edible shellfish is based upon total and faecal coliform counts, but little work has been done to determine the incidence of antibiotic resistance among coliform bacteria from such sources.

6.2. Sea Water Contact

Antibiotic resistant pathogens can be transmitted to humans through sea water during accidental ingestion, inhalation or by direct exposure of ears, eyes, nose or wounded soft tissue. Although sewage contamination has long been recognized as a significant risk factor in acquiring illnesses after sea water exposure, sewage-borne pathogens are primarily viral rather than bacterial in origin (Cabelli *et al.*, 1982; Griffin *et al.*, 2001). Invasive bacterial infections acquired in marine environments have primarily been attributed to marine endemic species including gamma-proteobacterial strains related to *Aeromonas, Halomonas, Pseudomonas, Shewanella,* and *Vibrio.* In beaches with high recreational activities, human-shed *Staphylococcus* or *Streptococcus* can cause minor wound and ear infections (Charoenca and Fujioka, 1993; Thomas and Scott, 1997). Other bacterial infections that have been reported after exposure to marine or estuarine waters include leptospirosis (Thomas and Scott, 1997) and skin granulomas caused by water-borne *Mycobacterium marinum* (Dobos *et al.*, 1999). Near-drowning experiences in marine environments bring seawater into the lungs and can result in pneumonia (Ender and Dolan, 1997; Thomas and Scott, 1997). Such infections have been reported for marine indigenous pathogens including *Legionella bozemanii, Francisella philomiragia, Klebsialla pneumonia* and several *Vibrio* and *Aeromonas* species (Ender and Dolan, 1997). Although the range of infectious doses for wound and skin infections is not known and the degree of exposure is difficult to estimate, the danger may potentially be high. Fifty per cent mortality was observed for artificially wounded rats exposed to ~10^7 CFUs of marine and clinical isolates of *Aeromonas hydrophila, V. parahaemolyticus,* and *V. vulnificus* (Kueh *et al.*, 1992). In the same study, similar mortalities were observed in rats exposed to 1 ml aliquots of sea water from multiple sites, suggesting a high degree of indigenous sea water-associated microbial virulence.

6.3. Aerosol Exposure

The first case of Legionnaires Disease in 1976 demonstrated the importance of airborne transmission of the water-borne bacterial pathogen *Legionella pneumophila* (McDade *et al.*, 1977). Transmission of bacterial disease by marine aerosols has not been documented but should be considered as a potential route of infection. Studies have shown that *Mycobacterium* species are enriched in aerosols from natural waters (Wendt *et al.*, 1980; Parker *et al.*, 1983) and additional respiratory disease agents, which have been detected in sea water, include *F. philomiragia, Legionella* spp., *Acinetobacter calcoaceticus,* and *K. pneumoniae* (Grimes, 1991; Ender and Dolan, 1997). In general, infectious doses for

respiratory agents are small, e.g. 5-10 organisms for *Mycobacterium tuberculosis* infection. In addition, aerosols, generated in coastal environments by wave activity, can transmit algal toxins to humans (Van Dolah, 2000) and cause viruses to become airborne (Baylor *et al.*, 1977). Thus, marine aerosols may be an unrecognized factor in the transmission of diseases from marine environments.

6.4. Marine Zoonoses

Zoonoses are naturally transmissible diseases from animals to humans. Warm-blooded marine mammals harbour and are afflicted by a wide variety of pathogens posing zoonotic risk to humans including *Brucella, Burkholderia, Clostridium, Helicobacter, Mycobacterium, Rhodococcus*, and *Salmonella* species (Bernardelli *et al.*, 1996; Harper *et al.*, 2000; Tryland, 2000; Aschfalk and Muller, 2001 and Aschfalk *et al.*, 2002). Tuberculosis, a chronic respiratory disease caused by *Mycobacterium* species including *M. tuberculosis* and *M. bovis*, has afflicted natural and captive populations of marine mammals (Bernardelli *et al.*, 1996; Montali *et al.*, 2001) and transmission from seal to man has been reported (Thompson *et al.*, 1993). Brucellosis, a systemic infection, is transmitted to humans from infected animals, meat or dairy products in many parts of the world. Brucellosis has also been observed in a wide range of marine animals including dolphins, porpoises, whales, seals and otters (Tryland, 2000; Foster *et al.*, 2002). The zoonotic potential of these marine *Brucella* species has been recognized after three incidents of infection involving a researcher handling a marine isolate (Brew *et al.*, 1999) and two cases of neurobrucellosis attributed to a marine *Brucella* strain in Peru (Sohn *et al.*, 2003).

The transmission of disease between farmed and wild fish populations is one of many concerns regarding the sustainability of aquaculture practices (Garrett *et al.*, 1997; Naylor *et al.*, 2000). The zoonotic potential of farmed fish environments has also been recognized on several occasions. The fish pathogen, *Streptococcus inae* (Zlotkin *et al.*, 1998; Colorni *et al.*, 2002), caused an outbreak of infection in fish farmers in British Columbia (Weinstein *et al.*, 1996, 1997). Additional health hazards of fish handlers include infections with *A. hydrophila, Edwardsiella tarda, E. rhusopathiae, M. marinum*, and *Vibrio* species (Lehane and Rawlin, 2000). In addition, several currently emerging pathogens of fish populations are closely related to human pathogens (Fryer and Mauel, 1997; Rhodes *et al.*, 2001; Starliper, 2001). Recently, *Serratia liquefaciens* was identified as an agent of deadly systemic hospital infections in humans (Grohskopf *et al.*, 2001) and in the same year was identified as a pathogen of farmed Atlantic salmon (Starliper, 2001).

7. CONCLUSION

It is apparent that given sufficient drug exposure and time, resistance will develop to all known antimicrobial drugs. As a result, conservative, appropriate use of antibiotics in ensuring human health, agriculture and aquaculture activities are absolutely necessary to prolong effective use of these drugs.

To control the spread of multi-drug resistant organisms, the following preventive measures are suggested:

1. Eliminate or reduce the amount of anti-microbial used in animal feed, aquaculture and agriculture practices.
2. Complete treatment of sewage, industrial, and aquaculture effluents to minimize the concentration of antibiotics and antibiotic resistant bacteria released into the environment.
3. Periodic monitoring of MAR strains and antibiotic residual concentrations in marine waters receiving discharges from land.
4. Complete treatment of ballast waters to prevent the spread of MAR strains from one location to another.
5. Replace the use and dependence on antibiotics and chemical agents in aquaculture practices with the use of vaccines and probiotics.
6. Restricted and control use of antibiotics in medical practices.
7. Ensure proper clinical use practices of antibiotics to minimize releasing partially inhibited yet alive cells which have survived partial exposure.

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Current Trends in Antibiotic Resistance in Infectious Diseases

This book contains ten chapters which cover current trends on antibiotic resistance in different parts of the world. Some of the chapters are dedicated to specific type of bacteria like marine and cholera associated microorganisms. Whereas rest of the chapters are mainly focused on the mechanism of drug resistance with special reference to beta lactamases. Since most of the antibiotics used to treat infections belong to β-lactam group which is lactam ring (β-lactam) or penam is a lactam with a heteroatomic ring structure, consisting of three carbon atoms and one nitrogen atom. A lactam is a cyclic amide.

The main focus of this book is to understand the different molecular markers responsible for developing resistance against this group of antibiotics. CTX-M family of enzyme which hydrolyzes third generation of cephalosporins preferably, cefotaxime, belongs to the category of Extended-spectrum β-lactamases (ESBLs). These types of enzymes are emerging among Gram-negative bacteria; predominantly *Klebsiella pneumoniae*, *Escherichia coli* and other species in different parts of the world. In the current scenario, the CTX-M family includes almost 89 variants. The bla_{TEM}, and bla_{SHV} are another important class of β-lactamases, most prevalent among enterobacteriacae which are also discussed in this book.

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