CHAPTER 2

Therapeutic Limitations due to Antibiotic Drug Resistance: Road to Alternate Therapies

Ashima Kushwaha Bhardwaj*, Kittappa Vinothkumar, Neha Rajpara, Priyabrata Mohanty and Braj Mohan Ram Narayan Singh Kutar

Department of Human Health and Diseases, Indian Institute of Advanced Research, Koba Institutional Area, Gandhinagar 382 007, Gujarat, India

Abstract: The antibiotics are destined for obsolescence as microbes would find a way to deal with them either by innate or by acquired genes. It is truly said that the power of bacteria should never be underestimated. There is a constant race between the humans for design and use of new drugs and the acquisition of genes by bacteria to render these novel drugs harmless. Situation has worsened with the indiscriminate use of antibiotics in human and animal health, agriculture, aquaculture and poultry. There have been reports of extremely drug resistant (XDR), totally drug resistant (TDR) bacteria and superbugs that have complicated the treatment of infectious diseases. Methicillin–resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *S. aureus* (VRSA) recognized as the bane of hospitals are some of the most dreaded bugs. This chapter discusses various mechanisms of multiple drug resistance (MDR) in bacteria and the limitations of antibacterial chemotherapy due to MDR. Various innate and acquired mechanisms of drug resistance like integrons, SXT elements, efflux pumps and quinolone resistance mechanisms are described in details. Some of the important databases related to these genetic factors have also been described here. The possibility of attacking the virulence of bacteria rather than the bug itself in order to circumvent the crisis of MDR has been discussed. It further highlights some of the novel strategies such as efflux pump inhibition and quorum sensing inhibition as anti-virulence strategies. It is advocated that this never-ending war with bacteria would probably require multifaceted approach combining antibacterial, antivirulent regimes in addition to the constant search for novel drug targets and newer drugs by the pharmaceutical companies. Success of these strategies would involve cumulative and strenuous efforts from public, policy makers, research community, clinicians and pharmaceutical companies.

Keywords: Databases, efflux pumps, government policies, inhibitors, integrons, multidrug resistance, phage therapy, plasmids, quinolone resistance, quorum sensing, SXT elements, virulence, vaccines.

***Author's address:** Department of Human Health and Diseases, Indian Institute of Advanced Research, Koba Institutional Area, Gandhinagar 382 007, Gujarat, India; Tel: +91-079-30514235; Fax: +91-079-30514110; Email: ashima.bhardwaj@gmail.com

INTRODUCTION

This chapter highlights the causes and implications of antimicrobial resistance. This text is documented at a time when the crisis due to resistant bacteria looms large in front of mankind and Centers for Diseases Control and Prevention (CDC), World Health Organization (WHO) have seriously recognized the impending catastrophe and The Lancet Infectious Diseases launches a Commission, entitled *Antibiotic resistance – the need for global solutions* [1-3]. Antimicrobial resistance is a global concern and has serious social, economic and clinical consequences. When any microorganism like bacterium, fungus, virus or parasite becomes resistant to an antimicrobial drug to which it was originally sensitive, that organism is said to display antimicrobial resistance. In case of a pathogen that may cause disease to humans, plants or animals, this property of drug resistance often renders the drug ineffective and leads to treatment failure. The problem is compounded when such organisms acquire resistance to a large array of antibiotics/antimicrobials/drugs which is known as multidrug resistance (MDR). This seriously hampers the treatment of diseases caused by these invincible microbes. In recent times, with indiscriminate use of antibiotics in human health, poultry, agriculture and aquaculture, lots of pathogens have accumulated a large battery of genes responsible for conferring these bugs resistance to almost all the drugs used clinically. This has spawned another generation of microbes that are extensively drug resistant (XDR) or totally drug resistant (TDR) and therefore, untreatable by all first-line and second-line drugs. For example, tuberculosis bacterium has been reported in all the three forms, MDR, XDR and TDR. According to WHO report, there were 6,30,000 (52.5%) cases of MDR-TB in 2011 out of a total of 12 million cases of TB (tuberculosis). XDR-TB has been reported from 84 countries and TDR-TB from countries like Iran, India, Italy and South Africa. These superbugs fail to respond to antimicrobial treatments resulting in higher treatment costs, prolonged hospital stay and high case fatality rates. Many ailments such as tuberculosis, malaria, influenza, diarrhoea, gonorrhea and nosocomial infections can no longer be vanquished due to the problem of antimicrobial resistance. Therefore, it becomes imperative to understand various factors that have led to the problem of antimicrobial resistance and assess different alternatives in the scenario of failed antibiotics. In this

chapter, the focus will be on antibiotic resistance mechanisms exhibited by bacteria and alternative strategies that could be ventured in case of ineffective antibiotics [1-3].

ANTIBIOTIC RESISTANCE

Development of antimicrobial resistance is a natural phenomenon that every organism undergoes for evolutionary fitness. Greater is the number and generations of antimicrobials, natural or manmade, faster is the development of new resistance mechanisms in a pathogen to counteract these drugs. Many bacteria including clinically significant pathogens have been reported to display MDR. Some of these pathogens are: *Mycobacterium tuberculosis* (*M. tuberculosis*), *Pseudomonas aeruginosa* (*P. aeruginosa*), *Klebsiella pneumoniae* (*K. pneumonia*e), *Escherichia coli* (*E. coli*), methicillin-resistant *Staphylococcus aureus* (MRSA), *Shigella dysenteriae* (*S. dysenteriae*) and *Vibrio cholerae* (*V. cholerae*). These organisms are found in hospitals as a source of nosocomial infections and they are also prevalent in communities leading to community-acquired infections. Antibacterial resistance gets aggravated due to a wide variety of genetic and non-genetic (like social, political, clinical malpractice) factors. Accordingly, to overcome this problem, multiple interventions are required at various levels.

GENETIC FACTORS/MECHANISMS GOVERNING MDR IN BACTERIA

Antibacterials kill or retard the bacteria by targeting the cellular processes or structures in bacteria which differ greatly from that of their host counterparts. The antimicrobial compounds that kill bacteria are called bactericidal while the others that merely retard the growth of bacteria are called bacteriostatic compounds. These drugs interfere with the vital housekeeping processes such as cell wall synthesis, protein, DNA and RNA synthesis or inhibiting the key enzymes of metabolic pathways [4]. To counteract these drugs, bacteria evolve different mechanisms and employ large battery of genes to affect these mechanisms. These resistance mechanisms could be intrinsic/innate (chromosome-borne) or acquired (borne on mobile genetic elements {MGE}). Though innate resistance allows a bacterium to adapt to the changing environment, it gets restricted to that particular

bacterium and only gets transferred to the progeny by a process called vertical gene transfer. In contrast to this, the acquired resistance leads to the resistance genes being disseminated quickly within different bacteria crossing the species and genera barriers. This process is carried out by horizontal gene transfer (HGT) and could be mediated through different vehicles like plasmids, integrons, transposons and mechanisms of transduction, conjugation and transformation. The other mechanisms through which bacteria mediate resistance to antibiotics are: chromosomal mutations at the antibiotic target sites, restricting the access of the antibiotics through porins and efflux pumps, enzymatically inactivating the antibiotics, modifying or protecting the antibiotic target and by hindering the activation of antibiotic [4-6]. Often, a bacterium counteracts an antibiotic by synergistic action of more than one of the above mentioned resistance mechanisms [7-10]. For example, resistance of *P. aeruginosa* to ticarcillin has been attributed to overexpression of outer membrane protein OprM, production of β-lactamase and overexpression of AmpC cephalosporinase [7]. Similarly, resistance to tetracycline is often a result of synergy between the efflux pumps (encoded by *tetA* to *tetG*, *tetK*, *tetL*), oxidation of tetracycline and cytoplasmic proteins (encoded by *tetM, tetO*, *tetQ*) that confer protection to ribosomes from tetracycline [5]. In many cases, acquisition of a single gene/single mutation can offer protection against many different classes of antibiotics. For example, the efflux pumps with specificity for many different antibiotics can lead to resistance for all these antibiotics on overexpression or mutations. Similarly, the enzyme rRNA methylase (encoded by *ermA*, *ermB*, *ermF*, *ermG*) methylates an adenine on 23S rRNA that lies within a region that binds to three classes of antibiotics; macrolides, lincosamides and streptogramins. Therefore, a single gene of this methylase confers resistance against these three structurally distinct classes of antibiotics [5].

It can be envisaged that the source of antibiotic resistance genes could be the bacteria that produce antibiotics. In such producers, these resistance genes provide protection to bacteria from the antibiotics produced by them. Another possible source of these resistance genes could be the organisms naturally resistant to some of the antibiotics. For example, *Lactobacillus* species do not use D-Ala-D-Ala dipeptide as part of their muramyl dipeptide and therefore, they are naturally

refractory to the glycopeptide antibiotic vancomycin. Vancomycin prevents crosslinking of peptidoglycan by binding to the D-Ala-D-Ala of the muramyl peptide. Resistance to vancomycin arises due to replacement of this peptide with D-Ala-D-lactate, a dipeptide that does not bind to vancomycin [5].

Bacteria possess an exquisite ability to adapt rapidly to the changing environments and this property is mediated by a large number of genetic elements that contribute to the genome plasticity. In the ensuing sections, two mobile genetic elements for MDR, integrons and SXT elements, are discussed in detail. Subsequently, efflux pumps have been explained as a general mechanism for MDR.

Integrons

Study of drug resistance mechanisms and their dissemination became important since discovery of MDR bacteria during mid 1950s [11]. In 1970s, in many cases, MDR was found to be associated with transmissible plasmids and/or with transposons [12, 13]. Integrons were discovered later in 1980s [14] and these mobile genetic elements harbouring antibiotic resistance gene cassettes are now known in clinical as well as agricultural and environmental samples [15-17]. The term chromosomal integron (CI) was introduced in late 1990s with the discovery of *V. cholerae* super integron [18]. CIs were found to be sedentary in nature and they were not involved in resistance phenotype. The evolutionary history of CI suggested that these elements helped in adaptation along with the change in environment as well as it was the main source of mobile integron's backbone and antibiotic resistance gene cassettes [19].

Structure of Integrons

Integron is a very common tool for antibiotic resistance gene capture and dissemination. It is the platform to acquire open reading frames (ORFs) by site-specific recombination and convert them to functional forms by their expression [19, 20]. Most of the integrons have three key components: 1) an Integrase (*intI*) belonging to tyrosine recombinase family; 2) a promoter (Pc) that directs the expression of the captured genes and 3) a primary recombination site (*attI*).

Additionally, all gene cassettes that incorporate in the integron share some specific characteristics at 3' end of the gene which are mostly imperfect repeats called *attC* (also called 59- base element) [19, 20].

Integrases

Integrases belong to site-specific recombinases known as tyrosine recombinases. Tyrosine recombinase includes a wide variety of enzymes that use a tyrosine residue as the nucleophile in their strand exchange reactions [21, 22]. It is involved in integration and excision or inversion of gene cassettes. The catalytic domain of integrase contains conserved amino acids Arg(R)-His (H)-Arg(R) and the nucleophilic tyrosine, Tyr (Y) [22, 23].

Promoter

Cassettes are generally promoterless but few reports revealed that some of the gene cassettes have their own promoter. For example, *cmlA* cassettes have both promoter and translational attenuation signals, *V. cholerae qnrVC* genes have their own internal promoter and the *qacE* cassettes have a weak promoter [24-26]. The expression of majority of promoterless cassettes is hence dependent on proximity of an external promoter located either in the integrase gene or on *attI* site [27-29].

Primary Recombination Site (attI) and 59-Base Element (attC)

Integrase recognizes and recombines two types of sites that have different structures, the *attI* type (non-palindromic) of site found in the integrase gene and *attC*/59-base elements (palindromic) found on the gene cassettes. The *attI* and *attC* sequences are complex attachment sites that include the crossover site and additional binding sites and integrase monomers act as accessory factors at these additional sites [30-32]. The *attI* sites are located at the end of the 5' conserved region (5'CS) of integrons and their sequences vary considerably. The *attC* sites share a common set of characteristics that enable them to be identified despite the diversity of their sequences and sizes [30, 33]. They are characterized by a palindrome of variable length and sequence between the RYYYAAC (R= Purines; Y= Pyrimidine) inverse core site and the GTTRRRY core site [33]. The

size of these recombination sites vary in length from 57 to 141 bp [34]. Mostly gene cassettes are found integrated in variable region of integrons or they can exist as covalently closed circular intermediates. The integrase recognizes *attC* and *attI* sites. In the event of integration *attI*×*attC* recombination occurs and that allows expression of genes that are downstream to the promoter. In excision, *attC*×*attC* recombination occurs [20, 35].

Types of Integrons

Two types of integrons are mobile integrons and chromosomal integrons.

Mobile Integrons (MI)

Integrons associated with mobile DNA elements and primarily involved in the spread of antibiotic resistance genes correspond to the mobile integrons [20]. The bacteria of same species or different species use these elements as natural genetic vehicles to transfer a wide array of genes including antibiotic resistance genes. More than 130 different antibiotic resistance cassettes have been identified in MIs [16, 36]. These cassettes have resistance genes for the majority of antibiotic classes like β-lactams, aminoglycosides, chloramphenicol, trimethoprim, streptomycin, quinolones, rifampin, erythromycin and antiseptics of the quarternary ammonium compound family [16, 19, 36]. Five classes of MIs have been well defined to date based on integrase sequences with ~40-58% identities [19, 20]. In this chapter, only three integron classes (class 1, 2 and 3) have been described.

Class 1 integrons are the most widespread and well characterized. They are widely distributed in animal and human clinical strains of Gram–negative bacteria [37, 38] and also in some of the Gram–positive bacteria [39-42]. Class 1 integrons consist of two conserved segments (CS) at 5′- and 3′ ends, separated by a variable region that usually comprises of one or more gene cassettes (Fig. **1**). The 5′CS region contains the integrase gene (*intI1*), the integration site (*attI1*) and a promoter region (Pc) that allows expression of any number of gene cassettes inserted at the *attI1* site in a suitable orientation. The 3′CS region usually comprises of *qacE*Δ1 encoding resistance to quaternary ammonium compounds

and *sul1* encoding resistance to sulphonamides [34, 43]. Class 1 integrons are embedded within larger transposon Tn 21 [12].

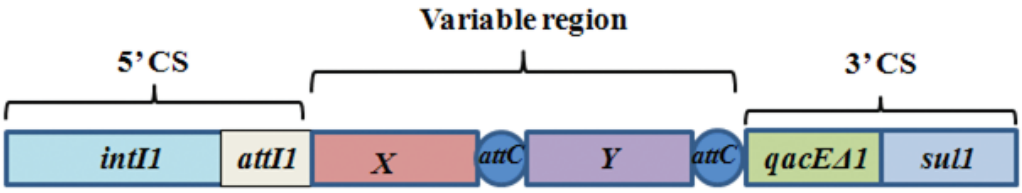

Fig. (1). Structure of a class 1 integron. Integrons consist of a gene *intI1* encoding a site-specific recombinase called "integrase" belonging to tyrosine-recombinase family and a recombination site *attI1* into which the exogenous gene cassettes (X and Y) harbouring the recombination site *attC* are inserted through site-specific recombination. In the 5'conserved sequences (5'CS), a promoter Pc located within *intI1* drives transcription of the captured genes. *qacEΔ1* and *sul1* are conserved regions in 3'conserved sequences (3'CS) which contribute resistance to ethidium bromide and sulfonamides.

Class 2 integrons are associated with Tn7 transposons. They have been found in several species of Gram-negative bacteria isolated from human, animal and environmental sources [37, 44]. Class 2 integrons have also been shown to carry three resistance gene cassettes, *dfrA1, sat1* and *aadA1*, that confer resistance to trimethoprim, streptothricin and streptomycin/spectinomycin, respectively [45]. The gene encoding class 2 integrase contains a nonsense mutation in codon 179 (ochre 179) and thereby it yields a truncated, non-functional protein which can be recovered by a single mutation. Mutation of the ochre179 codon to glutamic acid encoding codon produces an integrase with full recombinase activity [45]. It is not clear whether the cassette recombination in different Tn7 derivatives is due to natural suppression of the ochre 179 codon producing an active integrase or due to trans-acting recombination activity of other integrase which recognizes and recombines the *attI2* site [45, 46]. In addition, functional class 2 integrases have been reported in some instances [47-49]. In one case, class 2 integron was associated with four non-antibiotic resistance gene cassettes while in second case, class 2 integron carried *dfrA14* and a novel lipoprotein signal peptidase gene cassettes [47, 48]. In a recent report, a functional class 2 integron carrying *dfrA14* and three novel gene cassettes with unknown functions was found in 38 clinical isolates of *Proteus mirabilis* [49].

Class 3 integron was first reported in *Serratia marcescens* strain in 1995 [50] and characterized later in 2002 [51]. The configuration of the three potentially definitive features of the class 3 integrons, the *intI3* gene, the adjacent *attI3* recombination site and the P_c promoter that directs transcription of the cassettes was similar to that found in class 1 integron module. The *IntI3* integrase was active and able to recognize and recombine known types of *IntI*-specific recombination sites, the *attI3* site in the integron and different cassette-associated 59-base sites. Both integration of circularized cassettes into the *attI3* site and excision of integrated cassettes were catalyzed by *IntI3* [51]. Class 3 integron with its genes encoding blaGES-1 and OXA/AAC(6')-Ib responsible for the β-lactam and aminoglycoside resistance have been reported [50, 52].

Chromosomal Integrons (CIs)

Chromosomal Integrons or super-integrons (SIs) are located on the chromosomes of large number of bacteria [18, 53-55]. SIs are distinguished from conventional integrons by their size, placement of promoters, chromosomal location, and the nature of gene cassettes they carry [54]. It has been proposed that MDR integrons arose from SIs by the entrapment of *intI* genes and their related *attI* sites on MGEs such as transposons. The gene cassette reservoirs of SIs provide a source of gene cassettes that are recruited by multi-resistant integrons [56].

CIs have been found in bacteria belonging to families such as *Vibrionaceae*, *Pseudoalteromonas*, *Xanthomonadaceae*, *Alteromonadaceae*, *Pseudomonadaceae* and *Spirochaetales* [19, 20]. All of them share general characteristics like they are large (>20 gene cassettes and upto 200) and have homology between the *attC* sites of their endogenous cassettes. The first SI was discovered in *V. cholerae* chromosome II in a clustered region spanning 126 kb. It harbored 214 ORFs out of which the functions for 179 cassettes have not been assigned [56].

The difference between MIs and CIs is as follows [56]:

1. MIs contain <20 gene cassettes while SIs/CIs contains >20 gene cassettes.

2. In MIs, the cassettes typically code for antibiotic resistance genes whereas those of SIs are mainly unknown functions.

3. MIs are associated with mobile elements while the SIs coevolved with their host genomes which strongly suggest that SIs are sedentary.

4. The *attC* sites of the gene cassettes of MIs are highly variable in length and sequence while the *attC* sites of CI gene cassettes are closely related and species-specific.

5. Finally, there is evidence that not all of the gene cassettes but only few associated with CIs are significantly expressed.

Gene Cassettes

As described in the earlier section, more than 130 gene cassettes in MIs are known for antibiotic resistance genes [20]. For example, resistance to chloramphenicol is due to acetylation of the antibiotic (*catB* gene) and for the aminoglycosides, due to modification of antibiotic by acetylation (*aacA* and *aacC* genes) and adenylation (*aadA* and *aadB* genes). The β-lactamases encode three distinct families; class A (*blaP* genes), class B metallo β-lactamase (bla_{IMP} genes) and class D (*oxa* genes) which inactivate the β-lactam drugs. The *dfrA* and *dfrB* genes code for dihydrofolate reductase conferring trimethoprim resistance. Apart from antibiotic resistance genes, many unknown ORFs have also been reported and these ORFs are assigned letters in the order of their identification like *orfA*, *orfC* and *orfD* [34].

In contrast to MIs, CIs contain highly diverse cassettes, mostly of unknown functions. In 2007, 1677 cassettes were identified by the analysis of Vibrionales genome [57]. Among these, 75% of the cassette pool corresponded to accessory genes of unknown functions. Remaining 25% of the cassettes contained genes like phage-related proteins, toxin-antitoxin (TA) systems, acetyltransferases, DNA modification systems, virulence and experimentally confirmed restriction modification systems, lipases, dNTP pyrophosphohydrolases, polysaccharide biosynthesis and sulphate–binding proteins [58-60]. CIs also carry several resistance gene arrays. Many *dfr* cassettes in different environmental isolates of *V. splendidus* have been found while *catB9*, *carb7*, *carb9* (encoding carbenicillin resistance) and *qnr* (encoding resistance to quinolones) cassettes have been

identified in *V. cholerae* CI [56, 61-64]. Altogether, CIs are involved not only in acquisition of antibiotic resistance genes but also widely in the adaptation of bacteria in different environments [20, 35].

Toxin- Antitoxin (TA) Genes

The gene content of SI can be ~3% of the bacterial genome content [54, 65]. The cassette array of SI implies either existence of selective pressure to maintain the gene cassettes or mechanisms that promote their persistence in the absence of selection [20]. These addiction modules are selfish genetic elements. Two classes of addiction modules are known *i.e.* toxin/antitoxin (TA) and restriction methylation systems (RMS) [66-68]. TA loci are commonly found on plasmids or within prophages where they have been found to enhance plasmid and phage maintenance by preventing the multiplication of plasmid-free or phage-free progeny in bacterial populations. TA system consists of two ORFs and is organized as an operon. One ORF codes for stable toxin while the other codes for unstable antitoxin. Disruption in expression of TA system leads to the accumulation of free toxin due to loss of antitoxin neutralizing activity and inhibits the multiplication of bacterial growth (devoid of plasmids/ prophages). The TA systems have been proposed to stabilize chromosomal regions by preventing the accidental deletions, when located in unstable segments like MGEs. There are reports of SIs containing the TA cassettes. Thirteen TA cassettes have been reported in the *V. choerae* N16961 SI [54, 69, 70]. Functional analysis of TA cassettes in SI was also carried out in other groups of Vibrios like *V. vulnificus, V. metschnikovii* and *V. fischeri* [70-72]. Similarly, another addiction module RMS consists of long half-life restriction enzymes and comparatively short half-life modification methylases which maintain plasmid stability by post segregational killing [67]. The RMS acquired by bacterium begins to depend on this system for its survival, as the methylase protects the host genome along with RMS cassettes by methylating the specific sequences that could be recognized and cleaved by restriction enzyme. Loss or disruption of expression of RMS systems results in cell death. Thus even in the absence of antibiotic exposure, the resistance conferring gene cassettes linked with this RMS system will be maintained [67]. *Xanthomonads* and *Pseudomonads* SIs contained

RMS systems which may stabilize the cassette array in the absence of selection [70, 73].

SXT Element

SXT element (deriving its name due to encoded resistance to sulfa and trimethoprim), an Integrative and Conjugative Element (ICE), is a conjugative, self-transmissible integrating element that shows similarity to conjugative transposons. It is an important vehicle for spreading of antibiotic resistance in bacteria like *V. cholerae*, *Providencia alcalifaciens* and *P. rettgeri* [74, 75]. ICEs are a group of MGEs that contain programmes to determine their excision and integration and play important role in genome flexibility of numerous Gram-positive and Gram-negative bacteria [76]. These elements can integrate into the bacterial genomes, replicate as part of host chromosome, get excised and then disseminate to new host genomes by conjugation (Fig. **2**). ICEs help bacteria to adapt to new ecological conditions, to colonize in new niches, to survive in stress conditions like antibiotic exposure and play a very important role in reshaping bacterial genomes [77]. SXT element is also known as constin (conjugable, self-transmissible, integrating element) that helps in bacterial adaptation, evolution and expansion [78]. This MGE harbors a diverse array of genes such as antibiotic resistance genes, genes for complex degradation pathways for toxic compounds and genes for tolerance to heavy metals. Unlike plasmids, SXT elements are not capable of autonomous replication, so they mingle into the host chromosome for their replication and get transferred intracellularly and intercellularly [79]. ICEs combine within them, the properties of plasmids as well as phages. They are transferred to another bacterium through conjugation like a plasmid. Similar to phages, they have the property of integrating into the host genomes and getting excised under certain circumstances. SXT was identified for the first time in *V. cholerae* O139 serotype of MO10 isolated from Madras, India in 1992, where it was responsible for epidemic condition like the one caused by O1 serotype of *V. cholerae* [74]. Because this element was identified in *V. cholerae* MO10, the name was given SXT^{MO10} [74, 80] and it was found to encode resistance property for chloramphenicol, streptomycin, sulfamethoxazole and trimethoprim [74]. Apart from this report, SXT element was also reported in non-O1, non-O139 strains isolated before 1992 from Varanasi [81]. These strains were resistant to co-

trimoxazole, trimethoprim, streptomycin, furazolidone and ampicillin. The presence of SXT^{MO10} was observed in other O139 serotypes isolated earlier, whereas this element was not present in O1 serotype of *V. cholerae* [82]. *V. cholerae* O1 El Tor that reappeared dominantly by 1994 in Indian subcontinent showed resistance property for the four antibiotics characteristic of O139 isolate, where the corresponding genes were transferred through ICE. This element was designated as SXT^{ET} [80, 83, 84]. Since 1994, the *V. cholerae* O1 strains carrying SXT element have been reported from various geographic locations like Bangladesh, Mozambique, Laos and India [82, 84-87]. Concurrent with emergence of SXT-harboring O1 strains, the *V. cholerae* O139 strains have been reported with antibiotic resistance profiles different from the earlier O139 strains. These recent O139 strains showed resistance to streptomycin but were found to be sensitive to trimethoprim and sulfamethoxazole [88-90]. Those ICE elements which belong to the same ICE family usually share the same integration site and augment intra-species variability. There are various hot regions in bacterial genome that provide a dramatic illustration of how ICEs generate intra-species diversity at particular locus and inter-genus locus variability [79]. The recombinase and insertion sequences facilitate ICE evolution by acquisition and dissemination of genomic islands due to the effect of variable conditions [91]. Though the SXT element was discovered in 1992 and sequenced, the putative ORFs are still not eloquently characterized [92]. SXT^{MO10}, SXT^{ET}, SXT^{HN1}, SXT^{KN14}, SXT^{MCV09} and SXT^{LAOS} are the known SXT elements which bear different antibiotic resistance genes (http://db-mml.situ.edu.cn/ICEberg).

Origin of SXT Element

Deciphering the evolutionary history of SXT element is much difficult because there may be many recombination processes that seem to have taken place in past, between plasmid, phages and other ICEs. As there is great diversity among ICEs (total reported till date 460), it seems that they do not come from common ancestor but arose independently. DNA sequence-based study of SXT element revealed that this element of approximately 100 kb was composed of genes that could have been derived from plasmids, bacteriophages and many additional diverse unknown sources. The comparison between % GC content of SXT^{MO10} element (47.1%) and *V. cholerae* genome (47.6%) showed almost similar value

indicating that SXT element could have been derived from *V. cholerae* genome though it also contained several regions with significantly different GC content [84, 92]. This again indicated that SXT has captured its gene content from varied sources. For example, an insertion with GC content of 42% observed in the transfer region showed sequence similarity to Ti plasmid from *Agrobacterium tumifaciens* [92].

Structure of SXT Elements

Typically, the SXT elements show modular organization and harbor three modules responsible for their maintenance, dissemination and regulation [77]. Maintenance module consists of *Int* gene encoding a recombinase that catalyses recombination between *attP* site on SXT and an *attB* site on the target bacterial chromosome. Recombination occurs through an extra chromosomal circular intermediate of SXT generating two junctions *attL* and *attR* (Fig. **2a**). The intermediate can only express its function on being integrated into the chromosomal DNA. SXT also encodes a protein Xis required for excision of this element from the host genome [93]. The recombinases could most often be tyrosine recombinases like in integrons and could sometimes be serine recombinases like in Tn5397 from *Clostridium difficile* [94]. Apart from *Int, Xis, attB* and *attP*, ICEs may also contain additional mechanisms to ensure their stability through bacterial generations even in the absence of factors favoring their selection. For the dissemination of ICE, they encode proteins that promote conjugation between the donor and the recipient bacteria. Conjugative transfer is initiated at a *cis*-acting 299 bp locus called the Origin of transfer (OriT) that is nicked by a putative relaxase called TraI to produce single-stranded DNA. This DNA gets translocated to the recipient bacterium through a mating pore [95]. This single-stranded DNA gets converted into double-stranded DNA in the host, re-circularized and finally gets recombined/integrated into the host genome [95]. The transfer of ICE elements therefore could be mostly in the form of single-stranded DNA and also sometimes in the form of double-stranded DNA like in the case of pSAM2 from *Streptomyces ambofaciens* [96]. Regulation of ICE mobility could be induced by various environmental or stress factors that promote their dissemination to new host providing these hosts the ability to survive these stress conditions.

Factors Responsible for Maintenance, Acquisition and Dissemination of SXT Element

As mentioned in earlier sections, the SXT element basically requires three functionally conserved key genetic components: integration and excision component, conjugation component and regulation component. This conserved backbone can acquire other genes through insertion sequences, transposons and recombinases. The integration of SXT element in bacterial chromosome is site-specific unlike the other conjugative transposons that can integrate into many different sites in the bacterial genome. Integration and excision at 5'end of *prfC* region encoding peptide chain releasing factor 3 involved in termination of translation, occurs through non-replicative circular intermediate [97]. The SXT-encoded recombinase Int is mainly responsible for integration and excision from chromosome and is activated by SetC and SetD but Int alone does not promote efficient excision (Fig. **2b**). SetC and SetD, two loci of SXT element, which are similar to master activators of flagellar transcription FlhC and FlhD, are required for *int* expression by regulating host promoter [79, 92]. A repressor molecule SetR from *V. cholerae* SXT, homologous to lambda repressor cI, controls the dissemination of SXT DNA. ICE loss frequency increased when *mosT* and *mosA* genes were deleted [95]. MosT and MosA toxin-antitoxin proteins promote the maintenance of SXT element [98-100]. Although, SXT element's low frequency loss occur even in the absence of *mosT* and *mosA* genes, it suggests that SXT element may encode additional factors to promote its conservation [101]. As described above, conjugative transfer of SXT element is dependent on SXT relaxase TraI that binds covalently to well conserved SXT *oriT* present between *s003* and *rumB* [102]. During SOS responses, repression by SetR is lifted leading to dissemination of SXT element to a new host [77]. Mitomycin C treatment of cells harboring SXT elements has been shown to induce transfer of this element 400-folds [103]. Hence, the DNA damaging agents Mitomycin C and quinolone antibiotics promote the spread of SXT and equivalent ICEs [103]. *prfC* region, the major integration module of *V. cholerae* and other γ-proteobacteria, is also responsible for integration of closely related ICEs including R391, R997 and pMERPH. In the absence of *prfC* region, SXT can preferentially integrate in others alternative sites, such as the 5' end of *pntB* which encodes a pyrimidine

nucleotide transhydrogenase [79, 104, 105]. The Recombination Directionality Factor (RDF)-like factor which is encoded by novel SXT Xis (excisionase) facilitates more prominently excision of SXT element [77, 79].

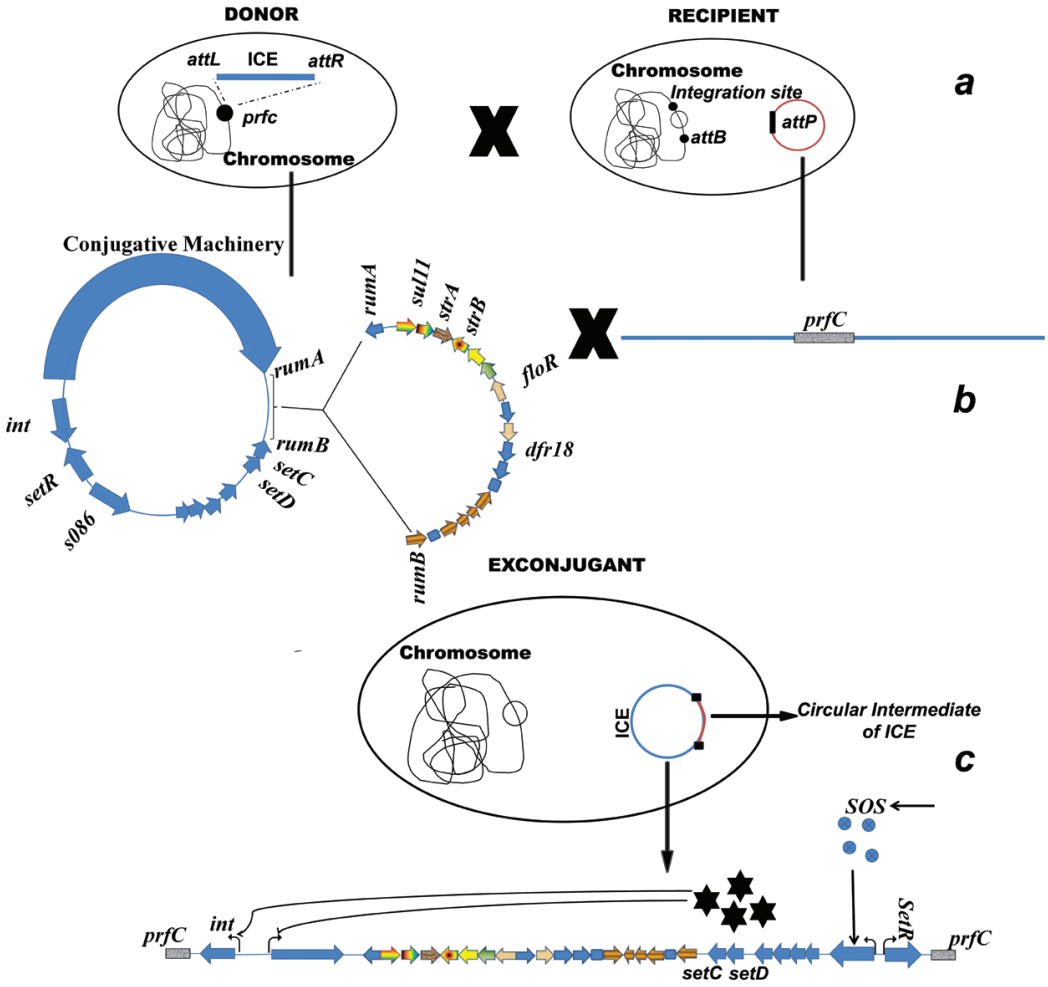

Fig. (2). Horizontal gene transfer in bacterial population containing SXT element. a. Donor bacterium transferring chromosomal ICE SXT element to recipient bacterium by conjugation. **b**. Circular intermediate of SXT element contain conjugative machinery, UV repair DNA polymerase genes (*rumA*, *rumB*), regulatory genes (*int*, *setC*, *setD*, *setR*). Between *rumA* and *rumB*, antibiotic resistance genes *sulII*, *strA*, *strB*, *dfr18* are located. This circular intermediate gets integrated at *prfC* (encoding peptide chain releasing factor 3) region of chromosomal DNA in bacterium; **c**. Effect of SOS response on SXT regulation and expression: SOS response in bacterial cell mediates auto-proteolysis of setR due to activation of recombinase A. This in turn activates *setC* and *setD* leading to increased expression of integrase gene *int* (responsible for integration and excision).

SXT/R391Family

Till date, a total of 28 ICE families have been defined based firstly on integrase similarity and secondarily on core structure synteny [106]. SXT/R391 is the biggest and most widespread of these 28 families and has SXT and R391 elements as its original members. Some other previously identified families also included Tn916, Tn4371, CTnDOT/ERL and ICE6013 [106]. R391 was reported from a clinical isolate of *P. rettgeri* in South Africa in 1972 where it encoded resistance to mercury and kanamycin [107]. The mechanisms of R391 and SXT integration into the 5' end of *prfC* gene and their excision were found to be similar [104]. Nucleotide sequence analysis of 99.5 kb SXT and 89 kb R391 revealed close similarity between these elements which shared about 65 kb of DNA. This conserved sequence included the machinery for mobility of these elements including the genes for conjugative transfer, integration, excision and regulation of these events [75]. The SXT/R391 family was then defined by Burrus *et al.* in 2006 where they proposed that any ICE that encodes an integrase gene closely related to *int*SXT and that integrates into *prfC* be considered part of the SXT/R391 family of ICEs [85]. In addition the *tra* genes, which encode the ICE conjugation apparatus, are also a defining feature of this family of ICEs. The ICE*Vfl*Ind1 of this family was reported to facilitate excision of genomic islands including some pathogenicity islands from three species of Vibrios and their conjugative transfer by recognition of a similar OriT in these islands as that of the SXT/R391 family [108]. This finding has again emphasized the importance of ICEs in genome plasticity/evolution. These ICEs have five hotspots (HS1-HS5) for DNA insertion and four minimal gene set modules: *int-xis* (integration and excision module); *mob* (DNA mobilization and processing module); *mfp* (mating pair formation module); *reg* (regulation module) which are required for integrity of these elements [101]. On the basis of acquisition of another member, SXT/R391 family was divided in two groups S and R. The S group SXT/R391 limits the acquisition of another S group but did not limit the acquisition of R group SXT/R391 ICE [109]. The TraG and entry exclusion protein Eex are inner-membrane proteins that promote exclusion activity of SXT/R391 ICEs [109].

Efflux Pumps

Efflux pumps are one of the major determinants of MDR in various pathogenic microorganisms. They play variety of important biological roles across all kingdoms of life from prokaryotes to eukaryotes with functions ranging from simple transport to secretion of a diverse range of secondary metabolites as a defense against herbivores and microbial pathogens in plants [110]. They also play a vital role in bacterial survival and pathogenicity. Based on sequence similarity with known transporters, it has been estimated that 15–20% of *E. coli* or *Saccharomyces cerevisiae* genome codes for these type of proteins [111]. The size of genomes have been found to be directly proportional to the number of efflux pump genes harbored by them indicating that these proteins are crucial for cell survival [112-114].

Efflux pumps are universal transport systems present both in antibiotic susceptible and resistant bacterial strains. Efflux pump-encoding genes could be present in the chromosome or could reside on MGEs like plasmids, transposons and integrons, which allow faster dissemination of these genes in the bacterial population [6, 15, 115-117]. These pumps enable microbes to extrude structurally diverse antimicrobials, facilitating their survival in toxic environments. Efflux pumps also have important physiological functions like bile tolerance in enteric bacteria, colonization, invasion and survival in host [113, 115, 118, 119]. Efflux as a mechanism of antibiotic resistance was first described against tetracyclines but now numerous efflux pumps have been discovered and fully characterized in terms of their structure and degree of resistance they confer towards variety of substrates [113, 115, 120-124]. Some of the efflux pumps like TetA and CmlA are specific for the antibiotics tetracycline and chloramphenicol respectively, but a large number of pumps like MexAB-OprM, NorA and BmrA can recognize and efflux out a diverse array of structurally unrelated compounds [6]. The latter type are called MDR pumps [115]. Efflux pumps confer relatively low to moderate degree of drug resistance (1- to 64-fold increase in minimum inhibitory concentration {MIC}) that makes their clinical relevance debatable [125-127]. However, different types of efflux pumps in synergy with other known drug resistance mechanisms like quinolone resistance proteins, β-lactamases and mutations in topoisomerase genes, contribute immensely towards MDR leading to

clinically significant MIC values [8, 10]. These pumps have also been shown to play vital role in the antibiotic resistance displayed by biofilms [128]. Efflux is a dynamic process and regulation of efflux is still not completely understood. These efflux pumps genes are regulated by a master operon which overexpresses efflux pump genes but in turn also downregulates porin activity. Chromosomally encoded local and global regulatory proteins controlling the expression of MDR pumps have been studied extensively in bacteria [111].

Classification and Structure of Efflux Pumps

Efflux pumps usually consist of a monocomponent protein with several transmembrane spanning domains. However, in Gram-negative bacteria, which are protected by an outer membrane, efflux transporters can be organized as multicomponent systems [129]. These efflux pumps could either be primary transporters that utilize energy derived from ATP hydrolysis, or they could be secondary transporters that utilize proton motive force (PMF) [111]. These efflux pump genes are classified based on sequence homology and utilization of energy [6, 115]. There are essentially five different families of efflux pump proteins in bacteria; ABC (ATP-binding cassette) transporters, SMR (small multidrug resistance) pumps of the drug/metabolite transporters (DMT) superfamily, MFS (major facilitator superfamily), RND (resistance nodulation division) family exclusive to Gram-negative bacteria and MATE (multidrug and toxic compound extrusion) pumps of multidrug/oligosaccharidyl-lipid/polysaccharide flippases (MOP) superfamily. The characteristics of each pump are described below.

ABC Transporters

The ABC-type efflux pumps are symporters that transport a diverse array of substrates where extrusion occurs by energy derived from ATP hydrolysis. In these transport proteins, a transport channel is made by two transmembrane domains (TMDs) that are composed of alpha helical membrane spanning regions [130]. These TMDs show variability in different ABC pumps. TMDs work in conjunction with two highly conserved nucleotide-binding domains (NBDs) which bind to ATP and hydrolyse it to produce energy facilitating the transport process [130]. NBDs are comprised of Walker A and B motifs characteristic of all

the ATP-binding proteins. They also carry within them a signature motif (LSGGQ) unique to the transporter family of ATP-binding proteins. The Vga (E) variant protein in *Staphylococcus* spp. conferring resistance to pleuromutilins, lincosamides and streptogramin A and LmrA in *Lactobacillus* spp. are members of this family of transport proteins [6, 131]. Though the role of ABC pumps in drug resistance in eukaryotes has been deciphered (P-glycoprotein), their role still remains dubious in prokaryotes in terms of MDR. They have been shown to play a major role in the MDR of cancer cells [6].

MFS Pumps

MFS comprises a diverse family of secondary transporters. These pumps could be uniporters mediating transport of their substrates without parallel ion movement, symporters that carry out transport with concomitant movement of ions in the same direction as the substrate or they could be antiporters where the substrate transport is coupled with the movement of ions in the opposite direction [132]. MFS pumps comprise of 250-400 amino acid residues spanning 12-14 transmembrane segments (TMS) [133]. These pumps play an active role in antibiotics export and belong to drug/ proton antiporters (DHA) superfamily which is subsequently divided into three major sub-families *i.e.* DHA1, 2 and 3 [134]. While DHA1 and 2 are widely distributed in prokaryotes and eukaryotes transporting a large array of drugs, DHA3 are exclusively found in bacteria and are specifically involved in antibiotic transport. DHA3 pumps have been shown to transport macrolides and tetracycline and have been reported from Gram-positive as well as Gram-negative bacteria. NorA of *S. aureus*, PmrA of *Streptococcus pneumoniae* and EmeA of *Enterococcus faecalis* are some of the well studied MFS pumps [135-137].

SMR Pumps

SMR family of pumps are prokaryotic transport systems consisting of homodimeric or heterodimeric structures having 100-120 amino acid residues with four transmembrane α-helices [138, 139]. Energy in the form of PMF provides the driving force for drug transport. There are very few reports of members of this family of transporters conferring resistance to antibiotics. EmrE

of *E. coli* [140] and AbeS of *Acinetobacter baumannii* [141] are members of this family of transporter proteins.

RND Pumps

Efflux pumps of this class have been shown to play a significant role in conferring MDR in Gram-negative bacteria [129]. They are proton antiporters that use the proton gradient across the membrane to power efflux exchanging one proton for one drug molecule. They have tripartite organization where the efflux pump in cytoplasmic membrane works in conjunction with a periplasmic adapter protein and an outer membrane channel. This allows any drug/substrate of these pumps to be expelled directly outside the cell rather than in the periplasmic space. MexAB-OprM of *P. aeruginosa* and AcrAB-TolC of *E. coli* are the most studied transporters of this family of efflux pumps where MexB and AcrB are the RND pumps, OprM and TolC are the outer membrane proteins and MexA and AcrA are the periplasmic adapter proteins. Some of these RND pumps such as AcrB display wide substrate specificity and efflux out not only the antibiotics but also dyes, detergents and solvents. The genes encoding the RND efflux pumps are organized as well regulated operons and the outer membrane proteins may or may not be co-located with the other genes in the operons [113].

MATE Pumps

MATE family of efflux pumps includes functionally characterized multidrug efflux systems. The prototype member was NorM from *V. parahaemolyticus* [142]. Subsequently, several homologues from other closely related bacteria were reported that function by a drug:Na^+/H^+ antiport mechanism. These include a putative ethionine resistance protein of *S. cerevisiae*, a cationic drug efflux pump from *Arabidopsis thaliana* and the DNA damage-inducible protein F (DinF) of *E. coli* [121]. These proteins are ~450 amino acid residues long and exhibit 10-12 putative TMS. They arose by an internal gene duplication event from a primordial 6 TMS-encoding genetic element. The family is conserved in bacteria, archaea and all eukaryotic kingdoms and includes hundreds of functionally uncharacterized but sequenced homologues [143]. MATE family proteins exhibit similar topological features as MFS but form a distinct group due to relatively low

degree of homology at the level of amino acid residues. NorM from *E. coli* and *V. cholerae*, YdhE from *E. coli* and VFH and VFD from *V. fluvialis* are some of the examples of MATE pumps [115, 142, 144, 145]. These are the most recently identified group of efflux pumps assessed for their role in multidrug resistance. They transport fluoroquinolones, aminoglycosides, and cationic dyes.

Regulation of Efflux Pump Expression

Complex regulatory operons/mechanisms control the expression of proteins that are responsible for influx and efflux of the drugs thus maintaining the intracellular concentration of these compounds. The *mar* (multiple antibiotic resistance) locus in *E. coli* is known to accumulate mutations leading to the acquisition of MAR phenotype. While the *marR* gene encodes a repressor for this operon and accumulates mutations, the *marA* gene product is known to activate a plethora of genes related to antibiotic resistance and oxidative stress. MarA regulator controls the expression of porins and efflux pumps and the expression of this regulator is in turn controlled by some antibiotics. For example, imipenem that is not a substrate of an efflux pump in *Enterobacter aerogenes* results in expression of the gene encoding *marA* regulator and alters the permeability of the membrane for some other antibiotics leading to increased resistance towards chloramphenicol, quinolones and tetarcyclines [146]. Therefore, even though *marRA* does not encode a multidrug efflux system/porin, the *marRAB* locus confers resistance to compounds like tetracycline, chloramphenicol, fluoroquinolones, nalidixic acid and rifampin because it controls the expression of other loci important in mediating drug resistance *e.g.* the porin OmpF and *acrAB* gene for AcrAB efflux pump [121].

Role of Porin and Efflux Pump Mutations in MDR

The bacterial cell envelope acts as a semipermeable membrane composed of porins and efflux pumps. These porins and efflux pumps together help in determining the effective concentration of antibiotics inside a bacterium. Thus, presence of their number determines the membrane permeability and in turn decides the fate of the bacterium *i.e.* antibiotic susceptible or resistant microorganism. Innate resistance to various antibiotics in many microbes like

P. aeruginosa can be attributed to low membrane permeability. Therefore, susceptibility of a bacterium is a direct correlation of mutations affecting the expression and function of porins as well as efflux pumps [134].

Porins are proteins that act as channels for uptake of nutrients and a variety of other compounds including antibiotics. These were first reported from *E. coli* [147]. Mutations in porins could have variable effects on their structure and functions such as complete loss of porins, change in the size of these channels or their conductance and their altered expression. These consequently result in reduced uptake of antibiotics into bacterial cells leading to decrease in cell death. Such porin-related mutations have been shown to accentuate resistance towards various antibiotics. Loss of OmpF porin from *E. coli* was shown to confer resistance towards β-lactams and subsequently role of porins in MDR was established in other bacterial species [148]. Loss of a porin could be due to deletion in its gene, a point mutation resulting in translation termination, an insertion element disrupting the porin gene and mutations in the regulatory genes or the promoter regions [149, 150]. As described above, mutations may affect the expression of porins, but some of the mutations could also alter the function of porin channels without affecting their expression. For example, a mutation Asp116Ala in the putative antibiotic-binding site of OmpU porin in *V. cholerae* was shown to reduce uptake of cephalosporins and increase the bacterial resistance towards these drugs [151]. Apart from the antibiotic-binding site, the scaffold provided by other parts of porin also plays a vital role in its function. The imipenem binding sites of OprD porin of *P. aeruginosa* requires the intact scaffold provided by loops 2 and 3 [152], while regulation of porin size is achieved by external loops 5, 7 and 8 [153].

As described above, similar to porins, efflux pumps are also the major determinants of antibiotic concentration inside a bacterium. Therefore, mutations in these efflux pumps would affect the resistance status of the host bacterium. These mutations could be localized in the structural genes, their promoter regions or in the genes encoding regulatory proteins [154, 155]. The clinical isolates of *P. aeruginosa* obtained from cystic fibrosis patients were found to have mutations in the MexY structural gene. The resulting amino acid change increased the efflux

activity of this RND pump leading to resistance towards many antibiotics that were the substrates for MexXY pump [154].

AN INTERESTING EXAMPLE OF CONTRIBUTION OF DIFFERENT MECHANISMS: RESISTANCE TO QUINOLONES

Resistance mechanisms for different classes of antibiotics vary. β-lactamases inactivate the β-lactam group of antibiotics by hydrolyzing the lactam ring. Aminoglycosides are inactivated by the enzymatic modification of the antibiotics by phosphoryl, adenyl, or acetyl groups. Chloramphenicol is rendered ineffective by the modifying enzyme chloramphenicol acetyl transferase that adds an acetyl group to the antibiotic [5]. Apart from these mechanisms specific for a particular class of antibiotics, all the antibiotics can also be made ineffective by combination of several other general mechanisms such as mutations in porins or efflux pumps. In this section, quinolone class of antibiotics has been discussed in details *vis-à-vis* their structure and different drug resistance mechanisms that operate in synergy to decide the quinolone resistance phenotype.

The emergence of drug resistance, since the introduction of antibiotics had made the humans to continuously seek for new antibiotics which overcome the evolving pathogens. This paved way for the development of various synthetic and semi-synthetic antibiotics with improved efficacy and extended target coverage. Among different synthetic antibiotics like sulfonamides, quinolones and oxazolidinones, quinolones gained popularity because of their wider application, broader spectrum of activity and drug safety [156-158]. In addition, it was hypothesized that resistance would not be mounted against the synthetic compounds which are not seen by bacteria in natural ecosystem. In this section, the focus would be on quinolone class of antibiotics and the bacterial resistance machinery against these widely used synthetic antibiotics.

Quinolones are a class of antibiotics which specifically kill bacteria by inhibiting the synthesis of nucleic acids. The parent compound of quinolones, nalidixic acid, was derived from the antimalarial drug chloroquine in 1962 [159, 160]. Nalidixic acid is composed of naphthyridine ring having ethyl and methyl group attached to its N1 and C7 position respectively along with keto and carboxyl group attached

to its C4 and C3 positions respectively (Fig. **3**). Though it had narrow spectrum antibacterial activity, it was widely used for urinary tract infections and diarrhoea until the introduction of broad spectrum fluoroquinolones [161, 162]. Fluoroquinolones were derived from nalidixic acid by the introduction of a fluorine atom at C6 position of naphthyridine ring and also replacing the N8 by a carbon atom (Fig. **3**) [163].

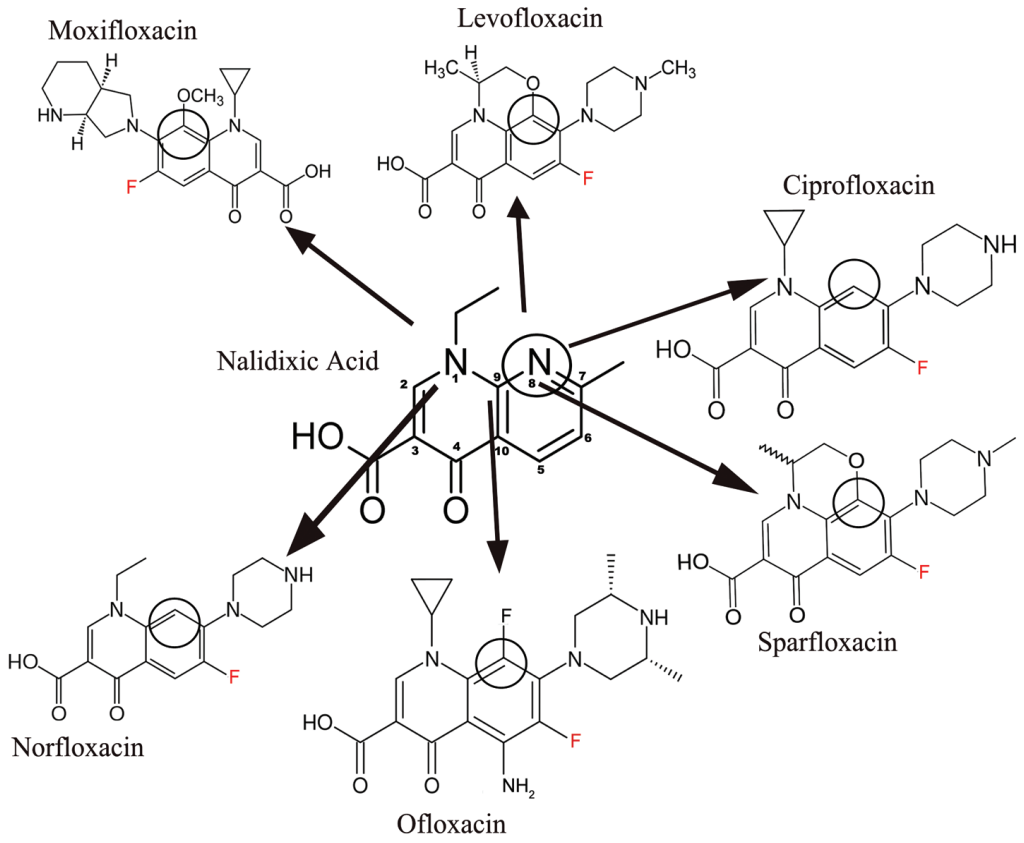

Fig. (3). Structures of nalidixic acid and some of the fluoroquinolones. Derivation of fluoroquinolones from parent quinolone (nalidixic acid) by the addition of a fluorine atom (shown in red) at C6 position of naphthyridine ring and by replacing N8 by a carbon atom (shown inside circles)

All the available quinolones are grouped into four generations based on their spectrum of activity [159, 164]. The first generation quinolones like nalidixic acid and cinoxacin were active against aerobic Gram-negative bacteria, but showed

little activity against aerobic Gram-positive bacteria or anaerobes. The second generation quinolones include fluoroquinolones such as norfloxacin, ciprofloxacin, ofloxacin, lomefloxacin and levofloxacin which showed broader Gram-negative spectrum and moderately increased Gram-positive spectrum. Piperazine moiety at C7 position of quinolone nucleus is the general feature of second generation quinolones which increased activity against *P. aeruginosa*. Sparfloxacin, gatifloxacin and grepafloxacin fall under third generation quinolones that showed greater potency against Gram-positive organisms. A superior coverage against pneumococci and anaerobes were achieved by fourth generation quinolones like trovofloxacin, moxifloxacin, gemifloxacin and garenoxin [159, 160, 164]. Although many of the quinolone derivatives were removed from the market because of some safety concerns, remaining drugs which are proved as safe, gained widespread use due to its favorable pharmacokinetics and broad antimicrobial spectra [159].

Quinolone Targets and Their Action

Quinolones target two enzymes DNA gyrase and topoisomerase IV which are essential for the vital activities of bacterial cell like DNA replication, transcription, recombination and repair. DNA gyrase is a special type II topoisomerse which is present in prokaryotes only and it introduces negative supercoiling rather than removing it [165]. During replication and transcription, the double stranded DNA are separated by helicase action that causes a reduction in linking number which in turn results in positive supercoiling in front of the replication fork. DNA gyrase removes the positive supercoiling ahead of the replication fork by introducing negative supercoiling and thereby renders an uninterrupted movement of replication fork. The topoisomerase IV enzyme is known for unlinking of daughter chromosomes after replication (decatenation). DNA gyrase is a tetrameric protein having two A (GyrA) and two B (Gyr B) subunits which wrap DNA into a positive supercoil. The active site of the enzyme consists of tyrosine, which breaks the phospodiester bond of a duplex DNA and forms phospho-tyrosine bond through its hydroxyl group. The other end of the DNA is also held by the enzyme to form a protein bridge. Another region of DNA is passed through the nick created by DNA gyrase and then the nick would be resealed [162, 165]. Both DNA gyrase and topoisomerase IV create double strand

break and allow double strand passage and they require ATP for this action. The main difference in the action of these two enzymes is that gyrase wraps DNA around itself while topoisomerase IV does not which may lead to the functional differences [162].

It was evidenced that quinolones form a ternary complex with topoisomerase enzymes and DNA and cease the enzyme activity. This complex is called as cleaved complex as it contains broken DNA. As discussed above, type II topoisomerase enzymes cause a double strand break and allow the passage of another duplex through the nick. But the reversible binding of quinolones with the DNA-enzyme binary complex allows the enzyme to generate the double strand break only and not the passage of another duplex [166, 167]. Quinolones were found to interact with both the DNA and the enzyme in the cleaved complex. The drugs, with the help of aromatic rings stack against the DNA bases (-1 and + 1 base pairs) at the site of cleavage and thus cause a misalignment of DNA at both the sides of the break which eventually prevents the religation of the cleaved DNA. Similarly, helix-4 of the GyrA or ParC which harbors quinolone resistance determining region (QRDR) of the enzymes seems to interact with drug [168-173].

Though binding of quinolones to the enzyme-DNA complex leads to inhibition of nucleic acid synthesis, it does not cause lethality to the cells. The binding of quinolones just causes a bacteriostatic action as the formation of cleaved complex is reversible. The lethality to the cell is caused as a result of chromosome fragmentation and cell death induced by ROS [174]. The chromosome fragmentation occurs in two ways, one is protein synthesis-dependent as it involves proteases or nucleases in releasing DNA breaks from the cleaved complex and the other is protein synthesis-independent where quinolones facilitate the dissociation of gyrase subunits and release the double strand break [175-177]. The former pathway can be inhibited by chloroamphenicol and so the first generation quinolones like nalidixic acid fail to kill the cells in presence of chloroamphenicol whereas second generation quinolones like ciprofloxacin are not influenced by it [162, 174, 178]. The chromosome fragmentation eventually triggers the accumulation of highly toxic ROS which amplifies the lethal action of the drug ultimately causing the cell death [179-181].

Quinolone Resistance Mechanisms

Development of resistance towards quinolones started right with the introduction of first generation quinolone, nalidixic acid in 1962. The increased use of this drug led to the increased development of resistance [182]. Earlier it was believed that target mutations and efflux pumps were the possible mechanisms of quinolone resistance. Later that belief was disproved due to discovery of various molecular factors like target-protecting proteins, quinolone-modifying enzymes and efflux pumps [182, 183]. Quinolone resistance could be mediated by either chromosome-borne or plasmid-borne genetic elements. The chromosome-borne genetic factors involve, (i) mutations in the DNA gyrase (*gyrA* and *gyrB*) and (or) topoisomerase IV (*parC* and *parE*) genes, (ii) chromosomal efflux pumps genes and (iii) chromosomal quinolone resistance (*qnr)* genes. The plasmid-mediated quinolone resistance (PMQR) has also been described with three mechanisms, (i) a quinolone-resistance/topoisomerase-protection mechanisms encoded by the *qnr* genes, (ii) a ciprofloxacin-modifying enzyme encoded by *aac(6')-Ib-cr* gene [184]; and (iii) plasmid borne-efflux pumps [185, 186]. These PMQRs are transferable traits and also play a main role in quinolone resistance. In the following sections, all these mechanisms are discussed in detail.

Mutations in the Topoisomerase Genes

Mutations in the genes of topoisomerase enzymes are the main cause of quinolone resistance. Such spontaneous mutations occur as a result of replication error in a bacterium, at rates as high as 1 in 10^6 to 1 in 10^9 [187]. A mutant bacterium having alteration in topoisomerase enzymes could withstand antibiotic stress and evolves as a resistant bug. Mutations tend to cluster in regions called the QRDRs of subunits of DNA gyrase or topoisomerase IV which results in reduced drug affinity of those enzymes [161, 182, 188, 189]. Mutations in *gyrA, gyrB, parC* and *parE* genes have variable effects on MICs in different species of bacteria. In Gram-negative bacteria, high level quinolone resistance is mainly due to the mutations in the genes encoding gyrase subunits, *gyrA* and *gyrB* (mainly in *gyrA*), whereas mutations in genes encoding topoisomerase IV subunits *parC* and *parE* are prevalent in Gram-positive bacteria [183]. Mutations conferring resistance typically occur in stepwise manner. Generally, the initial mutation occurs in *gyrA* (in case of Gram-negative bacteria) or

parC (in case of Gram-positive bacteria) genes [161, 162]. The first mutation helps to select the bacteria in quinolone stress by reducing the susceptibility of topoisomerase to the drug and allows accumulation of more mutations in the subunits of same or other target enzyme. The accumulation of multiple mutations in the drug target facilitates the development of high-level resistance to quinolones in bacteria [161, 162, 182, 190]. The type of bacterial species and the kind of quinolone used determine the order in which mutations occur and the quantum of change in MIC [191]. Clinical failure of quinolones can occur as a result of many such bacterial mutations. The mutations at amino acid positions 83 and 87 of GyrA and positions 80 and 84 of ParC have been reported as a cause for reduced susceptibility of bacteria towards quinolones [9, 87, 162, 174, 192, 193]. The substitution of hydrophilic amino acid (serine) by hydrophobic residues (leucine or isoleucine), or substitution of acidic residue (aspartic acid) by basic amino acid (asparagine) or substitution of negatively charged amino acid (glutamic acid) by positively charged residue (lysine) are the well documented substitutions which reduce the susceptibility of the target enzymes to quinolones. These residues are known to interact with the drug at quinolone binding pocket (QBP), the region where quinolones interact with both QRDR of enzyme and cleaved DNA [194]. The alteration in the residues of QRDR of topoisomerase enzymes causes a conformational change in QBP, which eventually prevents the binding of drug in the pocket [194].

Generally, these mutations occur as a replication error prior to the antibiotic exposure and at the time of antibiotic pressure, it helps the cells to resist the drug beyond the concentration required to kill wild type cells. The wild type cells fail to form colony at or above MIC. The selective enrichment of resistant mutants occurs only above MIC and the concentration where the colony recovery of mutants ceases is called mutant prevention concentration (MPC). Additional mutation is required for the bacteria to withstand the concentration beyond MPC, which is a rare phenomenon. So the drug concentration range which favors the selection of mutants, between MIC and MPC is called Mutant selection window [195]. As described above, the resistance conferring mutations occur in stepwise manner and at each step of acquisition of mutations, the values of MICs and

MPCs increase [196, 197]. In other words, selection window increases at each step of mutation acquisition. Hence, mutant selection window is important to optimize antimicrobial dose regimens and to avoid the emergence of resistant mutants [195].

Efflux Pumps (Chromosome- and Plasmid-Borne)

The second resistance mechanism involves expression/over expression of efflux pumps that transport quinolones and other antibiotics out of the cell. Efflux pumps are ubiquitous and are encoded either by chromosomal genes or by genes associated with mobile genetic elements. These genes are responsible for intrinsic resistance under constitutive expression and cause low to moderate level of quinolone drug resistance under induced or activated conditions [161]. Mutations that occur in the regulatory elements of efflux pumps lead to overexpression of pumps which ultimately causes increased efflux activity [198-201]. Quinolone-specific efflux pumps have been reported and characterized (like SmrA, PmrA, NorA, NorM, PmpM, AcrB, VcrM, VcmA, BmrA, MepA, VCH and VFH) from different bacterial species [115, 145, 161, 202]. The MATE family efflux pumps like NorM, VCH, VFH are known to effectively efflux out hydrophilic quinolones like norfloxacin, ciprofloxacin and ofloxacin and not the hydrophobic quinolones such as sparfloxacin, nalidixic acid and moxifloxacin [145, 190, 202]. Resistance due to efflux pumps causes only low to moderate level of resistance but they favor the emergence of resistance mutants by rendering the surviving ability to the cells at suboptimal concentration of antibiotics [125, 161,190].

Two plasmid-mediated quinolone transporters (OqxAB and QepA) have been described [117, 186, 203-206]. The presence of *qepA* in *Enterobacteriaceae* and *Vibrionaceae* was reported from different parts of the globe [203, 207-209]. QepA is a 511-amino acid protein belonging to MFS transporters and shown to efflux out mainly norfloxacin, ciprofloxacin, nalidixic acid and also other compounds like erythromycin, acriflavine and ethidium bromide. The RND family pumps OqxAB confer resistance to Olaquindox (a quinoxaline derivative), nalidixic acid and ciprofloxacin [204-206, 210, 211].

Qnr Genes (Chromosome- and Plasmid-Borne)

Qnr proteins belong to pentapeptide repeat family and are capable of protecting DNA gyrase from quinolone action. These proteins are characterized by five semi-conserved tandem repeat motifs represented by [Ser, Thr, Ala or Val] [Asp or Asn] [Leu or Phe] [Ser, Thr or Arg] [Gly] [210-212]. Qnr proteins consist of two domains of pentapeptide repeats separated by a single amino acid, usually glycine [211, 212]. Qnr acts by protecting DNA gyrase and topoisomerase IV from quinolones by binding to the enzyme prior to the binding of DNA [213]. These proteins mimic the structure of DNA and they compete with the DNA for enzyme binding. As Qnr occupies the DNA binding site of the enzyme, it prevents the binding of DNA to the enzyme and hence the number of enzyme-DNA complexes, the target of quinolone is reduced. As a result of this, the formation of cleaved complex is minimized and eventually cells are protected from the lethal action of quinolones [214, 215]. Several *qnr* genes have been widely reported from *Enterobacteriaceae* and *Vibrionaceae* families and *Vibrionaceae* family was found to be a possible reservoir for Qnr-like quinolone resistance determinants [216]. So far, five families of *qnr (qnrA, qnrB, qnrC, qnrD* and *qnrS*) have been reported in plasmids among the bacterial species [210, 211]. Both *qnrA* and *qnrS* genes encode 218-amino acid proteins and exist as seven and nine alleles respectively. The *qnrC* gene encodes 221-amino acid protein whereas *qnrD* encodes 214-amino acid peptide and the allelic forms of these two genes were not reported so far. The 214-amino acid protein encoding *qnrB* genes are found to exist in seventy three allelic forms [http://www.lahey.org/qnrStudies/] [210, 211, 217].

Genes for pentapeptide repeat proteins with sequence similarity to plasmid-borne Qnr proteins have been reported on the chromosomes of both Gram-positive and Gram-negative bacteria [211]. The chromosome borne *qnr*-like genes were largely reported in *Vibrionaceae* family (*V. vulnificus*, *V. fisheri* and *Photobacterium profundum*) [216, 218, 219]. *qnrVC1* isolated from *V. cholerae* O1 from a cholera epidemic in Brazil was found as gene cassette in chromosomal class 1 integron [25]. The origin of these *qnr* genes is likely to be the chromosomes of aquatic environmental organisms. Accumulation of quinolones in the environment enriched the organisms having *qnr* genes and that acted as a

reservoir from where other pathogenic organisms acquired these genes [210, 211]. A recent study has shown that the *qnrVC5* gene of chromosomal origin was associated with a transferrable plasmid from a clinical isolates of *V. fluvialis* [9]. This study showed that these genes are of chromosomal origin and circulating among the bacterial community through plasmids. Qnr proteins are found to have functional similarity with other well-studied pentapeptide repeat proteins like MfpA and McbG having amino acid identity 18.9% and 19.6% respectively with that of QnrA. The organisms producing the microcin B17, a topoisomerase poison, also produce McbG to protect its own DNA gyrase from the toxic effect of microcin B17 [220, 221]. So it is evident that the pentapeptide repeat proteins are generally evolved for protecting topoisomerases from the naturally occurring toxins that inhibit those enzymes and also evolved to protect the enzymes from other topoisomerase inhibiting agents like quinolones. Though higher-level *qnr*-mediated resistance has not been reported, they could help the isolates to attain clinical breakpoint of resistance in combination with the other mechanisms [8, 9, 193, 222].

Ciprofloxacin Modifying Enzyme (AAC (6')-Ib-Cr)

AAC(6')-Ib-cr, a variant aminoglycoside acetyl transferase, capable of reducing ciprofloxacin activity in addition to modifying aminoglycosides, is carried on plasmids and more prevalent than Qnr proteins [184]. This enzyme has acquired the ability to inactivate quinolones (ciprofloxacin and norfloxacin) by N-acetylating the amino nitrogen on its piperazinyl group. Two amino acid changes (Trp102Arg and Asp179Tyr) rendered the ability of the enzyme to additionally inactivate quinolones apart from aminoglycosides. The effect on MIC by AAC (6')-Ib-cr is less than that conferred by Qnr protein and the drug spectrum covered by this enzyme is also small (ciprofloxacin and norfloxacin only).

It is reasonably well documented that quinolone resistance in pathogenic bacteria through intrinsic and acquired traits causes a major health problem. The synergistic action of all these chromosomal and plasmid-borne factors helps the pathogen acquire higher-level resistance towards quinolones, as described by many researchers [8, 222-224].

OTHER FACTORS RESPONSIBLE FOR MDR

There are many other social/clinical/policy-related factors that lead to the emergence and dissemination of antibiotic resistant bacteria at a particular geographical location. These include the indiscriminate use of antibiotics, poor surveillance systems for various epidemics/pandemics, absence of comprehensive and coordinated response by government in case of spread of a serious infection, lack of preparedness in terms of efficient diagnostics, prevention and therapeutic tools [3]. In addition to this, the pharmaceutical companies have lost interest in the development of new antibiotics as this research is no longer lucrative. As anti-infective drugs are taken for shorter times till the infection persists, the companies refrain from investments on these pharmacologically active agents as compared to blockbuster drugs for lowering the cholesterol levels, for hypertension, diabetes *etc*. which are taken for prolonged periods and mostly lifelong. Accordingly, the government funding in this area has also been diminishing. There has been a drastic cutdown on antibiotic discovery programmes [225]. This amounts to the use of same old antibiotics in clinics and hospitals leading to development of bacterial resistance against them.

ANTIBIOTICS INDUCE SOS RESPONSE LEADING TO THE EVOLUTION OF ANTIBIOTIC RESISTANCE MECHANISMS

Before we embark on our journey to understand various strategies to combat MDR bacteria, it must be understood that the challenge of a bacterial cell with an antibiotic is similar to an SOS response. A bacterium will resort to a series of changes to overcome the effect of a drug that challenges its existence. Any type of horizontal gene transfer through conjugation, transformation and transduction or any type of antibiotic challenge induces SOS response through events mediated by single-stranded DNA, RecA protein and LexA repressor [35]. The LexA repressor has been shown to bind the promoter of the integrase of an integron. RecA activation leads to autoproteolysis of LexA repressor that keeps the SOS regulon in the repressed state under normal conditions. During SOS, this repressor gets inactivated leading to the expression of a diverse array of genes that were repressed by LexA. Integrases are one such genes that gets activated during SOS leading to the expression of gene cassettes harbored by integrons [35]. Similar to

the integrons, the activation of SXT, their excision, integration or dissemination events are triggered during SOS response. The expression of SXT integrase requires two transcriptional activators, setC and setD which are in turn controlled by the repressor setR rather than LexA. During SOS response, when single-stranded DNA is bound to recA, setR concentrations are depleted which results in the activation of SXT integrases and formation of hybrid ICE elements [226]. This clearly shows that SOS response leads to reshaping of the bacterial genomes through integrons as well as ICE elements. The direct regulation of SOS response by antibiotics itself has also been described [227]. The regulation of expression of *qnrB2* (a quinolone resistance determinant) through SOS response is induced by ciprofloxacin in LexA/RecA-dependent manner. Even sub-inhibitory concentration of ciprofloxacin was found to cleave LexA repressor so that it was prevented from binding on the LexA binding site present in the promoter region of *qnrB2* gene [227].

DATABASES OR TOOLS RELATED TO ANTIBIOTIC RESISTANCE MECHANISMS

As described in earlier sections, genes related to various mechanisms and factors responsible for drug resistance have been compiled by various workers for easy referencing. Compilation of data in form of databases is freely available on web. These databases and tools help a researcher to share the knowledge and to systematically analyze their data in easier way. The databases encompassing various genetic elements especially integrons, ICEs, efflux pumps and quinolone resistance mechanisms have been described below.

INTEGRALL (http://integrall.bio.ua.pt)

It is a database and search engine for integrons, integrases and gene cassettes found in integrons. It is a web-based platform developed by microbiologists and computer scientists. Initially in the year of 2009, the database contained more than 4800 integron sequences out of which ~70% corresponded to uncultured bacteria and 27% belonged to γ-proteobacteria. Remaining 3% constituted integron sequences from ά-, β-, δ- and ε-Proteobacteria, Actinobacteria, Firmicutes, Cyanobacteria *etc*. Thus, though integrons have a broad host range, higher

occurrence of integrons has been reported in γ-proteobacteria. The database provides a public genetic repository for integron sequence data, their nomenclature, genetic contexts and molecular arrangements [228]. As of 31st December 2013, the database consists of 6777 entries of integrons, 1498 integrase genes, 8522 gene cassettes from 119 genera and 250 species.

Annotation of Cassette and Integron Data (ACID) (http://integron.biochem.dal.ca/ACID/login.php)

It compiles and annotates integron-integrase genes and non-coding cassettes-associated *attC* recombination sites and all publicly available sequence information regarding these genetic elements. Manually curated open access database information was used for automated detection, annotation of integrons and their gene cassettes. ACID enables future sequence data to be incorporated easily. This database allows its users to annotate and save their data. They can also send the data to curators for its addition to the main database. In the first version of ACID, 5622 gene cassettes and 471 integrase sequences have been documented and new sequences are continuously updated [229].

Repository of Antibiotic Resistance Cassettes (RAC) (http://www2.chi.unsw.edu.au/rac)

Archive of gene cassettes which include alternative gene names are made available by RAC databases. Information regarding gene cassettes help to determine new gene cassettes. The automatic annotation engine allows users to easily and accurately access and annotate cassette arrays in bacterial DNA sequences. It also provides a process for assignment of unique name for newly sequenced antibiotic resistance cassettes in mobile resistance integrons consistent with existing nomenclature systems [230]. RAC now has a conglomeration of 387 antibiotic resistance gene cassettes.

Integron Analysis and Cassette Identification (XXR) (http://mobyle.pasteur.fr/cgi-bin/portal.py?forms=xxr)

The program detects the *attC* sites of integron gene cassette arrays. The software utilises the data from previously known sequences of integrons to predict putative cassette structures [70].

Antibiotic Resistance Genes Database (ARDB) (http://ardb.cbcb.umd.edu)

The database consolidates most of the publicly available antibiotic resistance genes and provides a reliable annotation with rich information, resistance profile, mechanism of action, ontology, Clusters of Orthologus Groups of proteins (COG) and Conserved Domain Database (CDD) annotation [231]. It also provides external links to sequence and protein databases. The information provided by the database can be used for further identification of the resistance genes of newly sequenced genes, genomes or metagenomes. As of 31st December 2013, ARDB contains a total of 23,137 genes, 380 types of gene cassettes for 249 antibiotics from 1737 species and 267 genera. It also contains pre-annotated 2881 vectors/plasmids and 632 genomes conferring resistance to various antibiotics.

ICEberg (http://db-mml.sjtu.edu.cn/ICEberg/)

On 13th August 2011, this database was mainly dedicated to ICEs which incorporated in bacterial genomes. This is a PostgreSQL-based database; that facilitates resourceful knowledge for ICEs like their integrative conjugative machinery, putative ORFs, antibiotic drug resistance gene cassettes and virulence determinants [106]. ICEberg provides information about predicted as well as experimentally proved ICE-related data. As of 31st December 2013, the database has a collection of 460 ICEs.

Insertion Sequence [IS] Finder (www-is.biotoul.fr)

This is another database dedicated to Insertion Sequences [IS], a type of short DNA sequences that act as transposable genetic elements [232]. IS finder is the tool for IS elements which are found in various mobile genetic elements like bacteriophages, conjugative transposons, integrons, unit transposons, composite transposons and insertion sequences (ISs). IS finder allows researchers to have coherent nomenclature for IS. It also includes detailed information about the IS of the repository like its DNA sequences, ORFs, end sequences, target sites of these elements, their origin, distribution and bibliography. It also imparts knowledge about the updated comprehensive grasping and the phylogeny of ISs [232]. The latest updated database constitutes 4115 insertion sequences.

Pathogenicity Islands Database (PAIDB) (http://www.gem.re.kr/paidb)

PAIDB is a comprehensive database for all those reported genetic elements whose products are essential to the process of disease development [233]. These PAIs horizontally transfer among microbes and contribute to the evolution of pathogenicity. This database provides convenient graphical presentation for name, host strain, function, insertion site and associated GenBank accessions which are helpful for phylogenetic as well as bioinformatic analysis. The latest version of PAIDB (31st December, 2013) has 112 kinds of PAIs, 889 GenBank accessions, 2681 virulence genes and total 7842 ORFs from 497 pathogenic strains. Also it has 743 PAI-like regions from 115 pathogenic strains having atleast one PAI-like region and 259 PAI-like regions from 77 non-pathogenic organisms with unconfirmed pathogenicity.

A Classification of Mobile Genetic Elements (ACLAME) (http://aclame.ulb.ac.be)

ACLAME is a database which provides classification and collection of Mobile Genetic Elements (gene sequences and proteins) from phages, transposons and plasmids. This database was first released in 2004. At that time, database classified 5069 MGE-associated proteins from 119 DNA bacteriophages into over 400 functional families. This database is publicly accessible in which TRIBE-MCL, a graph-theory-based Markov clustering algorithm was used to classify MGEs and proteins. Different evolutionary database versions like 0.1, 0.2, 0.3 and 0.4 have been released, in which 0.4 is the latest version which contains 122154 proteins from 2326 MGEs of 811 host organisms. Proteins have been clustered into families that are as follows; Prophages with 6822 clusters, Plasmids with 18228 clusters, Viruses and prophages with 16057 clusters and Viruses with 11503 clusters with a total of 32919 clusters. Evolutionary Cohesive Modules (ECMs) were generated for phages, which share replication, lysis/lysogeny, DNA packaging, and head and tail morphogenesis with reticulate relationship. These ECMs, helpful in studying phylogenicity, are stored in the ACLAME database [234]. They are accessible on the web site through the MGE viewer, following the link 'ECM'. The advance 0.4 version of ACLAME database is now running under the PostgreSQL relational database management system (RDBMS) version 8.3 (http://www.postgresql.org) [235].

Type-2 Toxin-Antitoxin Loci Database (TADB) (http://bioinfo-mml.sjtu.edu.cn/TADB/)

It is a comprehensive database for type 2 toxin-antitoxin loci distributed in bacterial and archaeal genomes. This database contains unique compilation of both predicted and experimentally supported Type 2 TA gene pairs identified within 1240 prokaryotic genomes and details of over 240 directly relevant scientific publications [236]. TADB provides a web-interface, allowing users to view an entire genome's TA loci repertoire within the context of the whole replicon and to access individual pages dedicated to each TA locus pair, toxin and antitoxin as required. TADB allows researchers to gain insight into the cognate TA proteins that are either hypothesized or proven to play vital role in stabilization of horizontally acquired genetic elements. As of 31st December, 2013, 6757 TA loci found in 750 genomes have been organized into the 44 toxin-antitoxin domain pair groupings.

Transporter Classification Database (TCDB) (http://www.tcdb.org)

TCDB is an online, curated repository of comprehensive database containing sequences, classification, structural, functional and evolutionary information about transporters from various living organisms. Originally in 2006, the database was a congregative repository for factual information compiled from >10 000 references with ~3000 representative transporters and putative transporters classified into >400 families. The database categorizes transporters as Enzyme Nomenclature on Recommendations of the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology [237]. As efflux pumps are integral part of transporters, TCDB can be considered as a conglomerate of all known and putative efflux pumps. The database offers several different methods for accessing the data including step-by-step access to hierarchical classification, sequence information or TC (Transporter Classification) number and full-text searching. The database is based on functional ontology algorithm that facilitates powerful query searches and yields valuable data in a quick and easy way. The TCDB website also provides several tools specifically designed for analyzing the unique characteristics of transport proteins. TCDB is not only a repository of curated information about classifying

newly identified membrane proteins; it also serves as a genome transporter annotation tool. As of 31st December, 2013, it contains over 10000 published references of about 5600 unique protein sequences which are classified into over 600 transporter families based on the TC system.

Databases Available for Quinolone Resistance Genes

Since the discovery of first PMQR resistance gene *qnrA1* in 1998, a considerable number of *qnr* genes and other PMQR genes have been discovered and reported in last decade. The *qnr* genes reported from several parts of the globe lacked a standard nomenclature or numbering system which created a chaos. In order to avoid the ambiguity and to systematically name the *qnr* genes of varying sequences, Jacoby *et al*., attempted to frame the criteria based on which the genes could be classified [217]. They have created a database where all the newly found *qnr* alleles can be verified and numbered accordingly (http://www.lahey.org/qnr Studies/). It is having a collection of 71 alleles of QnrB as of 31st, December, 2013. Similarly, in order to find the *qnr* genes in a fragmented nucleotide sequence of metagenomic data set, a tool/software was developed by Systems Biology and Bioinformatics group of University of Gothenburg, Sweden [238] (http://bioinformatics.math.chalmers.se/qnr/index.html). They have developed this tool in order to understand the role of environment as reservoir of these kinds of genes and to focus on their routes of transfer.

STRATEGIES TO COMBAT THE PROBLEM OF MDR

From the above discussion, it might appear that the antibiotics are no longer the magic bullets that they were once thought to be. It also leads to an apprehension in community that we are at the end of antibiotic era. As described in the sections above, bacteria employ various tactics to keep them alive in the varying environments. An insight into these mechanisms can lead to the development of various strategies to circumvent the problem of MDR. The strategies could be antibacterial meant to kill the bacterium itself (whether antibiotic resistant or antibiotic susceptible) or they could be antivirulent. In antivirulent regimes, instead of attacking the vital life processes of bacteria (antibacterial), it is aimed to target the virulence factors elaborated by bacteria. This results in control of the

pathogenicity caused by these bugs. In the subsequent sections, the antibacterial, antivirulent and other general strategies are discussed in light of their use in combating the diseases caused by MDR pathogens.

Antibacterial Therapies

Some of the antibacterial therapies have been reviewed that target transcriptional regulators, toxin-receptor binding or replication initiation in the bacterial cell [225, 239]. Kibdelomycin, a novel inhibitor of type IIA topoisomerase of *S. aureus* has been proposed to control the bacteria as well as its drug resistance. This antibiotic is produced by a new member of the genus *Kibdelosporangium* and found to inhibit the ATPase activity of topoisomerase enzymes [240]. NXL 101, an inhibitor of topoisomerase IV was found to be effective against Gram-negative bacteria with mutations in the gyrase enzyme [225]. A large number of loci and protein-coding genes have been identified as drug targets to control bacterial growth [241, 242]. Some of the other antibacterial therapies would be described in the ensuing sections.

Phage Therapy

Phage therapy has been used to cure a large number of bacterial infections such as cholera, typhoid and plague [239, 243, 244]. MDR strains, where most of the antibiotics fail to work, could be killed by the phages. Phages kill the pathogenic bacteria reducing the toxin load that leads to the control of disease transmission. Especially interesting in this case are superficial infections easily accessible for phages. These infections include infected wounds in case of diabetes and burns. Either single phage drugs or phage cocktails could be used in such cases. The bottleneck in phage therapy is to find the phages that could treat the variant strains of the same pathogen like *S. aureus* and the apprehension that the phage resistant strains may emerge in due course of time. Phage therapy in humans is still a debatable issue for the fear of toxicity of phage-based drugs. Most of the phage therapy research has therefore been carried out in veterinary medicine and funding for its human application has been limited A phage enzyme used for dissolving anthrax bacteria has been shown to be effective in animal experiments [245]. A phage enzyme has also been used to dissolve *Listeria monocytogenes* in a highly

specific manner in order to keep cheese rinds free of the bacteria. This bacterium spreads through contaminated vegetables and dairy products and is harmful as it also multiplies in the refrigerator [245]. A phage preparation for *Xanthomonas campestris* infections in tomatoes is available for purchase in the US. Inspite of many bottlenecks described above, there have been some success stories of phage therapy in human trials [243]. The phages were reported to be effective in treating children with dysentery in Georgia in 1963. The phase I and phase II clinical trials for safety and efficacy of phages were reported in 2005 and 2009 respectively. In 2009 trial, phages were shown to be effective in treating chronic drug-resistant ear infections of *P. aeruginosa* [243].

Phage therapy has its own advantages and disadvantages, which are described below [245].

Advantages

a. Phages are highly specific for the bacterium they infect and therefore, they do not interfere with the growth of other microbiota and thus do not cause selection of antibiotic resistance traits in them. This means that the therapy would have no side effects like diarrhoea or secondary infections which are common during antibiotic treatments.

b. Phages are generally innocuous to humans and animals and therefore, they can also be used for combating harmful bacteria in fattening animals and food.

c. Bacteria that become resistant to one kind of phage do not acquire resistance to the other type of phage and therefore, are not invincible.

d. Since nature is full of phages, it is easier to find new phages as compared to finding new antiobiotics.

e. Phages could provide an inexhaustible supply of reagents that can rapidly evolve and can be genetically modified to meet the challenges of antibiotic resistant bacteria.

f. As described above, phage products like lysins can also be used to treat bacteria.

g. Phage therapy is effective for antibiotic resistant as well as sensitive bacteria.

Disadvantages

a. There is a paucity of data to support phage therapy in humans though it has been extensively tried in case of animals.

b. Resistance of bacteria to phages could again be a problem in successful phage therapy.

c. Phages could prove ineffective in case of infections caused by the bacteria that take refuge inside the human cells and therefore may be inaccessible to the phages that are larger in size as compared to the small drug molecules.

d. Phages injected in the bloodstream are recognized by human immune system as foreign and this elicits immune responses against these phages followed by their disposal outside the human body. Humoral and cellular immune responses generated against therapeutic phages could therefore compromise their efficacy.

e. Administration of therapeutic phages is more difficult than administration of antibiotics. Hence it requires special skills and training for the physicians.

f. A lot of research still needs to be done to assess the shelf life of phages.

Vaccines

Vaccination is another antibacterial strategy that would control bacteria irrespective of their resistance to antibiotics. Vaccination interrupts transmission of the causative organism and the communities may develop herd immunity. Vaccines could also be antivirulent for example passive immunization in case of diphtheria. Here, the antibodies neutralise the diphtheria toxin and save the patients. In case of tetanus, the antibodies bind to and neutralize the circulating

tetanus toxin leading to the survival of patient. Similarly, for prevention of cholera, oral cholera vaccines are used. In case of *V. cholerae*, the cholera toxin (CT), flagella, fimbriae and lipopolysaccharides have been shown to be the antigens involved in protective immunity. Though cholera pathogenicity has been attributed to CT, the protective immune responses have been shown to be antibacterial rather than antitoxic [246, 247]. The vaccine formulations for this pathogen involved recombinant B subunit killed whole cell (rBS-WC) vaccine and the live attenuated CVD 103-HgR vaccine. One of the vaccines recommended by WHO is Dukoral which consists of mixture of virulent *V. cholerae* cells belonging to both the classical and El Tor biotype and an inactive B subunit of cholera toxin. Whole cell killed bivalent vaccines mOrvac and Shanchol have resulted from a technology transfer from Sweden to India (Shantha Biotech, Hyderabad) and Vietnam (National Institute of Hygiene and Epidemiology, Hanoi). These vaccines are comprised of whole cell killed *V. cholerae* serogroups O1 and O139, and do not contain recombinant B subunit, due to which they do not need to be reconstituted in a buffer solution [248]. A vaccine not only limits the total number of cases but could also offer additional benefit of lowering the resistant bacteria. Pneumococcal conjugate vaccine has been shown to be highly effective in controlling resistant *Streptococcus pneumonia*e [249, 250]. Vaccine for *Haemophilus influenzae* has been successful in treating meningitis. Therefore, vaccination could prove to be an effective tool to contain the infections caused by MDR bacteria thus not only facilitating the containment and emergence of infections but also making organ transplantations and cancer chemotherapy more successful and safe.

Antivirulent Therapies-New Horizons

Quorum Sensing Inhibition (QSI)

Quorum sensing (QS) is a process that bacteria employ for ensuring that they are present in sufficient numbers to elicit a biological response to any external stimulus. QS involves generation of signal molecules upto certain threshold concentration and their recognition by the receptors in bacterial cell. This signal transduction leads to the expression of a diverse array of genes that are utilized by bacterium for various processes like biofilm formation, virulence, spore

formation, evasion of host defenses, swarming and motility, to name a few [251]. QS has been studied in many bacteria including *V. fischeri*, *V. harveyi*, *V. cholera*e, *S. aureus*, *P. aeruginosa* and *E. coli*. The signal molecules produced during QS are called autoinducers (AIs) and there are many types of QS systems known in microorganisms based on the kind of AI employed in each [251, 252]. For Autoinducer type 1 (AI-1) system, N-acyl homoserine lactone (AHL) class of molecules act as signal molecules. These molecules are composed of a homoserine lactone (HSL) ring with an acyl chain that varies in the chain length, the degree of saturation and the number of oxygen substitutions. Though AHLs with small fatty acid chains can freely diffuse through the bacterial cell membrane, AHLs with long fatty acid side chains require efflux pumps to permeate outside the cell. AI-2 system was discovered in marine bioluminescent bacteria *V. harveyi*. It utilizes a receptor kinase network and the signal molecule made up of complex, multi-ringed, cyclical furanosyl molecules containing a boron atom. These interconvertible furanosyl molecules are derived from spontaneous cyclisation of DPD (S-4, 5-dihydroxy-2, 3-pentanedione) due to high reactivity of its 2, 3-dicarbonyl motif. Bacteria use AI-2 signals from other bacterial species to hijack their signal system and AI-2 is therefore considered as a mode for inter-species communication while AI-1 is used as intra-species communication. AI-3 is composed of two component receptor kinase intracellular signaling complex where the signal molecule is probably similar to catecholamines. AI-3 is also involved in interspecies and inter-kingdom communication [253]. QS in Gram-positive bacteria utilizes short cyclical autoinducing peptides (AIPs) as signal molecules. These AIPs are synthesized as propeptides that are further modified before being transported out of the bacterial cell by transport systems [254]. *S. aureus* is divided into four specificity groups based on the identity of AIP [255]. This system is referred to as accessory gene regulator system (agr) in *Staphylococci* and as *Enterococcus faecalis* regulator system (frs) in *Enterococci* [256, 257]. *Agr* locus consists of two operons, RNAII and RNAIII. While RNAII constitutes *agrA* to *agrD* genes involved in the synthesis of AIP system, the transcribed product of RNAIII functions as regulatory RNA sequence controlling genes of the virulome. Apart from these four QS systems described above, other systems are also known to act in synergy with these systems and utilize the signal molecules like diketopiperazines (DKPs)

and quinolone signals (PQS) in *P. aeruginosa* [251, 253]. In *V. cholerae*, the CAI-1 (Cholerae Auto Inducer-1) system comprising of (S)-3-hydroxytridecan-4-one, acts in synergy with AI-2 pathway [258].

QSIs, also called quorum quenchers (QQ) are attractive alternatives for inhibiting QS and its related processes to control not only the MDR bacteria but also different strains and serogroups of the pathogens [259-262]. QSIs do not threaten bacteria with life-or-death situation and therefore do not suffer from the problem of resistance of bacteria against them. QSI also offers the advantage of high specificity as QS is only found in bacterium and not in the human host. These inhibitors could either be natural or man-made and manifest their activity by interfering with any of the processes involved in QS. These could be:

a. Generation of the signal molecule/AI.

b. Activity of the AI.

c. Detection of AI by its receptor.

The QS systems and QSIs have been extensively reviewed and can be referred for more details [251, 253, 259-268]. Furanones are one of the most well studied QSIs. The natural compound (5Z)-4-bromo-5-(bromomethylene)-3-butyl-2(5H)-furanone also called furanone 1 has been shown to inhibit swarming and biofilm formation in *E. coli* at concentrations non-inhibitory to planktonic growth [269]. AHL as well as AI-2 based QS systems are inhibited by furanones as these compounds are structural mimics of QS signals (lactones and tetrahydrofuran rings) in both these QS systems [269]. The enzymes AHL lactonase, AHL-acylase and paraoxonases (PONs) degrade the QS signals by either hydrolyzing the lactone ring of AHL, by hydrolyzing the amide bond of AHLs producing the fatty acid component and the homoserine lactone respectively. Small molecule like triclosan inhibits enoyl-ACP reductase required for synthesis of an intermediate in AHL biosynthesis [270]. Another molecule closantel inhibits histidine kinase sensor [271]. Some compounds have been designed that are analogues of the signals generated during AI-1 or AI-2 [272, 273]. They compete with native signals for binding to the receptor thus inhibiting QS. For example, library of HSL

analogues has been screened for AHL inhibitors and the QSI compounds found by this method inhibited the expression of Green Fluorescent Protein (GFP) by 50% [274, 275]. Boronic acids and DPD analogues have been used as AI-2 antagonists [276-279]. Garlic extract was used as QSI in a first human clinical trial with 26 cystic fibrosis patients. The extract was shown to improve the lung function, weight and symptom score [280]. A recent study has shown that a sulfur-rich compound from garlic extract called ajoene is involved in QSI [281]. Synthetic peptides, antibodies and antibiotics have also been used in several cases as QSIs. Synthetic AIP targeting all four AIP-types of *S. aureus* were designed as universal inhibitors [282]. As described above, as RNAIII product plays a regulatory role in mediating quorum sensing and biofilm formation in staphylococci, an RNAIII-inhibiting peptide was shown to inhibit the *agr*-mediated biofilm formation in drug resistant *S. epidermidis* thus acting as a QSI [283]. Antibodies generated against QS signals can also be used as QSIs as they would block the QS signal and its activity. Antibodies against AI-1 signal molecules have been shown to disrupt QS and render protection in mice against lethal lung infection by *P. aeruginosa* [284]. Many antibiotics like macrolides, ceftazidime, ciprofloxacin, azithromycin have been used to inhibit QS in *P. aeruginosa* [285, 286]. This indicated that antibiotics also interfere with the process of QS and biofilm formation thus directly influencing bacterial virulence.

The advantage of QSI is that they do not kill the pathogen directly but act in synergy with the antibiotics and host immune system to control the bacterial virulence. QSIs have been shown to be useful in controlling plant pathogens, nematodes and pulmonary infection in mice models [251]. Apart from controlling the virulence of the bacteria, they offer additional advantages like softening of the bacterial biofilms to make them susceptible to antibiotics and the host immune system.

For anti-virulence strategies, one could also think of using inhibitors of the toxin receptor as a therapeutic agent [239]. Also, molecules like virstatin have been tested for controlling the virulence and intestinal colonization of *V. cholera*e [287]. Virstatin acts by inhibiting the transcriptional regulator ToxT and downregulates the expression of cholera toxin and toxin corregulated pilus.

Efflux Pump Inhibition

Efflux of antibiotics to decrease the intracellular concentration of these drugs by efflux pumps has been a major determinant of drug resistance exhibited by the bacteria. Efflux pump inhibition has been pursued as a promising approach to restore the efficacy of those antibiotics that are substrates for the efflux pumps [6, 288, 289]. EPIs have been used as adjuvants in antibiotic therapy and also as diagnostic tools for detection of antibiotic resistance due to the efflux pumps. As these pumps have been shown to be crucial for bacterial survival, virulence and pathogenicity, EPIs could be used to control the bacterial virulence [113, 115]. A variety of EPIs have been derived from natural sources, screening of libraries of chemical compounds and secondary evaluation of current therapeutics [288, 289]. For achieving inhibition of efflux pumps, the strategies devised have been described below along with the examples of EPIs that have been designed based on that strategy:

Designing New Antibiotics or Modification of the Existing Antibiotics in Such a Way that they are Refractory to Recognition by Efflux Pumps

Glycylcyclines and ketolides have lower affinity for the efflux pumps. Tigecycline bypasses MFS pumps specific for tetracycline [290]. Telithromycin bypasses MefA/E and AcrAB systems [291]. Levofloxacin, moxifloxacin and gatifloxacin are not affected by NorA and PmrA efflux pumps [6].

Interfering with the Assembly/Functioning of the Efflux Pumps

In case of tripartite efflux pumps like RND pumps, blocking the outer membrane channel can lead to the inhibition of pump activity [292, 293].

Interfering with the Regulatory Steps in the Expression of the Efflux Pump Genes so that Expression of the Efflux Pumps Declines

The membrane permeability of a bacterial cell is often under complex regulatory mechanisms that control the expression of the porins and the efflux pumps simultaneously to achieve certain standards of permeability. These regulators termed as Mar regulators can be targeted to control the efflux pump expression.

For example, MarA regulator that controls the membrane permeability in *E. aerogenes* regulates the expression of both the porins as well as AcrAB-TolC efflux pump, and can be affected by imipenem. Though this antibiotic is not a substrate for this efflux pump, in the presence of this drug, the bacterium becomes resistant to quinolones, tetracycline and chloramphenicol thus leading to cross resistance [146]. Mutations in Mar regulator often causes resistance to many classes of antibiotics [294]. Interference with these regulatory steps therefore could be used to decrease the expression of efflux pumps thus restoring the antibiotic activity.

Blocking the Energy Required by the Efflux Pumps to Operate

Energy decouplers can be used as a general mechanism to dissipate the energy gradients driving the efflux pumps [288, 295]. As most of the efflux pumps utilize the PMF as their energy source, any compound that dissipates this PMF will act as an inhibitor of the efflux pump [295, 296]. Examples include Carbonyl Cyanide m-ChloroPhenyl-hydrazone (CCCP), valinomycin and dinitrophenol (DNP). However, these compounds do not directly bind the efflux pumps to cause their inhibition. They dissipate the PMF by modifying the trans-membrane electrochemical potential. This class of molecules have not been used clinically or patented due to cytotoxicity issues [297].

Competitive/Non-Competitive Inhibition of Efflux Pumps

These inhibitors are beneficial in many ways clinically as they not only circumvent the problem of bacterial resistance to antibiotics by inhibiting efflux pumps, they also reverse the acquired resistance associated with the overexpression of efflux pumps and suppress the emergence of mutations leading to resistance [295, 297-300]. Example of competitive inhibitor is MC-207, 110 (Phenylalanine Arginyl β-Naphthylamide/PAβN). This compound has been shown to decrease the frequency of the emergence of highly levofloxacin resistant *P. aeruginosa* strains and reduced the intrinsic resistance of the bug to levofloxacin 8-folds [301-303]. MC-207, 110 is a competitive inhibitor of the efflux pumps and acts by binding to the same pocket or at a site closer to the antibiotic substrate binding site [295, 300]. The compound has not only restored

the activity of levofloxacin but has also been found to potentiate the activity of other antibiotics like oxazolidinones, chloramphenicol, rifampicin, macroliodes/ ketolides [289, 304]. Also, it has been shown to be effective not only for *P. aeruginosa*, but also for *K. pneumoniae*, *C. jejuni*, *E. coli*, *S. typhimurium* and *E. aerogenes* [296, 302, 305-307]. To summarise, PAβN appears to be a promising inhibitor with a broad host as well as antibiotic range and an effective mode of efflux pump inhibition. The derivatives of PAβN have been produced by substitution of amino acid or use of D-amino acids [302, 308-310]. As their toxicity has limited their clinical applications, to circumvent this problem, MC-04, 124 compound has been designed with lesser toxicity and higher stability [311].

Blocking the Efflux Pump Protein or Gene

This falls under the category of biological inhibition of efflux pumps. Efflux pumps could be deactivated with the means of specific antibodies [293]. A monoclonal antibody was synthesized and used to block the E2 loop of the extracellular domain of OprM pump of MexAB-OprM tripartite pump in *P. aeruginosa*. This antibody/its variant without Fc domain/ humanized antibody were administered with the antibiotic, and an increase in the efficacy of this antibiotic was observed. These formulations could also contain a pharmaceutically acceptable carrier along with the antibiotic and the antibody and could be used to inhibit the OprM subunit in both MexAB-OprM and MexXY-OprM.

Alternatively, the genes encoding these pumps or their regulators could be blocked using antisense strategies. The antisense approach has been shown to work for AcrAB efflux pump in *E. coli* and has also been patented [312, 313]. These antisense oligonucleotides hybridise with nucleic acids encoding efflux pump AcrB or with nucleic acids regulating the expression of an efflux pump (*marA*, *rob* or *soxS*). Other EPIs included ribozymes directed against the above mentioned genes or antibodies to the efflux pump or proteins that regulate the expression of this efflux pump.

Both QSIs and EPIs can be used as adjuvants with the antibiotics to increase their efficacy [6, 251]. Still, caution needs to be exercised in using these reagents for

controlling bacterial virulence as they suffer from some disadvantages. For example, emergence of resistance to QSIs or EPIs cannot be ruled out [6, 251]. QSIs based on lactone rings like AHL-analogues could present the problem of low stability due to the degradation of lactone ring by lactonases [314].

Other General Strategies

To successfully circumvent the problem of MDR, awareness and concerted efforts are required from the community, clinicians, pharmaceutical industry, governments and scientists. Some of the general strategies to be employed by them are described below:

- Improved water, sanitation and hygiene could help to control the water- and food-borne infections. Continuous surveillance is very critical to determine the prevailing patterns of antibiotic resistance in a given geographical location and thus to assess which of the antibiotics are still active. It is also important to continuously monitor the changes in the antibiotic sensitivity patterns in that location to keep pace with the ever changing pathogens [3].

- Representative strains with MDR phenotypes should be studied carefully in the laboratories to understand the kind of resistance mechanisms adopted by bacteria.

- In addition, we need to improve the existing antibiotics and develop new antibiotics which are inevitably costlier than the already existing drugs. The situation of funding in the area of antibiotic research and development needs to be improved to provide incentives to big and small pharmaceutical companies and research labs for carrying out research in this field.

- One of the other interventions for reversing antibiotic resistance is to relieve the antibiotic pressure in the ecological niches of bacteria like environmental water in case of *V. cholerae* and animal reservoirs in case of *Campylobacter jejuni*. This way, the bacteria would shed the extra genetic baggage that it required to deal with these antibiotics. Appropriate policies should be devised to stop the indiscriminate use of antibiotics after consultation of government agencies, pharmaceutical industries, researchers

and clinicians. It should be seen that the antibiotics used in animals are different from those used in humans. In addition to this, the use of antimicrobials in the case of upper respiratory tract infections or other infections of viral origin should be discouraged as should be the empirical therapy and broad spectrum antimicrobials-based therapy [315].

- Novel drug targets combined with new drugs, efficient diagnostic techniques and vaccine could provide a new hope to curb these pathogens [315].

- In this era, use of bioinformatics tools like *in silico* target identification, design of small drug molecules, and docking studies to predict their binding to target could aid in achieving this aim faster. Some companies like Intercell are employing anti-genome technology to identify new targets for vaccines and drugs. Projects have been initiated to identify novel targets in *S. aureus*, *S. pneumoniae* and *S. pyogenes* [315]. The advent of technologies based on genomics, proteomics, combinatorial chemistry and high throughput screening could lead to success stories inspite of the large funds required for them.

- The governments should realize the seriousness of impending disaster due to MDR bacteria and urgency of the situation due to their limitations in controlling the diseases caused by MDR bacteria [315]. Accordingly, the new policies should be made to deal with this problem. For example, American Society of Microbiology (ASM) set up a task force on antimicrobial resistance in 1995, The CDC published their "Guidelines for the Evaluation of Surveillance Systems" in 1998 and CDC also issued a Public Health Action Plan to combat Antimicrobial resistance in June 2000. The Infectious Diseases Society of America produced a shocking report in 2004 to draw the attention of the government towards the problem of MDR and to draw the attention of dwindling funds in research and development of new anti-infective agents by pharmaceutical industries. The report was titled "Bad Bugs, No Drugs: As Antibiotic Discovery Stagnates------- A Public Health Crisis Brews". Similarly, the UK Department of Health issued an "Antimicrobial Resistance Strategy and Action Plan' in 2000. An efficient

surveillance system has been set up in Europe by the name of European Antimicrobial Resistance Surveillance System (EARSS) (http://www.rivm.nl/earss). In India, national policy has been made for containment of antimicrobial resistance by Directorate General of Health Services (2011) when the superbug carrying New Delhi metallo-beta lactamase was reported in a Swedish patient of Indian origin. A task force was constituted to work on various aspects related to national surveillance system for antibiotic resistance, enhancing regulatory provisions for use of antibiotics in human, veterinary and industrial use, to enhance the rational use of antibiotics and strengthen the diagnostic methods for antimicrobial resistance monitoring. Health ministry has also approved an antimicrobial resistance programme that will be monitored and reviewed by The National Centre for Disease Control (NCDC). Global Antibiotic Resistance Partnership (GARP) develops different policy proposals on antibiotic resistance for low-and middle-income countries. Phase 1 of GARP has been initiated in India, Kenya, South Africa and Vietnam. In addition to this endeavour, in August 2012, the annual conference of Clinical Infectious Disease Society was held at Chennai, India. In this meeting of medical societies of India which also had many national and international representatives, a roadmap was made to tackle the problem of antimicrobial resistance in India. The document resulting from the discussions held at this meeting was named "Chennai Declaration". The recommendations made in Chennai Declaration would be considered by Indian Ministry of Health for formulating a national antibiotic policy.

CONCLUDING REMARKS

Considering the resilience of bacteria, it is imminent that other strategies and avenues apart from antibiotics be explored to control the MDR bacteria. As described in the section above, a multifaceted approach is perhaps required to vanquish these bugs. To conclude this chapter, the statement by Dr. Joshua Lederberg seems appropriate to describe the current situation.

The future of microbes and mankind will probably unfold as episodes of a suspense thriller that could be entitled "Our wits *versus* their genes"

ACKNOWLEDGEMENTS

The laboratory is supported by the grants from the Department of Biotechnology (DBT), Ministry of Science and Technology, Government of India (No. BT/PR/11634/INF/22/104/2008), Gujarat State Biotechnology Mission (GSBTM), Department of Science and Technology, Government of Gujarat (No. GSBTM/MD/PROJECTS/SSA/1535/2013-14) and Indian Council of Medical Research, New Delhi, India (No. the grant AMR/49/11-ECDI). N.R. is a recipient of Senior Research Fellowship from Indian Council of Medical Research, New Delhi, India and K.V. is a recipient of Junior Research Fellowship from the GSBTM grant mentioned above. P. M. is a recipient of ICMR senior research fellowship (No. F/815/2011-ECD-II) and B. M. R. N. S. K. is a recipient of senior research fellowship from CSIR (No. 09/988/(0003)/2010-EMR-I). The authors thankfully acknowledge The Puri Foundation for Education in India for providing infrastructure facilities.

CONFLICT OF INTEREST

The authors confirm that this chapter contents have no conflict of interest.

DISCLOSURE

Parts of this book chapter have been previously published in two articles by the authors of this chapter. These articles are: Recent Patents on Anti-infective Drug Discovery, Volume: 7; Issue: 1; Pages 73-89 (17); and Recent Patents on Anti-infective Drug Discovery, Volume: 8; Issue: 1; Pages 68-83 (16).

REFERENCES

[1] Antibiotic resistance: a final warning. Lancet 2013; 382: 1072.
[2] Antibiotic resistance threats in the United States, 2013. Centers for Diseases Control and Prevention 2013; 11-28.
[3] Sack DA, Lyke C, McLaughlin C*, et al.*, Antimicrobial resistance in shigellosis, cholera and campylobacteriosis. World Health Organization 2001; 1-20.
[4] Willey JM, Sherwood L, Woolverton CJ*, et al.*, 7th. Prescott, Harley, and Klein's microbiology. McGraw-Hill Higher Education 2008; 835-58.
[5] Salyers AA, Whitt DD, 2nd. Bacterial pathogenesis: a molecular approach. ASM Press 2002; 168-84.

[6] Bhardwaj AK, Mohanty P. Bacterial efflux pumps involved in multidrug resistance and their inhibitors: rejuvinating the antimicrobial chemotherapy. Recent Pat Antiinfect Drug Discov 2012; 7: 73-89.

[7] Cavallo JD, Plesiat P, Couetdic G*, et al.* Mechanisms of beta-lactam resistance in *Pseudomonas aeruginosa*: prevalence of OprM-overproducing strains in a French multicentre study (1997). J Antimicrob Chemother 2002; 50: 1039-43.

[8] Baranwal S, Dey K, Ramamurthy T*, et al.* Role of active efflux in association with target gene mutations in fluoroquinolone resistance in clinical isolates of *Vibrio cholerae*. Antimicrob Agents Chemother 2002; 46: 2676-8.

[9] Singh R, Rajpara N, Tak J*, et al.* Clinical isolates of *Vibrio fluvialis* from Kolkata, India, obtained during 2006: plasmids, the *qnr* gene and a mutation in gyrase A as mechanisms of multidrug resistance. J Med Microbiol 2012; 61: 369-74.

[10] Pazhani GP, Chakraborty S, Fujihara K*, et al.* QRDR mutations, efflux system & antimicrobial resistance genes in enterotoxigenic *Escherichia coli* isolated from an outbreak of diarrhoea in Ahmedabad, India. Indian J Med Res 2011; 134: 214-23.

[11] Mitsuhashi S, Harada K, Hashimoto H*, et al.* On the drug-resistance of enteric bacteria. 4. Drug-resistance of *Shigella* prevalent in Japan. Jpn J Exp Med 1961; 31: 47-52.

[12] Liebert CA, Hall RM, Summers AO. Transposon Tn21, flagship of the floating genome. Microbiol Mol Biol Rev 1999; 63: 507-22.

[13] Coppo A, Colombo M, Pazzani C*, et al. Vibrio cholerae* in the horn of Africa: epidemiology, plasmids, tetracycline resistance gene amplification, and comparison between O1 and non-O1 strains. Am J Trop Med Hyg 1995; 53: 351-9.

[14] Stokes HW, Hall RM. A novel family of potentially mobile DNA elements encoding site-specific gene-integration functions: integrons. Mol Microbiol 1989; 3: 1669-83.

[15] Rajpara N, Patel A, Tiwari N*, et al.* Mechanism of drug resistance in a clinical isolate of *Vibrio fluvialis*: involvement of multiple plasmids and integrons. Int J Antimicrob Agents 2009; 34: 220-5.

[16] Partridge SR, Tsafnat G, Coiera E*, et al.* Gene cassettes and cassette arrays in mobile resistance integrons. FEMS Microbiol Rev 2009; 33: 757-84.

[17] Stokes HW, Nesbo CL, Holley M*, et al.* Class 1 integrons potentially predating the association with tn402-like transposition genes are present in a sediment microbial community. J Bacteriol 2006; 188: 5722-30.

[18] Mazel D, Dychinco B, Webb VA*, et al.* A distinctive class of integron in the *Vibrio cholerae* genome. Science 1998; 280: 605-8.

[19] Mazel D. Integrons: agents of bacterial evolution. Nat Rev Microbiol 2006; 4: 608-20.

[20] Cambray G, Guerout AM, Mazel D. Integrons. Annu Rev Genet 2010; 44: 141-66.

[21] Esposito D, Scocca JJ. The integrase family of tyrosine recombinases: evolution of a conserved active site domain. Nucleic Acids Res 1997; 25: 3605-14.

[22] Nunes-Duby SE, Kwon HJ, Tirumalai RS*, et al.* Similarities and differences among 105 members of the Int family of site-specific recombinases. Nucleic Acids Res 1998; 26: 391-406.

[23] Demarre G, Frumerie C, Gopaul DN*, et al.* Identification of key structural determinants of the IntI1 integron integrase that influence *attC* x *attI1* recombination efficiency. Nucleic Acids Res 2007; 35: 6475-89.

[24] Stokes HW, Hall RM. Sequence analysis of the inducible chloramphenicol resistance determinant in the Tn1696 integron suggests regulation by translational attenuation. Plasmid 1991; 26: 10-9.
[25] da Fonseca EL, Vicente AC. Functional characterization of a Cassette-specific promoter in the class 1 integron-associated *qnrVC1* gene. Antimicrob Agents Chemother 2012; 56: 3392-4.
[26] Guerineau F, Brooks L, Mullineaux P. Expression of the sulfonamide resistance gene from plasmid R46. Plasmid 1990; 23: 35-41.
[27] Levesque C, Brassard S, Lapointe J*, et al.* Diversity and relative strength of tandem promoters for the antibiotic-resistance genes of several integrons. Gene 1994; 142: 49-54.
[28] Collis CM, Hall RM. Expression of antibiotic resistance genes in the integrated cassettes of integrons. Antimicrob Agents Chemother 1995; 39: 155-62.
[29] Jove T, Da Re S, Denis F*, et al.* Inverse correlation between promoter strength and excision activity in class 1 integrons. PLoS Genet 2010; 6: e1000793.
[30] Gravel A, Fournier B, Roy PH. DNA complexes obtained with the integron integrase IntI1 at the attI1 site. Nucleic Acids Res 1998; 26: 4347-55.
[31] Collis CM, Kim MJ, Stokes HW*, et al.* Binding of the purified integron DNA integrase Intl1 to integron- and cassette-associated recombination sites. Mol Microbiol 1998; 29: 477-90.
[32] Collis CM, Hall RM. Comparison of the structure-activity relationships of the integron-associated recombination sites *attI3* and *attI1* reveals common features. Microbiology 2004; 150: 1591-601.
[33] Stokes HW, O'Gorman DB, Recchia GD*, et al.* Structure and function of 59-base element recombination sites associated with mobile gene cassettes. Mol Microbiol 1997; 26: 731-45.
[34] Recchia GD, Hall RM. Gene cassettes: a new class of mobile element. Microbiology 1995; 141 (Pt 12): 3015-27.
[35] Baharoglu Z, Garriss G, Mazel D. Multiple pathways of genome plasticity leading to development of antibiotic resistance. Antibiotics 2013; 2: 288-315.
[36] Fluit AC, Schmitz FJ. Resistance integrons and super-integrons. Clin Microbiol Infect 2004; 10: 272-88.
[37] Goldstein C, Lee MD, Sanchez S*, et al.* Incidence of class 1 and 2 integrases in clinical and commensal bacteria from livestock, companion animals, and exotics. Antimicrob Agents Chemother 2001; 45: 723-6.
[38] Labbate M, Case RJ, Stokes HW. The integron/gene cassette system: an active player in bacterial adaptation. Methods Mol Biol 2009; 532: 103-25.
[39] Martin C, Timm J, Rauzier J*, et al.* Transposition of an antibiotic resistance element in mycobacteria. Nature 1990; 345: 739-43.
[40] Nandi S, Maurer JJ, Hofacre C*, et al.* Gram-positive bacteria are a major reservoir of Class 1 antibiotic resistance integrons in poultry litter. Proc Natl Acad Sci USA 2004; 101: 7118-22.
[41] Nesvera J, Hochmannova J, Patek M. An integron of class 1 is present on the plasmid pCG4 from gram-positive bacterium *Corynebacterium glutamicum*. FEMS Microbiol Lett 1998; 169: 391-5.
[42] Shi L, Zheng M, Xiao Z*, et al.* Unnoticed spread of class 1 integrons in gram-positive clinical strains isolated in Guangzhou, China. Microbiol Immunol 2006; 50: 463-7.

[43] Rouch DA, Cram DS, DiBerardino D*, et al.* Efflux-mediated antiseptic resistance gene *qacA* from *Staphylococcus aureus*: common ancestry with tetracycline- and sugar-transport proteins. Mol Microbiol 1990; 4: 2051-62.

[44] Uyaguari MI, Scott GI, Norman RS. Abundance of class 1-3 integrons in South Carolina estuarine ecosystems under high and low levels of anthropogenic influence. Mar Pollut Bull 2013; 76: 77-84.

[45] Hansson K, Sundstrom L, Pelletier A*, et al.* IntI2 integron integrase in Tn7. J Bacteriol 2002; 184: 1712-21.

[46] Collis CM, Kim MJ, Stokes HW*, et al.* Integron-encoded IntI integrases preferentially recognize the adjacent cognate attI site in recombination with a 59-be site. Mol Microbiol 2002; 46: 1415-27.

[47] Barlow RS, Gobius KS. Diverse class 2 integrons in bacteria from beef cattle sources. J Antimicrob Chemother 2006; 58: 1133-8.

[48] Marquez C, Labbate M, Ingold AJ*, et al.* Recovery of a functional class 2 integron from an *Escherichia coli* strain mediating a urinary tract infection. Antimicrob Agents Chemother 2008; 52: 4153-4.

[49] Wei Q, Hu Q, Li S*, et al.* A novel functional class 2 integron in clinical *Proteus mirabilis* isolates. J Antimicrob Chemother 2014; 69: 973-6.

[50] Arakawa Y, Murakami M, Suzuki K*, et al.* A novel integron-like element carrying the metallo-beta-lactamase gene *blaIMP*. Antimicrob Agents Chemother 1995; 39: 1612-5.

[51] Collis CM, Kim MJ, Partridge SR*, et al.* Characterization of the class 3 integron and the site-specific recombination system it determines. J Bacteriol 2002; 184: 3017-26.

[52] Correia M, Boavida F, Grosso F*, et al.* Molecular characterization of a new class 3 integron in *Klebsiella pneumoniae*. Antimicrob Agents Chemother 2003; 47: 2838-43.

[53] Clark CA, Purins L, Kaewrakon P*, et al.* The *Vibrio cholerae* O1 chromosomal integron. Microbiology 2000; 146 (Pt 10): 2605-12.

[54] Heidelberg JF, Eisen JA, Nelson WC*, et al.* DNA sequence of both chromosomes of the cholera pathogen *Vibrio cholerae*. Nature 2000; 406: 477-83.

[55] Drouin F, Melancon J, Roy PH. The IntI-like tyrosine recombinase of *Shewanella oneidensis* is active as an integron integrase. J Bacteriol 2002; 184: 1811-5.

[56] Rowe-Magnus DA, Guerout AM, Mazel D. Bacterial resistance evolution by recruitment of super-integron gene cassettes. Mol Microbiol 2002; 43: 1657-69.

[57] Boucher Y, Labbate M, Koenig JE*, et al.* Integrons: mobilizable platforms that promote genetic diversity in bacteria. Trends Microbiol 2007; 15: 301-9.

[58] Nield BS, Willows RD, Torda AE*, et al.* New enzymes from environmental cassette arrays: functional attributes of a phosphotransferase and an RNA-methyltransferase. Protein Sci 2004; 13: 1651-9.

[59] Robinson A, Wu PS, Harrop SJ*, et al.* Integron-associated mobile gene cassettes code for folded proteins: the structure of Bal32a, a new member of the adaptable alpha+beta barrel family. J Mol Biol 2005; 346: 1229-41.

[60] Smith AB, Siebeling RJ. Identification of genetic loci required for capsular expression in *Vibrio vulnificus*. Infect Immun 2003; 71: 1091-7.

[61] Fonseca EL, Dos Santos Freitas F, Vieira VV*, et al.* New *qnr* gene cassettes associated with superintegron repeats in *Vibrio cholerae* O1. Emerg Infect Dis 2008; 14: 1129-31.

[62] Le Roux F, Zouine M, Chakroun N, *et al.* Genome sequence of *Vibrio splendidus*: an abundant planctonic marine species with a large genotypic diversity. Environ Microbiol 2009; 11: 1959-70.

[63] Melano R, Petroni A, Garutti A, *et al.* New carbenicillin-hydrolyzing beta-lactamase (CARB-7) from *Vibrio cholerae* non-O1, non-O139 strains encoded by the VCR region of the *V. cholerae* genome. Antimicrob Agents Chemother 2002; 46: 2162-8.

[64] Petroni A, Melano RG, Saka HA, *et al.* CARB-9, a carbenicillinase encoded in the VCR region of *Vibrio cholerae* non-O1, non-O139 belongs to a family of cassette-encoded beta-lactamases. Antimicrob Agents Chemother 2004; 48: 4042-6.

[65] Chen CY, Wu KM, Chang YC, *et al.* Comparative genome analysis of *Vibrio vulnificus*, a marine pathogen. Genome Res 2003; 13: 2577-87.

[66] Gerdes K, Gultyaev AP, Franch T, *et al.* Antisense RNA-regulated programmed cell death. Annu Rev Genet 1997; 31: 1-31.

[67] Kobayashi I. Behavior of restriction-modification systems as selfish mobile elements and their impact on genome evolution. Nucleic Acids Res 2001; 29: 3742-56.

[68] Van Melderen L, Saavedra De Bast M. Bacterial toxin-antitoxin systems: more than selfish entities? PLoS Genet 2009; 5: e1000437.

[69] Pandey DP, Gerdes K. Toxin-antitoxin loci are highly abundant in free-living but lost from host-associated prokaryotes. Nucleic Acids Res 2005; 33: 966-76.

[70] Rowe-Magnus DA, Guerout AM, Biskri L, *et al.* Comparative analysis of superintegrons: engineering extensive genetic diversity in the *Vibrionaceae*. Genome Res 2003; 13: 428-42.

[71] Szekeres S, Dauti M, Wilde C, *et al.* Chromosomal toxin-antitoxin loci can diminish large-scale genome reductions in the absence of selection. Mol Microbiol 2007; 63: 1588-605.

[72] Christensen-Dalsgaard M, Gerdes K. Two *higBA* loci in the *Vibrio cholerae* superintegron encode mRNA cleaving enzymes and can stabilize plasmids. Mol Microbiol 2006; 62: 397-411.

[73] Vaisvila R, Morgan RD, Posfai J, *et al.* Discovery and distribution of super-integrons among pseudomonads. Mol Microbiol 2001; 42: 587-601.

[74] Waldor MK, Tschape H, Mekalanos JJ. A new type of conjugative transposon encodes resistance to sulfamethoxazole, trimethoprim, and streptomycin in *Vibrio cholerae* O139. J Bacteriol 1996; 178: 4157-65.

[75] Beaber JW, Burrus V, Hochhut B, *et al.* Comparison of SXT and R391, two conjugative integrating elements: definition of a genetic backbone for the mobilization of resistance determinants. Cell Mol Life Sci 2002; 59: 2065-70.

[76] Ceccarelli D, Spagnoletti M, Bacciu D, *et al.* ICEVchInd5 is prevalent in epidemic *Vibrio cholerae* O1 El Tor strains isolated in India. Int J Med Microbiol 2011; 301: 318-24.

[77] Burrus V, Waldor MK. Shaping bacterial genomes with integrative and conjugative elements. Res Microbiol 2004; 155: 376-86.

[78] Osborn AM, Boltner D. When phage, plasmids, and transposons collide: genomic islands, and conjugative- and mobilizable-transposons as a mosaic continuum. Plasmid 2002; 48: 202-12.

[79] Burrus V, Waldor MK. Control of SXT integration and excision. J Bacteriol 2003; 185: 5045-54.

[80] Hochhut B, Lotfi Y, Mazel D, *et al.* Molecular analysis of antibiotic resistance gene clusters in *Vibrio cholerae* O139 and O1 SXT constins. Antimicrob Agents Chemother 2001; 45: 2991-3000.

[81] Mohapatra H, Mohapatra SS, Mantri CK*, et al. Vibrio cholerae* non-O1, non-O139 strains isolated before 1992 from Varanasi, India are multiple drug resistant, contain *intSXT*, *dfr18* and *aadA5* genes. Environ Microbiol 2008; 10: 866-73

[82] Amita, Chowdhury SR, Thungapathra M*, et al.* Class I integrons and SXT elements in El Tor strains isolated before and after 1992 *Vibrio cholerae* O139 outbreak, Calcutta, India. Emerg Infect Dis 2003; 9: 500-2.

[83] Alam M, Hasan NA, Sadique A*, et al.* Seasonal cholera caused by *Vibrio cholerae* serogroups O1 and O139 in the coastal aquatic environment of Bangladesh. Appl Environ Microbiol 2006; 72: 4096-104.

[84] Toma C, Nakasone N, Song T*, et al. Vibrio cholerae* SXT element, Laos. Emerg Infect Dis 2005; 11: 346-7.

[85] Burrus V, Marrero J, Waldor MK. The current ICE age: biology and evolution of SXT-related integrating conjugative elements. Plasmid 2006; 55: 173-83

[86] Dalsgaard A, Forslund A, Sandvang D*, et al. Vibrio cholerae* O1 outbreak isolates in Mozambique and South Africa in 1998 are multiple-drug resistant, contain the SXT element and the *aadA2* gene located on class 1 integrons. J Antimicrob Chemother 2001; 48: 827-38.

[87] Kutar BM, Rajpara N, Upadhyay H*, et al.* Clinical isolates of *Vibrio cholerae* O1 El Tor Ogawa of 2009 from Kolkata, India: preponderance of SXT element and presence of Haitian *ctxB* variant. PLoS One 2013; 8: e56477.

[88] Mitra R, Basu A, Dutta D*, et al.* Resurgence of *Vibrio cholerae* O139 Bengal with altered antibiogram in Calcutta, India. Lancet 1996; 348: 1181.

[89] Sinha S, Chakraborty R, De K*, et al.* Escalating association of *Vibrio cholerae* O139 with cholera outbreaks in India. J Clin Microbiol 2002; 40: 2635-7.

[90] Yam WC, Yuen KY, Wong SS*, et al. Vibrio cholerae* O139 susceptible to vibriostatic agent 0/129 and co-trimoxazole. Lancet 1994; 344: 404-5.

[91] Schubert S, Dufke S, Sorsa J*, et al.* A novel integrative and conjugative element (ICE) of *Escherichia coli*: the putative progenitor of the Yersinia high-pathogenicity island. Mol Microbiol 2004; 51: 837-48.

[92] Beaber JW, Hochhut B, Waldor MK. Genomic and functional analyses of SXT, an integrating antibiotic resistance gene transfer element derived from *Vibrio cholerae*. J Bacteriol 2002; 184: 4259-69.

[93] Lewis JA, Hatfull GF. Control of directionality in integrase-mediated recombination: examination of recombination directionality factors (RDFs) including Xis and Cox proteins. Nucleic Acids Res 2001; 29: 2205-16.

[94] Wang H, Roberts AP, Lyras D*, et al.* Characterization of the ends and target sites of the novel conjugative transposon Tn5397 from *Clostridium difficile*: excision and circularization is mediated by the large resolvase, TndX. J Bacteriol 2000; 182: 3775-83.

[95] Wozniak RA, Fouts DE, Spagnoletti M*, et al.* Comparative ICE genomics: insights into the evolution of the SXT/R391 family of ICEs. PLoS Genet 2009; 5: e1000786.

[96] Grohmann E, Muth G, Espinosa M. Conjugative plasmid transfer in gram-positive bacteria. Microbiol Mol Biol Rev 2003; 67: 277-301.

[97] Hochhut B, Waldor MK. Site-specific integration of the conjugal *Vibrio cholerae* SXT element into *prfC*. Mol Microbiol 1999; 32: 99-110.

[98] Van Melderen L, Saavedra De Bast M. Bacterial toxin-antitoxin systems: more than selfish entities? PLoS Genet 2009; 5: e1000437.

[99] Magnuson RD. Hypothetical functions of toxin-antitoxin systems. J Bacteriol 2007; 189: 6089-92.
[100] Hayes F. Toxins-antitoxins: plasmid maintenance, programmed cell death, and cell cycle arrest. Science 2003; 301: 1496-9.
[101] Wozniak RA, Waldor MK. Integrative and conjugative elements: mosaic mobile genetic elements enabling dynamic lateral gene flow. Nat Rev Microbiol 2010; 8: 552-63
[102] Ceccarelli D, Daccord A, Rene M*, et al.* Identification of the origin of transfer (oriT) and a new gene required for mobilization of the SXT/R391 family of integrating conjugative elements. J Bacteriol 2008; 190: 5328-38.
[103] Beaber JW, Hochhut B, Waldor MK. SOS response promotes horizontal dissemination of antibiotic resistance genes. Nature 2004; 427: 72-4.
[104] Hochhut B, Beaber JW, Woodgate R*, et al.* Formation of chromosomal tandem arrays of the SXT element and R391, two conjugative chromosomally integrating elements that share an attachment site. J Bacteriol 2001; 183: 1124-32.
[105] Pembroke JT, MacMahon C, McGrath B. The role of conjugative transposons in the *Enterobacteriaceae*. Cell Mol Life Sci 2002; 59: 2055-64.
[106] Bi D, Xu Z, Harrison EM*, et al.* ICEberg: a web-based resource for integrative and conjugative elements found in Bacteria. Nucleic Acids Res 2012; 40: D621-6.
[107] Coetzee JN, Datta N, Hedges RW. R factors from *Proteus rettgeri*. J Gen Microbiol 1972; 72: 543-52.
[108] Daccord A, Ceccarelli D, Burrus V. Integrating conjugative elements of the SXT/R391 family trigger the excision and drive the mobilization of a new class of Vibrio genomic islands. Mol Microbiol 2010; 78: 576-88.
[109] Marrero J, Waldor MK. The SXT/R391 family of integrative conjugative elements is composed of two exclusion groups. J Bacteriol 2007; 189: 3302-5.
[110] Van Bambeke F, Glupczynski Y, Plesiat P*, et al.* Antibiotic efflux pumps in prokaryotic cells: occurrence, impact on resistance and strategies for the future of antimicrobial therapy. J Antimicrob Chemother 2003; 51: 1055-65.
[111] Poole K. Efflux-mediated resistance to fluoroquinolones in gram-negative bacteria. Antimicrob Agents Chemother 2000; 44: 2233-41.
[112] Lehtinen J, Lilius EM. Promethazine renders *Escherichia coli* susceptible to penicillin G: real-time measurement of bacterial susceptibility by fluoro-luminometry. Int J Antimicrob Agents 2007; 30: 44-51.
[113] Piddock LJ. Multidrug-resistance efflux pumps - not just for resistance. Nat Rev Microbiol 2006; 4: 629-36.
[114] Ren Q, Paulsen IT. Comparative analyses of fundamental differences in membrane transport capabilities in prokaryotes and eukaryotes. PLoS Comput Biol 2005; 1: e27.
[115] Piddock LJ. Clinically relevant chromosomally encoded multidrug resistance efflux pumps in bacteria. Clin Microbiol Rev 2006; 19: 382-402.
[116] Butaye P, Cloeckaert A, Schwarz S. Mobile genes coding for efflux-mediated antimicrobial resistance in Gram-positive and Gram-negative bacteria. Int J Antimicrob Agents 2003; 22: 205-10.
[117] Zhao J, Chen Z, Chen S*, et al.* Prevalence and dissemination of *oqxAB* in *Escherichia coli* isolates from animals, farmworkers, and the environment. Antimicrob Agents Chemother 2010; 54: 4219-24.

[118] Bina JE, Mekalanos JJ. *Vibrio cholerae tolC* is required for bile resistance and colonization. Infect Immun 2001; 69: 4681-5.

[119] Bina XR, Provenzano D, Nguyen N*, et al. Vibrio cholerae* RND family efflux systems are required for antimicrobial resistance, optimal virulence factor production, and colonization of the infant mouse small intestine. Infect Immun 2008; 76: 3595-605.

[120] McMurry L, Petrucci RE, Jr., Levy SB. Active efflux of tetracycline encoded by four genetically different tetracycline resistance determinants in *Escherichia coli*. Proc Natl Acad Sci USA 1980; 77: 3974-7.

[121] Poole K. Efflux-mediated antimicrobial resistance. J Antimicrob Chemother 2005; 56: 20-51.

[122] Borges-Walmsley MI, McKeegan KS, Walmsley AR. Structure and function of efflux pumps that confer resistance to drugs. Biochem J 2003; 376: 313-38.

[123] Higgins MK, Bokma E, Koronakis E*, et al.* Structure of the periplasmic component of a bacterial drug efflux pump. Proc Natl Acad Sci USA 2004; 101: 9994-9.

[124] He X, Szewczyk P, Karyakin A*, et al.* Structure of a cation-bound multidrug and toxic compound extrusion transporter. Nature 2010; 467: 991-4.

[125] Lomovskaya O, Lee A, Hoshino K*, et al.* Use of a genetic approach to evaluate the consequences of inhibition of efflux pumps in *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 1999; 43: 1340-6.

[126] Mazzariol A, Cornaglia G, Nikaido H. Contributions of the AmpC beta-lactamase and the AcrAB multidrug efflux system in intrinsic resistance of *Escherichia coli* K-12 to beta-lactams. Antimicrob Agents Chemother 2000; 44: 1387-90.

[127] Lynch IJ, Martinez FJ. Clinical relevance of macrolide-resistant *Streptococcus pneumoniae* for community-acquired pneumonia. Clin Infect Dis 2002; 34 Suppl 1: S27-46.

[128] Askoura M, Mottawea W, Abujamel T*, et al.* Efflux pump inhibitors (EPIs) as new antimicrobial agents against *Pseudomonas aeruginosa*. Libyan J Med 2011; 6:

[129] Nikaido H. Multidrug resistance in bacteria. Annu Rev Biochem 2009; 78: 119-46.

[130] Moussatova A, Kandt C, O'Mara ML*, et al.* ATP-binding cassette transporters in *Escherichia coli*. Biochim Biophys Acta 2008; 1778: 1757-71.

[131] Li J, Li B, Wendlandt S, *et al*. Identification of a novel *vga(E)* gene variant that confers resistance to pleuromutilins, lincosamides and streptogramin A antibiotics in staphylococci of porcine origin. J Antimicrob Chemother. 2014; 69: 919-23.

[132] Marger MD, Saier MH, Jr. A major superfamily of transmembrane facilitators that catalyse uniport, symport and antiport. Trends Biochem Sci 1993; 18: 13-20.

[133] Paulsen IT, Skurray RA. Topology, structure and evolution of two families of proteins involved in antibiotic and antiseptic resistance in eukaryotes and prokaryotes--an analysis. Gene 1993; 124: 1-11.

[134] Fernandez L, Hancock RE. Adaptive and mutational resistance: role of porins and efflux pumps in drug resistance. Clin Microbiol Rev 2012; 25: 661-81.

[135] Yoshida H, Bogaki M, Nakamura S*, et al.* Nucleotide sequence and characterization of the *Staphylococcus aureus norA* gene, which confers resistance to quinolones. J Bacteriol 1990; 172: 6942-9.

[136] Gill MJ, Brenwald NP, Wise R. Identification of an efflux pump gene, *pmrA*, associated with fluoroquinolone resistance in *Streptococcus pneumoniae*. Antimicrob Agents Chemother 1999; 43: 187-9.

[137] Lee EW, Chen J, Huda MN*, et al.* Functional cloning and expression of *emeA*, and characterization of EmeA, a multidrug efflux pump from *Enterococcus faecalis*. Biol Pharm Bull 2003; 26: 266-70.
[138] Chung YJ, Saier MH, Jr. SMR-type multidrug resistance pumps. Curr Opin Drug Discov Devel 2001; 4: 237-45.
[139] Bay DC, Rommens KL, Turner RJ. Small multidrug resistance proteins: a multidrug transporter family that continues to grow. Biochim Biophys Acta 2008; 1778: 1814-38.
[140] Venkatraman J, Nagana Gowda GA, Balaram P. Structural analysis of synthetic peptide fragments from EmrE, a multidrug resistance protein, in a membrane-mimetic environment. Biochemistry 2002; 41: 6631-9.
[141] Srinivasan VB, Rajamohan G, Gebreyes WA. Role of AbeS, a novel efflux pump of the SMR family of transporters, in resistance to antimicrobial agents in *Acinetobacter baumannii*. Antimicrob Agents Chemother 2009; 53: 5312-6.
[142] Morita Y, Kodama K, Shiota S*, et al.* NorM, a putative multidrug efflux protein, of *Vibrio parahaemolyticus* and its homolog in *Escherichia coli*. Antimicrob Agents Chemother 1998; 42: 1778-82.
[143] Kuroda T, Tsuchiya T. Multidrug efflux transporters in the MATE family. Biochim Biophys Acta 2009; 1794: 763-8.
[144] Begum A, Rahman MM, Ogawa W*, et al.* Gene cloning and characterization of four MATE family multidrug efflux pumps from *Vibrio cholerae* non-O1. Microbiol Immunol 2005; 49: 949-57.
[145] Mohanty P, Patel A, Kushwaha Bhardwaj A. Role of H- and D- MATE-type transporters from multidrug resistant clinical isolates of *Vibrio fluvialis* in conferring fluoroquinolone resistance. PLoS One 2012; 7: e35752.
[146] Bornet C, Chollet R, Mallea M*, et al.* Imipenem and expression of multidrug efflux pump in *Enterobacter aerogenes*. Biochem Biophys Res Commun 2003; 301: 985-90.
[147] Nakae T. Identification of the outer membrane protein of *E. coli* that produces transmembrane channels in reconstituted vesicle membranes. Biochem Biophys Res Commun 1976; 71: 877-84.
[148] Harder KJ, Nikaido H, Matsuhashi M. Mutants of *Escherichia coli* that are resistant to certain beta-lactam compounds lack the *ompF* porin. Antimicrob Agents Chemother 1981; 20: 549-52.
[149] Sanbongi Y, Shimizu A, Suzuki T*, et al.* Classification of OprD sequence and correlation with antimicrobial activity of carbapenem agents in *Pseudomonas aeruginosa* clinical isolates collected in Japan. Microbiol Immunol 2009; 53: 361-7.
[150] Kohler T, Michea-Hamzehpour M, Henze U*, et al.* Characterization of MexE-MexF-OprN, a positively regulated multidrug efflux system of *Pseudomonas aeruginosa*. Mol Microbiol 1997; 23: 345-54.
[151] Pagel M, Simonet V, Li J*, et al.* Phenotypic characterization of pore mutants of the *Vibrio cholerae* porin OmpU. J Bacteriol 2007; 189: 8593-600.
[152] Ochs MM, Bains M, Hancock RE. Role of putative loops 2 and 3 in imipenem passage through the specific porin OprD of *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 2000; 44: 1983-5.
[153] Huang H, Jeanteur D, Pattus F*, et al.* Membrane topology and site-specific mutagenesis of *Pseudomonas aeruginosa* porin OprD. Mol Microbiol 1995; 16: 931-41.

[154] Vettoretti L, Plesiat P, Muller C*, et al.* Efflux unbalance in *Pseudomonas aeruginosa* isolates from cystic fibrosis patients. Antimicrob Agents Chemother 2009; 53: 1987-97.
[155] Olliver A, Valle M, Chaslus-Dancla E*, et al.* Overexpression of the multidrug efflux operon *acrEF* by insertional activation with IS1 or IS10 elements in *Salmonella enterica* serovar *typhimurium* DT204 *acrB* mutants selected with fluoroquinolones. Antimicrob Agents Chemother 2005; 49: 289-301.
[156] Walker RC. The fluoroquinolones. Mayo Clin Proc 1999; 74: 1030-7.
[157] Saravanos K, Duff P. The quinolone antibiotics. Obstet Gynecol Clin North Am 1992; 19: 529-37.
[158] Brown SA. Fluoroquinolones in animal health. J Vet Pharmacol Ther 1996; 19: 1-14.
[159] Bolon MK. The newer fluoroquinolones. Med Clin North Am 2011; 95: 793-817.
[160] Andriole VT. The quinolones: past, present, and future. Clin Infect Dis 2005; 41 Suppl 2: S113-9.
[161] Van Bambeke F, Michot JM, Van Eldere J*, et al.* Quinolones in 2005: an update. Clin Microbiol Infect 2005; 11: 256-80.
[162] Drlica K, Zhao X. DNA gyrase, topoisomerase IV, and the 4-quinolones. Microbiol Mol Biol Rev 1997; 61: 377-92.
[163] Ball P. Quinolone generations: natural history or natural selection? J Antimicrob Chemother 2000; 46 Suppl T1: 17-24.
[164] O'Donnell JA, Gelone SP. The newer fluoroquinolones. Infect Dis Clin North Am 2004; 18: 691-716, x.
[165] Watson JD, Baker TA, Bell SP *et al*. Molecular biology of the gene, 5th ed.; Cold Spring Harbour Laboratory Press 2004; pp. 115-22
[166] Shen LL, Kohlbrenner WE, Weigl D*, et al.* Mechanism of quinolone inhibition of DNA gyrase. Appearance of unique norfloxacin binding sites in enzyme-DNA complexes. J Biol Chem 1989; 264: 2973-8.
[167] Kampranis SC, Maxwell A. The DNA gyrase-quinolone complex. ATP hydrolysis and the mechanism of DNA cleavage. J Biol Chem 1998; 273: 22615-26.
[168] Morais Cabral JH, Jackson AP, Smith CV*, et al.* Crystal structure of the breakage-reunion domain of DNA gyrase. Nature 1997; 388: 903-6.
[169] Berger JM, Gamblin SJ, Harrison SC*, et al.* Structure and mechanism of DNA topoisomerase II. Nature 1996; 379: 225-32.
[170] Laponogov I, Sohi MK, Veselkov DA*, et al.* Structural insight into the quinolone-DNA cleavage complex of type IIA topoisomerases. Nat Struct Mol Biol 2009; 16: 667-9.
[171] Laponogov I, Pan XS, Veselkov DA*, et al.* Structural basis of gate-DNA breakage and resealing by type II topoisomerases. PLoS One 2010; 5: e11338.
[172] Bax BD, Chan PF, Eggleston DS*, et al.* Type IIA topoisomerase inhibition by a new class of antibacterial agents. Nature 2010; 466: 935-40.
[173] Wohlkonig A, Chan PF, Fosberry AP*, et al.* Structural basis of quinolone inhibition of type IIA topoisomerases and target-mediated resistance. Nat Struct Mol Biol 2010; 17: 1152-3.
[174] Drlica K, Hiasa H, Kerns R*, et al.* Quinolones: action and resistance updated. Curr Top Med Chem 2009; 9: 981-98.
[175] Malik M, Zhao X, Drlica K. Lethal fragmentation of bacterial chromosomes mediated by DNA gyrase and quinolones. Mol Microbiol 2006; 61: 810-25.

[176] Ikeda H, Aoki K, Naito A. Illegitimate recombination mediated *in vitro* by DNA gyrase of *Escherichia coli*: structure of recombinant DNA molecules. Proc Natl Acad Sci USA 1982; 79: 3724-8.

[177] Ikeda H, Kawasaki I, Gellert M. Mechanism of illegitimate recombination: common sites for recombination and cleavage mediated by *E. coli* DNA gyrase. Mol Gen Genet 1984; 196: 546-9.

[178] Collin F, Karkare S, Maxwell A. Exploiting bacterial DNA gyrase as a drug target: current state and perspectives. Appl Microbiol Biotechnol 2011; 92: 479-97.

[179] Dwyer DJ, Kohanski MA, Hayete B*, et al.* Gyrase inhibitors induce an oxidative damage cellular death pathway in *Escherichia coli*. Mol Syst Biol 2007; 3: 91.

[180] Kohanski MA, Dwyer DJ, Collins JJ. How antibiotics kill bacteria: from targets to networks. Nat Rev Microbiol 2010; 8: 423-35.

[181] Kohanski MA, Dwyer DJ, Hayete B*, et al.* A common mechanism of cellular death induced by bactericidal antibiotics. Cell 2007; 130: 797-810.

[182] Jacoby GA. Mechanisms of resistance to quinolones. Clin Infect Dis 2005; 41 Suppl 2: S120-6.

[183] Hernandez A, Sanchez MB, Martinez JL. Quinolone resistance: much more than predicted. Front Microbiol 2011; 2: 22.

[184] Robicsek A, Strahilevitz J, Jacoby GA*, et al.* Fluoroquinolone-modifying enzyme: a new adaptation of a common aminoglycoside acetyltransferase. Nat Med 2006; 12: 83-8.

[185] Perichon B, Courvalin P, Galimand M. Transferable resistance to aminoglycosides by methylation of G1405 in 16S rRNA and to hydrophilic fluoroquinolones by QepA-mediated efflux in *Escherichia coli*. Antimicrob Agents Chemother 2007; 51: 2464-9.

[186] Yamane K, Wachino J, Suzuki S*, et al.* New plasmid-mediated fluoroquinolone efflux pump, QepA, found in an *Escherichia coli* clinical isolate. Antimicrob Agents Chemother 2007; 51: 3354-60.

[187] Sanders CC. Mechanisms responsible for cross-resistance and dichotomous resistance among the quinolones. Clin Infect Dis 2001; 32 Suppl 1: S1-8.

[188] Yoshida H, Bogaki M, Nakamura M*, et al.* Quinolone resistance-determining region in the DNA gyrase *gyrA* gene of *Escherichia coli*. Antimicrob Agents Chemother 1990; 34: 1271-2.

[189] Kakinuma Y, Maeda Y, Mason C*, et al.* Molecular characterisation of the quinolone resistance-determining regions (QRDR) including *gyrA*, *gyrB*, *parC* and *parE* genes in *Streptococcus pneumoniae*. Br J Biomed Sci 2012; 69: 123-5.

[190] Dougherty TJ, Beaulieu D, Barrett JF. New quinolones and the impact on resistance. Drug Discov Today 2001; 6: 529-36.

[191] Bearden DT, Danziger LH. Mechanism of action of and resistance to quinolones. Pharmacotherapy 2001; 21: 224S-32S.

[192] Fu Y, Zhang W, Wang H*, et al.* Specific patterns of *gyrA* mutations determine the resistance difference to ciprofloxacin and levofloxacin in *Klebsiella pneumoniae* and *Escherichia coli*. BMC Infect Dis 2013; 13: 8.

[193] Chowdhury G, Pazhani GP, Nair GB*, et al.* Transferable plasmid-mediated quinolone resistance in association with extended-spectrum beta-lactamases and fluoroquinolone-acetylating aminoglycoside-6'-N-acetyltransferase in clinical isolates of *Vibrio fluvialis*. Int J Antimicrob Agents 2011; 38: 169-73.

[194] Heddle J, Maxwell A. Quinolone-binding pocket of DNA gyrase: role of GyrB. Antimicrob Agents Chemother 2002; 46: 1805-15.
[195] Drlica K, Zhao X. Mutant selection window hypothesis updated. Clin Infect Dis 2007; 44: 681-8.
[196] Eliopoulos GM, Gardella A, Moellering RC, Jr. *In vitro* activity of ciprofloxacin, a new carboxyquinoline antimicrobial agent. Antimicrob Agents Chemother 1984; 25: 331-5.
[197] Li X, Zhao X, Drlica K. Selection of *Streptococcus pneumoniae* mutants having reduced susceptibility to moxifloxacin and levofloxacin. Antimicrob Agents Chemother 2002; 46: 522-4.
[198] Wang H, Dzink-Fox JL, Chen M*, et al.* Genetic characterization of highly fluoroquinolone-resistant clinical *Escherichia coli* strains from China: role of *acrR* mutations. Antimicrob Agents Chemother 2001; 45: 1515-21.
[199] Cohen SP, McMurry LM, Hooper DC*, et al.* Cross-resistance to fluoroquinolones in multiple-antibiotic-resistant (Mar) *Escherichia coli* selected by tetracycline or chloramphenicol: decreased drug accumulation associated with membrane changes in addition to OmpF reduction. Antimicrob Agents Chemother 1989; 33: 1318-25.
[200] Alekshun MN, Levy SB. Regulation of chromosomally mediated multiple antibiotic resistance: the *mar* regulon. Antimicrob Agents Chemother 1997; 41: 2067-75.
[201] Cohen SP, Yan W, Levy SB. A multidrug resistance regulatory chromosomal locus is widespread among enteric bacteria. J Infect Dis 1993; 168: 484-8.
[202] Morita Y, Kataoka A, Shiota S*, et al.* NorM of *Vibrio parahaemolyticus* is an Na(+)-driven multidrug efflux pump. J Bacteriol 2000; 182: 6694-7.
[203] Cattoir V, Poirel L, Nordmann P. Plasmid-mediated quinolone resistance pump QepA2 in an *Escherichia coli* isolate from France. Antimicrob Agents Chemother 2008; 52: 3801-4.
[204] Hansen LH, Johannesen E, Burmolle M*, et al.* Plasmid-encoded multidrug efflux pump conferring resistance to olaquindox in *Escherichia coli*. Antimicrob Agents Chemother 2004; 48: 3332-7.
[205] Hansen LH, Sorensen SJ, Jorgensen HS*, et al.* The prevalence of the OqxAB multidrug efflux pump amongst olaquindox-resistant *Escherichia coli* in pigs. Microb Drug Resist 2005; 11: 378-82.
[206] Hansen LH, Jensen LB, Sorensen HI*, et al.* Substrate specificity of the OqxAB multidrug resistance pump in *Escherichia coli* and selected enteric bacteria. J Antimicrob Chemother 2007; 60: 145-7.
[207] Kim ES, Jeong JY, Choi SH*, et al.* Plasmid-mediated fluoroquinolone efflux pump gene, *qepA*, in *Escherichia coli* clinical isolates in Korea. Diagn Microbiol Infect Dis 2009; 65: 335-8.
[208] Ma J, Zeng Z, Chen Z*, et al.* High prevalence of plasmid-mediated quinolone resistance determinants *qnr*, *aac(6')-Ib-cr*, and *qepA* among ceftiofur-resistant *Enterobacteriaceae* isolates from companion and food-producing animals. Antimicrob Agents Chemother 2009; 53: 519-24.
[209] Amin AK, Wareham DW. Plasmid-mediated quinolone resistance genes in *Enterobacteriaceae* isolates associated with community and nosocomial urinary tract infection in East London, UK. Int J Antimicrob Agents 2009; 34: 490-1.
[210] Rodriguez-Martinez JM, Cano ME, Velasco C*, et al.* Plasmid-mediated quinolone resistance: an update. J Infect Chemother 2011; 17: 149-82.

[211] Strahilevitz J, Jacoby GA, Hooper DC*, et al.* Plasmid-mediated quinolone resistance: a multifaceted threat. Clin Microbiol Rev 2009; 22: 664-89.
[212] Vetting MW, Hegde SS, Fajardo JE*, et al.* Pentapeptide repeat proteins. Biochemistry 2006; 45: 1-10.
[213] Tran JH, Jacoby GA. Mechanism of plasmid-mediated quinolone resistance. Proc Natl Acad Sci USA 2002; 99: 5638-42.
[214] Tran JH, Jacoby GA, Hooper DC. Interaction of the plasmid-encoded quinolone resistance protein Qnr with *Escherichia coli* DNA gyrase. Antimicrob Agents Chemother 2005; 49: 118-25.
[215] Tran JH, Jacoby GA, Hooper DC. Interaction of the plasmid-encoded quinolone resistance protein QnrA with *Escherichia coli* topoisomerase IV. Antimicrob Agents Chemother 2005; 49: 3050-2.
[216] Poirel L, Rodriguez-Martinez JM, Mammeri H*, et al.* Origin of plasmid-mediated quinolone resistance determinant QnrA. Antimicrob Agents Chemother 2005; 49: 3523-5.
[217] Jacoby G, Cattoir V, Hooper D*, et al. qnr* Gene nomenclature. Antimicrob Agents Chemother 2008; 52: 2297-9.
[218] Saga T, Kaku M, Onodera Y*, et al. Vibrio parahaemolyticus* chromosomal *qnr* homologue VPA0095: demonstration by transformation with a mutated gene of its potential to reduce quinolone susceptibility in *Escherichia coli*. Antimicrob Agents Chemother 2005; 49: 2144-5.
[219] Kim HB, Wang M, Ahmed S*, et al.* Transferable quinolone resistance in *Vibrio cholerae*. Antimicrob Agents Chemother 2010; 54: 799-803.
[220] Heddle JG, Blance SJ, Zamble DB*, et al.* The antibiotic microcin B17 is a DNA gyrase poison: characterisation of the mode of inhibition. J Mol Biol 2001; 307: 1223-34.
[221] Garrido MC, Herrero M, Kolter R*, et al.* The export of the DNA replication inhibitor Microcin B17 provides immunity for the host cell. Embo J 1988; 7: 1853-62.
[222] Srinivasan VB, Virk RK, Kaundal A*, et al.* Mechanism of drug resistance in clonally related clinical isolates of *Vibrio fluvialis* isolated in Kolkata, India. Antimicrob Agents Chemother 2006; 50: 2428-32.
[223] Rushdy AA, Mabrouk MI, Abu-Sef FA*, et al.* Contribution of different mechanisms to the resistance to fluoroquinolones in clinical isolates of *Salmonella enterica*. Braz J Infect Dis 2013; 17: 431-7.
[224] Zhu JM, Jiang RJ, Kong HS*, et al.* Emergence of novel variants of *gyrA*, *parC*, *qnrS* genes in multi-drug resistant *Klebsiella* caused pneumonia. Zhonghua Liu Xing Bing Xue Za Zhi 2013; 34: 61-6.
[225] Gould IM, Bal AM. New antibiotic agents in the pipeline and how they can help overcome microbial resistance. Virulence 2013; 4: 185-91.
[226] Baharoglu Z, Mazel D. *Vibrio cholerae* triggers SOS and mutagenesis in response to a wide range of antibiotics: a route towards multiresistance. Antimicrob Agents Chemother 2011; 55: 2438-41.
[227] Da Re S, Garnier F, Guerin E*, et al.* The SOS response promotes *qnrB* quinolone-resistance determinant expression. EMBO Rep 2009; 10: 929-33.
[228] Moura A, Soares M, Pereira C*, et al.* INTEGRALL: a database and search engine for integrons, integrases and gene cassettes. Bioinformatics 2009; 25: 1096-8.
[229] Joss MJ, Koenig JE, Labbate M*, et al.* ACID: annotation of cassette and integron data. BMC Bioinformatics 2009; 10: 118.

[230] Tsafnat G, Copty J, Partridge SR. RAC: Repository of Antibiotic resistance Cassettes. Database (Oxford) 2011; 2011: bar054.
[231] Liu B, Pop M. ARDB--Antibiotic Resistance Genes Database. Nucleic Acids Res 2009; 37: D443-7.
[232] Siguier P, Perochon J, Lestrade L*, et al.* ISfinder: the reference centre for bacterial insertion sequences. Nucleic Acids Res 2006; 34: D32-6.
[233] Yoon SH, Park YK, Lee S*, et al.* Towards pathogenomics: a web-based resource for pathogenicity islands. Nucleic Acids Res 2007; 35: D395-400.
[234] Lima-Mendez G, Van Helden J, Toussaint A*, et al.* Reticulate representation of evolutionary and functional relationships between phage genomes. Mol Biol Evol 2008; 25: 762-77.
[235] Leplae R, Lima-Mendez G, Toussaint A. ACLAME: a CLAssification of Mobile genetic Elements, update 2010. Nucleic Acids Res 2010; 38: D57-61.
[236] Shao Y, Harrison EM, Bi D*, et al.* TADB: a web-based resource for Type 2 toxin-antitoxin loci in bacteria and archaea. Nucleic Acids Res 2011; 39: D606-11.
[237] Saier MH, Jr., Tran CV, Barabote RD. TCDB: the Transporter Classification Database for membrane transport protein analyses and information. Nucleic Acids Res 2006; 34: D181-6.
[238] Boulund F, Johnning A, Pereira MB*, et al.* A novel method to discover fluoroquinolone antibiotic resistance (*qnr*) genes in fragmented nucleotide sequences. BMC Genomics 2012; 13: 695.
[239] Fazil MH, Singh DV. *Vibrio cholerae* infection, novel drug targets and phage therapy. Future Microbiol 2011; 6: 1199-208
[240] Phillips JW, Goetz MA, Smith SK*, et al.* Discovery of kibdelomycin, a potent new class of bacterial type II topoisomerase inhibitor by chemical-genetic profiling in *Staphylococcus aureus*. Chem Biol 2011; 18: 955-65.
[241] Katara P, Grover A, Kuntal H*, et al. In silico* prediction of drug targets in *Vibrio cholerae*. Protoplasma 2011; 248: 799-804.
[242] Barh D, Kumar A, Misra AN. Genomic Target Database (GTD): a database of potential targets in human pathogenic bacteria. Bioinformation 2010; 4: 50-1.
[243] Keen EC. Phage therapy: concept to cure. Frontiers Microbiol 2012; 3:1-3.
[244] Marcuk LM, Nikiforov VN, Scerbak JF*, et al.* Clinical studies of the use of bacteriophage in the treatment of cholera. Bull World Health Organ 1971; 45: 77-83.
[245] Häusler T, Viruses *vs* superbugs : a solution to the antibiotics crisis? Macmillan 2006; pp. 248-60.
[246] Levine MM, Nalin DR, Craig JP*, et al.* Immunity of cholera in man: relative role of antibacterial *versus* antitoxic immunity. Trans R Soc Trop Med Hyg 1979; 73: 3-9.
[247] Holmgren J, Clemens J, Sack DA*, et al.* New cholera vaccines. Vaccine 1989; 7: 94-6.
[248] Cholera, 2012. Wkly Epidemiol Rec 2013; 88: 321-34.
[249] Stephens DS, Zughaier SM, Whitney CG*, et al.* Incidence of macrolide resistance in *Streptococcus pneumonia*e after introduction of the pneumococcal conjugate vaccine: population-based assessment. Lancet 2005; 365: 855-63.
[250] Kyaw MH, Lynfield R, Schaffner W*, et al.* Effect of introduction of the pneumococcal conjugate vaccine on drug-resistant *Streptococcus pneumoniae*. N Engl J Med 2006; 354: 1455-63.

[251] Bhardwaj AK, Vinothkumar K, Rajpara N. Bacterial quorum sensing inhibitors: attractive alternatives for control of infectious pathogens showing multiple drug resistance. Recent Pat Antiinfect Drug Discov 2013; 8: 68-83.
[252] Williams P. Quorum sensing, communication and cross-kingdom signalling in the bacterial world. Microbiology 2007; 153: 3923-38.
[253] Lowery CA, Dickerson TJ, Janda KD. Interspecies and interkingdom communication mediated by bacterial quorum sensing. Chem Soc Rev 2008; 37: 1337-46.
[254] Jayaraman A, Wood TK. Bacterial quorum sensing: signals, circuits, and implications for biofilms and disease. Annu Rev Biomed Eng 2008; 10: 145-67.
[255] Ji G, Beavis R, Novick RP. Bacterial interference caused by autoinducing peptide variants. Science 1997; 276: 2027-30.
[256] Gov Y, Borovok I, Korem M*, et al.* Quorum sensing in *Staphylococci* is regulated *via* phosphorylation of three conserved histidine residues. J Biol Chem 2004; 279: 14665-72.
[257] Mylonakis E, Engelbert M, Qin X*, et al.* The *Enterococcus faecalis fsrB* gene, a key component of the *fsr* quorum-sensing system, is associated with virulence in the rabbit endophthalmitis model. Infect Immun 2002; 70: 4678-81.
[258] Wei Y, Perez LJ, Ng WL*, et al.* Mechanism of *Vibrio cholerae* autoinducer-1 biosynthesis. ACS Chem Biol 2011; 6: 356-65.
[259] Sintim HO, Smith JA, Wang J*, et al.* Paradigm shift in discovering next-generation anti-infective agents: targeting quorum sensing, c-di-GMP signaling and biofilm formation in bacteria with small molecules. Future Med Chem 2010; 2: 1005-35.
[260] Galloway WR, Hodgkinson JT, Bowden SD*, et al.* Quorum sensing in Gram-negative bacteria: small-molecule modulation of AHL and AI-2 quorum sensing pathways. Chem Rev 2011; 111: 28-67.
[261] Rasmussen TB, Givskov M. Quorum sensing inhibitors: a bargain of effects. Microbiology 2006; 152: 895-904.
[262] Fulghesu L, Giallorenzo C, Savoia D. Evaluation of different compounds as quorum sensing inhibitors in *Pseudomonas aeruginosa*. J Chemother 2007; 19: 388-91.
[263] Asad S, Opal SM. Bench-to-bedside review: Quorum sensing and the role of cell-to-cell communication during invasive bacterial infection. Crit Care 2008; 12: 236.
[264] Williams P, Winzer K, Chan WC*, et al.* Look who's talking: communication and quorum sensing in the bacterial world. Philos Trans R Soc Lond B Biol Sci 2007; 362: 1119-34.
[265] Dong YH, Wang LY, Zhang LH. Quorum-quenching microbial infections: mechanisms and implications. Philos Trans R Soc Lond B Biol Sci 2007; 362: 1201-11.
[266] Miller MB, Bassler BL. Quorum sensing in bacteria. Annu Rev Microbiol 2001; 55: 165-99.
[267] Rutherford ST, Bassler BL. Bacterial quorum sensing: its role in virulence and possibilities for its control. Cold Spring Harb Perspect Med 2012; 2(11).
[268] Pan J, Ren D. Quorum sensing inhibitors: a patent overview. Expert Opin Ther Pat 2009; 19: 1581-601.
[269] Chan YY, Chua KL. The *Burkholderia pseudomallei* BpeAB-OprB efflux pump: expression and impact on quorum sensing and virulence. J Bacteriol 2005; 187: 4707-19.
[270] Hoang TT, Schweizer HP. Characterization of *Pseudomonas aeruginosa* enoyl-acyl carrier protein reductase (FabI): a target for the antimicrobial triclosan and its role in acylated homoserine lactone synthesis. J Bacteriol 1999; 181: 5489-97.

[271] Stephenson K, Yamaguchi Y, Hoch JA. The mechanism of action of inhibitors of bacterial two-component signal transduction systems. J Biol Chem 2000; 275: 38900-4.
[272] Lyon GJ, Mayville P, Muir TW*, et al.* Rational design of a global inhibitor of the virulence response in *Staphylococcus aureus*, based in part on localization of the site of inhibition to the receptor-histidine kinase, AgrC. Proc Natl Acad Sci USA 2000; 97: 13330-5.
[273] Smith KM, Bu Y, Suga H. Induction and inhibition of *Pseudomonas aeruginosa* quorum sensing by synthetic autoinducer analogs. Chem Biol 2003; 10: 81-9.
[274] Suga H, BU Y. Combinatorial libraries of autoinducer analogs, autoinducer agonist and antagonist, and methods of use thereof. US20080027115 (2008).
[275] Suga H, Smith KM. Molecular mechanisms of bacterial quorum sensing as a new drug target. Curr Opin Chem Biol 2003; 7: 586-91.
[276] Guo M, Gamby S, Nakayama S*, et al.* A pro-drug approach for selective modulation of AI-2-mediated bacterial cell-to-cell communication. Sensors (Basel) 2012; 12: 3762-72.
[277] Ganin H, Tang X, Meijler MM. Inhibition of *Pseudomonas aeruginosa* quorum sensing by AI-2 analogs. Bioorg Med Chem Lett 2009; 19: 3941-4.
[278] Li M, Ni N, Chou HT*, et al.* Structure-based discovery and experimental verification of novel AI-2 quorum sensing inhibitors against *Vibrio harveyi*. ChemMedChem 2008; 3: 1242-9.
[279] Lowery CA, Park J, Kaufmann GF*, et al.* An unexpected switch in the modulation of AI-2-based quorum sensing discovered through synthetic 4, 5-dihydroxy-2, 3-pentanedione analogues. J Am Chem Soc 2008; 130: 9200-1.
[280] Smyth AR, Cifelli PM, Ortori CA*, et al.* Garlic as an inhibitor of *Pseudomonas aeruginosa* quorum sensing in cystic fibrosis--a pilot randomized controlled trial. Pediatr Pulmonol 2010; 45: 356-62.
[281] Jakobsen TH, van Gennip M, Phipps RK*, et al.* Ajoene, a sulfur-rich molecule from garlic, inhibits genes controlled by quorum sensing. Antimicrob Agents Chemother 2012; 56: 2314-25.
[282] McDowell P, Affas Z, Reynolds C*, et al.* Structure, activity and evolution of the group I thiolactone peptide quorum-sensing system of *Staphylococcus aureus*. Mol Microbiol 2001; 41: 503-12.
[283] Balaban N, Giacometti A, Cirioni O*, et al.* Use of the quorum-sensing inhibitor RNAIII-inhibiting peptide to prevent biofilm formation *in vivo* by drug-resistant *Staphylococcus epidermidis*. J Infect Dis 2003; 187: 625-30.
[284] Miyairi S, Tateda K, Fuse ET*, et al.* Immunization with 3-oxododecanoyl-L-homoserine lactone-protein conjugate protects mice from lethal *Pseudomonas aeruginosa* lung infection. J Med Microbiol 2006; 55: 1381-7.
[285] Tateda K, Standiford TJ, Pechere JC*, et al.* Regulatory effects of macrolides on bacterial virulence: potential role as quorum-sensing inhibitors. Curr Pharm Des 2004; 10: 3055-65.
[286] Skindersoe ME, Alhede M, Phipps R*, et al.* Effects of antibiotics on quorum sensing in *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 2008; 52: 3648-63.
[287] Hung DT, Shakhnovich EA, Pierson E*, et al.* Small-molecule inhibitor of *Vibrio cholerae* virulence and intestinal colonization. Science 2005; 310: 670-4.
[288] Van Bambeke F, Pages JM, Lee VJ. Inhibitors of bacterial efflux pumps as adjuvants in antibiotic treatments and diagnostic tools for detection of resistance by efflux. Recent Pat Antiinfect Drug Discov 2006; 1: 157-75.

[289] Zechini B, Versace I. Inhibitors of multidrug resistant efflux systems in bacteria. Recent Pat Antiinfect Drug Discov 2009; 4: 37-50.
[290] Chopra I. New developments in tetracycline antibiotics: glycylcyclines and tetracycline efflux pump inhibitors. Drug Resist Updat 2002; 5: 119-25.
[291] Chollet R, Chevalier J, Bryskier A*, et al.* The AcrAB-TolC pump is involved in macrolide resistance but not in telithromycin efflux in *Enterobacter aerogenes* and *Escherichia coli*. Antimicrob Agents Chemother 2004; 48: 3621-4.
[292] Zgurskaya HI, Nikaido H. Multidrug resistance mechanisms: drug efflux across two membranes. Mol Microbiol 2000; 37: 219-25.
[293] Yoshihara, E., Inoko, H. Method or agent for inhibiting the function of efflux pump in *Pseudomonas aeruginosa*. US7985410B2 (2011).
[294] Kern WV, Oethinger M, Jellen-Ritter AS*, et al.* Non-target gene mutations in the development of fluoroquinolone resistance in *Escherichia coli*. Antimicrob Agents Chemother 2000; 44: 814-20.
[295] Pages JM, Masi M, Barbe J. Inhibitors of efflux pumps in Gram-negative bacteria. Trends Mol Med 2005; 11: 382-9.
[296] Mallea M, Chevalier J, Eyraud A*, et al.* Inhibitors of antibiotic efflux pump in resistant *Enterobacter aerogenes* strains. Biochem Biophys Res Commun 2002; 293: 1370-3.
[297] Mahamoud A, Chevalier J, Alibert-Franco S*, et al.* Antibiotic efflux pumps in Gram-negative bacteria: the inhibitor response strategy. J Antimicrob Chemother 2007; 59: 1223-9.
[298] Marquez B. Bacterial efflux systems and efflux pumps inhibitors. Biochimie 2005; 87: 1137-47.
[299] Lynch AS. Efflux systems in bacterial pathogens: an opportunity for therapeutic intervention? An industry view. Biochem Pharmacol 2006; 71: 949-56.
[300] Lomovskaya O, Bostian KA. Practical applications and feasibility of efflux pump inhibitors in the clinic--a vision for applied use. Biochem Pharmacol 2006; 71: 910-8.
[301] Lomovskaya O, Warren MS, Lee A*, et al.* Identification and characterization of inhibitors of multidrug resistance efflux pumps in *Pseudomonas aeruginosa*: novel agents for combination therapy. Antimicrob Agents Chemother 2001; 45: 105-16.
[302] Renau TE, Leger R, Flamme EM*, et al.* Inhibitors of efflux pumps in *Pseudomonas aeruginosa* potentiate the activity of the fluoroquinolone antibacterial levofloxacin. J Med Chem 1999; 42: 4928-31.
[303] Coban AY, Ekinci B, Durupinar B. A multidrug efflux pump inhibitor reduces fluoroquinolone resistance in *Pseudomonas aeruginosa* isolates. Chemotherapy 2004; 50: 22-6.
[304] Mamelli L, Amoros JP, Pages JM*, et al.* A phenylalanine-arginine beta-naphthylamide sensitive multidrug efflux pump involved in intrinsic and acquired resistance of *Campylobacter* to macrolides. Int J Antimicrob Agents 2003; 22: 237-41.
[305] Hasdemir UO, Chevalier J, Nordmann P*, et al.* Detection and prevalence of active drug efflux mechanism in various multidrug-resistant *Klebsiella pneumoniae* strains from Turkey. J Clin Microbiol 2004; 42: 2701-6.
[306] Mazzariol A, Tokue Y, Kanegawa TM*, et al.* High-level fluoroquinolone-resistant clinical isolates of *Escherichia coli* overproduce multidrug efflux protein AcrA. Antimicrob Agents Chemother 2000; 44: 3441-3.

[307] Baucheron S, Imberechts H, Chaslus-Dancla E*, et al.* The AcrB multidrug transporter plays a major role in high-level fluoroquinolone resistance in *Salmonella enterica* serovar *typhimurium* phage type DT204. Microb Drug Resist 2002; 8: 281-9.

[308] Renau TE, Leger R, Flamme EM*, et al.* Addressing the stability of C-capped dipeptide efflux pump inhibitors that potentiate the activity of levofloxacin in *Pseudomonas aeruginosa*. Bioorg Med Chem Lett 2001; 11: 663-7.

[309] Renau TE, Leger R, Yen R*, et al.* Peptidomimetics of efflux pump inhibitors potentiate the activity of levofloxacin in *Pseudomonas aeruginosa*. Bioorg Med Chem Lett 2002; 12: 763-6.

[310] Renau TE, Leger R, Filonova L*, et al.* Conformationally-restricted analogues of efflux pump inhibitors that potentiate the activity of levofloxacin in *Pseudomonas aeruginosa*. Bioorg Med Chem Lett 2003; 13: 2755-8.

[311] Watkins WJ, Landaverry Y, Leger R*, et al.* The relationship between physicochemical properties, *in vitro* activity and pharmacokinetic profiles of analogues of diamine-containing efflux pump inhibitors. Bioorg Med Chem Lett 2003; 13: 4241-4.

[312] Oethinger M, Levy SB. Methods of reducing microbial resistance to drugs. US6346391B1 (2002).

[313] Oethinger, M., Levy S. B. Methods of screening for compounds that reduce microbial resistance to fluoroquinolones. US8012711B2 (2011).

[314] Chun CK, Ozer EA, Welsh MJ*, et al.* Inactivation of a *Pseudomonas aeruginosa* quorum-sensing signal by human airway epithelia. Proc Natl Acad Sci USA 2004; 101: 3587-90.

[315] Finch R, Hunter PA. Antibiotic resistance--action to promote new technologies: report of an EU Intergovernmental Conference held in Birmingham, UK, 12-13 December 2005. J Antimicrob Chemother 2006; 58 Suppl 1: i3-i22.