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Antibiotic resistance in *Vibrio cholerae:* Understanding the ecology of resistance genes and mechanisms

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ABSTRACT

The unique genetic makeup and remarkable competency of *Vibrio cholerae* are the key factors that help the cholera pathogen adapt rapidly to adverse environmental conditions and resist the detrimental effect of antimicrobial agents. In the last few decades, *V. cholerae* that causes acute watery diarrhoeal disease cholera has emerged as a notorious multidrug resistant (MDR) enteric pathogen. Although chromosomal mutations can contribute to antimicrobial resistance (AMR), the frequent acquisition of extrachromosomal mobile genetic elements (MGEs) from closely/distantly related bacterial species are major players in *V. cholerae* drug resistance. Whole genome sequence analysis of clinical and environmental *V. cholerae* strains revealed that the genome of most of the recent isolates harbour integrating conjugative elements (ICEs), plasmids, superintegron, transposable elements and insertion sequences, which are the key carriers of genetic traits encoding antimicrobial resistance function. Different antimicrobial resistance genes identified in *V. cholerae* can contribute in antibiotic resistance by facilitating one of the following three mechanisms; (i) reduced permeability or active efflux of the antibiotics, (ii) alteration of the antibiotic targets by introducing post-transcriptional/translational modifications and (iii) hydrolysis or chemical modification of antibiotics. Here, we present an overview of the present insights on the emergence and mechanisms of AMR in *V. cholerae.*

1. Introduction

Vibrio cholerae, a comma shaped bacillus autochthonous to the aquatic environment that infects humans through contaminated water or food, is the causative agent of cholera, a self-limiting acute diarrhoeal disease. In the treatment of cholera along with oral and intravenous rehydration therapy, antibiotics may also be administered as part of the treatment regimen to reduce the stool volume, duration of diarrhoea/excretion of *V. cholerae* and volume of rehydration fluid uptake. Over the years, several antimicrobials such as tetracycline, fluoroquinolones and azithromycin have been effectively used in the treatment of cholera patients [1]. However, in the recent years, treatment failures are often seen with the recurrent emergence of antimicrobial resistant *V. cholerae* [2]. Recently, there has been an increasing concern about the emergence of AMR in *V. cholerae* (Fig. 1). The drug resistant *V. cholerae* can withstand the detrimental effects of most, if not all, antibiotics commonly used in the treatment of diarrhoeal and other bacterial infectious diseases.

Antibiotics, the chemical compounds that inhibit bacterial multiplication (bacteriostatic) or kill the bacterial species (bactericidal), are mostly natural products, synthesized by the bacterial or fungal species as chemical weaponries to kill off other microbes in the nearby microenvironment and keep the balance in the microbial communities in natural ecosystems [3]. Targets of the antibiotics are generally unique to bacteria or significantly different from their eukaryotic counterpart and are essential for bacterial growth and survival. Most of the antibiotics work broadly by inhibiting (i) cell wall synthesis or assembly (ii) interrupting cell membrane integrity (iii) preventing DNA, RNA and protein syntheses and (iv) interrupting essential cellular metabolic pathways (Fig. 2). Specificity of antibiotics towards microbial machineries is the key for its usage in clinical practice to prevent and cure microbial infections and their therapeutic implementation revolutionized the history of medicine. However, since its introduction, it was recognized that antibiotics have two distinct properties: First, the rapid and desired one of inhibiting the growth of microbes by interfering with the essential functions

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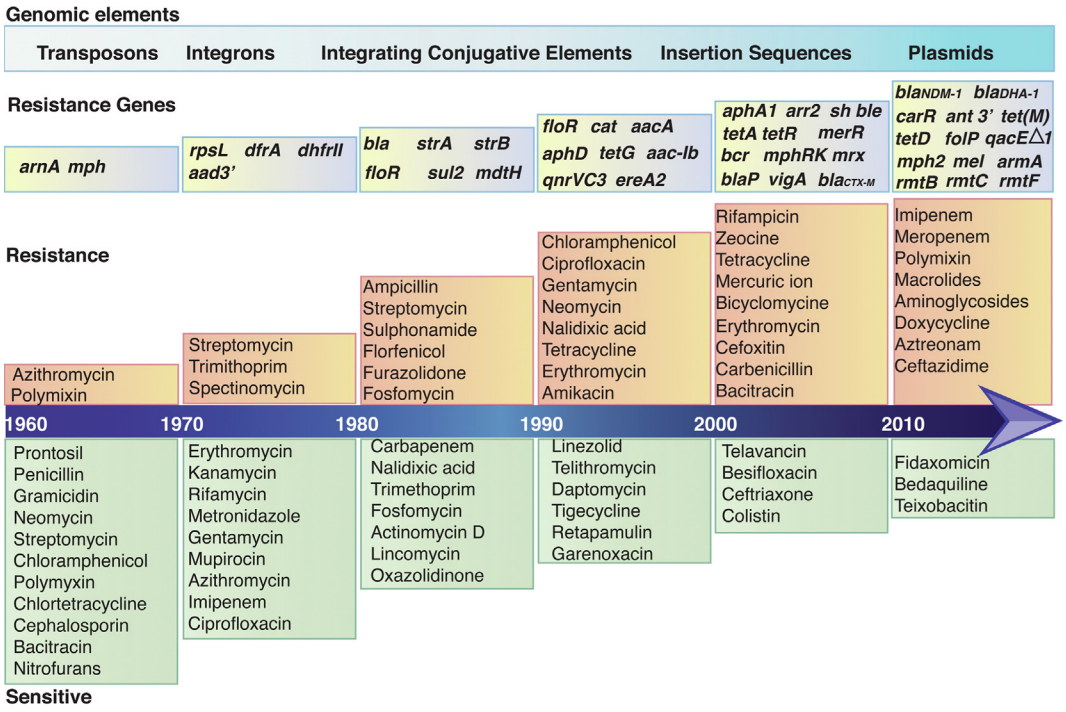

Fig. 1. Antibiotic susceptibility/resistance genes encoding resistance function and genetic elements carrying the resistance genes in *V. cholerae*. The picture shows that *V. cholerae* acquired resistance functions against almost all the commonly used antibiotics over time. Most of the resistances are due to acquisition of MGEs that are physically linked with the antibiotic resistance genes. Relevant information was obtained from multiple sources including http://arpcard.mcmaster.ca, and references from [28,29,30,31].

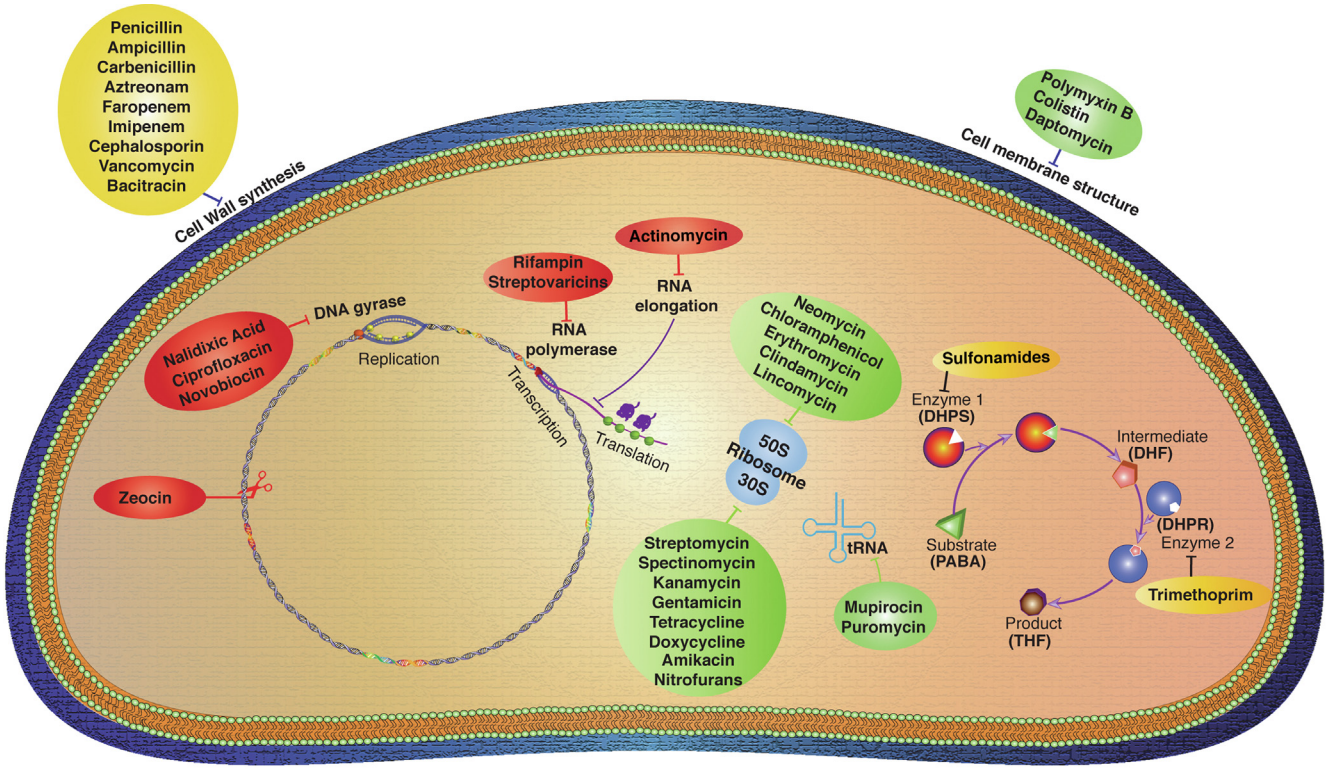

Fig. 2. Major targets of antimicrobial agents in bacterial cells. The figure shows different antimicrobials have different modes of action. Antimicrobials could inhibit synthesis or assembly of bacterial cell wall, alter membrane integrity, inhibit nucleic acid metabolisms or inhibit the other metabolic processes essential for survival. Inhibition of functions might be reversible or irreversible [76,80].

or cellular process and the second, the undesired one, which promotes the emergence of AMR pathogens by providing a suitable environment for their growth by eliminating sensitive variants. The first effective antibiotic sulfonamide was discovered in 1932, put into clinical trials in 1935 and resistance was reported in the late 1930s [4]. Similarly for penicillin, resistance was

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reported in the mid 1940s, within two years of its introduction in clinical practice [5].

Emergence of resistance against antibiotics is a natural evolutionary process and several factors including environment, density of microbial community in a specific habitat, usage pattern of antibiotics in health, animal farming, food and agriculture sectors can influence the frequency of emergence [6]. A number of processes like transformation, conjugation, transduction and fusion of outer membrane vesicles (OMVs) that mediate the horizontal gene transfer (HGT) are major pathways of rapid emergence of AMR pathogens [7]. Environmental factors, specifically compounds that induce bacterial SOS response and modulate HGT, are crucial for exchange of genetic materials including AMR genes between bacterial species [8,9]. The emergence of multidrug resistance (MDR) among enteric pathogens was first detected in the early 1960s [10,11]. In the last few decades, AMR *V. cholerae* has evolved rapidly and spread across the globe as a result of overuse and misuse of antibiotics in different sectors (Fig. 1). Extensive drug resistant (XDR) and MDR *V. cholerae* genomes are enriched with mobile genetic elements (MGEs) linked with resistance genes and could potentially transmit the resistance traits to other bacterial pathogens. Rapid dissemination of resistance among bacterial pathogens, including *V. cholerae* is now a major threat to public health as well as pharmaceutical companies involved in new antimicrobial development. Here, we reviewed the emergence and spread of antimicrobial resistance in *V. cholerae*, the ecology of antibiotic resistance genes, mechanisms of antibiotic resistance and genomic elements involved in the dissemination of antimicrobial resistance.

2. Antibiotics in the treatment of cholera

Antibiotic treatment in cholera patients is recommended after restoration of the initial fluid deficit and prevention of vomiting. Between the 1940s and 1960s, the early antibiotics effectively used in the treatment of cholera include streptomycin [12,13] and chloramphenicol [14,15]. Use of tetracycline in the treatment of cholera was demonstrated in Calcutta in 1962 [16]. Furazolidone was considered as an alternative to tetracycline in treating children with cholera [14] due to their comparable results in the various clinical trials [17,18]. Sulphamethoxazole-trimethoprim- (SXT) was introduced in the treatment of cholera during 1970s [19]. In Lima, Peru, the therapeutic regimes SXT and tetracycline performed equally well in cholera patients [20]. Tetracycline, chloramphenicol and SXT were equally effective in eliminating *V. cholerae* from cholera patients [21–23]. Azithromycin and ciprofloxacin are also used for cholera patients to reduce the severity and duration of the disease [2]. In the cholera control program, chemoprophylaxis is not generally recommended due to unnecessary expenditure and the emergence of resistant organisms against specific drugs that has been used to treat the patients.

3. Emergence of antimicrobial resistance in *V. cholerae*

Emergence of MDR and XDR *V. cholerae* is an excellent example of bacterial evolution in the recent time. During 1960s, resistance against one or a few antibiotics reported in *V. cholerae* was mostly due to the acquisition of spontaneous mutations in the drug's target like DNA gyrase, topoisomerase, β-subunit of RNA polymerase (RpoB) and small subunit ribosomal protein12 [24,25]. Recent studies suggest that the emergence of MDR and XDR *V. cholerae,* however, is mainly facilitated by the horizontal gene transfer (HGT) via self-transmissible, autonomously replicating plasmids or integrative IMGEs including **I**ntegrating **C**onjugative **E**lements (ICEs), **I**nsertion **S**equences (IS) and transposable genetic elements [26,27]. Emergence of MDR and XDR *V. cholerae* by accumulating spontaneous mutations in the target gene is unusual, since the frequency of spontaneous mutation in multiple genes per genome is very low. Nevertheless, accumulation of multiple mutations may also impact the fitness of bacteria in the absence of antibiotics.

The MDR *V. cholerae* isolates belonging to serogroup O1 displaying resistance against tetracycline, streptomycin and chloramphenicol was first reported during 1970 [28]. Subsequently, during the last five decades, resistance profile of *V. cholerae* has changed intensely [29]. *V. cholerae* isolates remained susceptible to most of the antibiotics used in the treatment of cholera/diarrhoea till early 1970s. The emergence of tetracycline resistant *V. cholerae* O1 from a huge cholera epidemic in Tanzania that had been reported during 1977–78 was mainly due to the prophylactic use of the drugs [28]. This resistance was found to be due to the presence of a mega plasmid belonging to an unstable incompatibility complex C (IncC) [30]. During 1994, death of about 12,000 Rwandan refugees in Goma, Eastern Zaire was attributed due to tetracycline resistant *V. cholerae* O1 [31]. In this worst outbreak, tetracycline and doxycycline were the drugs used in the treatment of cholera patients. During 1988–89, the classical biotype isolates from the southern coastal region of Bangladesh were found to be resistant to tetracycline, whereas the El Tor biotype isolated from the same region were sensitive [32]. Even the newly emerged *V. cholerae* serogroup O139 in 1993 from Karachi, Pakistan was reported to be resistant to tetracycline [33]. A drastic increase in resistance against ampicillin, nalidixic acid, chloramphenicol and tetracycline started to appear from early 1990 [34]. The recent trend shows that most of the clinical isolates of *V. cholerae* are resistant against almost all routinely used antibiotics [26,35]. The antimicrobial resistance encoding functions are also being identified as in self-transmissible plasmids. Emergence of MDR *V. cholerae* belonging to serogroup O139 was first described in 1996. Resistance was shown to be due to a ~100-Kb ICE called SXT element that carries multiple resistance genes against **S**ulfamethoxazole, **T**rimethoprim and **S**treptomycin. Subsequently it was discovered that several environmental *V. cholerae* strains isolated during 1986 also harboured SXT element in their genome and displayed resistance against ampicillin, SXT, streptomycin and furazolidone [36]. Besides these, mobile integron physically linked with conjugative plasmids or transposons also disseminate multiple resistance encoding functions to the clinical and environmental *V. cholerae* isolates across the globe.

4. Genomic insights into antimicrobial resistant *V. cholerae*

The *V. cholerae* genome consists of two non-homologous, asymmetrical, circular chromosomes (Ch1 and Ch2) with more than 4000 open reading frames (ORFs) involved in essential cellular functions and other metabolic and signaling pathways that provide fitness to the cholera pathogen in specific environmental condition [37]. More than 3600 ORFs in the *V. cholerae* chromosomes are part of the core genome and contribute to the cellular functions including replication, transcription, translation, cellular metabolic pathways, cell membrane and cell wall biosynthesis. Several proteins involved in the somatic antigen biosynthesis, regulatory functions, nutrients and metabolite transport, chemotaxis, DNA mobility, pathogenicity, antibiotic and heavy metal resistance and unknown functions are identified in the *V. cholerae* genome with specific genomic signatures that diverge from common proteobacterial ancestors [37]. Nearly, ~5–10% of the genomic content of *V. cholerae* consists of flexible gene pools that have been acquired by HGT from closely or distantly related bacterial species and provide fitness to the cholera pathogens to compete with other microbial species living in the same ecosystem [38].

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The acquired functions in the MGEs contribute in intestinal colonization [39], toxin production [40] phage dissemination [41,42] disease development [43], AMR [27,44], chromosome dimer resolution [45] and fitness [46,47]. Ch1 of the current *V. cholerae* strains comprised of ~2.96 mega bases (mb) and may harbour four to five Genomic Islands (GIs), three to four prophages, one ICE and hundreds of gene cassettes [37,48]. The acquired gene pools in the GIs and prophages encode functions that are essential for intestinal colonization (using toxin co-regulated pilus), virulence (ToxT), toxin-production (cholera toxin) and disease development [37]. Most of the ICEs are physically linked with multiple ORFs that provide AMR functions to *V. cholerae* [49].

Ch2 of the *V. cholerae* is ~1.07 mb in size and most of the ORFs carry unknown gene functions encoded by the hypothetical or conserved hypothetical genes. Few genes encode subunit proteins for ribosome (L20 and L35) that are essential for viability. The acquired gene pools in the Ch2 are part of the CTX-prophage, TLC, GIs or gene cassettes captured by the integron. Several gene cassettes in the Integron island are involved in AMR [50]. The gene cassettes detained by the Integron island is generally devoid of promoter element and their transcription is mainly initiated from a strong promoter of the Integron island [51].

5. Ecology of antimicrobial resistance genes in *V. cholerae*

Comprehensive genomic studies of pathogenic and non-pathogenic bacterial species and the Comprehensive Antibiotic Resistance Database (CARD) catalogues 175,753 alleles with potential resistance functions against 249 clinically important antibiotics (https://card.mcmaster.ca). A single isolate of *V. cholerae* may harbour as many as 40 different AMR encoding genes that can confer resistance against 22 antibiotics representing nine different classes of antimicrobial drugs [26]. Irrespective of their geographical origins, both pathogenic and non-pathogenic *V. cholerae* can acquire AMR to promote their subsistence. Most of the AMR genes reported in toxigenic *V. cholerae* are acquired through HGT and are physically-linked with replicative and/or integrative MGEs. HGT allows bacteria to evolve very fast by introducing multiple fitness factors even in the single event of acquisition [27]. Several studies have reported that the MGEs are preexisting source of AMR genes and widely distributed in the genome of environmental as well as human associated microbial species [52]. There are six major classes of MGEs often found in the genome of pathogenic and non-pathogenic bacteria namely, transposons, gene cassettes and integrons, genomic islands, plasmids, bacteriophages and ICEs [52]. Most of these MGEs mediate the exchange of AMR genes between pathogens, or between pathogenic and commensal bacterial population living commonly or transiently in similar host or environmental niches. In *V. cholerae*, AMR genes are often physically linked with transposons, ICEs, integrons or plasmids [26].

5.1. Integrating conjugative elements (ICEs)

ICEs are widely distributed self-transmissible IMGEs that encode all the functions essential for their mobility and integration into the bacterial chromosome [53,54]. Soon after its discovery, ICEs drew attention of scientific communities as it contributed to several antibiotic and heavy metal resistances and their important role in bacterial motility and biofilm formation [53]. ICEs have many similarities with plasmid and bacteriophages. Similar to plasmids, ICEs transfer via conjugation and like phages, these elements integrate into host chromosome and replicate passively by exploiting chromosomal replication machineries [55]. ICEs played important role in the emergence of multidrug resistant *V. cholerae* [56]. In *V. cholerae* and other bacteria, ICEs first generate ssDNA in the donor cell and transfer- to the recipient through the type IV secretion system. The initiation of extrachromosomal ssDNA-ICE formation starts at the origin of transfer (*oriT*), recognized and covalently linked with the 5′ end of the DNA relaxase. The linear ssDNA-ICEs covalently linked with the DNA relaxase get transferred to the recipient cell and relaxase mediates the circularization of ssDNA-ICEs. Complementary strand is synthesized before its integration into the host chromosome. Integration of circular dsDNA-ICEs into the host chromosome is mostly mediated by the tyrosine recombinase called integrase [57]. The integrases contain highly conserved Arg-His-Arg-Tyr catalytic tetrad in the carboxy-terminal end of the protein. The integration is site-specific (*attP* × *attB*) and reversible. The ICEs integrate at the 5′ end of *pfrC*, which encodes peptide chain release factor 3, a protein essential for survival and multiplication of *V. cholerae* [58]. ICEs integration is reversible and like integration, excision is also mediated by the tyrosine or serine recombinases.

Most of the ICEs have a modular structure and can be divided into multiple modules associated with distinct functions including, (i) integration/excision module, (ii) replication/DNA processing module, (iii) DNA secretion module, (iv) regulation module and (v) auxiliary modules. The auxiliary modules often harbour evolutionarily important traits, including antimicrobial resistance genes, virulence or toxin encoding genes, heavy metal resistance genes and genetic traits involved in the alternative catabolic pathways (Table 2).

Multiple antibiotic resistance genes carrying ICEs, namely SXT and R391, were originally reported from *V. cholerae* (MO10) and *Proteus rettgeri*, respectively [56,59]. The name SXT was given because of its contribution to help bacteria to sustain and grow in the presence of streptomycin, sulfamethoxazole and trimethoprim. The SXT^{MO10} encodes resistance against chloramphenicol, streptomycin, and SXT [56]. SXT/R391 ICEs possibly emerged first from the Asian continents during the late 1980s and subsequently spread into several clinically important bacterial species in different countries [60]. Till date, more than 60 ICEs have been identified in *V. cholerae* and categorised them within the SXT/R391 family because of its functional modules and secondary functions, including antimicrobial resistance and heavy metal tolerance [61].

After its first report in *V. cholerae* O139 isolates in India, at least three more ICEs belonging to the same family, ICE*Vch*Ind5, ICE*Vch*Moz10 and ICE*VchHai1* have been reported in India, Mozambique and Haiti, respectively [62]. Currently, ICEs of the SXT-R391 family have been spread across the globe and are detected in almost every *V. cholerae* O1 isolates. The recent comprehensive genomic studies suggested that ICE of the SXT-R391 family, possibly appeared between the wave-1 and wave-2 of the cholera epidemic during 1978–1984. The ICE*Vch*Ind5 has been widely prevalent for several years in the *V. cholerae* O1 clinical strains isolated from India. Genomic studies indicated that *V. cholerae* O1 and O139 isolates independently acquired the ICE*Vch*Ind5 and SXT, respectively. Currently, ICE*Vch*Ind5 was documented as the most predominant ICE among clinical *V. cholerae* O1 isolates circulating across the globe [63]. The genome of *V. cholerae* O1 isolates that caused Haitian cholera outbreak in 2010, harboured ICE*Vch*Hai1 and its auxiliary module encodes resistance against trimethoprim (*dfrA1*), sulfamethoxazole (*sul2*), streptomycin (*strAB*) and chloramphenicol (*floR*). ICEs with a similar set of antimicrobial resistance genes have also been reported in India (1994–2005) and Bangladesh (1998), Nepal (1994) and Nigeria (2010) [63].

5.2. Integrons

Integrons are genetic system that captures exogenous ORFs by site-specific recombination and convert the integrated cassette into a functional gene by providing an active transcription platform

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[64]. Integrons use their own integrase (IntI) for cassette integration. All the reported integrons have three basic modules viz., (i) integrase encoding gene, (ii) specific integration site (*attI*) and (iii) a functional promoter (P_c) for integrated elements. The integron-encoded integrase catalyzes the site-specific integration between double stranded host chromosome (*attC*) and folded single-stranded exogenous cassette (*attI*). All the integrons identified so far can be categorized into two classes: the mobile integrons and superintegrons. The mobile integrons are often linked with the MGEs (insertion sequences, transpososns, ICEs, conjugative plasmids) and are responsible for the dissemination of antibiotic resistance genes in bacterial cells. In *V. cholerae*, the SXT element harbours a trimethoprim resistance gene, which is physically linked with a mobile integron [60]. Antibiotic resistance genes encoding resistance against trimethoprim, β-lactams, aminoglycosides, erythromycin, chloramphenicol, rifampicin, fosfomycin, quinolones, and several antiseptic compounds have been reported physically linked with the mobile integrons [44,6].

Superintegrons are part of bacterial chromosomes [65,66]. In *V. cholerae*, superintegron is identified in the Ch2 without any association with MGE. The superintegron of the first whole genome sequenced *V. cholerae* strain N16961 contained more than 210 ORFs, mostly with hypothetical or toxin-antitoxin functions and contribute to ~3% of the total genomic content. Other than antibiotic resistance, the genes linked with the superintegron mostly provides fitness to *V. cholerae* and help during the survival under stressed conditions. The DNA elements captured at the integron contain hundreds of genes with known or unknown functions and constitute a significant portion of the *V. cholerae* genome. The importance of integrons is primarily recognized because of its contribution in capturing antibiotic resistance genes, but the association of several other genes linked with fitness and virulence of bacteria suggests that role of superintegron in bacterial evolution is not restricted only at the antibiotic resistance regiment.

5.3. Plasmids

Plasmids, the extrachromosomal genetic elements, proficient in autonomous replication, have largely been attributed in HGT and key players in microbial evolution [67]. The contribution of plasmids in drug resistance and virulence in *V. cholerae* was recognized much earlier than that of ICEs and integrons [68]. Their existence in the genome of both clinical and environmental *V. cholerae* isolates belonging to serogroups O1, O139 and non-O1, non-O139 has widely been reported since the 1960s [69]. The plasmids reported from different *V. cholerae* are very heterogeneous in terms of their size (4 to 200-Kb), mode of dissemination (conjugative, non-conjugative) and contribution to *V. cholerae* biology (toxin acquisition, virulence, adhesion, chromosome partitioning, dimer resolution, antibiotic and heavy metal resistance). A single plasmid can confer resistance against more than 6 different drugs (ampicillin, streptomycin, gentamicin, tetracycline, chloramphenicol and SXT) in *V. cholerae* and other enteric pathogens [70]. The sequencing analysis of the plasmids isolated from the clinical *V. cholerae* revealed that the multiple resistance determinants (*aac (3)-IIa, bla*$_{CMY-2}$, *bla*$_{CTX-M-2}$, *bla*$_{TEM-1}$, *bla*$_{NDM}$, *sh ble, dfrA15, mphA, arr3, aadA16, sul1, strAB, floR* and *tetA* are of plasmid origin and sometimes physically linked with IS elements. The *sh ble-bla*$_{NDM1}$ allele conferring resistance against bleomycin and imipenem was revealed to be physically linked with the insertion sequence (IS) element (*IS*30) and integrated in the mega plasmid reported in *V. cholerae* and other pathogens [71]. Plasmids conferring resistance against multiple antibiotics are usually large (>40-Kb), conjugative and maintained at low copy numbers in the host bacteria. Genes encoding resistance to chloramphenicol, tetracycline, erythromycin, aminoglycosides, SXT and cephalosporins were identified in the IncA mega plasmid (pVC1447) of *V. cholerae* O139 isolated in China during 2000–2006 [72].

Due to the excessive use of fluoroquinolones such as norfloxacin and ciprofloxacin from late 1980s, several resistance mechanisms have been developed in Gram-negative bacteria. The emergence of plasmid-mediated quinolone resistance (PMQR) has been reported since 1998. The first plasmid-mediated gene involved in quinolone resistance (currently known as *qnr*A1) was reported in *Klebsiella pneumoniae*. Subsequent to this the other plasmid genes such as *qnrA, qnrB, qnrC, qnrD, qnrS*, and *qnrVC* have also been reported in several Gram-negative pathogens. These genes code for proteins of the pentapeptide repeat family that protects DNA gyrase and topoisomerase IV from quinolone inhibition. *qnrVC1* and *qnrVC3* were reported from a clinical strain of *V. cholerae* isolated from Brazil in 1998 and Bangladesh in 2005, respectively [73]. These *qnrVC* genes conferred transferable quinolone resistance and were carried by the SXT-ICE of *V. cholerae*. Unlike in members of the Enterobacteriaceae, the AAC(6′)-Ib-cr has been reported rarely in *V. cholerae* [74]. Functionally, *aac(6′)-Ib* encodes acetyltransferase and active on aminoglycosides as amikacin, kanamycin, and tobramycin.

6. Mechanisms of antimicrobial resistance in *V. cholerae*

Although, AMR pathogens from all three domains of life (bacteria, viruses and eukaryotes) are of major concern, three classes of bacterial pathogens of public health importance, belonging to phyla Proteobacteria, Firmicutes, and Actinobacteria have emerged as major threats in the developed and developing countries [75]. Several species of Gram-negative bacteria belonging to phylum Proteobacteria that are linked with a wide range of health disorders, including diarrhoea, urinary tract infections, respiratory tract infections, pneumonia and sepsis have emerged as life threatening MDR and PDR pathogens. Most of the isolates have been reported to be resistant against several antibiotics- β-lactams, quinolones, aminoglycosides, tetracyclines, SXT, polymixins and macrolides routinely used in clinical practice to treat Gram-negative pathogens.

Unregulated and profuse use of antibiotics simply promoted the evolution and spread of resistant variants. Bacterial species, including *V. cholerae* can acquire resistance to antimicrobial compounds by any of the following seven mechanisms. (i) modification of the antibiotic target site, (ii) antibiotic target replacement, (iii) antibiotic target protection, (iv) inactivation of the antibiotic by hydrolysis or chemical modification, (v) preventing access to the target site by changing membrane permeability, (vi) actively exporting antibiotics from bacterial cell, and (vii) resistance by absence of antibiotic target [76,77]. Both, spontaneous mutation and horizontal gene transfer could contribute in all the resistance mechanisms. Accumulation of spontaneous mutations in the target ORF or a sequence of its regulatory region can confer resistance by inhibiting antibiotic binding or by over producing target molecules. Resistance through spontaneous mutations is common in intracellular pathogens [78]. Antimicrobial resistance in *V. cholerae* is mostly due to acquisition of resistance genes from closely or distantly related microbial species through horizontal gene transfer [26]. The principal mechanisms of resistance in *V. cholerae* are summarized in Fig. 3 and discussed in the following sections.

6.1. Resistance due to structural changes in the antibiotic target

Genetic modifications in the structural gene: Most antibiotics have high affinity for their native targets, and thus can inhibit the indigenous essential cellular functions. Interactions between antibiotics and their targets are specific and rely on the chemical

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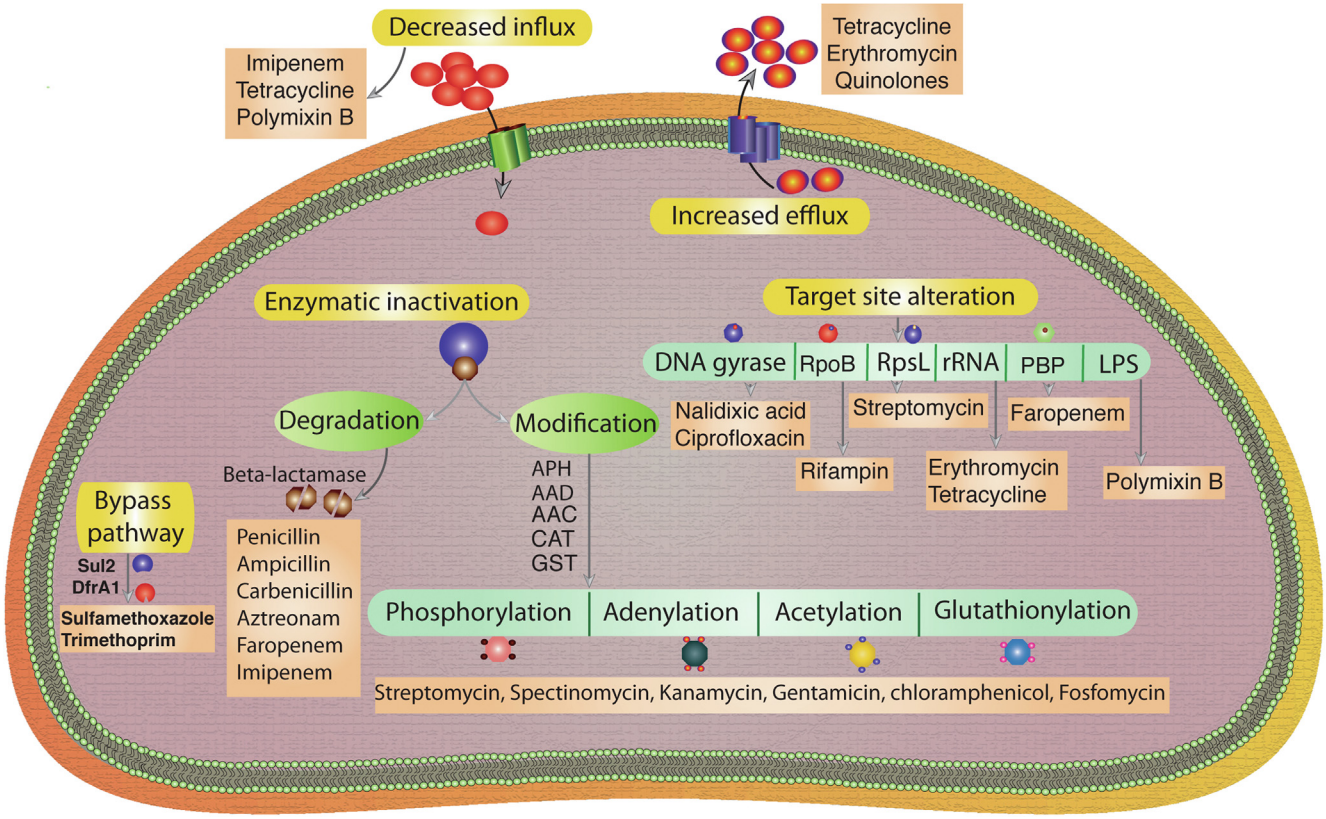

Fig. 3. Mechanisms of antibiotic resistance in *Vibrio cholerae* and other bacteria. Bacteria can develop resistance to antimicrobials by two simple mechanisms; by altering the target sequence (vertical transmission) or by acquiring resistance-encoding genes from other bacterial species (horizontal gene transfer). The acquired resistance traits can provide resistance by changing membrane permeability, enzymatic degradation or modification of antimicrobial drugs or by modifying the drug targets. They may also provide an alternative metabolic pathway or actively pump out antimicrobial compounds from the cytosol [87,88,106,107].

nature of specific residues of target molecules. Accumulations of point mutations in the target-encoding gene change the composition of the target molecule, and thus alter antibiotic-target interactions milieu and reduce antibiotic efficiency [79]. Such mechanism of resistance is widely reported in *V. cholerae* against routinely prescribed antibiotics that inhibit (i) DNA replication and repair by affecting *parC* and *gyrB* gene functions (in the quinolone resistance-determining region) [80] (ii) transcription by affecting *rpoB* encoded protein (rifampicin), (iii) protein synthesis by affecting *rpsL* gene function (streptomycin) (iv) cell wall biosynthesis by affecting *pbp* gene function (β-lactam), and (v) several metabolic enzymes by affecting *katG, embB, mshA* functions (SXT). In *V. cholerae*, quinolones resistance is mainly due to the accumulation of point mutations in *gyrA* and *parC* genes encoding topoisomerase II and IV, respectively [81,82]. Substitution mutations in genes of topoisomerase II and IV (Ser-83-Ile in *gyrA* and Ser-85-Leu in *parC*) of *V. cholerae* O1 also reduced susceptibility to ciprofloxacin [83].

Modification of target by acquired functions:

Presence or absence of RNA modification enzymes like rRNA methyltransferase that transfer a methyl group to the specific nucleotides of 16S or 23S rRNA confer resistance against several aminoglycosides and macrolides antibiotics routinely used in clinical practice to cure Gram-negative and Gram-positive bacterial infections [6]. Several genes (*aviR, cfr, emtA, ermC ermAM*) encoding 23S rRNA methyltransferases confer resistance against phenicols, lincosamides, oxazolidinones, pleuromutilins and streptogramin-A antibiotics in Gram-negative bacteria**.** Interestingly, the absence of certain rRNA methyltransferases also leads to antibiotic resistance. For example, Ksg methyltransferase transfer a methyl group to the 16S rRNA in *E. coli* and responsible for kasugamycin sensitivity. Its absence in *Escherichia coli* confers kasugamycin resistance. Similarly, inactivation of *rulC* gene confers significant resistance to clindamycin, linezolid and tiamulin in *V. cholerae* and other enteric pathogens.

6.2. Resistance due to reduced permeability or active ejection of antibiotics

The optimal intracellular level of an antibiotic is essential for its effectiveness to cure an infection. In bacteria, two major factors can prevent this event (i) state of cell membrane permeability and (ii) selective pumping out of antibiotics through efflux pump proteins.

Reduced membrane permeability to chemical compounds is an important defense mechanism for bacteria, which prevents several antibiotics from entering into the cytoplasm where drug target sites are mostly situated [85]. In Gram-negative bacteria, the outer membrane forms a major permeability barrier. Lipopolysaccharides in Gram-negative bacteria, including *V. cholerae*, *Pseudomonas aeruginosa, Salmonella enterica* etc., acts as a strong permeability barrier, making these bacteria intrinsically resistant to several antibiotics such as polymixin B, erythromycin, azithromycin and rifamycin. Alterations in the expression pattern of outer membrane porins and modifications of the membrane lipid barrier can also reduce permeability of antibiotics into several folds. Reduction, loss or replacement of outer membrane channel proteins, such as porins, can reduce the permeability of selective antibiotics. Several Gram-negative bacteria, including *V. cholerae* showed resistance to carbapenems, tetracycline, fluoroquinolones, aminoglycosides and chloramphenicol due to functional changes or loss of porins.

Genes encoding efflux pump proteins are ubiquitously present in the genome of all bacteria and the encoded proteins can actively transport different antimicrobial compounds by utilizing energy

from ATP or transmembrane ion gradients [86]. They may be part of the chromosome or may also be present in the extrachromosomal genetic elements like plasmids. In Gram-negative bacteria, efflux pump can be categorized into single component transporters or multicomponent transporters. In terms of substrate recognition, efflux pumps can be very specific like the tetracycline efflux pumps or may export a range of structurally distinct compounds. Till now, six different classes of drug efflux proteins have been reported in *V. cholerae* and other bacterial species: (i) ATP-binding cassette (ABC) efflux protein can recognize and pump out β-lactams, macrolides, aminoglycosides, nalidixic acid, tetracyclines and SXT antibiotics. There are four ABC transporter encoding loci in the genome of N16961 (ii) The resistance-nodulation-cell division (RND) efflux protein can selectively remove ampicillin, chloramphenicol, streptomycin and tetracycline antibiotics. Six RND-encoding loci have been identified in the Ch1 (five) and Ch2 (one) of the *V. cholerae* strain N16961. *V. cholerae* RND pumps could confer resistance against several antibiotics (iii) Major facilitator (MFS) efflux protein is reported in large numbers of Gram-negative pathogens including *V. cholerae*, *E. coli, Shewanella*. In the genome of N16961 there are eleven loci that encode MFS efflux pump proteins (iv) Small multidrug resistance (SMR) family. Only one locus is identified in the genome of N16961 that encodes SMR family efflux pump proteins (v) Multidrug and toxic compound extrusion (MATE) families can confer resistance against multiple antimicrobial compounds derived from β-lactams, macrolides, aminoglycosides, nalidixic acid, chloramphenicol SXT scaffolds. The genome of N16961 has six MATE transporter encoding loci. (vi) Proteobacterial antibacterial compound efflux family (PACE).

6.3. Resistance due to inactivation of the antibiotics

Like other bacteria, *V. cholerae* can destroy or modify antibiotic scaffolds by hydrolyzing the core structure or transferring a chemical group to the scaffolds (Table 1). Chemical modification of antibiotics by using enzymatic function of acquired genetic traits is the most common mechanism of drug resistance in pathogenic bacteria including *V. cholerae*. Generally, *V. cholerae* acquire such enzymatic functions through HGT and become resistant against multiple antibiotics.

6.3.1. Inactivation of antibiotics by hydrolysis

The most well studied antibiotic resistance mechanism in *V. cholerae* and other Proteobactera is the inactivation of β-lactam antibiotics by β-lactamases [87]. β-lactams are most prescribed antibiotics across the globe. β-lactam ring is common among all the β-lactam antibiotics, including penicillins, cephalosporins, carbapenem and monobactams. The hydrolysis of β-lactam ring by metallo-β-lactamase or serine-β-lactamase confer β-lactam resistance in most of the bacterial species. In the β-lactam sensitive bacterial cell, the electrophilic β-lactam ring of β-lactam antibiotics offers a site for nucleophilic attack to the active site serine residue of cell-wall biosynthesis enzyme penicillin binding proteins (PBPs) and inactivate the enzymes by forming a slowly hydrolyzing covalent β-lactam-PBP intermediate [76]. The enzyme β-lactamases inactivate β-lactam group of antibiotics by hydrolyzing its β-lactam ring. Like PBPs, the β-lactamases can form a covalent enzyme-β-lactam intermediate through its active site Ser residue and form an acyl-enzyme intermediate. However, unlike PBPs, hydrolysis of the acyl-enzyme intermediate structure is fast in β-lactamases and this results inactivation of the antibiotics efficiently in the periplasm and the cytoplasm of the resistant bacteria. Recently, antibiotic resistance due to hydrolysis of antibiotic scaffolds has also been reported for (i) fosfomycin epoxide ring (ii) macrolide macrocycle lactone ring and (iii) amidohydrolysis of bacitracin undecaprenyl pyrophosphate.

6.3.2. Inactivation of antibiotics by chemical modifications

Resistance due to enzymatic modification of antibiotic scaffolds due to the covalent transfer of chemical groups is a very common mechanism in *V. cholerae* and other Proteobacteria. Thousands of enzymes that are involved in drug resistance by modifying antibiotic structure have been identified [87]. These enzymes can inactivate antibiotics by one of the following seven modifications (i) O-phosphorylation, by transferring a phosphate group to the antibiotic scaffold from ATP or GTP. Resistance due to phosphorylation has been reported for fosfomycin, chloramphenicol, viomycin, macrolide and rifampicin antibiotics (ii) O-nucleotidylylation, generally modified the antibiotics by transferring adenosine monophosphate (AMP). Resistance against several aminoglycosides and lincosamide antibiotics are often conferred by nucleotidylylation (iii) O-glycosylation, the addition of glycosyl moiety to the macrolide and rifampin antibiotics are common mechanisms of AMR (iv) O-ribosylation, inactivation of rifampin by the addition of ADP-ribose from NAD is also well-known mechanism AMR (v) O- and N-acetylation, AMR due to transferring an acetyl group to the antibiotic from acetylCoA. Several antibiotics like chloramphenicol, fluoroquinolone, streptothricin and other aminoglycosides get inactivated in the pathogens due to acetylation. (vi) hydroxylation, inactivation of an antibiotic due to the addition of a hydroxyl group (vii) sequestration, resist an antibiotic action by forming a chemical complex that inhibits its access to the

Table 1
Antibiotic inactivation by hydrolysis or chemical modification in *V. cholerae* and other bacterial species.

Mechanisms	Genes/Proteins	Antibiotics	Organisms	References
Hydrolysis	bla_{NDM}, bla_{SHV}, bla_{TEM}, bla_{CTX}, bla_{KPC}, bla_{IMP}, bla_{SPM}, bla_{VIM} *fosA, fosB* *ereA, ereB, bahA*	Beta-lactam Fosfomycin Macrolide Bacitracin	*V. cholerae, E. coli, K. pneumoniae, A. baumannii*	[26,87,88]
Linearization	*vgbA-C*	Streptogramin	*S. aureus*	[89]
Glycosylation	*mgtA, gimA, rgt*	Macrolide, Rifampicin	Actinomycetes	[90]
ADP-ribosylation	*arr1-5, arr7-8, aadA16,*	Rifampicin, Streptomycin, Spectinomycin	*V. cholerae, M. tuberculosis*	[26,27,91]
Nucleotidylation	*ant*(6), *ant*(9), *ant*(2′-4′)	Aminoglycosides	*V. cholerae, E. coli, K. pneumoniae, A. baumannii*	[26,92]
Phosphorylation	*fomA-C, cmlV, aph, mphA-G, rphA, rphB*	Fosfomycin, Chloramphenicol, Macrolide, Aminoglycoside, Rifampin	*V. cholerae, E. coli, K. pneumoniae, A. baumannii*	[26,93,94,95]
Acetylation	*cat, aac(6′)-1b, sat-1, aac(1′), aac(2′), aac(3), aac(6′), apmA, vatA-F, vatH-I,*	Chloramphenicol, Fluoroquinolone, Streptomycin, Aminoglycosides, Streptogramin	*V. cholerae, E. coli, K. pneumoniae, A. baumannii*	[26,96,97]
Hydroxylation	*rox, tetX*	Rifampicin	*B. fragilis*	[98,99]
Sequestration	*sh ble*	Bleomycin	*V. cholerae, E. coli, K. pneumoniae, A. baumannii*	[26,100,101]

Table 2
Different ICEs reported in clinical and environmental *V. cholerae* species across the globe.

ICE	Integration site	Size (kb)	Host	Key fitness traits	References
SXT^{MO10}	*prfC*	99.5	*V. cholerae* O139	*floR, strBA, sul2, dfr18* Toxin-antitoxin system	[56]
ICE*Vch*Ban8	tRNA-Ser	105.790	*V. cholerae* non O1/O139	Toxin- antitoxin system	[53]
ICE*Vch*Ind4	*prfC*	95.491	*V. cholerae* O139 Ind 4	*floR, strBA, sul2* Toxin-antitoxin system	[101]
ICE*Vch*Ind5	*prfC*	97.847	*V. cholerae* O1 Ind 5	*floR, strBA, sul2, dfrA1, AraC* family transcription regulator, glyoxylase abx resistance	[27]
ICE*Vch*Ban5	*prfC*	102.131	*V. cholerae* O1 Ban 5	*floR, strBA, sul2, dfrA1, AraC* family transcription regulator, glyoxylase abx resistance	[58]
ICE*Vch*Ind 6	*prfC*	90.0	*V .cholerae* O1	*dfrA1*	[102]
ICE*Vch*Ban9	*prfC*	106.124	*V. cholerae* O 1 MJ-1236	*floR, strBA, sul2, dfrA1, AraC* family transcription regulator, glyoxylase abx resistance, ATP dependent Lon protease	[103]
ICE*Vch*Moz10	*prfC*	104.495	*V. cholerae* O1 B33	*floR, strBA, sul2, tetA, AraC* family transcription regulator, glyoxylase abx resistance, ATP dependent Lon protease	[104]
ICE*Vch*Mex1	*prfC*	82.839	*V. cholerae* Mex1non O1/O139	Fic family protein, diguanylate cyclase, restriction modification system	[105]

target. More specific information including the gene, bacterial species where it was detected and relevant references are provided in Table 1. Several bifunctional enzymes that modify aminoglycoside antibiotics have broad substrate specificity and can inactivate most clinically important drugs [77]. In *V. cholerae* and other proteobacterial pathogens N'-acetylation at the 6′-position of aminoglycoside antibiotics is widely reported resistance trait. The transfer of acetyl group in the aminoglycoside is catalyzed by the acetyl-CoA dependent acteyltransferase. Recent findings have demonstrated that these enzymes can also modify fluoroquinolone antibiotics like ciprofloxacin and norfloxacin [84]. Enzymes involved in inactivation of an antibiotic by preventing access of the antibiotics to the target site are also spreading rapidly in the *V. cholerae* through transposon and plasmids [85]. Acidic bleomycin resistance protein sequesters the basic antibiotic bleomycin and prevents DNA cleavage by blocking its access to the substrate. Similarly, enzymes related to O-phosphorylation, O-nucleotidylylation, O-glycosylation and O-ribosylation are also reported in clinical and environmental *V. cholerae* isolates (Table 1). Inactivation of an antibiotic by transferring a hydroxyl group (–OH) to the scaffold is also emerging among bacterial species [84]. Recent work has demonstrated that the *rox* gene of Actinobacterial species *Nocardia farcinica* encodes a rifampicin monooxygenase that can convert rifampicin into 2′-N-hydroxy-4-oxo-rifampicin and reduces its antimicrobial activity several folds [86].

7. Conclusion

V. cholerae has extraordinary fitness capacity to adjust rapidly to the changing environment. The MGEs play pivotal role in *V. cholerae* adaptation, including antibiotic resistance, virulence, disease development and survival in hostile environment. The ICEs, integrons and conjugative plasmids primarily attribute the multidrug resistance phenotypes in cholera pathogens. Although, large numbers of MGEs have been identified in the genome of MDR *V. cholerae*, these elements are not only restricted in the genome of cholera pathogens. The recent progresses in comparative genomic studies suggest that a large number of environmental and clinical isolates of highly pathogenic or opportunistic pathogens harbored MGEs in their genome. The MGEs present in the genome of *V. cholerae* are highly dynamic and could potentially propagate to other bacterial species through HGTs. Since, most of these MGEs integrate into *V. cholerae* chromosomes by exploiting tyrosine recombinases, their stability could be reduced by promoting expression or activities of the integrases that will help the elements to get excised from the chromosome of cholera pathogens. This could potentially be explored to reverse drug resistance in MDR *V. cholerae* and other enteric pathogens.

8. Contributors

Conceived and designed the review: BD; Wrote the manuscript: BD, JV, PK. Edited the review AG, TR. All authors read and approved the review.

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Declaration of Competing Interest

All authors declare that they have no conflicts of interest.

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