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Evolution of super-drug resistant microbial strains: mechanisms and strategies for containment

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Review Article

ABSTRACT

Super drug resistance (PDR / EDR) in microbial strains has been a continuous phenomenon in nosocomial and miscellaneous infections. The load of these bugs has inflated over worldwide. Microbes evolve such phenomena involving mutational processes, hyper performance of pumping out systems, synthesis of secretory saccharides, bioaccumulation and directed flagella based shifting resulting out of hypo-doses of drug stimulation. Further, the prevalence of Enterobacteriaceae member strains has been witnessed producing Extended Spectrum β-Lactamases (ESBLs) and Carbapenemases. Drug resistance in viral entities has also posed challenge for public health programs. About 20% people die of viral hepatitis in one of Pakistan provinces. A counter malarial acrine drug is being tried against proteinaceous infectious particles (causing many transmissible neuro-diseases). These epigenetical agents have been a source of concern for our planet regarding food safety issues (e.g. infected meat). No doubt, man has made significant achievements in effective and neo-antimicrobials research, one wonders why not a single infectious agent has been completely knocked out. Our group has been focusing on ascertaining the basis of antibiotic deferring processes acquired by (indigenous) clinical strains. Accordingly, sub-lethal doses of the drug result in development of hyper-resistances. The bacteria have evolved the molecular genetical basis (and other parameters) for acquiring the resistance. For the containment and eradication of globally evolving MDR bacteria, it is crucial to understand and implement certain strategies/agents such as, probiotics, CRISPRs, bacteriophages, nanotechnology and phytochemicals.

Keywords: Super drug resistance, ESBLs, antimicrobial drugs, sub-lethal drug induced resistance, crossed resistance.

INTRODUCTION

Antibiotics constitute naturally occurring antimicrobials or metabolic products of bacteria and fungi. These substances reduce competition for nutrition and space. The microbes that produce antibiotics include *Streptomyces, Bacillus, Penicillium, Cephalosporium* spp (Sethi *et al*., 2013). Residues of tetracycline were traced in *Homosapien* skeletons in pre-historic Sudanian Nubia (350–550 CE). The drug presence in bones is only possible after post exposure to tetracycline-laced material(s) in the diet of the ancient people. The exposition to antibiotics during pre-antibiotic period has been seen out of cures opted for complementary drugs with particular reference to Chinese medicine. The historic narrative of drug deferring genetic factors may be explained via phylogeny and that could suggest the longer period sustenance of varied resistance genes even prior to drug era. Phylogenic bases of metallo-beta-lactamases and serine proteases suggested the origin of these enzymes earlier than two thousand million years and quite a few serine β-lactamases

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were similarly located on extra-chromosomal elements. The initial approaches of Paul Ehrlich met with sulfa discovery success (e.g. Prontosil) and was subjected to testing (by Gerhard) for the counter of microbes. Infact, Prontosil acted as precursor for furthering the activated compounds (Aminov, 2010).

The "antibiotics era" began with Paul Ehrlich and Alexander Fleming (the Laureates). "Magic bullet" concept was floated by Ehrlich that is targeting selectivity (of microorganisms that cause diseases instead of the cells of the host) by Aniline and other dyes. Sexually transmitted diseases (STDs) (like syphilis) was cured (with low efficiency) using salts of mercury but also leaving behind the bad effects. Further, Salvarsan & Neosalvarsan (with low toxicity) constituted the drugs of choice till "penicillin" took their place during 1940s (Marketed by Hoest) (Pelczar *et al*., 1986). Selman Waksman coined the word "antibiotics" as a chemical substance produced by microbes that suppresses or kills other microorganisms. He was responsible for the disclosure of Streptomycin (Davies & Davies, 2010). About 80% of antibiotics have been extracted from Streptomyces (Barka *et al*., 2016). A precise scheme for the historical development of antibiotics is shown in figure1.

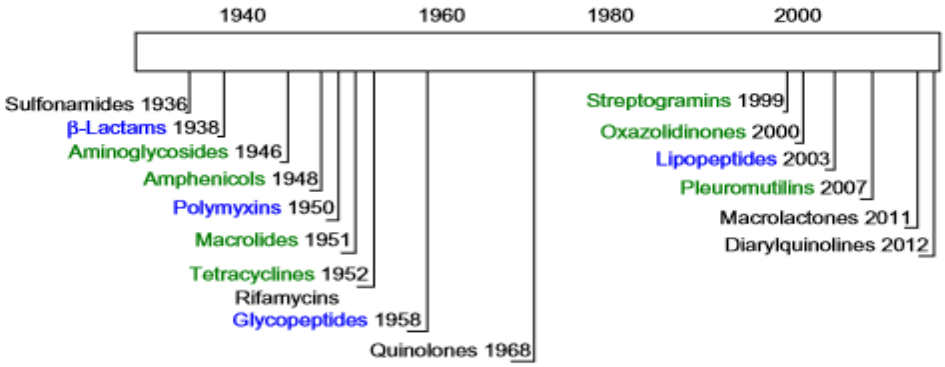

Fig. 1: Development of antibiotic era (Fair and Tor, 2014)

Antibiotics should be soluble, show tissue stability with selective / stable toxicity, non-resistance acquisition, normal shelf life, not showing allergy and be cost effective. Antibiotics should be exclusively (possibly) toxic for bacteria (with bactericidal or bacteriostatic activities) but be patient friendly (Tortora *et al*., 2004; Gould, 2016). Antibiotics are usually grouped on the basis of their strategy to encounter bacteria. Various classes of antibiotics are listed in Table I.

Table I: Various classes of antibiotics on the basis of their mechanism of action (Nelson *et al*., 2019)

Mechanism	Class of antibiotic	Selected drug examples
Inhibitors of cell wall synthesis (beta-lactams)	Penicillins	Penicillin G, Methicillin, Ampicillin
	Cephalosporins	Cefalotin, Cefaloxin, Cefotan
	Carbapenems	Ertapenem, Meropenem,
	Monobactams	Aztreonam
	Vancomycin	Vancomycin
	Bacitracin	Bacitracin
Disruptors of cell membranes	Polymyxins	Polymyxin B, Polymyxin E
Nucleic acid synthesis inhibitors	DNA synthesis inhibitors	Nalidixic Acid, Ciprofloxacin
	RNA polymerase inhibitors	Rifampin
Protein synthesis inhibitors	30s subunit—aminoglycosides	Gentamicin, Streptomycin
	Tetracyclines	Tetracycline, Doxycycline
	50s subunit—macrolides	Erythromycin, Clarithromycin
	Chloramphenicol	Chloramphenicol
	Lincosamide	Clindamycin
Folic acid synthesis inhibitors	Sulfonamides/Trimethoprim	Sulfamethoxazole, Trimethoprim
	Pyrimethamine	Pyrimethamine
Mycolic acid synthesis inhibitors	Isoniazid	Isoniazid

Some factors should be taken into consideration while deciding to opt for an antibiotics: (1) Where did the patient fall ill (travel or exposure!), (2) Anatomical origin of the infection (and spread thereafter) and the causative agent, (3) Recent antibiotic therapy of the patient, presence of underlying diseases, hospital flora, culture data (current and past), (4) Risk for drug resistant pathogens, antibiotics administered within last 90 days and presence of risk factors for resistance, (5) Current hospitalization of ≥5 days, (6) Immunosuppressive disorders / or therapy (Gidal & Barnett, 2018).

After the discovery of antibiotics in 1929, they have been extensively adopted in animals and human medications to hamper bacterial ailments. Their superfluous use has necessarily escalated the degree of resistance in bacteria globally (Ali *et al*., 2018). The relevant figure of deaths are frightening, touching upto 50,000 fatalities per annum in Europe and USA (Simlai *et al*., 2016; Jansen *et al*., 2018). The extensive use, discriminative pressure and injudicious application of antibiotics is mainly responsible for discriminating evolution of pathogenic and non-pathogenic bacteria defiant to presently used antibiotics, thus causing widely distribution of resistance genes in the environment (Tello *et al*., 2012; Nitsch-Osuch *et al*., 2016). Antimicrobial defiance or resistance has evolved into an international issue, after the first communiqué of its first emergence in Pakistan, India, the United States, United Kingdom, Japan and Canada (Rios *et al*., 2016). This antibiotic defiance can takes place in various fashions relying mainly upon attained and discriminative genetic alterations or infusion of foreign genes. Many processes of resistance have surfaced recently, containing modification of drug target (e.g. DNA gyrase), inhibition of quinolones (by aminoglycoside N-acetyltransferase), enhanced efflux (outflow of a drug from bacteria), preservation of target by DNA fastening peptides (Qnr family), and hindrance of 30S component of ribosome (by aminoglycosides) (Redgrave *et al*., 2014; Munita & Arias, 2016; Kapoor *et al*., 2017). Few of such alterations were previously determined like, change in the chemical structure of antibiotics (Alekshun & Levy, 2007), reduction in the conc. of antimicrobial at the spot of its activity (Gonzalez-Bello, 2017; Willers *et al*., 2017), alterations in the targets of antibiotics (Sieradzki & Markiewicz, 2004), and changes in membrane permeability (Hao *et al*., 2018). Some processes of reduced permeability in *P. aeruginosa* do not comprise expression of porins instead of surface alteration which are linked with Polymyxin B resistance (Falagas & Kasiakou, 2005). Activity of antimicrobials like Penicillins, Tetracyclines, Macrolides and Glycopeptides may also diminish because of their altered targets (Poehlsgaard and Douthwaite, 2005; Wu *et al*., 2005).

Resistance has necessarily been expended in Gram negative bacteria and Gram positive bacteria as a result of discriminatory stress of antibiotics in 20 years. Such broadened resistance is absolutely important for healthcare system (Lautenbach *et al*. 2001; Evans *et al*., 2007; Patel at al., 2008; Gasink *et al*., 2009; Hu *et al*., 2010; Tumbarello *et al*., 2012; Pouch *et al*., 2015; Thaden *et al*., 2017). In the widest view, knowing the antibiotic resistance systems can illustrates the surge of antibiotic resistance and its dissemination. The knowledge of resistance mechanisms is essential for pharmaceutical industry because various new gents have surfaced to bypass resistance mechanisms in bacteria. Their application in combo with antibiotics will be crucial to prevent antibiotic resistance (Marshall *et al*., 2017). This review article is aimed to elaborate the evolution of drug resistance, antibiotic resistance mechanisms in Gram negative and Gram positive bacteria and the possible solutions and novel strategies (new antimicrobials) to minimize the degree of resistance.

Antibiotic resistance mechanisms and evolution

Living organisms have the tendency to adapt and obviously bacteria cannot be left out. Antibiotic resistance has been regarded as a major risk to well being of mankind during the ongoing century by the World Health Organization. Approximately, 7 lacs lose their life every year out of diseases caused by antibiotic resisting microbes and many more contracting infections. Antibiotics presence allows super-bugs to flourish. A common reason responsible for the evolution of drug resistant strains includes rapid frequency of non-induced mutations. Such mutants are selective for some drugs (Fair and Tor, 2014). Misuse, overuse and over the counter procurement of antibiotics in the agricultural and medical areas are responsible to the problem related to the antibiotic resistance. It is supposed that 70% of pathogenic bacteria are now resistant to at least one or more antibiotics (inclusive of our study) (Rasool *et al*., 2019). Various mechanisms of drugs and their targets in bacterial cells are depicted in fig 2.

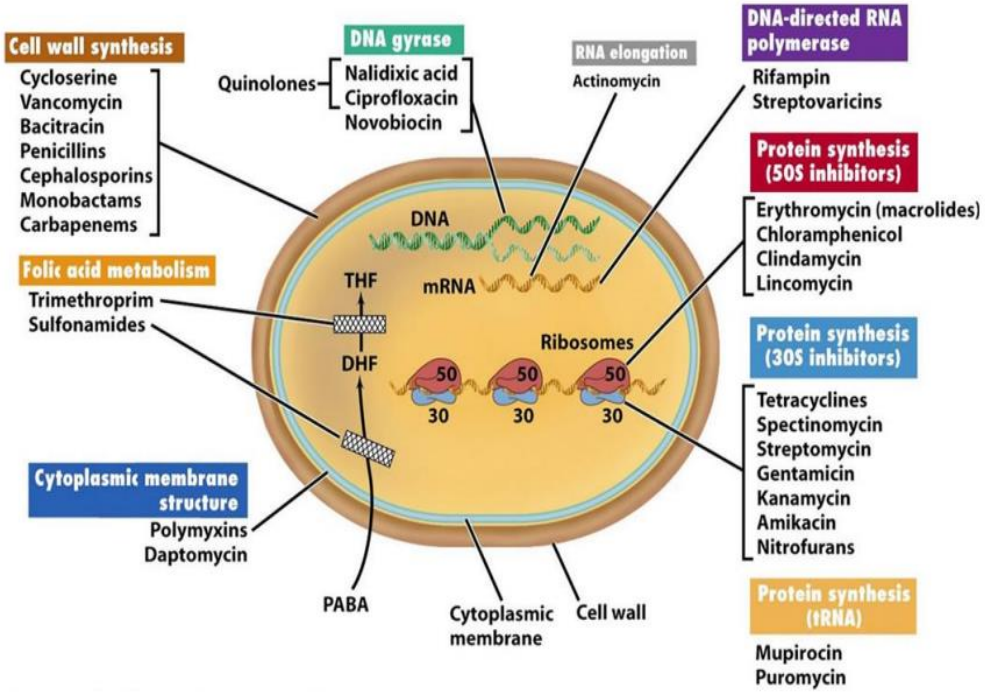

Fig. 2: A simplified presentation of various approaches of antibiotics activity (Etebu and Arikekpar, 2016)

Multi-drug resistant organism is described as having attained resistance to an antibiotic in 3 or >3 antibiotic groups (Magiorakos *et al*., 2012). Extensively drug resistance is described as sensitivity to a minimum of 1 antibiotic in total or <2 antibiotic classes (i.e., bacteria stay sensitive to at least 1or 2 antimicrobial classes) (Magiorakos *et al*., 2012; Eichenberger and Thaden, 2019). This term is referred as resistant to all antibiotics in all antibiotic classes. These types of bacterial strains have acquired the ratio of resistance that actually none of the antimicrobial choices are offered to cure them (Magiorakos *et al*., 2012). The resistant microbial strains carry on multiply themselves that end up *in toto* resistant clones. A fast track enhancement of drug resistance is caused by mutational process and pressure oriented evolution (Anthony *et al*., 2010).

Misuse-excessive use of antibiotics leads to the emergence of resistant clones. It is possible that such drugs are prescribed which stand ineffective against the common flu viruses. Such drugs also wipe out the resident normal flora. While, resistant opportunistic bacteria could sustain and continue to multiply. Tertiary care facility centers may act as “reservoir” to cater the resistant strains. Hospital is a place and paradise where resistance can develop rapidly. The human communities exist with unhealthy status; enhanced clustering of microorganisms and a bulk of them are extreme pathogens (Rasool, 2016). Considerable amounts of different antibiotics are constantly in use. Antibiotic resistance can be transferred by bacterial swapping genes. This can be easily accomplished in a hospital setting. Health care workers (who don’t observe infection control norms) also promote the drug resistance. Plasmids containing genes for resistance can integrate into the chromosome and form resistance islands. These genes accumulate and are stably maintained and transferred (also possess the tendency of jumping) (Davies, 1994).

Bacteria exploit several mechanisms to acquire antibiotic-resistance e.g. inactivation of the antibiotic, outer membrane permeability barrier, efflux pumping of the antibiotic, modification of the antibiotic target(s), alteration of the pathway etc. (Casson and Giordono, 2009; Maviglia *et al*., 2009). Many resistant bugs follow a scheme (fig 3) to render the drugs ineffective.

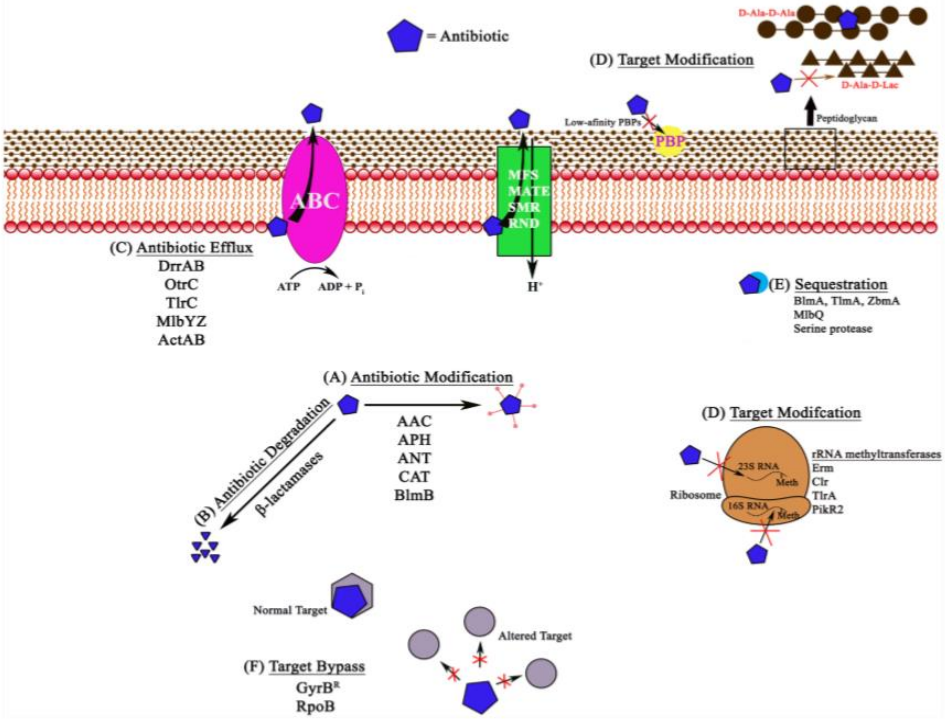

Fig. 3: Mechanisms of acquisition of antibiotic resistance (Peterson and Kaur, 2018)

Antibiotics get inactivated

Enzymes are responsible for breaking down of the drugs e.g. β-lactamase which is released in the space between cell wall and cell membrane and destroys the drug as it follows its targeted locations (Zeng and Lin, 2013). Reportedly, >190 variants of beta-lactamases exist e.g. specific for *Escherichia coli* and *Staphylococcus. aureus* etc. referred as extended spectrum β-lactamases (ESBLs) e.g. TEM, SHV and CTX-M, and carbapenemases. These enzymes are subjected to disrupt beta-lactam ring of beta-lactams (Rasool, 2016; Farzana *et al*., 2013). AmpC cephalosporinase encoded by chromosome in *Acinetobacter baumannii* and has ability to breakdown cephalosporins. *A. baumannii* also produce oxacillinase (OXA-51) which disintegrates carbapenem and penicillins (Corvec *et al*., 2003; Turton *et al*., 2006). Procurement of carbapenemases (OXA-23, OXA-40 and OXA-58) has been witnessed on plasmids, transposons (Tn2006 and Tn2007) and chromosome in *A. baumannii* and is responsible for hospital acquired outbreaks globally (Poirel & Nordmann, 2006; Corvec *et al*., 2007; Poirel *et al*., 2010; Olaitan *et al*., 2013; Merino *et al*., 2014). Other carbapenemases like KPC, SIM, VIM, NDM and IMP has also been reported in such bacteria. Over-expression, inherent occupation and gaining of antibiotic degrading enzymes like AmpC through conjugation grant antimicrobial defiance in *P. aeruginosa* (Potron *et al*., 2015; Emily and Joshua, 2019). PSE-1 and PSE-4 (*Pseudomonas* specific enzyme) confer resistance only to *Pseudomonas* limited penicillins, While GES-1 and 2 (Guiana extended spectrum), PER-1 (*Pseudomonas aeruginosa* RNL-1) and VEB-1 (Vietnam extended-spectrum beta-lactamase) contribute in resistance against monobactams and cephems in addition to *Pseudomonas* limited penicillins but are inefficient against carbapenems. Metallobetalactamases (MBLs) like SPM (Sao Paulo metllo beta-lactamase), IMP, VIM (Verona Integron-borne Metallobetalactamase) and GIM (Germany imipenamase) can destroy all beta-lactams. Aminoglycoside modifying enzymes (AMEs) are the major cause of resistance against aminoglycosides in *Enterobacteriaceae* family (Table 2) (Nordmann

& Poirel, 2002; Rossolini & Mantengoli, 2005; Bush, 2010).

Table: 2 Nomenclature and classification (modified) of beta-lactamases (Jacoby, 2006)

Ambler Class	β-Lactamases	Active Site Agent	Examples	Substrates
A	Penicillinases	Serine	PSE TEM, SHV, CTX-M, VEB, PER, GES KPC, SME, IMI/NMC-A	Penicillins Penicillins, 3rd generation cephalosporins All β-lactams
B	Metallo-β-lactamases	Zinc	IMP, VIM, NDM, SPM, GIM	All β-lactams, except monobactams
C	Cephalosporinases	Serine	AmpC	Cephamycins, 3rd generation cephalosporins
D	Oxacillinases	Serine	OXA	All β-lactams, though class D enzymes have highly variable spectra of activity

Abbreviations: CTX-M, active against cefotaxime (CTX) and isolated in Munich (-M); GES, Guiana extended spectrum; GIM, German imipenemase; IMP, active on imipenem; KPC, Klebsiella pneumoniae carbapenemase; NDM, New Delhi metallo-β-lactamase; NMC, not metalloenzyme carbapenemase; OXA, oxacillinase; PER, Pseudomonas aeruginosa RNL-1; PSE, Pseudomonas specific enzyme; SHV, sulfhydrl reagent variable; SME, Serratia marcescens enzyme; SPM, Sao Paulo metallo- β -lactamase; VEB, Vietnamese extended-spectrum β-lactamase; VIM, Verona integron-encoded metallo-β-lactamase.

Efflux pumping of drugs

Such systems are active ousting watch-outs and need ATP. These pumps exist in the microbial cell membrane and the outer most layer of Gram-negatives bacteria. Such ousting maintains the drug level well below which will be toxic for bacterial cells. Genetic factors that regulate ousting systems are located on extra chromosomal genetic elements (e.g. Transposons, Insertion sequence (IS) elements and plasmids). Some of these DNA elements carry out "flip flop" activities i.e. mobilize themselves to new locations along the cellular genome of a single cell (Hurdle *et al*., 2011). Transposons are horizontally transferred to susceptible bacterial cells. Insertion sequences are also instrumental in resistance spread (Lix & Nikaido, 2009). *Pseudomonas aeruginosa* strains have different types of efflux pumps, (some are responsible for beta-lactam resistance). For example MexAB-OprM system when over expressed provides resistance against monobactams, ticarcillin, cefepime and some carbapenems (Keith, 2011; Pan *et al.*, 2016). Efflux systems participate in the development of MDR *P. aeruginosa* and other Gram negative bacilli. Multidrug efflux pump was also identified in *M. smegmatis* (Lix & Nikaido, 2009; Ankita *et al*., 2016).

The antibiotic producer microbes possess self-immunity systems e.g. transmembranous proteins flush out the denovo synthesized antibiotics in order not to allow their accumulation (a musical chair like activity). Otherwise, it would be a self-suicidal activity. Genes responsible to code for the efflux mechanisms are connected with the ones coding for the antagonistic metabolites. Actually, antibiotic synthesis genetic factors are switched on concomitant with the pumping genes i.e. the two systems are switched on simultaneously (Lix & Nikaido, 2009; Li *et al*., 2015). In *Enterobacteriaceae*, modified porin proteins and efflux pumps are also responsible for carbapenems resistance in addition to carbapenemases. AcrAB-ToIC pump reside in the resistance-nodulation-division (RND) family of efflux pumps and is a powerful resistance mechanism against betalactams, fluoroquinolones, tetracyclines and macrolides. Enhanced regulation of efflux pump further strengthens the resistance mechanism (Goessens *et al*., 2013).

Outer membrane (OM) permeation gate(s)

A number of microbes decrease membrane permeation to keep the drugs out. They switch off synthesis of porins along with miscellaneous membrane products e.g. as witnessed in streptomycin, tetracycline and sulfa-drug formulation resistance as the genes controlling porins undergo mutations. β-lactams (small water-soluble antibiotics) may have access to the cell interior through porins such as OmpF in *E. coli* and OprD enclosed in the OM of *aeruginosa.* Modifications in lipids and the porin proteins are responsible for antibiotic resistance by bacteria

(denEngelsen *et al*., 2009; Jose and Cesar, 2015). OprD grant access to basic amino acids and carbapenems through the outer membrane. Deprivation or impairment of OprD delivers resistance against meropenem and imipenem. Enhanced repression together with the impairment of OprD (due to mutations) yields resistance to carbapenems, quinolones, ureidopenicillin, ceftazidime, tetracyclines, carboxypenicillin and chloramphenicol (Livermore, 2001). Mutated OmpK35 and OmpK36 in conjunction with carbapenemases enhance the carbapenem resistance in representatives of *Enterobacteriaceae* (Cornaglia *et al*., 1995 Livermore, 1995).

Antibiotic receptors or targets are modified

Bacterial modification of drug receptors takes place for slipping out. A changed structuring of the receptor is ensured (while still functioning). It is achievable by mutating the genetic factors that code for the receptor protein or by a borrowed gene responsible for the changed receptor e.g. *Staph. aureus* that resist methicillin, analogous to penicillin, bound proteins which act as the attempted locations (Livermore, 2003; Monserrat-Martinez *et al*., 2019).The mode of resistance against methicillin depends upon chromosomal cassette SCCmec including mec A which is responsible for PBP2a with decreased binding capacity to beta-lactams. SSCmec IV and V are the variants with deficient resistance to various antibiotics and are reported in community acquired MRSA (DeLeo and Chambers, 2009). Betalactam resistance in *Streptococcus pneumoniae* is also linked with change in cpoA, murM and pdgA which produce glycosyltransferase, murein and GlcNAc respectively (Hakenbeck *et al*., 2012). Vancomycin resistant *Enterococci* (VRE) harbor vancomycin resistance genes clustered on transmittable and non-transmittable plasmids and transposons (Tn3 and Tn1546). The enzymes encoded by the these genes alter the last D-Alanin-D-Alanin peptidoglycan precursor (Courvalin, 2006; Kelley *et al*., 2015). Linezolid resistance is attributed by mutations in the domain V of central loop in 23rRNA of *E. coli* (Marshall *et al*., 2002). Flouroquinolones resistance mediated by their mutated targets (gyrA for topoisomerase IV and parC for DNA gyrase) has been disclosed in *P. aeruginosa* (Kato *et al*., 1992; Morais Cabral *et al*., 1997; Akasaka *et al*., 2001).

Bacterial ribosomes constitute the basic targeted locations for the incoming drugs (varied drugs modify ribosomes differently). Of course, rRNA modification results in acquiring the resistance. Some organisms exploit target modification along with efflux pumps (such resistance is fairly effective) (Livermore, 2003; Doi *et al*., 2016). Aminoglycoside resistance in *P. aeruginosa* is favored by the mutations in 16S rRNA (e.g. ArmA, RmtA and RmtD for 16S rRNA methylase) (Yokoyama *et al*., 2003; Doi *et al*., 2007; Gurung *et al*., 2010). Mutations in phoPQ and pmrAB are accountable for the altered LPS which promot colistin resistance (Olaitan, *et al*., 2014). Moreover, polymyxin/colistin resistance can be passed on between bacteria through plasmid bearing *mcr*-1 gene leading to altered target hence reduces the affinity between polymyxin and its target (Sun *et al*., 2018).

Altering the pathways

Some antimicrobials competitively disrupt metabolic cycles of microbes. Microorganisms may seize such approach through opting a substitute metabolic cycle. Approximately, seven percent of *Staph. aureus* chromosome comprises of genetic factors reserved for resistance to drugs. The non-pathogenic *B. subtilis* carries no such genes. A number of methicillin resistant *Staph. aureus* (MRSA) genes (conferring particular resistance) are reported (linked to varied resistance logistics) e.g. beta-lactamase and erythromycin resistances, synthesis of aminoglycosides along with efflux pumping systems being switched on (Canu *et al*., 2002).

Methicillin resistant *S. aureus* (MRSA), Vancomycin resistant *S. aureus* (VRSA) and Vancomycin resistant *Enterococci* (VRE)

Quite regularly, clinically threatening microbes are seen offering resistance to drugs. Consequently, MRSA and VRSA carry hyper-virulence for humans (as professional pathogens). MRSA and VRSA carry abundant resistance genes (with >25 extra gene crowding on plasmids that have the tendency to flip-flop). Several antibiotic-resistant bacteria are considered dangerous. MRSA and VRSA resistance pockets are present in *Staphylococcus aureus* and others. Vancomycin resistant *Enterococci* (VRE) strains share about 90 percent of total microbes that offer resistance to vancomycin (Kehrenberg *et al*., 2005). Furthermore, aminoglycoside, methicillin resistance and macrolide genes on plasmid have been noticed in association with exfoliative toxin B gene on single plasmid of MRSA (Hisatsune *et al*., 2013).

Relevant Researches at our end

Antibiotics at sub-inhibitory concentrations promote/induce mutations, cross-resistance and biofilm formation

Drug resistant bugs are steadily increasing on the global basis. Drug resistance stimulation by hypo-toxic levels of ampicillin can be the outcome of mutational rounds, cross resistance and adaptation to keep on surviving at intermediate level ampicillin. Research at our lab revealed about 16% *E. coli* and 17% *Salmonella* spp., (both of clinical origin) did develop irreversible ampicillin resistance (often their parent isolates were gradually treated to ampicillin of hypo-toxic levels. The percentages of *E. coli* strains and *Salmonella* spp., (out of the same lot of isolates) that opted adaptation to intermediate resistance concentration of ampicillin was approximately doubled. The biofilm formation (fig 4) by the non-reversible resistant, at various concentrations of ampicillin was also detected by scanning electron microscopy (SEM). Accordingly, sub-lethal concentrations (0.25 to 4 µg/ml) of ampicillin should be avoided because such exposure may enable bacteria to adapt to higher concentrations of ampicillin and provoke bacteria to develop cross resistance.

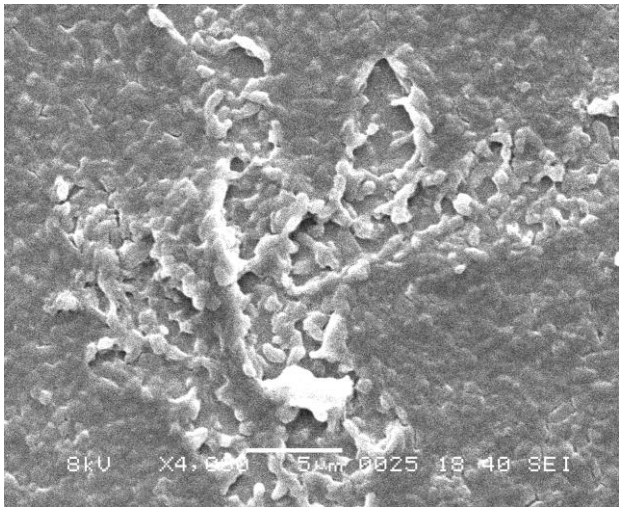

Fig. 4: Biofilm formation (*E. coli*) seen by SEM, 2016; Hoiby *et al*., 2010)

Detection of plasmid mediated *bla*-TEM ESBL gene in *E.* Coli

Plasmids were isolated (miniprep method) from 47 selected ESBL producing *E. coil* and subjected to PCR for the amplification of *bla*-TEM 1 (a type of ESBL gene), twenty nine (62%) plasmid preps showed about 848 bp amplified DNA product (*bla*-TEM 1) in agarose gel electrophoresis (fig 5) and were sequenced (fig 6) (Hoiby *et al*., 2010; Rasool, 2016).

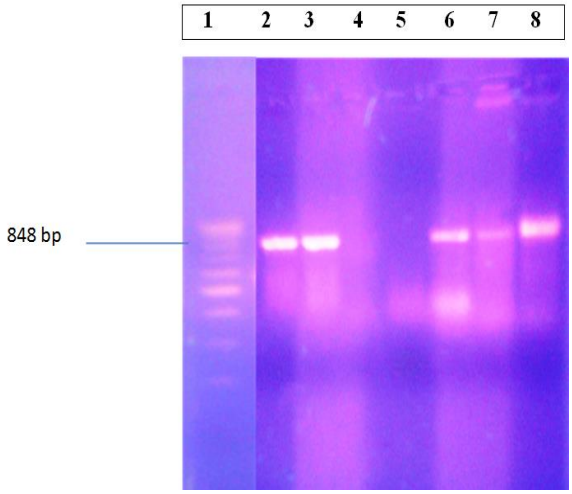

Fig. 5: Agarose gel electrophoresis of PCR product of ESBL enzyme gene (*bla*-TEM 1) (Rasool, 2016)

Key: Lane 1: 1 Kb Marker; 2, 3, 6, 7, 8: Amplified *bla*-TEM 1 gene PCR product

>160113-30_G12_6_TEM-1A.ab1 (Length 843bp)

TCGTCACCGCTCCTGCGGCATTTTGCTTCCTGTTTTTGCTCACCC AGAAA
CGCTGGTGAAAGTAAAAGATGCTGAAGATCAGTTGGGTGCACGAGTGGGT
TACATCGAACTGGATCTCAACAGCGGTAAGATCCTTGAGAGTTTTCGCCC
CGAAGAACGTTTTCCAATGATGAGCACTTTTAAAGTTCTGCTATGTGGCG
CGGTATTATCCCGTGTTGACGCCGGGCAAGAGCAACTCGGTCGCCGCATA
CACTATTCTCAGAATGACTTGGTTGAGTACTCACCAGTCACAGAAAAGCA
TCTTACGGATGGCATGACAGTAAGAGAATTATGCAGTGCTGCCATAACCA
TGAGTGATAACACTGCGGCCAACTTACTTCTGACAACGATCGGAGGACCG
AAGGAGCTAACCGCTTTTTTGCACAACATGGGGGATCATGTAACTCGCCT
TGATCGTTGGGAACCGGAGCTGAATGAAGCCATACCAAACGACGAGCGTG
ACACCACGATGCCTGCAGCAATGGCAACAACGTTGCGCAAACTATTAACT
GGCGAACTACTTACTCTAGCTTCCCGGCAACAATTAATAGACTGGATGGA
GGCGGATAAAGTTGCAGGACCACTTCTGCGCTCGGCCCTTCCGGCTGGCT
GGATTATTGCTGATAAATCTGGAGCCGGTGAGCGTGGGTCTCGCGGTATC
ATTGCAGCACTGGGGCCAGATGGTAAGCCCTCCCGTATCGTAGTTATCTA
CACGACGGGGAGTCAGGCGACTATGTATGAACGAAATAGACAGATCGCTG
AGAAGGGCCCACAAAAAATAAAAAAAAAATTTTTTTTTTGTG

Fig. 6: Sequencing of bla-TEM gene

By BLAST (NCBI) nucleotide sequence analysis of *bla*-TEM 1 gene (Fig. 6) indicated the identity (95-99%) with *bla*-TEM-116 gene (Rasool, 2016).

Contributing factors and possible solutions

Misuse and abusive usage of antimicrobial drugs have been a tremendous source for the evolution of drug resistant bacterial strains. Further,

noncompliance of nosocomial infective incidence containment guidelines, uneasy access to novel drugs and rapid easy global travel facilitation (turning the world into a global village) also contribute to infection spread outs (reportedly, 20% infections are contracted during air travel) (Wang *et al*., 2019). Super-infections are promoted as a result of excessive use of wide spectrum drugs (e.g. cephalosporins). Pathogenic strains fall into places where from usual or sensitive microorganism would be eliminated (fig 7). Infact, the infected persons are compromised by the antibiotics (e.g. GIT resident *Cl. difficile* may cause pathogenic superinfection). This anaerobic pathogen has acquired antibiotic resistance over the time and causes diarrhea (a tough clinical condition to handle) (Rasool & Ajaz, 2017).

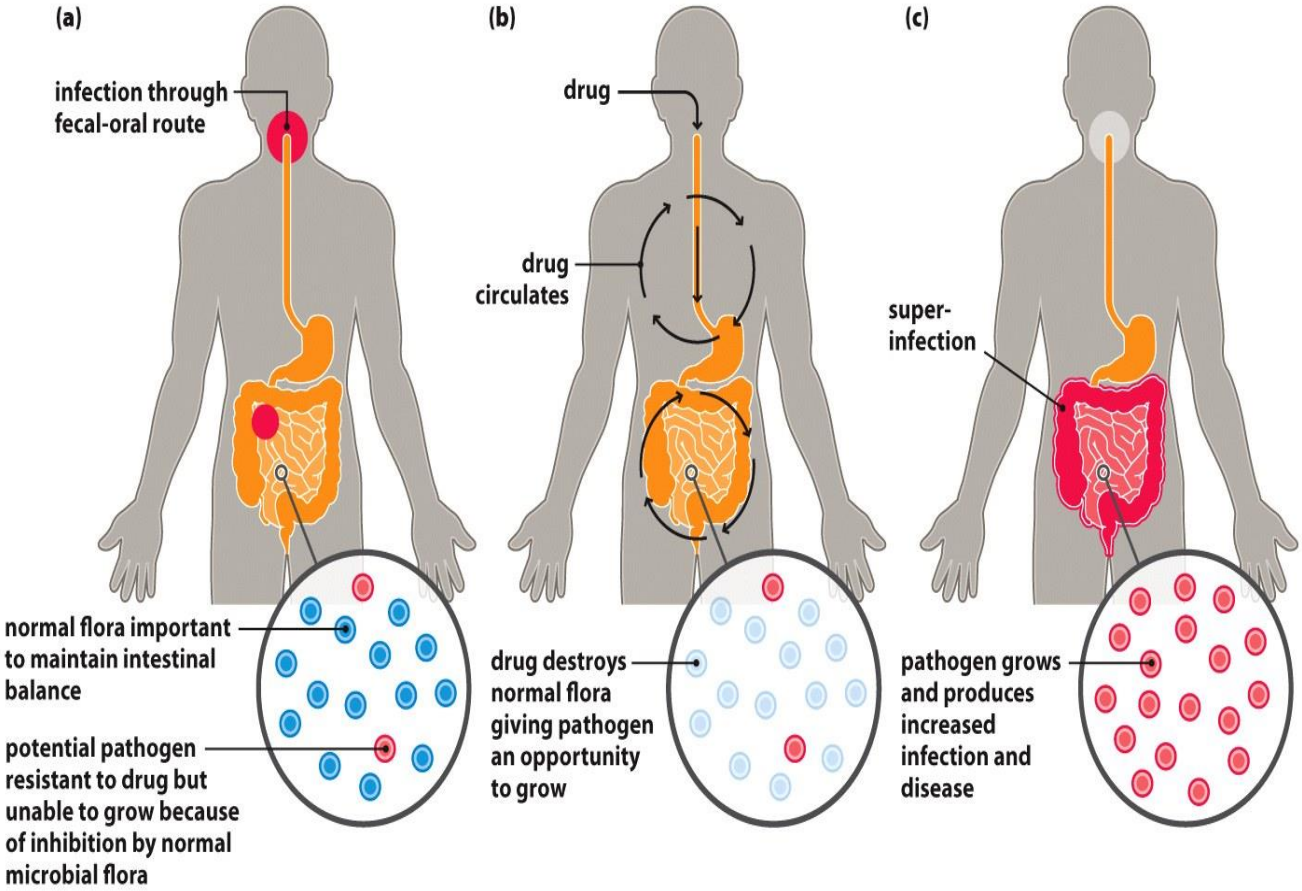

Fig. 7: Knock out of normal flora allures pathogens for dominance (Anthony *et al*., 2010)

New targets for antimicrobials

Comparing the metabolism cycles of resident flora and microbial pathogens with the antibiotics that target them could facilitate and indicate the new antibiotics / targets in pathogenic strains. The drug phosphonosulfonate lowers the human cholesterol (targeting squalene synthase) and inhibits the *Staphylococcus aureus* virulence contributing enzyme dehydrosqualene synthase (Gao *et al*., 2017). Novel staphyloxanthin inhibitors (with improved potency against MRSA) have been reported. Nanotechnology against drug resistant bacteria and material probiotic (human breast milk) supplementations are best choices. Other potential areas in microbial metabolism constitute fatty acid anabolism, cellular multiplication, synthesis of aminoacyl-tRNAs, protonic motive force (PMF), signal transmission sensing of quorum etc. Combined therapeutic approach involving coupled action of drugs along with antibiotics vitiating bacteriophage, is a confident and encouraging encounter approach for resistant bacteria (Ni *et al*., 2018).

Probiotics-postbiotics

The live microorganisms and their exclusive

products (enjoy GRAS rating) offer health benefits to the host. These living microbes exhibit their survivability along with attachment to mucous membrane of the gut with transient bioclustering. They effectively encounter the bugs of vast variety in medical-clinical therapeutics (challenged by rapidly emerging MDR / XDR microbial strains). A number of studies involving probiotics (such as *Saccharomyces boulardii* and *Lactobacillus rhamnosus* GG) have indicated a considerable reduction in >50% of antibiotic related diarrhea. *Lactobacilli reuteri* (producing reuterin) and species of *Bacillus* have been exploited as probiotics against pathogenic *Vibrio* spp. Reuteri also acts against miscellaneous microbial infections (Rasool *et al*., 2018). Reuterin carries an extended antimicrobial spectrum of bioactivity that downgrade the release of "proinflammatory" cytokines. It obstructs adsorption/adherence, to limiting the crowding of the pathogenic bacteria. Bacteriotherapy with *L. reuteri* helps to curer rotavirus gastroenteritis also. According to recent studies human breast milk has been found to have extended antimicrobial action over MDR pathogens (with possible anticancer activity as well). Recently, *ludgunensis* (resident of nostrils) produces ludgunin (bioacative peptide) which shows antagonistic action against MRSA and many other MDR strains (Simpson *et al*., 2015; Rasool and Ajaz, 2017; Kerry *et al*., 2018).

A strong link exists between livestock and human population. Vaccines should be preferred for animals to avoid resistance against antibiotics.. Field livestock and others are given to feed along with about 80 percent of drugs within the USA that are fed to humans. So is the case with poultry that needs to be antibiotic free industry. Alternatively, powerful commissions may draw the line between the antibiotics for poultry and farm animals and such antibiotics must not be prescribed for the human pathogenesis intervention (w.p.r. to developing countries). Instead of antibiotics phytochemicals can be used alternatively for the enhancement of poultry and livestock (Lillehoj *et al*., 2018). Education for credible vaccines development (vaccines are alternative to antibiotics) is essential. Vaccines are all and always effective but not the antibiotics. Marine microorganisms (algae, sponge and cyanobacteria) are rich source of bioactive compounds against human pathogens. Sidr honey is used against virulence genes of MRSA (inactivation of *cva* and *spa* genes) (Newman & Cragg, 2007). Similarly, biofilm formation on gallstone by *S. typhi* was deferred by Manuka Honey (Hannan *et al.*, 2018). MDR reversal activity by a rare dimericnaphthoquinone from *Diospyros lotus* was reported. Bioactive phenozine from *Ps. aeruginosa* against clinical isolates has been recorded (Gao *et al*., 2017).

CRISPRs

Clustered regularly interspaced short palindromic repeats (CRISPRs) are adjusted defence mechanism acquired from bacteria. CRISPR-Cas scheme uses RNA for target DNA identification and enzyme (Cas) for successive degradation of nucleic acid. This technique has antimicrobial activity and is now being applied to selectively destroy microorganisms and especially multidrug resistant bacteria (Sorek *et al*., 2013; Bikard *et al*., 2014; Gomaa *et al*., 2014; Hsu *et al*., 2014). Recently, genetically modified bacteriophages and nanoparticles are being practiced to dispence CRISPRs (Yan *et al*., 2015; Shen *et al*., 2018). However, some studies have revealed that resistant Shigella and *K. pneumoniae* may lessen the effect of CRISPR-Cas (Oliveira Santos *et al*. 2018, Chen *et al*. 2019). Recently, a successful study has been conducted to handle carbapenem resistant *K. pneumoniae* by two efficient novel DNA editing mechanisms like pCasKP-pSGKP and pBECKP. Both mechanisms could help in the cure of carbapenem resistant bacterial infections (Wang *et al*., 2018).

Nanotechnology to tackle multidrug resistant (MDR) bacteria

Nanotechnology is a crucial approach to formulate novel antibiotics because it employs nanometric-sized substances with tremendous affinity for the bacteria and compounds with enhanced bioavailability and absorption, improved muco-adherence, quick entry of drug into the cell. They may generate regulated discharge systems for encapsulated or surface ligated drugs delivery (Zaidi *et al*., 2017; Jamil and Imran, 2018). A recent progress in nanotechnology is the use of silver that influences the respiration of bacteria and stimulates the production of reactive oxygen species (ROs). Such nanoparticles can be applied in combination with antibiotics to modify cell wall synthesis and disintegration (Shahverdi *et al*., 2007; Kumar *et al*., 2018). Moreover, nanoparticles have proved promising treatment of infections as they can approach sites of microbial colonization (Zaidi *et al*., 2017).

Nanocages are small, emptied and absorbent chemical frameworks that are valuable in drug transit and distribution. They may be composed of polymers, metals and proteins having considerable strength to destroy MDR bacteria as better attachment, enhanced systemic circulation and aggregation at the site of infection (Wang *et al*.,

2016; Meeker *et al*., 2018). Gold nanocages have confirmed the bactericidal activity against *S. aureus* when injected locally and systemically (Wang *et al*. 2018). Apoferritin nanocages enclose streptomycin and deliver it at the site of infection (Ruozi *et al*. 2017).

Bacteriophages

They have the capability to encounter resistant bacteria hence are crucial substitute of presently used antibitics (Hagens & Loessner, 2010; Summers, 2012). Practices of phage for the treatment and eradication of MDR bacterial infections have been approved by Euorpean Medicines Agency (EMA) and Food and Drug Administration (FDA) (Rios *et al*., 2016). The combined strategy is potentially advantageous that is the use of designated phages to dispense CRISPR-Cas in bacteria to abolish MDR-bacteria (Balcão and Vila, 2015). Various companies have produced such systems including Eligo Bioscience and Locus Biosciences. Current advancement in biotechnology has improved the capacity to invade biofilms, enhance the phage efficiency, increased host range of phages and rendered a phage more specific and durable (Maura & Debarbieux, 2011; Rios *et al*., 2016; Harada *et al*., 2018).

Phytochemicals

Phytochemicals are plant derived biologically active chemicals having potential to reduce the evolution of drug resistance in bacteria (Rossiter *et al*., 2017). Encompassing the all possible choices, phytochemicals have found more potential to encounter drug resistant bacteria. They have antifungal, antioxidant and antibacterial effect and potentiate the ancient antibiotics to evade drug resistance and hence can be recovered for clinical use again (Barbieri *et al*., 2017). Piperine, an alkaloid, can reduce minimum inhibitory concentrations (MICs) of ciprofloxacin and kill the *Staphylococcus aureus* when use synergistically with ciprofloxacin (Khan *et al*., 2006). Similarly, piperine in combo with gentamicin can cure MRSA inflicted infections (Khameneh *et al*., 2015). Dictamnine, maculine and kousagine (Quinoline alkaloids) has demonstrated immense antimicrobial activity (Lin *et al*., 2006; Kuete *et al*., 2008). Respiration inhibition with decreased oxygen utilization is attributed by Alkyl methyl quinolones (Tominaga *et al*., 2002). Synergistic use of Reserpine with antibiotics increases the antimicrobial effect on *Micrococcus spp*., *Staphylococcus spp*., and Streptocccus spp., (Abdelfatah *et al*., 2015; Sridevi *et al*., 2017). Further, it inactivates efflux pumps like AdeABC in MDR *A. baumannii* and makes this bacterium sensitive (Jia *et al*., 2015). Sanguinarine is potentially effective against MRSA. It can cause the discharge of cell wall autolytic enzymes consequently, disruption of the cell. Moreover, under electron microscopy modifications in the pattern of septum synthesis were observed (Obiang-Obounou *et al*., 2011; Vandevelde *et al*., 2016).

CONCLUSIONS

The super drug resistant bacteria are on high emergence. They have evolved and acquired multiple strategies to conferment the effects of antibiotics. Therefore, the application of new systems and methods to evade MDR bacteria is mandatory, as scarcity of new drugs and consistent evolution of resistant bacterial strains. The scenario and various approaches explained in this review may contribute novel ideas for eradicating MDR bacteria. These strategies may include novel targets for antimicrobials, use of probiotics and prebiotics, CRISPRs, nanotechnology, bacteriophages and phytochemicals. All these approaches have been found effective and significant upto their extent in excluding the emergence and evolution of MDR bacteria.

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