HISTORY OF ANTIBIOTIC RESISTANCE

Microbial resistance is emerging faster than we are replacing our armamentarium of antimicrobial agents. Throughout millennia infections were treated with folk remedies like leaches, herbal extracts, wine, vinegar, silver etc, tender loving care or not at all. Many people died from common infections that today are easily treated with antibiotics. In 1900, at a time when infections were the leading cause of death in the United States, the average life expectancy at birth was approximately 47 years (46 for men and 48 for women). Childhood deaths secondary to infections were frequent. The major causes of death were smallpox, cholera, diphtheria, typhoid fever, plague, tuberculosis, typhus, scarlet fever, rheumatic fever, measles, mumps, pertussis, poliomyelitis, and syphilis. Many of these diseases also caused complications leading to severe disabilities (e.g., paralysis from polio, valvular heart disease from rheumatic fever, and neurological deficits and heart disease from syphilis) [1].

The first antibiotic, penicillin, was discovered in 1929 by Sir Alexander Fleming, who observed inhibition of staphylococci in the 19-th century. Ignaz Semmelweis in 1847 demonstrated that puerperal fever (also known as “childbed fever”) was contagious and that its incidence could be dramatically reduced by enforcing appropriate hand-washing behavior by medical care-givers [2]. Louis Pasteur first clearly demonstrated the connection between bacteria and disease [3]. In 1865, Joseph Lister performed first successful antiseptic surgery using carbolic acid [4]. Other antiseptics that followed include iodine, boric acid, alcohol and Dakin’s solution (sodium hypochlorite). These therapies saved many lives during the First World War and continue to be important today.

Sulfonamide drugs were the first antimicrobial drugs, and paved the way for the antibiotic revolution in medicine. The first sulfonamide, prontosil was discovered in 1932 in the laboratories of Bayer and by mid-1930th many sulfa drugs became available. As the first and only effective antibiotics available in the years before penicillin, sulfa drugs had a central role in preventing wound infections during the World War II [5]. Sulfonamides however have the potential to cause a variety of serious side effects, including urinary tract disorders, haemopoietic disorders, porphyria, and hypersensitivity reactions [6]. Resistance mutations have severely compromised the usefulness of these drugs [7].

The first antibiotic, penicillin, was discovered in 1929 by Sir Alexander Fleming, who observed inhibition of staphylococci on an agar plate contaminated by a Penicillium mold [8]. Antibiotics were introduced into commercial use in 1940s and the subsequent emergence of resistance, particularly to multiple drugs, has thwarted treatment of patients in the hospital and the community [9]. In 1960, the development of methicillin was described as an outstanding achievement as it was able to withstand destruction by penicillinase. One year later, in 1961, the first reports of methicillin-resistant Staphylococcus aureus (MRSA) appeared [10]. In healthcare facilities around the world, bacterial pathogens that express multiple resistance mechanisms are becoming common, com-
plicating treatment and increasing human morbidity and mortality [9]. For instance, in a study of patients at the F. I. Proctor Institute in San Francisco, CA from 1998 to 2006, it was found that less than 15% methicillin resistant Staphylococcus aureus (MRSA) ocular infections were sensitive to ciprofloxacin, a second generation broad spectrum fluoroquinolone [11]. According to the State of Pennsylvania 2005 Report, 14% of hospital Intensive Care Unit (ICU) infections were fatal and those infections accounted for $2.9 billion in increased costs.

A number of studies demonstrated that sub-lethal doses of non-therapeutic antibiotics result in the selection of resistant organisms in farm animals and workers due to their indiscriminate usage on farms (Testimony of Dr. Stuart B. Levy President, Alliance for the Prudent Use of Antibiotics before the Subcommittee on Health of the U.S. House Committee on Energy and Commerce July 14, 2010). As a consequence, there is an evolving unmet medical need for novel antimicrobial agents that are effective against existing resistant pathogens and that carry a low potential for the development of drug resistance [12, 13].

Unfortunately the pipeline of new antibiotics is getting dry. Between 1940 and 1962, more than 20 new classes of antibiotics were marketed. Since then, only two new classes have reached the market [14]. Part of the reason is that all “easy targets” are already discovered. Another problem is that infection is typically an acute, non-chronic decease lasting for weeks, not years. Therefore antibiotics rarely become “blockbuster drugs” making billions of dollars like high blood pressure or asthma drugs, resulting in declining interest from large pharmaceutical companies. According to WHO, current trends indicate that there may be no effective treatments for some diseases in next ten years as direct consequence of the development of resistance by many microorganisms to existing therapeutics (WHO fact sheet https://apps.who.int/inf-fs/en/fact194.html). A combined effort from government, public and industry is needed to reverse this trend.

ORIGINS AND EVOLUTION OF ANTIBIOTIC RESISTANCE

The development of resistance to one or several drugs is a result of the combination of the frequency of mutation, the number and type of mutations required to express resistance, the potency and concentration of the treating drug, and other factors including the use and overuse of antimicrobials that promote transfer of resistance by both vertical and horizontal mechanisms [12, 15, 16]. Soon after introducing antibiotics for human therapy it became evident that bacteria were able to develop resistance, not just as the consequence of mutations in the targets of antibiotics, but by horizontally acquiring genes conferring resistance to antimicrobials [17]. Since those genes were not present before in the human bacterial pathogens, the only suitable source for them was the environmental microbiota, and indeed the presence of R-factors (resistance plasmids) in environments without any previous record of contact with antibiotics was described in the first studies of antibiotic resistance in the field [18]. Recently the use of functional genomics and metagenomic techniques has demonstrated that natural ecosystems, including soils and human gut, contain a large number of elements that, upon transfer to a new host, confer resistance to antimicrobials [19, 20]. These include natural antibiotics, which are produced by the environmental microbiota, and synthetic antimicrobials, such as quinolones. An important question from an evolutionary point of view is the frequency or these resistance genes in their natural hosts [21]. Whereas for naturally produced antibiotics a protective role for resistance genes in the producer organisms (or those coexisting with producers [22]) might be foreseen [23], this explanation is not suitable for synthetic antibiotics such as quinolones. Indeed, it has been described that the origin of the quinolone resistance gene QnrA, which is now widespread in plasmids present in human pathogens, is the environmental non-antibiotic producer Shewanella algae [24]. Therefore a gene that confers resistance in a human pathogen does not necessary play the same role in its original host [25]. The finding that several proteins involved in basic bacterial physiology also contribute to antibiotic resistance [22, 26, 27] further supports the hypothesis that resistance genes, acquired through horizontal gene transfer by human pathogens, might have evolved in their original host to play a different role than antibiotic resistance. We can therefore distinguish two ages in the evolution of antibiotic resistance. The first age started billions of years ago and lasted until the use of antibiotics by humans. The resistance genes during this period have been typically chromosomally encoded and had evolved for different purposes. Some of them, as those found in antibiotic producers, likely evolved for detoxifying the original host from the antibiotic it produced, although a role in biosynthesis of the antibiotic itself has been proposed as well [23, 28]. Others, like β-lactamases, might be involved in the biosynthesis of the cell wall [29, 30], while others, like multidrug efflux pumps, might serve for different purposes including the transport of signaling molecules or extrusion of toxic compounds [31]. The second age started about 70 years ago with introduction of penicillin followed by many other classes of antibiotics. Once a gene is introduced in a new host in which it lacks its original biochemical and genetic context, its function is limited to antibiotic resistance. This change of function without changing the sequence of the gene itself is the consequence of the strong selective pressure exerted by antibiotics in the last seven decades. Two important aspects are emerging from the studies of natural resistome. First, the environmental microbiota contains a much larger number of resistance genes than those seen to be
acquired by bacterial pathogens [21, 32]. Furthermore, different ecosystems contain different resistance genes, meaning that there is a vast reservoir of potential resistance genes present in natural ecosystems. For example resistance genes are found in deep terrestrial subsurface [33], ice [34], and even the permafrost [35], which have not been in contact with human contaminants. Second, those genes present on mobile elements in human bacterial pathogens can be found nearly everywhere, including pristine ecosystems or wild animals not supposed to be in contact with antibiotics [25]. This indicates that pollution with antibiotic resistance genes is widespread and that resistance genes can persist even in the absence of antibiotic selection pressure. The analysis of historical soil archives has shown a consistent increase of antibiotic resistance elements in water microbiota and puts a focus on the effect that the presence of antibiotic resistance genes since 1940 [36], which is a clear prove of the contamination natural ecosystems by antibiotic resistance elements and the persistence of those elements. Tracking the origin of known resistance gene is a difficult task, however there are several successful examples. It has been determined that QnrA originated in S. algae [24] and that chromosomally encoded qnr genes are mainly present in water-dwelling bacteria [37]. This suggests that the source of transferrable quinolone resistance is the water microbiota and puts a focus on the effect that the use of quinolones in aquaculture might have had for the emergence and dissemination of these resistance elements [38].

MECHANISMS OF ANTIBIOTIC RESISTANCE

Mechanisms of resistance to antibiotics are divided into innate characteristics, described as intrinsic, and those that result from the acquisition of DNA (plasmids, transposons) by transformation and recombination.

Bacteria differ in their cell wall composition resulting in differences in their intrinsic susceptibility to antibiotics. Intrinsic resistance depends on the hydrophilicity of the antibiotic and is mediated by cell wall composition, formation of biofilms, efflux or by chromosomally mediated enzymatic inactivation [39]. As discussed above, bacteria can also acquire and disseminate antibiotic resistance determinants by horizontal transfer on plasmids, transposons and other genetic elements. These transferred mechanisms include efflux, modification of existing antibiotic targets, acquiring new targets and production of the enzymes that inactivates the antibiotic.

There are two major cell wall structures that often lead to impermeability in Gram-negative bacteria: lipopolysaccharide (LPS) composition and expression of outer membrane proteins, porins that restrict inward inflow of antibiotics and biocides. The core region of the LPS is strongly negatively charged and functions as a selective permeability barrier for negatively charged antibiotics resulting in decreased susceptibility. Porins are outer membrane proteins that permit the influx of nutrients and efflux of waste products. Antibiotics can function as substrates for porins. Decrease or loss of porin synthesis in nosocomial pathogens in combination with other resistance mechanisms result in multidrug resistant (MDR) bacteria [40].

Biofilms are bacterial sessile communities irreversibly attached to a substrate and embedded in a matrix of extracellular polymeric substances, the glycocalyx. Bacteria in biofilms are resistant to many antibiotics due to the slow growth, altered physiological state and delayed penetration of antibiotics through the glycocalyx. Diffusion of β-lactams and macrolides through the extracellular material is more rapid than of aminoglycosides [41].

Efflux pumps are transporter proteins involved in the removal of toxic substances from the interior of the cell to the external environment. Some efflux pumps are specific for a single drug or substrate while others are capable of transporting multiple substrates. Increased efflux results in sub-therapeutic intracellular concentrations of antibiotics and subsequent therapeutic failure. There are five major efflux pump families: the major facilitator superfamily (MFS), resistance nodulation cell division subfamily (RND), the small multidrug regulator subfamily (SMR), the ATP-binding cassette (ABC) family, and the multidrug and toxic effects (MATE) family. A proton motive force mediated by the counter flow of protons drives the MFS, RND, MATE and SMR families. The ABC family uses the hydrolysis of ATP by ATPase to provide the energy for active transport of antibiotics and other toxic molecules. The genes of efflux pumps can be intrinsic or acquired. The intrinsic efflux mechanism of resistance is chromosomally encoded and is activated by environmental signals or by mutation in regulatory genes [42]. For example in the RND superfamily, the mexAB-oprM operon in Pseudomonas aeruginosa regulates porin and pump genes. Mutations in the gene mexR encoding the repressor protein result in the reduced affinity for the promoter target and up-regulation of the mexAB-oprM operon. This three-component efflux pump provides an exit portal for quinolones, tetracycline, chloramphenicol, β-lactams and meropenem but not imipenem [43]. Acquired macrolide resistance mediated by efflux has been described in streptococci and is encoded by mefE in S. pneumonia and by mefA in Streptococcus pyogenes [44].

Modification of a target reduces the affinity of the target for the antibiotic. Target site modifications occur primarily by chromosomal mutation as in quinolone antimicrobials, and by enzymatic alteration of macrolide, glycopeptide, and β-lactam target sites. Quinolones target DNA gyrase and topoisomerase IV. The primary mechanism of resistance to this antimicrobial class is modification by mutations encoding single amino acid changes in these targets. Mutations are generally localized to the amino terminal domains of gyrA and parC, termed the quinolone resistance-
determining region (QRDR) [45] Although the presence of single mutation in the QRDR of gyrA results in high-level resistance to nalidixic acid, the presence of additional mutations in gyrA and/or another target such as parC is required to produce high levels of resistance to fluoroquinolones [46]. In Gram-positive bacteria, first step mutations leading to fluoroquinolone resistance occur in parC and second step mutations occur in GyrA [47]. Interestingly, first step mutations in gyrA do not cause an elevated MIC in S. aureus, suggesting that the primary target of the quinolone is topoisomerase IV [48]. β-lactam antibiotics kill S. pneumonia by targeting high-molecular weight penicillin binding proteins PBP1A, 1B, 2A, 2B and 2X. Mutations in these PBPs confer low-level to high-level resistance to β-lactam antibiotics depending on the number of mutations and PBPs involved [49]. Mutations in PBP2 or PBP2X mediated by amino acid changes in close proximity to the active-site region of the PBP result in low level resistance [50]. High-level resistance is the result of mutations in all five PBPs [49].

Enzymatic alteration of antibiotic targets results in reduced affinity of antibiotics for their microbial targets and is exemplified by resistance to macrolides through ribosomal methylase and resistance to vancomycin through reprogramming of the peptidoglycan termini. Macrolides such as erythromycin bind to the 50S subunit of the ribosome at the peptidyltransferase cavity in the proximity of the A and P loops, and near adenine 2058 of 23S rRNA [51]. Mono or dimethylation of the amino group in the adenine residue of 23S rRNA results in reduced affinity of the macrolide for its target site. Resistance to macrolides is mediated by erythromycin ribosome methylase (ERM), which is found on plasmids and transposons. Methylation of the 23S rRNA also results in cross-resistance to the lincosamide family and streptogramin B class of antibiotics. Cross-resistance to these antibiotic groups is known as MLSβ resistance phenotype [52]. Vancomycin is a glycopeptide antibiotic used to treat enterococci that cause endocarditis and methicillin resistant S. aureus (MRSA). Increased use resulted in the emergence of vancomycin resistant enterococci (VRE). The phenotypes conferring resistance to vancomycin are known as VanA and VanB. Enterococci containing the vanA phenotype are highly resistant to vancomycin (MIC ≥ 64 μg/mL) and resistant to teicoplanin (MIC ≥ 16 μg/mL). Strains carrying the VanB phenotype range from vancomycin susceptible to resistant but remain sensitive to teicoplanin. The VanA phenotype is resident on plasmids and transposons that mediate spread of the determinant while vanB appears to be on large chromosomal elements. VanA is inducible by vancomycin and teicoplanin and is controlled by VanS and VanR. The transmissibility of the vancomycin resistant elements to MRSA is of great concern. VanA has been detected in an MRSA with high level resistance to vancomycin (VRSA) [53]. Characterization of the vanA determinant shows five genes three of which are involved in target modification. These three genes, VanH, VanA and VanX, sequentially modify the peptidoglycan termini involved in cross-linking, N-acyl-D-ala-D-ala to N-acyl-D-ala-D-lactate. Affinity of vancomycin to this modified target is reduced 1000-fold. Van-H codes for a D-hydroxy acid dehydrogenase that synthesizes the D-lactate used by VanA, a ligase that mediates the preferential production of D-ala-D-lactate. VanX is a protein that acts specifically to cleave the natural peptidoglycan termini D-ala-D-ala thus preventing the competing synthesis of vancomycin-susceptible peptidoglycan. Other Van phenotypes mediate resistance to vancomycin using similar mechanisms [54].

Another mechanism of antibiotic resistance is acquisition of a new target with reduced affinity to antibiotic. An important example of such mechanism is MRSA where resistance to methicillin is mediated by the acquisition of a mobile element carrying a staphylococcal cassette chromosome mec (SCCmec) with three genes on it, mecrI-mecf-mecA [55]. The gene responsible for methicillin resistance, mecA, codes for a new penicillin binding protein, PBP2A, a bifunctional transglycosylase/transpeptidase with reduced affinity to β-lactams [56].

Another strategy used by bacteria to survive the action of antibiotics is the acquisition of enzymes that inactivate the antibiotic directly. β-lactams and aminoglycosides are examples of antibiotics inactivated by these mechanisms. The most important class of chromosomally encoded antibiotic inactivating enzymes is β-lactamases. These enzymes hydrolyze β-lactam antibiotics using two distinct mechanisms, one serine-based and the other metallo based. These mechanisms of action allow classification of β-lactamases into four major classes with classes A, C and D containing active-site serine enzymes and class B exhibiting metallo based mechanism of action. β-lactam antibiotics act by binding to PBPs, bifunctional transglycosylases/transpeptidases responsible for cross-linking of glycan strands and backbone peptide strands, respectively. The mechanistic and architectural similarities of class A, C and D β-lactamases to PBPs suggest a common evolution of serine-based β-lactamases from PBPs. The binding equilibrium between PBP, β-lactamase and the antibiotic determines the survival of the microorganism. This observation led to the development of β-lactamase inhibitors e.g. clavulanic acid, sublactam and tazobactam that are structural analogs and are co-administered with β-lactams [39]. Intrinsinc chromosomal resistance mediated by enzymatic inactivation of penicillin class antibiotics is exemplified by the class C β-lactamases produced by gram-negative pathogens including Citrobacter freundii, Enterobacter aerogenes, and P. aeruginosa. Most class B metalloenzymes are chromosomally encoded cephalosporinases. They are inducible or constitutively expressed in Stenotrophomonas maltophilia, Klebsiella pneumoniae and P. aeruginosa [57]. With the emergence of plas-
mid-mediated $\beta$-lactamase resistance, such as mediated by TEM-1 (named TEM after the patient from whom it was isolated (Temoniera) and SHV-1 (SulphHydryl Variable 1), new oxyimino-$\beta$-lactam parenteral antibiotics resistant to the hydrolysis by these $\beta$-lactamas were introduced into clinical practice (cephalosporins and monobactams). However with the continued use of these antibiotics, variants of existing $\beta$-lactams emerged that could hydrolyze the new antibiotics [58, 59]. These extended spectrum $\beta$-lactamas (ESBLs) are now known to be derivatives of TEM, SHV and OXA type $\beta$-lactamas that differ by one or more amino acid substitutions near the reactive sites of the enzyme [58]. Of particular concern is NDM-1, which stands for New Delhi metallo-beta-lactamase 1 and actually refers not to a single bacterial species but to a transmissible genetic element encoding multiple resistance genes that was initially isolated from a strain of Klebsiella obtained from a patient who acquired the organism in New Delhi, India. Subsequently, organisms in the Enterobacteriaceae family containing this genetic element (or variants thereof) have been found widely throughout India, Pakistan, and Bangladesh and are now turning up in Britain and many other countries around the world. The spread of these organisms has prompted widespread concern because some of them are resistant to all antimicrobial agents except the polymyxins [60]. The most clinically relevant mechanism of resistance to aminoglycosides is enzymatic modification, thus preventing recognition of the 16S RNA binding sites and subsequent inhibition of mRNA translation [61]. Enzymatic modifications result from N-acetylation, O-phosphorylation and O-adenylation of aminoglycoside radicals. Therefore, aminoglycoside-modifying enzymes are classified by their modifying reaction, their regiospecificity on the aminoglycoside ring structure, and by their specific isozyme sequence. Inactivation of the aminoglycoside by the aminoglycoside-modifying enzymes is mediated by the transfer of a functional group to the aminoglycoside: AAC transfers the acetyl group from acetyl-CoA to the NH$_2$ group, ANT transfers the nucleotide triphosphate, and APH transfers the phosphoryl group from ATP to the OH of NH$_2$ group [61].

**APPRAOHES TO COUNTER RESISTANCE**

Antibiotic stewardship has been suggested as one of the approaches to the problem which involve reduction of unnecessary antibiotic consumption, and preservation of existing agents [62]. Because culture results are often not available at the time of therapeutic decision-making, treatment success or failure depends upon up-to-date, accurate and local information on the prevalence of resistance among the pathogens. To this end, a number of surveillance programs have been initiated. For example the SENTRY Antimicrobial Surveillance Program was designed to monitor the predominant pathogens and antimicrobial resistance for both nosocomial and community-acquired infections globally by using validated, reference-quality identification and susceptibility testing methods performed in a central laboratory [63]. An example of a specific surveillance program is PROTEKT (Prospective Resistant Organism Tracking and Epidemiology for the Ketolide Telithromycin), a global program for monitoring resistance to telithromycin and disseminating the data to clinicians [64].

Some other approaches include salvaging the existing classes of antibiotics by co-administration of antibiotics with resistance inhibitors (e.g. $\beta$-lactamase + $\beta$-lactams, the only commercially successful combination to date [39], and efflux pump inhibitors [65]). Peptidomimetic compounds such as phenylalanine arginyl $\beta$-naphthylamide (PA$\beta$N) have been introduced as efflux pump inhibitors (EPIs); their mechanism of action is through competitive inhibition with antibiotics on the efflux pump resulting in increased intracellular concentration of antibiotic, hence, restoring its antibacterial activity. The advantage of EPIs is the difficulty to develop bacterial resistance against them, but the disadvantage is their toxic properties hindering their clinical application. Also in early development are natural products including defensin peptides [66], new antibacterial vaccines [67], and phage therapy (the latter approach had been explored in the former Soviet republic of Georgia since 1920s) [68]. Following a 40-year hiatus in discovering new classes of antibacterial compounds, three new classes of antibacterial antibiotics have been brought into clinical use: cyclic lipopeptides (such as daptomycin [69]), glycylcyclines (such as tigecycline [70]), and oxazolidinones (such as linezolid [71]). Several new antibiotics from known classes were introduced recently, mostly against Gram-positive bacteria only (e.g. telavancin [72]). “The drugs of last resort” against multidrug-resistant (MDR) Gram-negative pathogens P. aeruginosa and A. baumannii are polymixin B and colistin, old drugs with significant toxicity [73]. The complete sequencing of the genomes of many pathogenic bacteria has led to an explosion in knowledge about these organisms [74]. The genomics route has proven to be target rich, but has not led to the introduction of a marketed antibiotic as yet. Non-culturable bacteria may be an alternative source of new antibiotics [75]. “Old therapies” like antisepsis need to be revisited and used when possible for topical indications to spare the antibiotic use only for when it is really needed. To limit the use of antibiotics for topical indications, NovaBay is developing a new class of non-antibiotic topical antimicrobials with a novel mechanism of action. NVC-422, a stable analog of N-chlorotaurine (NCT), is the first representative of this new class of fast-acting, bactericidal antimicrobial agents (Aganocide compounds) active against a broad range of both Gram-positive and Gram-negative species, including drug resistant and MDR pathogens [76].
NVC-422 mechanism of action studies show that it kills bacteria and fungi and inactivates viruses by rapidly inactivating surface proteins via the preferential oxidative modification of sulfur-containing moieties (Met, Cys) [77]. This unique mechanism differentiates Aganocide compounds from traditional antibiotics by simultaneously attacking multiple targets on the surface of pathogens thereby making it virtually impossible for pathogens to develop resistance or be affected by existing mechanisms of resistance. Furthermore, for hundreds of millennia pathogens have been exposed to high concentrations of naturally-occurring NCT without any consequent development of resistance [78].

CONCLUSIONS

Antibiotic resistance is a growing unmet medical need. To prevent a public health crisis, novel families of antibiotics must enter the marketplace at regular intervals. Although analogues of existing families active against resistant bacteria prolong the life of each family for a number of decades, new classes of antimicrobials are urgently needed. Within the next 10 years, screening of whole bacteria against novel natural and chemical compound libraries may produce new antibiotics. Genomics, non-culturable bacteria and bacteriophages may also be a source of novel compounds. Antiseptics and non-antibiotic antimicrobials should be used where possible for topical indications to reduce unnecessary antibiotic consumption in medicine and agriculture.

REFERENCES