

Chapter 2

Development of Resistance to Antibiotics

Abstract Widespread use and misuse of penicillin and other antibiotics have resulted in development of resistance most antibiotics. The mechanisms by which microorganisms develop resistance to antibiotics are discussed. Topics covered include acquisition of point mutations and antibiotic resistance genes, methods of transfer of resistance genes between bacteria, and the advantages of synthetic antibiotics. Contribution of subtherapeutic use of antibiotics to resistance development and the response of the governments and other regulatory agencies to address the problem are also discussed.

2.1 Antibiotics Are No Longer Considered to be Miracle Drugs

Antibiotics, which were hailed as the miracle drugs that cured most infected people before, do not work in many cases today. This is because bacteria are increasingly becoming resistant to antibiotics at an alarming rate and the resistance is spreading throughout the world among all species of bacteria. The main reason for this resistance development is the excessive use of the antibiotics. After the discovery and introduction of penicillin people were so excited about its miracle properties in curing infections that the drug was not only available as over-the-counter medicine (one that does not require a prescription) but was also added to a large variety of household items such as ointments and cosmetics. Later the practice was banned and penicillin was made a prescription drug but by then there was already widespread resistance to the antibiotic.

It is not surprising that development of antibiotic resistant bacteria is a major concern in the scientific and medical community. This is evident from the amount of research that is being done on the subject. When significant amount of research has been done on an important scientific topic, journals usually publish review articles on the topic. So one way to determine the importance of a research topic is to count how many review articles are being published on that subject. To do that, one can search in the “PubMed” website of NCBI and search for “antibiotic resistance” or “antimicrobial resistance” reviews for each year. The website will display a list of all the review articles on antibiotic resistance that have been published in that

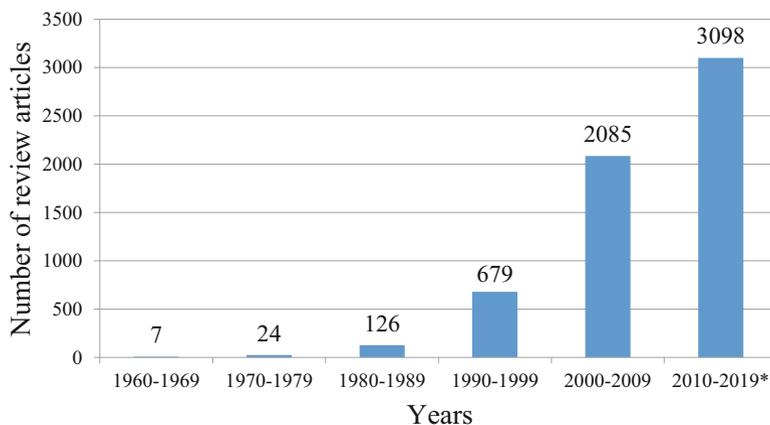


Fig. 2.1 Number of review articles published per decade. *The last bar for 2010–2019 is by extrapolation of the numbers for 2010–2015

year. Results of such a search are shown as a bar graph in Fig. 2.1. As can be seen from the graph, very little was known about antibiotic resistance in the 1960s but today it is an extremely important subject of study.

2.2 Detection of Antibiotic Resistance

How effective an antibiotic is is determined by its minimum inhibitory concentration (MIC), which is the minimum concentration of the antibiotic that can stop growth of a particular microorganism. The lower the MIC, the stronger is the antibiotic. The MIC is usually determined by either the “broth dilution” or “agar dilution” method. In broth dilution method, increasing amounts of the antibiotic are added to liquid growth medium (broth) in test tubes, which are then inoculated with the same number of cells. The minimum concentration of the antibiotic that prevents growth as seen visually by the lack of turbidity is designated as the MIC for the antibiotic for that microorganism. For the agar dilution method, varying concentrations of the antibiotic are added to the molten agar immediately prior to pouring the plates. Serial dilutions of the cells are then spread on the plates and MIC is determined as the minimum concentration of the antibiotic that prevents growth of colonies on the plates.

Another method that is easier and less expensive than the broth or agar dilution method is the Kirby–Bauer method, also known as the disk diffusion method [27]. In this method, cells are first spread on a plate and a filter paper disk containing a known amount of the antibiotic is placed in the center of the plate. As the antibiotic diffuses from the paper to the agar medium in the plate, a gradually decreasing concentration gradient of the antibiotic is created. After overnight incubation, an absence of growth observed around the disk indicates sensitivity to the antibiotic. The visibility of the

zone of inhibition can be dramatically improved by staining the cells with cationic dyes such as methylene blue [28]. The diameter of the zone of inhibition indicates the strength of the antibiotic but is only a qualitative indicator of the strength since the zone also depends on other factors such as the depth of the agar as well as size and water solubility of the antibiotic molecules. The MIC of the antibiotic is the concentration at the boundary of the zone of inhibition; however, it is not feasible to measure that concentration. An easier method for determining the MIC is the E-test in which a commercially available plastic strip containing a gradually decreasing and known concentration of an antibiotic is placed on an agar plate. The minimum concentration in the E-strip that shows no cell growth is the MIC. For a diagrammatic representation of the results of zone of inhibition and E-test see Fig. 3.24.

2.3 Classification of Antibiotic Resistance

Antibiotic resistance in bacteria can be of two types: intrinsic or acquired.

Intrinsic resistance: Intrinsic resistance is when by virtue of their structural or functional features, some bacteria are naturally resistant to some antibiotics without having prior exposure to the antibiotics. For example, gram-negative bacteria are intrinsically resistant to vancomycin, which is too large a molecule to cross the outer membrane (Sect. 3.3.3.4). Aerobic bacteria are intrinsically resistant to metronidazole, which requires an anaerobic environment to be reduced to its active form (Sect. 5.4). These and other examples will be discussed separately for each antibiotic in later chapters.

Acquired resistance: In a population of antibiotic sensitive bacteria, some cells may acquire the ability to be resistant to the antibiotic. Thus, unlike intrinsic resistance, which is effective in all cells of a certain species, acquired resistance can be observed in only a subpopulation of any bacterial species. Acquired resistance development can take place by two different mechanisms: (1) By point mutations and (2) by resistance gene acquisition.

2.4 Resistance Development by Point Mutations

Methods of development of point mutations can be of two types: natural methods and induced methods.

Natural methods: Replication errors: Many bacteria usually have a generation time of about 20 min, which means that the number of bacteria will double every 20 min. So one bacterium in 10 h will double 30 times to give 2^{30} or approximately one billion bacteria. This high number facilitates development of mutants. Before every cell division the chromosomal DNA has to be duplicated by a process called DNA replication. This copying of DNA is catalyzed by the enzyme DNA polymerase III, which binds

to the template DNA, brings in the correct complementary base from the surrounding medium and ligates (joins) it to the previous base in the chain. It does all this at the incredibly fast rate of about 1000 bases per second, which is the fastest known enzymatic polymerization reaction. Because of this very fast rate the enzyme makes some mistakes during replication. The error rate is about 10^{-5} , i.e., 1 in 10^5 . About 99% of these errors are corrected by the DNA polymerase itself. This is because the polymerase enzyme has a proofreading activity. Every time it incorporates a wrong base, it removes that and brings in the right base in its place. Since only 99% errors are corrected, 1% still remains. So the overall error rate in replication is 1% of $10^{-5} = 10^{-7}$. Each of these errors can give rise to a mutation. Besides the proofreading function of the DNA polymerase enzyme there are other repair enzymes that correct most of these errors after the replication process is completed giving an overall error rate of 10^{-9} . That means, out of every billion bases copied, one will be a mistake. Bacteria usually have about 3×10^6 base pairs of DNA in their genome. So everytime the bacterial cell multiplies, there is a $3 \times 10^6 \times 10^{-9} = 3 \times 10^{-3}$ probability of containing a mutation. This may not appear to be a high rate of mutation. However, because of the high number of bacteria present in an infected patient, the probability of a mutant being present in the population of bacteria becomes very significant. In a typical infection there can be about 100,000 bacterial cells per gram of tissue or per ml of urine. Assuming there are about 10^8 bacterial cells in an infected person, the number of mutants in this population will be about $3 \times 10^{-3} \times 10^8 = 3 \times 10^5$. Since these are random mutations, also known as spontaneous mutations, they can be expected to be equally distributed over the 3000 genes in the bacteria. So for every gene there will be about 100 bacteria that will have a mutation somewhere in the gene. It should be clarified here that all 100 mutations are not in the same bacterial cell but rather, there are 100 bacteria in the population having one mutation each in a particular gene. Actually the mutations are not uniformly distributed in all genes because mutations are more tolerated in some genes than in others. Mutations in housekeeping genes (those that are expressed in all cells all the time) are less tolerated because they code for proteins that perform indispensable functions.

Induced methods: Point mutations can also happen due to harsh environmental conditions such as strong ionizing radiations or oxidizing or alkylating chemicals. These methods are not relevant for resistance development in infecting bacteria because such harsh conditions do not exist in the host. However, recently it has been discovered that use of antibiotics by the host can induce generation of point mutations in the infecting bacteria. Lethal concentration of bactericidal antibiotics will of course kill the bacteria but if sublethal concentration of antibiotic is used, it triggers the formation of reactive oxygen species, which can cause mutations in the DNA [29]. Since this has relevance to use of antibiotics in farm animals, this phenomenon will be discussed further under “Subtherapeutic use of antibiotics” (Sect. 2.10.2).

Effect of point mutations: Most mutations (but not all) will result in a change in protein sequence (Sect. 6.1). Some of these changes (but not all) can affect the activity of the protein. For example, changes in the sequence at the active site of an enzyme can have an effect on its activity while mutations in the structural part of the enzyme will have less or no effect. Mutations do not always result in a loss of function; some

mutations can cause a gain of function. For example, a mutation can give the bacterium a selective advantage for survival even in the presence of an antibiotic. Such a mutant is said to be antibiotic resistant.

2.5 Selection for Resistance

In the absence of antibiotic in the growth medium the mutants and wild type bacteria will all grow at the same rate as they compete with each other for nutrients. However, if grown in the presence of antibiotic only the mutants that are resistant to the antibiotic will grow fast while all others will either die or grow slowly. Thus at the beginning there may have been only one mutant cell out of several million but at the end the culture will consist of 100 % antibiotic resistant cells. This process is called “selection.” Note that this selection process could take place because the mutant was resistant to the antibiotic at the concentration that was used. If a higher concentration was used, it is possible that all bacteria including the mutant would have died and so there would have been no selection for the mutant. The minimum concentration of an antibiotic that can stop the growth of bacteria is called its minimum inhibitory concentration (MIC). In the above example the wild type cells have a much lower MIC than the mutant. If the antibiotic concentration prescribed to the patient is not high enough to kill the mutant then a false sense of curing will result since the wild type bacteria, which constitutes the majority of the infecting bacterial population will be killed. However, this good feeling is temporary since the few resistant bacteria that survive will then multiply rapidly without any competition from wild type bacteria. Within a few days the mutants will be sufficiently high in number to continue the disease as before. The only difference now will be that the antibiotic will not work anymore against this infection. So it is important for the doctor to prescribe the right dose of the antibiotic that can kill all infecting bacterial cells.

Even if the doctor prescribed the right dose of antibiotic, selection for antibiotic resistant mutant can still take place if the patient failed to complete the full course of the prescription. After the antibiotic enters the bloodstream it is cleared from the body within a certain time either through urine or by degradation. So the concentration of the antibiotic decreases with time. In order to maintain the concentration at a level higher than the MIC, more doses need to be taken as prescribed. Within the first one or two doses most of the wild type bacteria will be killed giving the patient a false impression that the disease has been cured. However, the few resistant mutants will survive and unless more doses are taken, they will gradually multiply to bring back the symptoms of the disease. The resistant bacteria can then be transmitted to other people and thus increase the pool of antibiotic resistant bacteria in the environment. To stop the selection of antibiotic resistant bacteria it is the doctors’ responsibility to prescribe the right dose of the antibiotic and it is the patients’ responsibility to complete the prescribed dose of the antibiotic and not stop taking the remaining doses just because they start to feel better. Because of such misuse of antibiotics, resistance has developed to most antibiotics available. The more prescriptions a doctor writes for a certain antibiotic the more probable is the development of resistance to that antibiotic.

Misuse of antibiotics selects for gradually increasing level of antibiotic resistance: Point mutations usually confer low level resistance to the bacteria. However, the mutants that have low level resistance can develop new mutations that provide increased level of resistance. Probability of developing chromosomal mutations in any species is very low, about 10^{-9} replication error per cell per cell division (Sect. 2.4). Probability of developing two mutations in the same cell in a population of wild type bacteria is even lower, about 10^{-18} per cell. So direct acquisition of high level resistance by point mutations is very unlikely. However, the probability of developing the second mutation will be higher if improper use of the antibiotic has already resulted in selection for the first mutation which confers low level resistance since 100 % of the bacteria would then have the first mutation. The two mutations (the old and the new one) can act by the same or different mechanism. For example, the first mutation may decrease the binding of the antibiotic to the active site of the enzyme while the second mutation may decrease it even further. Another possibility is that the first mutation decreases the permeability of the antibiotic through the cell membrane while the second mutation may decrease the binding of the antibiotic to the active site. It is easy to treat low level antibiotic resistance since the bacteria can be killed by usual therapeutic doses of antibiotics. However, an incomplete dose of antibiotic will select for the low level resistant bacteria, which can later acquire a new mutations giving rise to gradual selection of high level antibiotic resistance which cannot be treated by the usual therapeutic dose of the same antibiotic which would have been sufficient before.

The development of antibiotic resistance is not a recent concept. Way back in 1945 in his Nobel Lecture Alexander Fleming had warned, “It is not difficult to make microbes resistant to penicillin in the laboratory by exposing them to concentrations not sufficient to kill them” [15]. He had also warned that misuse of penicillin could lead to selection of mutant bacteria that are resistant to the drug. This was not just a prediction; he actually produced these penicillin resistant mutants in his lab to demonstrate this. When penicillin V became available for oral use, there was a dramatic increase in use of the drug. In those days it was available without a prescription and was advertised to the public as a miracle drug. Easy access resulted in rampant misuse of the antibiotic. People were taking it even for diseases that were not caused by bacteria. Excessive use rapidly increased the development of resistance to the antibiotic.

2.6 Resistance Development by Resistance Gene Acquisition

Alexander Fleming’s prediction that bacteria can become resistant to antibiotics by point mutations has been proven by him as well as numerous others later. However, if point mutations were the only mechanism for resistance development then this would have been only a nuisance and not a serious health problem since the resistance level will be low and the bacteria can be killed by the usual dose of the antibiotic. Actually the situation has become more serious than what Fleming had

predicted. Today penicillin does not work for most infections. This has happened because of another mechanism of resistance development that is more important than point mutations and this was not known during Fleming's time. This mechanism of resistance development is by acquisition of resistance genes that already preexist in nature. Humans have been using antibiotics for only about 80 years since their first discovery but these natural antibiotics have been present in the environment for a billion years since the existence of bacteria, which produce them. This is a long time during which other bacteria have evolved by developing multiple mutations that have resulted in the formation of new genes coding for enzymes which degrade various antibiotics or make them ineffective. So long before humans started to use penicillin, there already existed bacterial strains that can make penicillin degrading enzymes. These enzymes are called **β -lactamases** since they can break the β -lactam ring in the structure of penicillin. We will study about the enzyme in more detail later (Sect. 3.3.2.5). Enzymes that degrade antibiotics are much more effective in providing resistance than point mutations in proteins. So if such a highly resistant bacterium exists in the infecting bacterial population, it will be selected for and the infection will not be cured. Under such circumstances a full course of a different antibiotic should then be taken. Until all the resistant bacteria are killed there will be a finite possibility of spreading the resistant bacteria to other people.

Plasmids, Transposons and Integrins: Antibiotic resistance genes usually reside in either the chromosome or plasmids or transposons or integrins. Plasmids are small (up to a thousand fold smaller than the chromosome) pieces of extrachromosomal DNA, usually circular. They can be present in multiple copies and use the cellular proteins for their replication. Replication is initiated at a specific site called the origin of replication (*oriV*). Of the various genes that are present in plasmids, one important gene that is usually present is resistance gene for some antibiotic. Many plasmids contain resistance genes for more than one antibiotic. Plasmids do not carry out any useful function for the cell and so they may be lost if one daughter does not receive a copy of the plasmid when the cell divides. If the particular antibiotic is present in the environment then the plasmid will be maintained in all cells since those daughter cells that do not receive a copy of the plasmid will be killed by the antibiotic. The process is called selection. Some plasmids have specific genes which ensure that each daughter cell always receives a copy of the plasmid during cell division even in the absence of antibiotic in the environment. These genes together form the "plasmid maintenance system."

Transposons (also known as insertion sequence (IS) elements) are small pieces of DNA that can insert into the chromosome mostly at random sites or in some cases, specific sites, and are commonly known as "jumping genes." They can also be excised from the chromosome and then inserted at a different location in the chromosome. Transposons were first discovered by Barbara McClintock, for which she was awarded the Nobel Prize in 1983. There are two main requirements for a transposon to function. It contains a direct or inverted repeat sequence at the two ends and the transposon sequence is preceded or followed by a gene sequence for the enzyme Transposase. The enzyme cleaves the DNA at the two repeat sequences

and inserts it, along with any DNA sequence in between, into the target DNA. Various genes including antibiotic resistance genes can be present in between the two ends of the transposon. There are two ways by which transposon insertions into the genome can confer antibiotic resistance to the bacteria. (1) The transposon may have an antibiotic resistance gene in it. So when it inserts at any region in the chromosome, the antibiotic resistance gene will be expressed. (2) If a transposon does not contain any resistance gene but inserts into a gene that is essential for proper functioning of an antibiotic then the cell will become resistant to the antibiotic. One common example of the latter type is by insertion into a gene for porins, which are needed for transport of the carbapenem antibiotics from the outside to inside the cell (Sect. 3.3.2.12). Note that most natural plasmids as well as some transposons contain resistance genes for more than one antibiotic. So improper use of any one of these antibiotics will select for the whole plasmid or transposon and thus, will automatically select for resistance to more than one antibiotic.

Similar to transposons, another type of mobile genetic elements is called integrons, which have the added ability to capture various genes such as antibiotic resistance genes from the DNA that they are inserted into [30]. So integrons can acquire resistance genes for multiple antibiotics. Unlike transposons, integrons do not have any direct or indirect repeats on two sides of the resistance gene. They contain an integrase gene that is needed for the insertion process.

Transfer of resistance genes between bacteria: If some bacteria in the infecting population contain an antibiotic resistance gene they can transfer the gene to other bacteria in the population that are sensitive to the antibiotic. These bacteria that acquire the resistance genes also become resistant. Transfer of genes between bacteria can take place in three ways: (1) *Bacterial Conjugation*. In 1946, Joshua Lederberg, who was awarded the Nobel Prize in 1958, discovered that bacteria can mate with each other and transfer DNA from one bacterium to another by a process called conjugation. The chromosomal DNA is usually not transferred (with some exceptions). Some plasmids, called “conjugative plasmids” are capable of being transferred by this method. Another type of plasmids called “mobilizable plasmids” contain some but not all the genes necessary for conjugative transfer. So these plasmids can be transferred only in the presence of another conjugative plasmid, which acts as a helper plasmid. In order to be transferred, one strand of the plasmid DNA is cut at a site called the origin of transfer (*oriT*). The cut single strand is transferred and then joined to make it circular [31]. The second strand is synthesized in both the donor and recipient strain. Thus, by the method of bacterial conjugation, more and more antibiotic sensitive bacteria can acquire antibiotic resistance genes and become resistant. (2) *Bacterial Transformation*. This is the process by which bacteria take in DNA from outside which is usually released from dead bacteria. Some bacteria can be artificially made transformable in the presence of added chemicals such as calcium chloride. Some, but not all bacterial species are capable of natural transformation. This takes place by an active process in which competence genes present in the bacteria are expressed and the proteins facilitate the process of transformation. Natural transformation can be of two types: nonspecific, in which case any DNA

can be taken in or specific, in which case only DNA from the same species can be taken in. The bacteria recognize DNA from the same species by the presence of an “Uptake Signal Sequence” (USS) that is repeated numerous times throughout the genome [32]. (3) *Transduction/Transfection*. This is the process by which DNA is transferred between bacteria using **bacteriophages** as intermediaries. Bacteriophages are viruses that infect bacteria. After infecting the cell one bacteriophage can multiply to give more bacteriophages which are then released from the bacteria and can then infect other bacteria. In the process of multiplication the bacteriophage can incorporate some of the bacterial DNA into their DNA and can then transfer the DNA to other bacteria that they infect. This way, antibiotic resistance genes are transferred between bacteria thus contributing to spread of resistance.

Note that transposons that are present in the plasmid or chromosome can also be transferred to other bacteria along with the plasmid or chromosomal DNA during transformation, conjugation or transduction. There are some transposons called conjugative transposons that are capable of transferring by the process of conjugation [33].

Antibiotic Resistance Pool: Taking insufficient dose, not completing the full course, taking the wrong antibiotic and taking antibiotics for viral infections such as common cold constitute misuse of antibiotics. Note that antibiotics which are antibacterials will not be able to cure viral infections. Misuse of antibiotics increases the population of antibiotic resistant bacteria (also known as “antibiotic resistance pool”). The more an antibiotic is used, the greater will be the resistance pool for that antibiotic. With time more and more bacterial strains will become resistant to the antibiotic. Historically, resistance to an antibiotic has been observed within a few years after its introduction into the market. The Center for Disease Control (CDC) estimates that, each year, nearly two million people in the USA acquire an infection while in a hospital (nosocomial infection, Sect. 1.1), resulting in 90,000 deaths. More than 70 % of the bacteria that cause these infections are resistant to at least one of the antibiotics commonly used to treat them. Table 2.1 shows the timeline for introduction and resistance development for some antibiotics. A more complete list

Table 2.1 Timeline for resistance development

Name	Year introduced	Year resistance first reported	Years taken for resistance development
Penicillin	1943	1940	−3
Tetracycline	1950	1959	9
Erythromycin	1953	1968	15
Methicillin	1960	1962	2
Gentamycin	1967	1979	12
Vancomycin	1972	1988	16
Imipenem	1985	1998	13
Levofloxacin	1996	1996	0
Linezolid	2000	2001	1

of antibiotics and resistance development to them can be found in the 2013 CDC report [34]. As can be seen in Table 2.1, resistance developed to most antibiotics within a few years. Although penicillin was discovered in 1928, it was not until 1943 that it was marketed. Resistance development to the antibiotic had already been reported in 1940, 3 years before it was marketed (Sect. 2.5).

2.7 Mechanism of Antimicrobial Resistance

Mechanisms of resistance development to antibiotics can be classified into two types. (1) Altering the target of the antibiotic such that it is no longer affected by the antibiotic [35]. (2) Decrease the concentration of the antibiotic to a level that is lower than the MIC such that it will not have a significant inhibitory effect on the bacteria. The low concentration can be achieved in three different ways: (a) Preventing entry of the antibiotic into the cell, (b) Pumping out the antibiotic after it enters the cell and (c) Degrading or inactivating the antibiotic by enzyme catalyzed chemical modification before it can bind to its target. The actual mechanism used depends on the type of bacteria as well as the type of antibiotic. For example, the mechanism of resistance development in gram-positive and gram-negative bacteria may be different because of the differences in their structures. Cell membranes act as selective barriers for various molecules including antibiotics. Gram-positive bacteria have one membrane (cytoplasmic) while gram-negatives have two membranes (one outer and one inner or cytoplasmic). Because of the double membrane, gram-negative bacteria have intrinsic resistance to many antibiotics. Entry of the antibiotic may be prevented by either the outer or the inner or both membranes. Entry of various molecules through membranes takes place through specific pores present in the membranes. Pores in some bacteria may be specific for transporting only positively charged molecules while those in other bacteria may be specific for only negatively charged molecules. If this does not match with the charge of the antibiotic then the bacteria will be resistant to that antibiotic. For details see the effect of penicillins on various gram-negative bacteria (Sect. 3.3.2.4). Resistance to tetracyclines is by pumping out the antibiotic after it enters the cell (Sect. 6.2.4). Resistance to β -lactam antibiotics can be by degradation of the antibiotics by enzymes called β -lactamases (Sect. 3.3.2.5). Resistance to aminoglycosides is also by modification of the drugs (Sect. 6.2.3). Resistance development to quinolones is by target modification (Sect. 5.3). More than one mechanism may be applicable for some antibiotics.

2.8 Synthetic Antibiotics

Semisynthetic antibiotics were made in the laboratory by chemically modifying natural antibiotics with the purpose of improving their properties. Some desired properties are broader spectrum of activity, less side effects, lower cost, greater shelf life or

bio-stability, and lower frequency of resistance development. The first example of semisynthetic antibiotic was tetracycline which was made by catalytic hydrogenation of chlorotetracycline (the Cl was replaced with H) at Pfizer laboratory.

Various modifications were made synthetically to the penicillin structure to obtain broader spectrum of activity as well as other improved properties. Some examples include amoxicillin, ampicillin, carbenicillin, and ticarcillin (Fig. 3.14). The modified drugs ampicillin (α -aminobenzylpenicillin) and amoxicillin, both semisynthetic penicillins, are important because they are effective against both gram-negative and gram-positive bacteria (broad spectrum), while penicillin G works only for gram-positive bacteria. One important semisynthetic penicillin is methicillin, which was introduced in the early 1960s. Methicillin is not degraded by β -lactamase (Sect. 3.3.2.10) and so it is effective against bacteria that are resistant to penicillin. However, resistance to methicillin has also increased over the years, especially in the case of infection by MRSA (methicillin resistant *Staphylococcus aureus*, Sect. 3.3.2.10) which has become a major concern today.

Advantage of Synthetic Antibiotics: Although most antibiotics are natural products or their semisynthetic derivatives, there are some antibiotics that are entirely man-made. The first chemically synthesized antibiotics were the sulfonamides. In later years several more synthetic antibiotics were developed including trimethoprim, nalidixic acid and its derivatives. Mechanisms of action of all these antibiotics are discussed in later chapters. Except for a few synthetic antibiotics, most other antibiotics are natural products made by other microorganisms namely bacteria and fungi, which have existed on this earth for millions of years. As discussed before (Sect. 2.6) mutations can develop within a day of bacterial growth. So in the millions of years many mutations have developed including those that have resulted in formation of genes for antibiotic resistance. Thus for all natural antibiotics there already exists resistance genes that can be transferred from one microorganism to another. Synthetic antibiotics on the other hand have been in existence for not more than 85 years since the first one was made in the 1930s. In this short period of time some point mutations may have developed but no gene is expected to be present in nature for resistance to these antibiotics. As discussed before, point mutations confer much weaker resistance than antibiotic resistance genes. So for pharmaceutical companies it is always more desirable to develop new synthetic antibiotics than to discover new natural antibiotics. However, unfortunately very few synthetic antibiotics have been developed or are in the pipeline.

Discovery of sulfonamides: In 1932, Gerhard Domagk, in Germany, was examining various dyes for their antibacterial activities. There was precedence for testing dyes as potential chemotherapeutic agents. That is how Ehrlich had discovered Trypan Red (Sect. 1.2). The reasoning for testing dyes is that they stain bacteria. So it was thought that dyes may interfere with their growth. Domagk tested thousands of dyes for antibiotic property and discovered the dye Prontosil, which had such property. He received the 1939 Nobel Prize for his discovery of Prontosil. Normally when scientists test potential new antibiotics, they will first test them on bacteria growing in a test tube (in vitro). If it works then they will test it in animals that have been infected

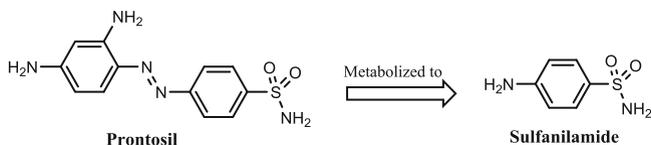


Fig. 2.2 The prodrug Prontosil metabolized to the antibiotic Sulfanilamide

with the bacteria (in vivo). However, when Domagk did the test, he did it simultaneously on bacteria growing in a test tube and he also injected these dyes in mice that were infected with bacteria. He made the strange discovery that one of the dyes, named Prontosil was effective against bacteria present in mice. However, in test tube assay the dye had no effect on the bacteria. So if he had relied only on test tube assay, his discovery would never have been made. Usually when new drugs are tested, many of them work in vitro but not in vivo and so those drugs will not be useful clinically. In this case it was just the opposite. The reason it worked only when administered to mice is because during metabolism in the mice the prontosil molecule was broken down to its smaller part called sulfanilamide (para-aminobenzenesulfonamide) which had the antibiotic property (Fig. 2.2). The dyeing property of the molecule was unrelated to its antibiotic property. Thus, prontosil can be classified as a “prodrug,” which is defined as a medication that is administered in inactive form and is converted to the active form by metabolic reaction in the body. Once the active part of the prontosil dye was determined, scientists synthesized various derivatives of sulfonamides and tested them for their antibiotic properties. Thus the first class of synthetic antibiotics, called “sulfa drugs,” was born and continues to be used even today.

Other synthetic antimicrobials: The second synthetic antibiotic to be made was trimethoprim. In the 1960s Hitchings and Elion explored the idea of developing drugs to target bacterial or viral DNA synthesis to cure infections. Their work resulted in the synthesis of trimethoprim and pyrimethamine which was used to treat malaria, meningitis, septicemia and a variety of other infectious diseases. They were awarded the 1988 Nobel Prize for their work [36]. Similar to the sulfonamides, trimethoprim inhibits the biosynthesis of folic acid in bacteria. In fact, for a long time the two drugs were administered together as combination drugs under the brand names Septra, Bactrim, etc. Trimethoprim is able to penetrate deep into tissues, which made it a drug of choice for diseases such as typhoid. The mechanism of action of trimethoprim is discussed in Sect. 4.3.6. Another group of synthetic antibiotics are nalidixic acid and its derivatives called fluoroquinolones which can be taken by mouth and still achieve high concentration in the blood. These are discussed in Sect. 5.3.

Multidrug resistant microorganism: Those microorganisms that are resistant to at least three out of the four antibiotic classes (those that affect the cell membrane, the cell wall, nucleic acid synthesis, and protein synthesis), are said to be multidrug resistant. Instances of infections by multidrug resistant microorganisms have been rapidly increasing and are of great concern because most of the available antibiotics

do not work against them. A group of bacteria known as ESKAPE are of particular concern. This group comprises of *Enterococcus faecium* (vancomycin resistant), *Staphylococcus aureus* (methicillin or vancomycin resistant), *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp., the latter four being carbapenem resistant. Some of these are discussed in later chapters.

New Antibiotics: We see that most of the antibiotics in use today were discovered in the first four decades since the discovery of penicillin. Bacteria have been found to develop resistance to all of them. The antibiotic crisis is made even worse by the fact that pharmaceutical companies are not developing many new antibiotics. Only two classes of synthetic antibiotics were developed in the past 50 years: fluoroquinolones (introduced in 1968) and oxazolidinones, a linezolid (introduced in 2000). The biggest advantage of totally new antibiotic is that there will be no resistance gene that neutralizes the effect of the antibiotic and point mutations conferring resistance to the antibiotic have not yet been selected for. Very few “truly new” antibiotics have been developed in the last three decades. Most “new” antibiotics developed are modified versions of already existing ones. Pharmaceutical companies are not investing much for research that can lead to developing new antibiotics because it is more profitable to develop drugs for fighting chronic problems such as high cholesterol or heart problems rather than antibiotics which are used by the patient for a short time only. Moreover, it is likely that the new antibiotic will lose effectiveness soon because of excessive use. The ideal use of a new antibiotic is to use it very little so that resistance development is delayed. However, that goes against any reasonable business model. Companies would want to make the maximum profit possible before the term of the patent expires. Solutions to these problems will be to significantly increase government funding and subsidizing of antibiotic research and manufacturing and revising patent validity period for new antibiotics.

2.9 Alternative Approaches for Studying Antibiotics

Fitness cost of antibiotic resistance: It is generally believed that antibiotic resistance makes bacteria weaker because the gain of antibiotic resistance will result in a loss of some function. For example, if a hypothetical antibiotic enters the bacterial cell through a certain pore in the cell membrane, resistance can develop by mutating the protein that makes the pore. However that will also affect the transport of nutrients that normally enter through that pore, thus decreasing the fitness of the bacteria. Similarly, if resistance develops by decreasing the binding of the corresponding enzyme to the antibiotic, that may also decrease the binding to the natural substrate. If the resistance is due to the presence of an antibiotic resistance gene inserted into the chromosome, it will be an extra burden on the cell to express that gene. If the antibiotic resistance gene is on a plasmid the cell will have to spend even more resources for not only expression of the resistance gene but also other genes that are on the plasmid. Also more energy and resources will be needed to replicate and maintain the plasmid.

Fitness cost of antibiotic resistance has been well documented. For example, resistance to fluoroquinolones has been shown to cause impaired motility in pseudomonads [37] and resistance to aminoglycosides is known to affect the structure of the ribosomes [38]. This fitness cost suffered by the bacteria is of benefit for us because resistant bacteria grow slower than nonresistant ones even in the absence of added antibiotic. Widespread use of antibiotics has resulted in a large increase in antibiotic resistant strains. The best way to control the spread of antibiotic resistance is by stopping or regulating use of the antibiotics. Since the fitness of the resistant strains is less than that of the nonresistant strains, with time, the proportion of resistant strain in the population will gradually decrease. A careful analysis of many mutations causing antibiotic resistance showed that most of these resistance mutations confer a fitness cost on the bacteria since most antibiotics target important cellular processes and resistance to them disrupts those processes [39].

Some antibiotic resistances can confer enhanced fitness on the bacteria: Contrary to the popular belief that antibiotic resistant strains are less fit than wild type strains and so can be easily eliminated by stopping use of the antibiotic, alarm bell has been sounded by reporting that this is not universally true for all antibiotic resistance. So better methods, such as developing vaccines against the bacteria are needed to tackle the problem of antibiotic resistance [40]. The authors reported that they found enhanced fitness in *Pseudomonas aeruginosa* because of antibiotic resistance. A transposon containing resistance gene for carbapenem was inserted in the *oprD* gene of the bacteria. Inactivation of the outer membrane porin (*oprD*) confers carbapenem resistance to the bacteria since the antibiotic normally enters the cell through this porin (Sect. 3.3.2.12). However it was observed that in addition to gaining the carbapenem resistance property, the bacteria at the same time acquired enhanced resistance to killing at acidic pH. The reason proposed for this enhanced fitness is that the inactivation of the *oprD* gene led to changes in transcription pattern of numerous genes, some of which may be the cause of the new beneficial property. This result is of immense concern because if antibiotic resistance develops during antibiotic therapy (due to inadequate concentration of antibiotic used), it may lead to increased fitness and virulence of the bacteria and may be more difficult to cure even if the particular antibiotic is no longer used by the patient.

Bacterial Persistence: Although antibiotic resistance development is the major reason why some bacteria are not killed by an antibiotic, another reason can be that some bacteria enter a slow growing physiological state in which antibiotics cannot kill them, a phenomenon first reported for *Staphylococcus aureus* [41]. However, it is believed that this applies to all bacteria. This phenomenon by which a population of antibiotic sensitive cells produces some transiently resistant cells is known as “persistence” [42]. Later the persister cells can switch back to the growing state after surviving the antibiotic treatment. Since persisters are only transiently resistant, when the cells multiply, the daughter cells will not be antibiotic resistant. This phenomenon of forming persisters is not dependent on use of the antibiotic but can happen anytime and the transient resistance developed is not just to the antibiotic being used but to all antibiotics. Thus, persister cells are transiently multidrug

resistant cells. This is because antibiotics usually inhibit active targets and thus in slow-growing or dormant bacteria the antibiotics cannot inhibit the targets.

Although this phenomenon applies to all bacteria, it does not create an alarming problem because the multidrug resistance is only transient and secondly, only a very small percentage of the bacterial population become persisters, so the body's immune system can effectively cope with this small number of resistant bacteria. However, the persisters are of serious concern in tuberculosis. The molecular mechanism of bacterial persistence has recently been reviewed [43] in which the authors summarize the role of the bacterial stress alarmone, 5'-diphosphate-3'-diphosphate guanosine (ppGpp) as a central regulator of persistence. An alarmone is an intracellular signal molecule that is produced in response to harsh environmental conditions.

Alternative view regarding function of antibiotics and antibiotic resistance genes: It is widely accepted that microorganisms secrete antibiotics to inhibit or kill other microorganisms in response to competition for limited resources. However, that concept has been challenged by some scientists [44, 45]. According to the authors, the natural concentrations of antibiotics made and secreted by microorganisms usually are much lower than the therapeutic concentrations needed for killing other neighboring microorganisms. It has been proposed that the principal roles of these so called "antibiotics" are cell-cell communication and not antibiosis and they also have regulatory effects on various pathways. After sensing these antibiotics, some of these pathways are upregulated (increased activity) and some are downregulated (decreased activity).

It is also widely accepted that the function of antibiotic resistance genes in microorganisms is to protect them from the effect of antibiotics. However, that theory has also been challenged by some scientists who propose that antibiotic resistance genes have a different function [46]. This suggestion stems from the observation that the expression levels of most antibiotic resistance genes are much higher than what is required to effectively combat the therapeutic doses of antibiotics that is normally used. In fact, as explained above, the actual concentrations of antibiotics present in nature is even less. What is the reason for this large excess of resistance strength? It was also observed that antibiotic resistance often persists despite reduction in the use of the antibiotics suggesting that the antibiotic resistance genes have an alternative physiological role. As an example the authors have pointed out that the tetracycline efflux transporter, Tet(L) as well as a multidrug efflux transporter, MdfA, both confer alkali tolerance to the bacteria besides the usual antibiotic resistance.

2.10 Antibiotic Use in Animals

Antibiotic use in animals is a major cause of resistance development. There are two types of use of antibiotics in animals.

2.10.1 *Therapeutic Use*

Similar to infections in humans, infections in animals are also cured with antibiotics. There are many times more farm animals than there are humans. According to the US Department of Agriculture report of 2010, more than ten billion animals (excluding fish) are raised and killed in the USA every year. Of these 91% are chickens raised for meat, 4.5% are chickens raised for eggs, 2.5% are turkeys, and 2% are cows pigs and other animals. This corresponds to an average of 28 land animals per person per year plus, according to another estimate, about 175 aquatic animals per person per year. Total number of land animals killed every year for food worldwide is about 65 billion. Use of antibiotics to cure infectious diseases in these animals is understandable but one should be aware that all use of antibiotics will contribute to increase in antibiotic resistance pool (Sect. 2.6).

Similar to animals, plants can also suffer from infections, which can be cured with antibiotics. These antibiotics are also sprayed on the plants, a process by which most of the antibiotics end up in the soil thereby increasing the resistance pool. Subtherapeutic use (see below, Sect. 2.10.2) of antibiotics in fish for growth promotion has been discontinued in Europe and North America; however, therapeutic use in fish is still a common practice. Since the antibiotics are added to fish food, the whole body of water gets contaminated with the antibiotic. This increases the antibiotic resistance pool and selects for antibiotic resistant bacteria in the water.

Antibiotic are also used for pets. According to estimates by the US Humane Society, about 62% of US households have at least one pet. They are also given antibiotics for treatment of bacterial infections. One can buy antibiotics from a pet store without a prescription. As a result there is misuse of these antibiotics. Since the antibiotics given for pets are the same as those given for humans, people often buy antibiotics for themselves from pet stores. This way they save money because antibiotics in pet stores are cheaper and also they avoid seeing a doctor. This kind of self-medication is potentially dangerous and also increases the antibiotic resistance pool.

Misleading safety standards: Fruits and vegetables are declared to be safe for consumption if antibiotic residue is below a certain limit. However, it is not the antibiotic residue that should be the only concern but the process by which the produce is obtained. If antibiotic was used during its growth, it will contribute to the antibiotic resistance pool even though all residues are later washed away from the produce. The resistant bacteria that are selected for due to the antibiotics can then transfer the resistance to other bacteria including those that infect humans.

According to rules, fish that have previously been treated with antibiotics, can be harvested and sold only after waiting for a certain period of time, known as the withdrawal period. The withdrawal time, which depends on the fish as well as the antibiotic used ensures that there will be no antibiotic residue left in the fish. However, once again it should be noted that the antibiotic residue should be only a minor concern, the greater concern should be the history of the fish and how much the farming has contributed to the antibiotic resistance pool.

2.10.2 *Subtherapeutic Use*

Contribution of therapeutic use of antibiotics to the antibiotic resistance problem actually appears to be insignificant when compared to another type of use of antibiotics in animals. This is known as subtherapeutic use and is not related to any infection. According to FDA reports, only about 20 % of the approximately 18,000 tons of antimicrobials sold in the USA are used by humans while the rest 80 % are used in animals [47, 48]. Of course our farm animals are not so sick that they will need this amount of therapeutic antibiotics. Most of this antibiotic is added to animal feed to increase their body weight, which means more profit for the farmers. Antibiotics used this way are also called antimicrobial growth promoters (AGPs). This phenomenon of growth promotion was an accidental discovery when scientists were testing random food additives to discover new vitamins. In 1948, Robert Stokstad and Thomas Jukes added cellular debris of *Streptomyces aureofaciens* to chicken feed, after the antibiotic chlorotetracycline was extracted from the bacterial culture and observed faster growth of the chicken. Initially they thought that it was due to vitamin B12 present in the additive but later it was understood that the growth promotion was due to small amounts of chlorotetracycline still remaining in the bacterial cell debris. Further studies confirmed the surprise discovery that addition of a small amount (much less than therapeutic dose) of chlorotetracycline increased the growth rate of farm animals. Soon it was found that the same effect was observed with many other antibiotics. Since only a small amount of antibiotic was sufficient to get this effect, the practice was allowed by the government and antibiotics for farm animals was allowed to be sold without a prescription. The simple logic was not considered that therapeutic use is only for a short time (about 10 days per infection) whereas subtherapeutic use is for lifetime of the animal.

The mechanism of growth promotion by subtherapeutic use of antibiotics is not clearly understood. It is possible that antibiotics kill bacteria that compete with beneficial bacteria in the intestines of the animals, thereby promoting growth of the animals. Although it is not clearly understood how antibiotics promote growth, one observation made is that the antibiotic does not have to enter the bloodstream to show the growth promoting effect because its site of action is in the intestines. Bacitracins, which are not absorbed through the intestinal walls are not used internally in humans but are used only as ointments for skin infections. However, it is one of the most commonly used growth promoting antibiotic in animals. Of all the bacitracin manufactured, 90 % is used for growth promotion in farm animals.

Negative aspects of subtherapeutic use: With time it was realized there is a big negative effect of the subtherapeutic use of antibiotics that far outweighs the minor monetary benefit of growth promotion. The constant exposure of the bacteria present in the animals to the antibiotics, selects for antibiotic resistant bacteria as explained in Sect. 2.5. If these bacteria are opportunistic pathogens, they may later infect the animals under conditions of weakened immune system. Or it may be possible that antibiotic resistant beneficial bacteria in the animals may transfer (Sect. 2.6) the antibiotic resistant genes to any infecting bacteria making it difficult to cure the disease.

Antibiotic resistant bacteria may be transferred from animals to humans both of which can be infected by the same pool of resistant bacteria. The spread of resistant bacteria from animals to humans can take place by any of the following ways: (a) by eating contaminated meat that is not cooked properly, (b) by every-day direct contact of farm workers with the animals, (c) by transfer of resistant bacteria from animal manure to soil then to plants and then to humans through the food chain, and (d) by transfer of bacteria from dead non-farm animals or farm animals who died because of disease, to the soil and then to plants and then to humans.

Call to ban subtherapeutic use of antibiotics: Because of the negative effects of subtherapeutic use, there has been calls to ban the practice particularly in the developed world where the practice was more prevalent. In 1969, the UK Government asked the Swann Committee to report on the use of antibiotics in both humans and animals. The committee concluded that AGPs contribute significantly to the development of antibiotic-resistant infections. The committee recommended that growth promotion in animals with antibiotics used for human therapy should be banned. The use of tetracycline and later penicillin as growth promoters was gradually phased out by the European Common Market in the 1970s [49]. Later the European Union banned the subtherapeutic use of avoparcin in 1997 and bacitracin, spiramycin, tylosin, and virginiamycin in 1999. In 2006, it banned subtherapeutic use of all antibiotics.

Following the ban by the European Union, numerous studies have shown that there has been a decline in the cases of antibiotic resistance [50]. It should be pointed out that although most studies show that banning subtherapeutic use of antibiotics has a positive effect, there is not a 100 % agreement on that conclusion. It is believed by some that subtherapeutic use has a prophylactic effect and is needed for proper health of the animals. Banning the practice will increase diseases in the animals which can then be passed on to humans [51].

In the USA in 1977, based on the recommendations of a 1970 FDA Task Force Report, the FDA proposed to withdraw drug approvals for subtherapeutic uses of penicillin and tetracyclines in animal feed [52]. These two drugs were chosen because of their importance in human medicine. However the proposal was criticized for lack of adequate evidence and US Congress directed FDA to hold the proposed withdrawal until further studies are conducted. In the meantime there have been numerous reports of antibiotic resistance (including multidrug resistance) development related to subtherapeutic use of antibiotics.

In 1999, in an open letter to the Commissioner of FDA, 53 eminent scientists from universities and research institutions throughout the USA urged that swift action be taken to protect the effectiveness of antibiotics by limiting their subtherapeutic use in agriculture [53]. In the letter they pointed out that although the FDA initiated proceedings to ban the subtherapeutic use of antibiotics in animal feed in 1977, that work was never completed while new research continued to demonstrate that subtherapeutic use increases antibiotic resistance in pathogens. Those resistant bacteria can be transferred to humans via contaminated food products or through direct or indirect contact with animals.

Transfer of resistance genes between bacteria puts humans at risk: The fact that subtherapeutic use of antibiotics increases the development of antibiotic resistance in bacteria has been demonstrated repeatedly in numerous studies. Some of these bacterial species can then infect humans who come in contact with the animals. In another scenario, the resistance genes can be transferred to other bacteria which can then infect humans. It was shown that when tylosin (a macrolide antibiotic, Sect. 6.2.6.1) was given to pigs for growth promotion it resulted in the appearance of erythromycin (also a macrolide) resistant enterococci in the pigs' guts and at the same time erythromycin resistant staphylococci was detected in the pigs' skin. These results demonstrate that use of one antibiotic can promote resistance to a different antibiotic and then the resistance can be transferred to another species of bacteria [54]. Bacteria that cause diseases in animals and plants may not infect humans. However, these bacteria may belong to the same family as those that infect humans. One example is the bacterial species *Erwinia* that causes fruit disease but does not infect humans. However, it is in the same family of bacteria (*Enterobacteriaceae*) as *E. coli*, *Salmonella* and *Shigella* which infect humans. Transfer of resistance genes between these species of bacteria has been well documented [55, 56].

It was shown in 1975 that adding low dose oxytetracycline in chicken feed resulted in the appearance of tetracycline-resistant *E. coli* in the intestinal flora of not only the chickens but also the farm workers who routinely handle the chickens [57]. Thus, this demonstrated the selection of antibiotic resistant bacteria in the chicken and then transmission of the bacteria from chicken to humans. Another example is the antibiotic enrofloxacin, which is currently approved by the FDA for the treatment of individual pets and domestic animals in the USA. Both therapeutic and subtherapeutic use of the antibiotic in chicken feed can result in development of resistance in *E. coli* which can then transfer the resistance to *Campylobacter*, another resident bacterial species present in chicken. The *Campylobacter* is harmless to the chickens but can infect humans. It is estimated that more than 80% of the chicken meat in the USA is contaminated with *Campylobacter*, which is the most common cause of food borne bacterial infection in the USA. If the bacteria have acquired resistance to enrofloxacin that was given to the chicken, the same resistance will also be effective against ciprofloxacin (Sect. 5.3), which is very similar to enrofloxacin and is the most widely used antibiotic for food borne illnesses in humans. One encouraging news came in 2005 when the FDA withdrew approval of Baytril (brand name for enrofloxacin) for use in water to treat flocks of poultry. The reason cited was that this practice was known to promote the evolution of fluoroquinolone-resistant strains of the bacterium *Campylobacter*, a human pathogen [58].

There is another well-known example of subtherapeutic use of one antibiotic resulting in resistance to other related antibiotics. A class of antibiotics called streptogramins is often used as an antibiotic of last resort when other antibiotics, including vancomycin have failed because of infection by multidrug resistant bacteria. One such antibiotic combination, Synercid was approved by FDA for human use in 1999. However, before its first use in humans, the effectiveness of Synercid had already been compromised because another streptogramin antibiotic, virginiamycin was already approved for use in animals and had been extensively used not just for

curing infections but to a much greater extent for growth promotion in animals. As a result, turkeys that were fed with virginiamycin were found to harbor bacteria that had developed resistance to Synercid even though they had not been previously exposed to Synercid [59]. Bacteria resistant to Synercid were detected in humans even before the antibiotic was first used in humans in Germany [60]. Thus virginiamycin resistant bacteria arising due to subtherapeutic use in animals had been transmitted to humans either through food or through people who handle the animals.

Sweden was the first country to ban subtherapeutic use of antibiotics in as far back as 1986. At that time this was done mainly out of concern for residues of antibiotics that remain in food due to their subtherapeutic use. However, antibiotic residue in food is actually a very minor concern because of the small amount of residual antibiotic. Also most of the residual antibiotic can be easily removed by washing the food and are also destroyed during cooking the meat. Of much greater concern is the fact that subtherapeutic use increases the antibiotic resistance pool, creates resistant mutants and what is even worse, results in resistance to antibiotics that are used in humans as described above. In 1998, the World Health Organization (WHO) called for a ban on the subtherapeutic use of those antibiotics that either (1) are used therapeutically in humans or (2) are known to select for cross-resistance to antibiotics used in humans. As mentioned above, by 1999, the European Union banned the use of some antibiotics used in animal feed because of concerns about cross resistance to antibiotics used in humans. In 2006, it banned subtherapeutic use of all antibiotics.

The USA and Canada stand out: By now most developed nations except the USA and Canada have banned the subtherapeutic use of some or all antibiotics. In the USA a slight progress has been made recently when the FDA issued not a ban but a nonbinding recommendation for voluntary withdrawal of medically important antibiotics from growth promotion [61]. This slight progress is too little too late while the problem of antibiotic resistance keeps increasing. The situation in Canada is the same if not worse. In Canada also there is denial by people in authority regarding any link between subtherapeutic use of antibiotic and development of antibiotic resistance. In some parts of Canada farmers do not need prescriptions to buy antibiotics even the ones that are important for human medicine. Like in the USA, in Canada also there is expected be self-regulation by farmers and drug manufacturers to use antibiotics sensibly. Leaving such important decisions to agencies that benefit financially from it is not expected to give the desired results.

Multidrug resistance development caused by subtherapeutic use: While some people continue to deny that subtherapeutic use of antibiotics causes development of antibiotic resistance, a recent publication has provided evidence for a direct link between the two. There are two methods of antibiotic resistance development that have been well established: (1) by selection of naturally occurring point mutations and (2) by resistance gene acquisition (Sect. 2.6). In the recent publication a new method of antibiotic resistance development has been described. It was shown that the presence of subtherapeutic level of an antibiotic induces generation of point mutations in the bacterial genome. Some of these mutations can confer resistance to other antibiotics that may not be related to the antibiotic that the cells have been

subjected to [29]. The authors have demonstrated that low levels of bactericidal antibiotics stimulate production of reactive oxygen species, which are known to cause mutations which result in emergence of resistance to various other antibiotics including multidrug resistance. Formation of reactive oxygen species in response to low concentrations of antibiotics has previously been shown to take place for quinolones, β -lactams, and aminoglycosides [23, 24, 62].

Whatever is the mechanism of development of multidrug resistant bacteria in animals, there is a definite threat of transfer to humans even if they are not in direct contact with the animals. The most vulnerable are those people who are already taking an antibiotic for some other unrelated infection. This is because the antibiotic kills all bacteria in the body including the beneficial ones. So the infecting multidrug resistant bacteria can more easily cause disease because (1) they are not killed by the antibiotic and (2) they face no competition from any resident bacteria in the body.

Growth promoting effect is less than what was previously thought: Subtherapeutic use of antibiotics in farm animals was banned by the European Union in 1999 and since then there has been a decline in the prevalence of antibiotic resistant bacteria [50]. This is encouraging news for proponents of a ban on subtherapeutic use of antibiotics. Productivity of Danish swine farms was monitored for 8 years before and 8 years after the ban. Stoppage of subtherapeutic use of antibiotics had no negative effect on pig productivity, in fact there was an increase in the number of pigs, and mortality rate of pigs remained constant [63]. This result challenges the “(mis) conception” that antibiotics have any significant growth promoting effect.

Recent reports show that the amount of growth promotion is about 1–2 % as opposed to 10 % that was originally reported in the 1950s [64]. Considering the diminishing returns, and the certainty of increasing antibiotic resistance, it needs to be decided whether it is worth the risk to obtain about 1–2 % increase in profit. Today the amount of antibiotic needed to obtain the level of growth increase supposedly obtained in the 1950s is gradually increasing to that of therapeutic doses. Thus, today it makes even more sense to ban all subtherapeutic use of antibiotics.

2.11 Prevention of Antibiotic Resistance Development

Every misuse of antibiotics contributes to development of antibiotic resistance. Antibiotics are misused by many people in most countries. People often request antibiotics from doctors for any disease because of the misconception that antibiotics, which are antibacterials, can cure viral infections such as common cold. Many times doctors agree to the patients' demands just to appear nice to their customers. Oftentimes antibacterial antibiotics are prescribed for viral infection in order to prevent secondary bacterial infection. There is also the misconception that antibiotics can do no harm. In many countries antibiotics and most other drugs are available without prescription, so people themselves decide that they need an antibiotic. In the developed countries this is theoretically not possible, but still many people manage to get antibiotics without a prescription. One source of antibiotics

is half used antibiotic dose from a previous infection. This creates double the problem. There is chance of resistance development after the first infection since the complete dose was not taken and then the second time, because of self medication the patient may be unnecessarily taking the antibiotics for a viral infection. Even if the antibiotics is the right one for the second infection, the patient will get only half the required dose, again increasing the chance of resistance development. Thus, everyone, including doctors, patients, as well as everyone involved in the subtherapeutic use of antibiotics in animals shares the responsibility for resistance development to antibiotics.

What is the solution to the problem? There is no simple solution. Doctors should stop overprescribing antibiotics. Certainly, the sale of antibiotics need to be regulated. In the poor countries this is not an enforceable solution because of the scarcity of doctors. Even if patients have access to a doctor, they may not be able to afford their fees. This will encourage the creation of a black market for antibiotics. A long term but more effective solution is to educate the people about the antibiotic resistance development problem. Subtherapeutic use is a major contributor and the farming industry needs to stop the practice. The government has a big role to play in the prevention of antibiotic resistance development. It costs millions of dollars for discovery, development, and clinical trials of new drugs. Once approved by the FDA, the companies have only a limited amount of time before the patent expires and they want to make as much money as possible in that short time. However, the best use of an antibiotic is to use it very little. The government needs to recognize this dilemma and change patent laws and tax laws so that the pharmaceutical companies will be willing to invest their time and money in developing new antibiotics. The government should also provide more grant money to scientists to develop new antibiotics. If enough action is not taken now, very soon the problem will reach a crisis situation.

The National Action Plan: According to Centers for Disease Control and Prevention (CDC), drug-resistant bacteria cause 23,000 deaths and two million illnesses each year in the USA. The threat of increasing antibiotic resistance has been taken seriously by the government. Recently, in a White House Press Release, March 27, 2015, President Barack Obama's office released a comprehensive plan to combat the rise of antibiotic resistant bacteria [65]. The National Action Plan for Combating Antibiotic Resistant Bacteria describes five goals, one of which is to slow the emergence of and prevent the spread of antibiotic resistance. According to the CDC, about half of all human antibiotic use is unnecessary. By the year 2020 it is expected that inappropriate use of antibiotics will be cut by 50% in outpatient settings and the use of medically important antibiotics for growth promotion in food-producing animals will be completely eliminated. Another goal of the Action Plan is to accelerate basic and applied research and develop new antibiotics. The federal funding for research on antibiotics has been nearly doubled in the President's FY 2016 Budget.



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