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„Antibiotic Treatment and the Risk of Antibiotic Resistance“

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Contents

Summary

Antibiotics are the most used drugs. Their importance is unambiguously very high in human medicine as well as in other areas such as farming and agriculture. Since the discovery of the first antibiotic drug, penicillin, on 1929 by Alexander Fleming, they have saved thousands of lives. Their application is not always easy and there is a lot of knowledge needed in order to be able to exactly know when they should be prescribed.

Antibiotic usage is very important for many patients. Unfortunately they are also often misused, mistakenly prescribed or therapy guidelines are not followed. This overuse and incorrect application leads to the development of resistance from various bacteria strains. Antibiotics are not used only on human medicine but also in farming and agricultural areas, where they are not only used in therapy but also as growth promoters.

The development of resistance differs depending on antibiotic groups such as tetracycline, β-lactam antibiotics, peptide antibiotics, macrolides etc. Resistance dispersion is an important issue. Transduction, transformation and conjugation have been elucidated in this diploma thesis together with the possibility of multi drug resistance development.

In Austria, like in the major European countries, the usage and prescription of antibiotics is strictly regulated. This is anyhow not the case for many countries of Southern Europe: Kosovo, Serbia, Albania etc. Although there might be regulations, they are not followed and neither pharmacists nor farmers fear any consequences.

Resistance development may have lethal consequences for many patients. This concerns also many international institutions and organizations and has been recognized as a very important issue to deal with. Many efforts have been started in order to slow down its development. Nevertheless there is a lot to accomplish in order to diminish resistance development and to have satisfactory results.

Zusammenfassung

Antibiotika sind eines der meist verwendeten Medikamente weltweit. Seit Alexander Flemming 1929 das erste Antibiotikum, Penicillin, entdeckte, sind Antibiotika unentbehrlich für die Menschheit und haben tausende Leben gerettet. Deren Anwendung ist nicht immer einfach, deshalb ist es enorm wichtig, genau informiertes und geschultes Personal zu haben um unnötigen Gebrauch zu verhindern.

Antibiotika Einsatz ist lebenswichtig für viele Patienten. Leider wird es oft aber missbraucht, unnötig verschrieben oder die Therapievorgaben werden nicht eingehalten. Durch diese übermäßige und unkorrekte Verwendung kommt es zur Resistenzentwicklung bei verschiedenen Bakterien-Stämmen. Nicht nur im humanmedizinischen Bereich ist das der Fall. Auch im landwirtschaftlichen Betrieb, ob in Tierhaltung oder der Agrikultur, kommen Antibiotika nicht nur in der Therapie sondern auch als Wachstumsfördernde Mittel zum Einsatz.

In dieser Arbeit wurde genauer auf Resistenz-Entwicklungen auf bestimmte Antibiotika-Klassen wie Tetracycline, β-Lactame. Peptid-Antibiotika, Makrolide etc., eingegangen. Weiteres wurden auch die wichtigsten Möglichkeiten der Resistenz-Entwicklung und Verbreitung vorgestellt. Transduktion, Transformation und Konjugation wurden genauer erläutert, gefolgt von Möglichkeiten der Multiplen Antibiotika Resistenz und deren Auftreten.

Die Verschreibung von Antibiotika ist in Österreich, wie in den meisten europäischen Ländern, streng geregelt. Leider ist dies in den Balkanländern wie z.B. Kosovo, Serbien, Albanien, etc., nicht der Fall. In diesen Ländern sind entweder keine Regelungen vorhanden oder sie werden vollkommen missachtet, ohne das Konsequenzen zu befürchten sind.

Resistenz gegen Antibiotika kann tödliche Folgen für viele Patienten haben. Dieses Problem wurde auch von verschiedenen Institutionen und Organisationen erkannt. Diese haben einiges in die Gänge gesetzt um die Resistenz-Entwicklung zu verlangsamen. Nichtsdestotrotz ist noch ein weiter und anspruchsvoller Weg zu beschreiten um zufriedenstellende Ergebnisse zu erreichen.

Abbreviations

ARG	Antibacterial Resistance Genes
CDDEP	Center for Disease Dynamics, Economics and Policy
EARS-Net	European Antibiotic Resistance and Surveillance network
ECDC	European Centre for Disease Prevention and Control
EMEA	Europian Medicines Agency
ESBL	Extended-Spectrum β-Lactamase
EUCAST	European Committee on Antimicrobial Susceptibility Testing
GTA	Gene Transfer Agents
HGT	Horizontal Gene Transfer
MAR	Multiple Antibiotic Resistance
MBC	Minimal bactericidal concentration
MDR	Multi Drug Resistance
MDR\TB	Multi-Drug Resistant Tuberculosis
MGEs	Mobile Genetic Elements
MIC	Minimal Antibiotic Concentration
MRSA	Methicillin Resistant Staphylococcus aureus
NAP-AMR	Austria's National Action Plan on Antimicrobial Resistance
NCBI	National Center for Biotechnology Information
NI-AMR	Austrian National Initiative on Tackling Antimicrobial Resistance
PBPs	Penicillin-Binding proteins
ROS	Reactive Oxygen Species
VRE	Vancomycin Resistant Enterococci
WHO	World Health Organization

1 Introduction

1.1 Discovery of antibiotics and the impact on health care

Antibiotics are antimicrobial drugs effective against bacteria. They may be natural, semi-synthetic or synthetic produced drugs. Many of antibiotics are formed naturally by microorganisms. They are developed as a result of struggle and competition for acquisition of nutrients and getting advantages to themselves but they might also be used as signal molecules for these organisms (Hibbing *et al.*, 2010). Most of antibiotics though are semi-synthetic or synthetic produced. The advantages compared to natural antibiotics might be, among others, the increase of efficacy and/or not susceptibility to antibiotic resistance that may have been developed to their natural precursors.

Since penicillin was discovered by Alexander Fleming on 1929 (Fleming, 1929), antibiotics have emerged to one of the most important drugs in the world. The next important milestone on the history of antibiotics was the discovery of the first antibiotic for tuberculosis treatment on 1943: streptomycine. The discovery of cephalosporine on 1948 was another crucial achievement in treatment of infectious diseases (Forth, Henschler and Rummel, 2009).

Infectious diseases and their live threatening dimension along the human history make the usage of antibiotics absolutely indispensable. Antibiotic application against various infectious diseases saved countless lives. In developing countries, antibiotics are generally most sold drugs (Cagri Buke *et al.*, 2003). They are important for medication of tuberculosis which affects almost one third of world population (Davies and Davies, 2010). Ostermann and colleagues made in their publication on 1995 clear how important antibiotics are for defeating the infections of open fractures (Ostermann, Seligson and Henry, 1995) . Furthermore antibiotics are used to prevent and cure bacterial infections which are serious complications after trauma or orthopedic implant surgery (Diefenbeck, Mückley and Hofmann, 2006). Antibiotics are widely used also in agricultures as well as aquacultures in order to prevent and treat possible diseases impacting the yield (Liu, Steele and Meng, 2017).

1.2 Mechanisms of action and classification of antibiotics

Antibiotics may be categorized in different ways. They may be classified in groups with different modes of action, different spectrum and nature of action or depending on their chemical structure. Antibiotics may affect bacteria cells in two ways: reversible stop or slow down bacterial growth (bacteriostatic) and irreversible damage or kill the bacteria with an efficiency of 99.9% (bactericide). Bacteriostatic antibiotics perform their activity primarly inhibiting ribosome function for example, tetracylines and macrolides (Kohanski *et al.*, 2007).Referring to target and mode of actiorin, antibiotics might be classified into (Forth, Henschler and Rummel, 2009; Lüllmann *et al.*, 2016):

- **Inhibitors of bacterial cell wall** by targeting the peptide building by transpeptidases (e.g. penicillines), Inhibition of correct transport of cell wall components (e.g. vancomycine) and inhibition of the synthesis of these components (e.g. fosfomycine);
- **Inhibitors of plasma membrane** by building pores in the membrane (e.g. Daptomycine) or interacting with phospholipids (e.g. polymyxin B);
- **Inhibition of protein biosynthesis** by blocking the formation of initiation complex (e.g. linezolid), leading to wrong aminoacyl tRNA attachements (e.g. aminoglycosides), blocking of ribosomial peptitytransferasa activity (e.g. chloramphenicol) or by inhibiting the translocation of peptides (macrolides);
- **Inhibition of tetrahydrofolate synthesis** (e.g. sulfonamides)
- **Interfere with bacterial DNA** (e.g. nitroimidazoles) or inhibiting the DNA gyrase (e.g. chinolones)
- **Inhibition of mRNA synthesis** by blocking the DNA dependent RNA polymerases (e.g. rifampicin).

According to their chemotherapeutic spectra, antibiotics may be classified into: narrow-spectrum antibiotics, extended-spectrum and broad-spectrum antibiotics. The first group, narrow-spectrum antibiotics, act in limited group of microorganisms, while the second type of antibiotics have a bit wider spectrum. They are effective against Gram-positive bacteria but also against a number of Gram-negative bacteria. Broad-spectrum antibiotic, on the other hand, are used across a broad range of bacteria.

Antibiotic classes that are formed according to the chemical structures are: β-lactam antibiotics, aminoglycosides, tetracyclines, amphenicols, macrolides, lincosamides, glycopeptides, etc.

β-Lactam antibiotics

As the name reveals, β -lactam antibiotics have in their structure a β -lactam ring which is crucial for their efficacy. β -lactam antibiotics show bactericide effects in irreversible inhibiting the enzymes involved in peptidoglycan synthesis. They covalently interact with penicillin-binding proteins (PBP) in both Gram-negative and Gram-positive bacteria as can be seen in Figure 1-1. This enzyme is very important for bacteria to build the cross-linkage in peptidoglycan chains (Forth, Henschler and Rummel, 2009; Bush and Bradford, 2016). Bacteria have distinct numbers of PBPs, ranging from three enzymes to eight per species(Bush and Bradford, 2016).

β-Lactam antibiotics are among most powerful drugs ever existed (Braga and Lackner, 2017). They are well tolerated, efficacious and therefore widely used(Bush and Bradford, 2016).

Bacteria may be resistant to this group by synthesizing an enzyme called β-lactamase which may cleave the β–lactam ring and make the antibiotic ineffective. These enzymes might act as penicillases (synthesized by *Staphylococcus*) or as cephalosporinases (synthesized by *Pseudomonas*) (Lüllmann *et al.*, 2016). β-lactam resistance was a big concern soon after the penicillin wide usage. The need for new antibiotics led to the Golden Age (ca. 1940-1980) of antibiotics. During this time diverse new classes of β-lactams along with several semi-synthetic antibiotics have helped to seize the resistance problem as well as spectrum of activity, antibiotic toxicity and other issues medicine had to handle with the first-generation antibiotics (Braga and Lackner, 2017). The newest discovery of selective binding of β-lactams (e.g. ceftaroline) to an allosteric section of PBPs from *S. aureus* leading to higher susceptibility of these organisms to the antibiotic (Bush and Bradford, 2016).

β-Lactams are currently the antibacterial agents that are mostly used to treat infectious diseases(Bush and Bradford, 2016). Depending on the chemical structure of that part of the molecule that bounds to the β-lactam component, β-lactam antibiotics may be categorized in (Forth, Henschler and Rummel, 2009):

- penicillins,
- cephalosporins,
- monobactams and
- carbapenems

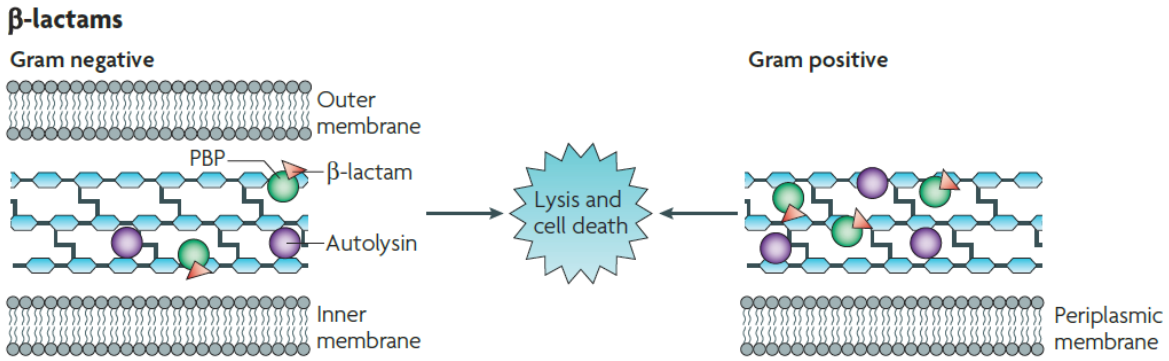

Figure 1-1 Mechanism of action of β-lactams against Gram-negative and Gram-positive bacteria. They inhibit transpeptidation whereas they covalently bind the penicillin-binding protein (PBPs) while maturing the peptidglucan strands. This leads to decrease in peptidoglycan synthesis. This combined with the increase on autolysins lead to lysis and cell death. (Kohanski, Dwyer and Collins, 2010)

Penicillins is the biggest subclass of this group. Penicillins are broad-spectrum antibiotics, easy to produce and therefore mostly used. The first penicillin discovered was penicillin G also called benzylpenicilline. It may be extracted from *Penicilliun notatum/ chrysogenum*.

The usage of penicillins is comprised due to the increasing number of β-lactamses that are able to inactivate the antibiotic. Therefore a monotherapy with penicillins is limited (Bush and Bradford, 2016). Penicillins are very sensitive to β-lactamases and therefore they are mostly used in combination with clavulanic acid which inhibits this enzyme (Forth, Henschler and Rummel, 2009). A monotherapy is only possible in cases if infections with

species that do not produce these enzymes e.g. Group A Streptococci or Treponema pallidum (Bush and Bradford, 2016).

There are at least four groups of penicillins: penicillin G derivatives, isoxazolylpenicillins, aminopenicillins and acylaminopenicillins. They differ in their chemical structure but also in the indication area they are used (Forth, Henschler and Rummel, 2009; Lüllmann *et al.*, 2016).

Penicillinase-resistant penicillins nafcillin and oxacillin have been used to treat infections with staphylococci that have acquired penicillin binding protein (Watkins and Bonomo, 2016).

Cephalosporins differ from penicillins concerning chemical structures. They possess a cephem basic structure which is a β-lactam ring with a six-membered ring with a sulfur atom (Bambeke *et al.*, 2016). Nevertheless, the mechanism of action is similar. They are classified into five groups or generations. The first generation cephalosporins are effective in treating infections with gram-positive cocci but lesser with gram-negative cocci (Watkins and Bonomo, 2016). Thus they have activity against *Streptococcus* and *Staphylococcus* (Forth, Henschler and Rummel, 2009). An improvement of activity against Gram-negative bacteria has been achieved in second generation. Some of the second generation cephalosporins such as cefotoxin ans cefoteta are also effective against anaerobes (Forth, Henschler and Rummel, 2009; Watkins and Bonomo, 2016).

Cephalosporins of third generation have even higher efficacy against Gram-negative bacteria than those of second generation. Meanwhile the cephalosporins of the fourth generation are also effective against *Pseudomonas aeruginosa* (Forth, Henschler and Rummel, 2009). They are effective actually against most important Gram-positive and Gram-negative bacteria in clinics. Ceftaroline belongs to fifth generation cephalosporins. It as an increased activity against methicillin-resistent *S.aureus* (MRSA) (Watkins and Bonomo, 2016).

The resistance of bacteria is increasing due to β-lactamases and carbapenemases (Forth, Henschler and Rummel, 2009). Some β-lactamases, although are not effective against cephalosporins with expanded-spectrum (e.g. ceftriaxone, cefotaxime and ceftazidime (Rossolini, Arena and Giani, 2017).

Thus a redefinition of the antimicrobial spectrum will be needed, particularly for Gram-negative bacteria (Watkins and Bonomo, 2016).

Carbapenems are another grouping within the β-lactam antibiotics. They are broad-spectrum antibiotics and stable against β-lactamase enzyme. They may be only used in parenteral therapies, because of their low resorption through the gastrointestinal tract (Lüllmann *et al.*, 2016). Imipenem, ertapenem, meropenem and doripenem are agents belonging to this categorization.

Monobactams include aztreonam the lead structure of which was sulfalezin, produced by Gram-negative bacteria (e.g., *Pseudomonas acidophila*) (Braga and Lackner, 2017). The difference to other β-lactams is the absence of the ring that is fused to the β-lactam ring (Vandavasi *et al.*, 2017). Monobactasm have the advantage of not being inactivated by β-lactamases (Li *et al.*, 2016).

Aztreonam shows activity against aerobic Gram-negative bacteria and it is stable to β-lactamase. It is not effective against anaerobics or Gram-positive bacteria (Lüllmann *et al.*, 2016; Ramsey and MacGowan, 2016; Watkins and Bonomo, 2016). It shows, like other β-lactams, bactericidal and time dependent efficacy (Ramsey and MacGowan, 2016). It may be used parenteral as substitution for patient that are allergic to penicillins or cephalosporins (Lüllmann *et al.*, 2016).

β-Lactamase inhibitors was an achievement in the 1970´s as a result of a broad research and natural product screening. Clavulanic acids was the first discovered and it was identified as e broad spectrum inhibitor. It was able to inhibit most recognized penicillases that were plasmid-encoded in enteric bacteria but also the staphylococcal penicillases (Bush and Bradford, 2016).

Glycopeptide antibiotics

Glycopeptide antibiotics are bactericide, large, stiff molecules. As their name reveals they consist of a peptide chain (heptapeptide) and sugars (Solenberg *et al.*, 1997; Allen and Nicas, 2003). They are natural products formed by a group of actinomycetes (Nicolaou *et*

al., 1999; Allen and Nicas, 2003). Their mechanism of action is inhibition of peptidoglycan synthesis of bacterial cell wall in a late stage by steric hindrance (Reynolds, 1989). This leads to failures in construction of the backbone glycan chains (catalysed by peptidoglycan polymerase) from the wall subunits (Reynolds, 1989; Allen and Nicas, 2003).

Glycopeptid antibiotics are a very important and lifesaving possibility to treating people infected with MRSA bacteria or with multiresistent *Enterococci*. (Forth, Henschler and Rummel, 2009; Lüllmann *et al.*, 2016). The first glycopeptid antibiotic discovered is vancomycine (Barna and Williams, 1984). The huge usage of vancomycne for decades in treatement of MRSA and Gram-positive bacterial infections had a tremendous impact in medicine. This was also the major factor that contributed to the resistance developement against vancomycine (Allen and Nicas, 2003).

Aminoglycosid antibiotics

Aminoglycoside antibiotics are broad-spectrum and bactericide antibiotics that are products of bacterial or fungal metabolism (Azucena and Mobashery, 2001). Their mechanism of action is disturbing protein synthesis by binding the aminoacyl-tRNA on its "A-site" of 16S rRNA which then leads to mistranslations of mRNA and to premature readout terminations (Figure 1-2) (Ryu and Rando, 2001; Llano-Sotelo *et al.*, 2002). These results in misfolded proteins that appear to activate membrane systems which lead to metabolic activation in the cell and changes in the membrane potential. These activities result in production of lethal hydroxyl radicals for the bacterial cell (Kohanski *et al.*, 2007). Aminoglycosides are broad-spectrum antibiotics mostly effective against Gram-negative bacteria. Unlike penicillin they are effective also against aerobic Gram-negative microorganisms such as *Enterobacteriaceae* and *Pseudomonas* spp.(Xie, Talaska and Schacht, 2011).

Streptomycine was the first discovered in the 1940s. It was isolated from *Streptomyces griseus* and it was very important for tuberculosis treatment. Semisynthetic aminoglycosides have emerged as modified natural products by chemists in order to expand their activities and furthermore to make them insensitive to modification by

bacterial resistance enzymes (Azucena and Mobashery, 2001). The following developed kanamycin, gentamicin, tobramycin and others have been also widely used (Talaska, Schacht and Fischel-Ghodsian, 2006). Aminglycosides are constantly used in clinical treatments for cystic fibrosis and emergent infections with multi-drug-resistant bacteria in many types of infections, e.g. urinary tract infections, tuberculosis and visceral leishmaniasis (Xie, Talaska and Schacht, 2011).

Aminoglycoside antibiotics may be used for systemic application on patients with sepsis and many other live threatening infections.

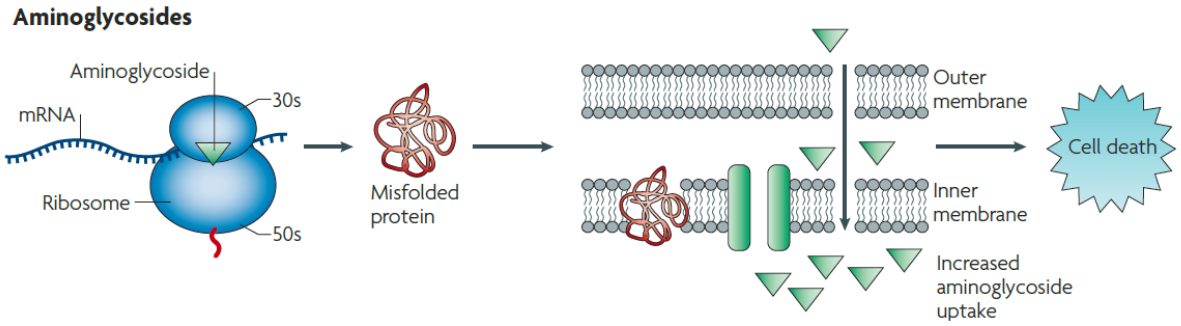

Figure 1-2 Aminoglycoside induced misincorporation of amino acids and thereby bacterial protein-misfolding. This has been associated with cell death (Kohanski, Dwyer and Collins, 2010).

Aminoglycosides are known to have a lower risk on developing antimicrobial resistance than e.g. cephalosporins. However they are not very often prescribed and this might be for two reasons: firstly their potentially nephrotoxicity and ototoxicity and secondly measurement of aminoglycosd serum concentrations is required which might be resource consuming and presumes knowledge and experience for assessing the results (Mehl *et al.*, 2017).

Macrolide antibiotics

Macrolide antibiotics are a large group of natural and semi-synthetic drugs. All of them consist of sugar substituents attached to 14- to 16-membered lactone rings (Hansen *et al.*, 2002). They include erythromycin and its derivatives such as roxithromycine,

azithromycin etc. Their application may be in oral and parenteral forms(Lüllmann *et al.*, 2016).

Macrolide antibiotics impede protein synthesis during elongation of polypeptide chain in ribosomes. They are effective against Gram-positive bacteria, *Legionella* spp., *Chlamydia* spp., *Mycoplasma* spp. and *Haemopilus influenza* (Ianaro *et al.*, 2000).

Bacteria acquire resistance to macrolide antibiotics through mutations (Hansen *et al.*, 2002). It is well documented that erythromycin usage is strongly correlated with the resistance development among group A *Streptococci* (Seppälä *et al.*, 1997). Macrolide resistance is transmissible through plasmids. The production of RNA methylases impede the efficacy of macrolides through methylations of their target (Forth, Henschler and Rummel, 2009; Lüllmann *et al.*, 2016).

Macrolide antibiotics are used in many cases to treat infections with *S. pyrogenes* in cases where patients are allergic to penicillines (Seppälä *et al.*, 1997). Furthermore Macrolide antibiotics are known to have anti-inflamatory effects, both local and systemic. Although the mechanisms which underlies these effects are not quite clear (Ianaro *et al.*, 2000).

Tetracyclines

Tetracyclines are, like macrolides, inhibitors of protein synthesis but they interfere with aminoacyl tRNA on the acceptor side. Tetracyclines count to the broad-spectrum agents. They do not only interfere with a range of Gram-negative and Gram-positive bacteria, but also with other organsims for instance mycoplasms, rickettsiae, chlamydiae and protozoan parasites (Chopra and Roberts, 2001). Anyhow some bacteria species are not susceptible to tetracyclines: *Proteus* species, *Providencia*, *Pseudomonas aeruginosa* and *Serratia marcensens* (Forth, Henschler and Rummel, 2009). Furthermore a number of other bacteria have developed a plasmid transmitted resistance. This not only because of high usage but also because of tetracycline application in livestock food. Tetracyclines are contraindicated in using in children under 12 years old (Lüllmann *et al.*, 2016).

First members of tetracyclines discovered in the late 1940s are chlortetracycline and oxytetracycline. They were produced by some species of the largest genus of Actinobacteria *Streptomyces, S. aureofaciens* (chlortetracycline) and *S. rimosus*

(oxytetracycline). Nevertheless, the most recently discovered subcalss of tetracyclines are called glycylcyclines (Chopra and Roberts, 2001).

Tetracyclines are antibiotics that have been extensively used in human and animal infection therapy because of their antimicrobial qualities and the lack of many side effects (Chopra and Roberts, 2001) prophylactically for the prevention of malaria caused by mefloquine-resistant Plasmodium falciparum (Chopra and Roberts, 2001).

Fluoroquinolones

Nalidixic acid is the first discovered fluoroquinolone which initially were called gyrase inhibiors. Some of the further developed antibiotics from this class are norfloxacine, ciprofloxacine, levofloxacine, gemifloxacine (Forth, Henschler and Rummel, 2009). As can be seen in Figure 1-3 the mechanism of action is the inhibition of topoisomerase. The efficacy of the drugs of this group might vary across the species range depending on drug generation (Kohanski, Dwyer and Collins, 2010).

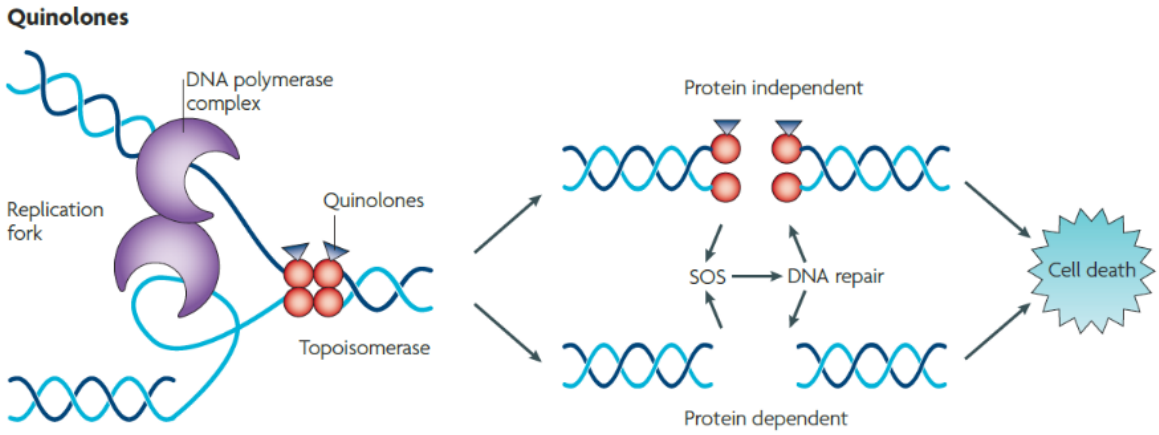

Figure 1-3 Action mechanism of quinolones in bacterial cells. They interfere with the topoisomerase II or IV while supercoiling the DNA. The consequence is breaking the formation of double-stranded DNA and cell death. This might happen depending on protein synthesis or not (Kohanski, Dwyer and Collins, 2010).

Fluoroquinolones of the new generation namely sparfloxacin, gatifloxacin, levofloxacin, moxifloxacin, trovafloxacin, gemifloxacin and rufloxacin are broad-spectrum antibiotics that have improved pharmacokinetic parameters (Srinavas *et al.*, 2008).

Fluoroquinolones have been mainly used for invasive infections which are associated with serotypes of Typhi and nontyphoidal *Salmonella enterica* (Booker *et al.*, 2005). The usage of fluoroquinolones as alternatives to β-lactam antibiotics on treating many bacterial infection (Srinavas *et al.*, 2008).

Peptide antibiotics

Antimicrobial peptides are ubiquitous natural products. They have been discovered in many organisms starting from bacteria, to plants, mammals and humans. Antimicrobial peptides are produced as part of the innate host defense in humans to protect lungs from developing respiratory infections (Bals *et al.*, 1998). Their antibiotic activity is based mainly on targeting membrane pore-forming mechanisms. In contrast to conventional antibiotics it is for microbes more difficult to develop resistance to this class of antibiotics (Sang and Blecha, 2008). Among peptide antibiotics, defensins, cysteine-rich peptides are the most characterized ones (Bals *et al.*, 1998).

1.3 Antibiotic applications

Antibiotics are used to treat or prevent various infectious diseases. Since antibiotics are one of the most used drugs and they are used in high dosage compared to other drugs, it is very important to assure that they are not toxic to the human organism (Livermore, 2011). Therefore it would be very important to assess the concentration of antibiotics in the human body and more importantly in the infection area. Since later one is however not possible, it is important to be able to know the antibiotic concentration in blood plasma. Still there is no guarantee that this correspond with the information of antibiotic concentration in the infected area. For some antibiotics, for example, quinolones and macrolides, it is already known that they accumulate in the infection area since they assemble in phagocytes (Forth, Henschler and Rummel, 2009).

Important for the application of antibiotic is understanding and evaluation the minimal inhibition concentration (MIC), which is the lowest concentration of antibiotics that have the bacteriostatic effect on bacteria (in-vitro). Another important parameter which describes the bactericide efficacy of antibiotics is the minimal bactericidal concentration (MBC), which is the lowest concentration of antibiotics that kills 99.9 % of the bacteria (in-vitro) (Forth, Henschler and Rummel, 2009; Lüllmann *et al.*, 2016).

As mentioned above the prophylactic use antibiotics is very important in disease prevention. The usage of antibiotics in prevention therapy is especially indicated in cases such as malaria, rheumatic fever, prophylaxes of endocarditis, surgery of colon area, and vagina hysterectomies. Additionally, preventive usage of antibiotics is very important for patient that have weak immune system e.g. AIDS patients (Forth, Henschler and Rummel, 2009).

The importance of targeted antibiotics therapy is undisputed. Thus the usage of antibiotics with narrow-spectrum antibiotics should be favored as often as possible (Forth, Henschler and Rummel, 2009). Inappropriate and excessive antibiotic use may affect the intestinal microbiota and may reduce the variability of it. This might be reversible or not, depending on many factors such as antibiotic spectrum, route and duration of administration, dosage etc. (Fernandes *et al.*, 2017).

Crucial for the successful treatment with antibiotics seems to be also correlated to the time point of their application. This was proven for patients using antibiotics against Enterococcal bloodstream infections which had a reduced mortality when using the antibiotics within the first 48 hours (Zasowski *et al.*, 2016). Moreover early diagnosis and treatment are highly important especially for severe sepsis cases. There is a correlation on increasing mortality with every hour of delay on antibiotic treatment (Mehl *et al.*, 2017).

Treating Community-Acquired Pneumonia which is the leading cause of death and hospitalization worldwide is of great importance (Postma *et al.*, 2015; Guillamet *et al.*, 2016). Preferred empirical treatments during successive periods of 4 months are treatments with β-lactam monotherapy, β-lactam combined with a macrolide, or fluoroquinolone monotherapy (Postma *et al.*, 2015). The usage of broad-spectrum

penicillines such as acylaminopenicilline are indicated for treatment infections of genitourinary tract, biliary tract, endocarditis etc. (Forth, Henschler and Rummel, 2009). Other indications for β-lactams are introduced in Figure 1-4. Ampicillin and amoxicillin are orally bioavailable and shoe improved activity against Gram-negative bacteriav(Bush and Bradford, 2016).

Third generation cephelosporins are used in the treatment of respiratory tract infections (Livermore, 2004). The monobactam aztreonam is very important for the treatment of cystic fibrosis on patient with chronic pulmonary infections caused by *Pseudomonas aeruginosa* that express extended-spectrum β-lactamases (ESBLs) (Vandavasi *et al.*, 2017).

Glycopeptide antibiotics or more specifically vancomycin and teicoplanine are useful for the treatment of infections with multi-resistant Gram-positive bacteria (Forth, Henschler and Rummel, 2009). Vancomycine is considered as the drug of last resort when it comes to clinical MRSA and *Enteroccocus* spp. (Allen and Nicas, 2003; Ruzin *et al.*, 2004).

The first discovered aminoglycoside antibiotics, streoptomycine is very important on treating tuberculosis, as already mentioned in Chapter 1.1, which again has to be combined with other anti-tuberculosis drugs. Newer aminoglycoside drugs such as genatmicine, tobramycine, netilmicine and amikacine are indicated in septic infections especially for nosocomial infections. Other indications might be endocarditis, pseudomonas infections, etc.(Forth, Henschler and Rummel, 2009). In industrialized countries aminoglycosides are used to treat life threatening sepsis in children, infections of urinary tract with complications, osteomyelitis and intra-abdominal infections. Furthermore they are used as alternatives to fluoroquinolones for instance in cases of resistance development (Xie, Talaska and Schacht, 2011).

Macrolide antibiotics (erythromycne, clarithromycine, roxithromycien, azithromycine) are important for the treatment if several infections, in many cases alternatives to penicillin or aminoglycosides. Some of these infections might be: tonsillitis, prophylactic medication for rheumatic fever, diphtheria, pyogenic infections of respiratory tract (sinusitis, otitis media etc.) and many other infectious diseases (Forth, Henschler and Rummel, 2009). It has been shown that there is a clear clinical profit of *P.aeruginosa* (Cysitc fibrosis) when

macrolides are applied (Ruzin *et al.*, 2004; Manniello *et al.*, 2017). They are effective against Gram-negative bacteria (Ianaro *et al.*, 2000) as introduced in the previous chapter.

Infection Site	β-Lactam Used
Skin/soft tissue infections	Cephalosporins, penicillins, carbapenem (ertapenem)
Head and neck infections	
Dental infections	Penicillins
Pharyngitis	Cephalosporins, penicillins
Sinusitis	Penicillins, cephalosporins
Meningitis	Cephalosporins (third- and fourth-generation agents), carbapenem (meropenem)
Lower respiratory tract infections	Penicillins, cephalosporins, carbapenems (especially for hospital-acquired infections)
Urinary tract infections	Penicillins, cephalosporins, monobactams, carbapenems (especially for infections due to multidrug-resistant gram-negative bacilli)
Intra-abdominal infections	Cephalosporins (in combination with agents with anaerobic activity), carbapenems, ureidopenicillin with β-lactamase inhibitor
Bone and joint infections	Penicillins, cephalosporins
Infective endocarditis	Penicillins, cephalosporins

Figure 1-4 Table of clinical use of β-lactam antibiotics by site of infection (Watkins and Bonomo, 2016).

Indications for tetracycline antibiotics are infections of respiratory tract, urinary tract, skin, intestinal (e.g. cholera), etc. (Forth, Henschler and Rummel, 2009).

Fluoroquinolones are used for the treatment of respiratory tract infections(Livermore, 2004), furthermore they are also used to treat topical infections, infections of eyes, as drops for ears and against Acne vulgaris (Forth, Henschler and Rummel, 2009). In the treatment of many bacterial infections they are used as alternatives to β-lactam antibiotics (Srinavas *et al.*, 2008).

Antibiotics are not only used for treatment or prevention of human diseases but also in animals and in agriculture (Figure 1-5). The usage of antibiotics in aquaculture is inevitable since the los in yield can be outrageous as it I known for China, as one of the greatest providers of aquaculture products (Liu, Steele and Meng, 2017). Food animals is another area where antibiotics are widely used (Landers *et al.*, 2012). The practice of tetracycline usage in veterinary medicine and in subtherapeutic levels in animal feed which is thought to promote animal growth, is quite common (Chopra and Roberts, 2001). Growth promoting effect of many antibiotics is thought to be, among others, due to the declined microbial metabolites that might act growth-depressing, increased uptake and use of nitrogen sources from nutritients, decreased microbial use of nutrients and restriction of infections (Gaskins *et al.*, 2002).

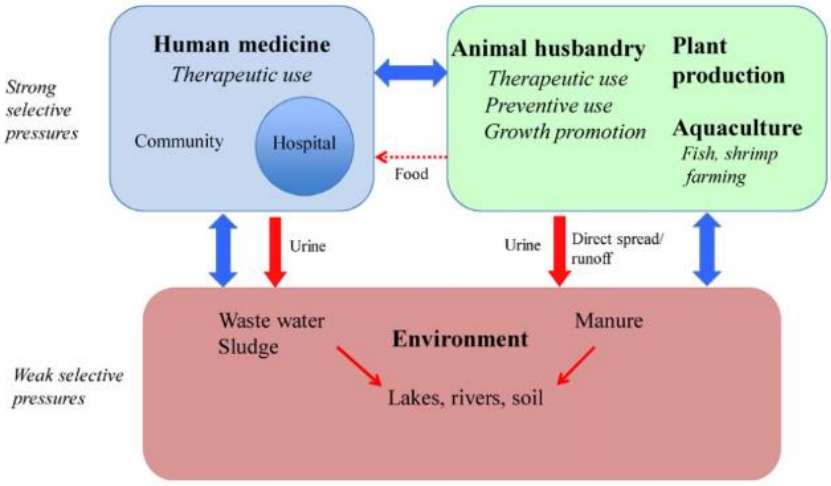

Figure 1-5 Flow of antibiotic usage (red arrows) and resistant bacteria (blue arrows) in different areas and situations (Andersson and Hughes, 2012).

2 Methods and materials

2.1 Literature

This diploma thesis is a literature research about antibiotics and the bacteria developed antibiotic resistance. The area about antibiotics covers their mechanisms of action, classification possibilities and applications. The part about antibiotic resistance covers almost all types of resistance mechanisms and their development in bacteria.

Another important part of this thesis is the broad and uncontrolled usage of antibiotics in southern Europe with a special attention to Kosovo and the possible ways Kosovo might benefit from other legislatives on how to manage this issue.

The literature used includes different pharmacology or medical textbooks and publications searched over NCBI, u:search from Vienna University or directly in the Library of Pharmacy and the Library of Medical University of Vienna. Further information was also collected from the internet such as the homepage of the WHO, ECDC, Austrian Ministry of Health.

3 Antibiotic resistance

3.1 Introduction to antibiotic resistance and its origination

The effectiveness of antibiotics in bacteria might be limited through various ways of bacterial attempts to protect themselves. Bacteria may respond to antibiotic treatment in tolerating, persisting or even be resistant to the antibiotic (Brauner *et al.*, 2016). `Tolerance` is the ability of microorganisms to endure short-term exposure to high antibiotic concentrations which might be acquired through a genetic mutation or evolved by environmental condition. Meanwhile `persistence` is the capability of a bacterial subpopulation to endure exposition to high antibiotic concentrations. Bacterial subpopulations use this transient mechanism that is non-hereditary, to survive lethal antibiotic concentrations (Rodríguez-Rojas *et al.*, 2013).

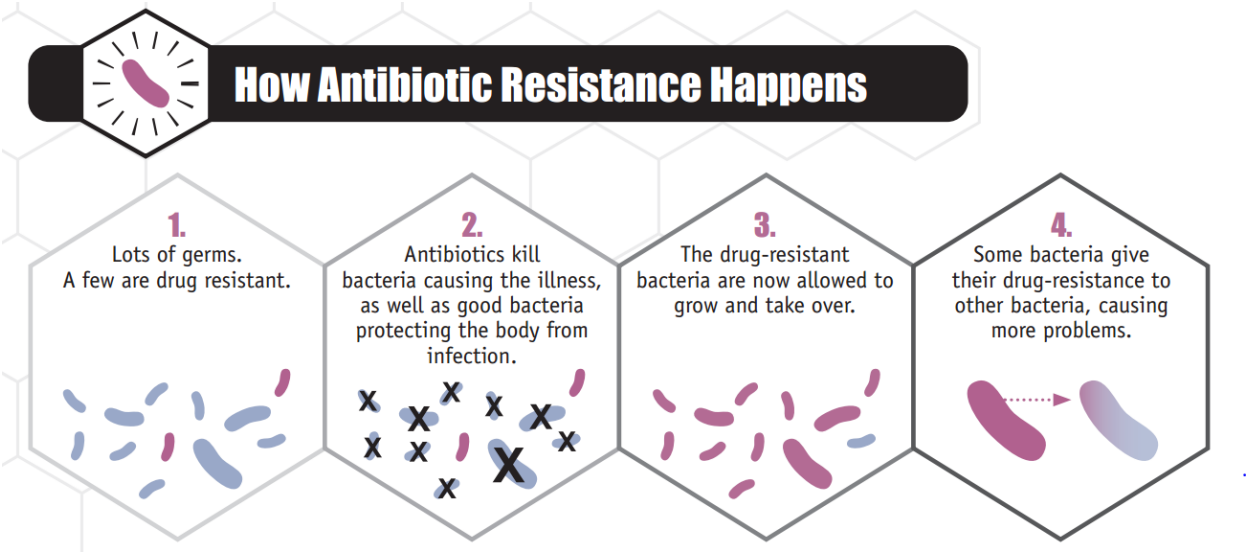

Figure 3-1 Antibiotic resistance development in bacteria subpopulations. (CDC, 2013)

Resistance on the other hand is the capability of bacteria or other microorganisms to develop under high antibiotic concentrations, irrespective of the time span (Brauner *et al.*, 2016). Resistance is typically inherited and connected to diverse molecular mechanisms. Resistance is quantified by the MIC which is, as described in the first chapter, the minimal

antibiotic concentration that inhibits bacterial growth (Forth, Henschler and Rummel, 2009). Figure 3-1 presents how resistant bacteria survive and grow.

Resistance may be active or adaptive. Active resistance is the result of an evolutionary pressure that allows the bacteria to counterattack against antibiotics in general or against a specific antibiotic class. Passive resistance on the other hand appears as a consequence of a regular adaptive process and not to a specific class of antibiotics (i.e. the outlying membrane of Gram-negative bacteria) (Wright, 2005).

Bacteria resistance to antibiotics is continuously increasing and infections with antibiotic resistant microbes is an escalating problem worldwide (Mehl *et al.*, 2017). These infections are serious human life threatening diseases even in several EU member states where the resistance to Gram-positive as well as Gram-negative bacteria reaches 25% or higher, according to the ECDC-EMEA Report on 2009 (ECDC, 2009). Moreover, in the low- and middle-income countries the spread of bacterial resistance comes through poor hygiene, polluted water, contaminated food but also higher susceptibility to infections as a result of chronic illnesses, malnutrition or immunosuppression (Merrett, Bloom and Wilkinson, 2016). These and other reasons may help the bacteria to sooner or later generate resistance mechanisms for almost all antibiotics (Aminov, 2010).

Resistance has been recognized as an important issue, and thus researched and reviewed in lots of publications. The management of each infection may be potentially complicated through bacterial resistance, regardless how mild the resistance at its first appearance might be (ECDC, 2009). Hughes and Andersson categorized the resistance in their review on 2017 into three types: intrinsic resistance (mechanisms intrinsic to bacterium that constrain the drug action e.g. slow uptake or extrusion through the efflux pumps), acquired resistance (conferred through gene transfer or mutation that leads to drug modifying or protecting the antibiotic target) and adaptive resistance (antibiotic-induced resistance, transient resistance increase due to antibiotic gene induction). The latter one comes as a consequence of the environmental antibiotic concentrations which again is the driving force for the natural selection of bacterial populations developing resistances (Andersson and Hughes, 2012).

The intrinsic resistance, also called “natural” resistance i.e. efflux mechanisms, has been presented to be species- or genus-specific that leads to decrease of antibiotic efficacy (Depardieu *et al.*, 2007). The process of pumping out the drug is energy consuming. It works through ATP hydrolysis or the mechanism of an ion antiport (Li and Nikaido, 2010). This type of resistance mechanism has been shown to be among Gram-positive cocci such as *S. epidermidis*, *S. aureus* and others such as *E. facalis*, *E. faesium* and *S. pneumonia*, but also Gram-negative bacilli, for example, *E. coli* and *P. aeruginosa* (Depardieu *et al.*, 2007).

Bacteria or populations that possess more than one of the above mentioned resistance mechanisms have the ability to be multi drug resistant (MDR) (Depardieu *et al.*, 2007). According to an ECDC-EMEA report in 2009, in Europe roughly 25.000 people die from MDR bacterial infections (ECDC, 2009). MDR bacteria are a big threat to global health (Bottery, Wood and Brockhurst, 2016). MDR appear via radical formation that is antibiotic related which leads to mutations that confer antibiotic resistance (Kohanski, DePristo and Collins, 2010).

From a historical view antibiotic resistance developed quite fast after the antibiotic discovery shown in Figure 3-2. Therefore scientists have started to work on finding new antibiotics that do not fall prey of that resistance or design new antibiotics that endure and have the needed efficacy despite the bacterial resistance. The increase of resistance to penicillin through the enzyme penicillinase led to the first designed antiresistant antibiotic in 1959 which was methicillin. It did not take longer than 3 years for *Staphylococcus aureus* to develop the methicillin resistance (methicillin resistant *Staphylococcus aureus*, MRSA) (Davies and Davies, 2010). Nevertheless the greatest impact on community health had the development of extended-spectrum-lactamases (ESBLs) together with carbapenemases (Kohira *et al.*, 2016). The enzymes ESBLs hydrolyse almost all penicillins and cephalosporins but they do not hydrolyse cephamycins or carbapenems (Giske *et al.*, 2013; Kohira *et al.*, 2016). Carbapenemases are enzymes that are able to

hydrolyse penicillins and most cephalosporins, they also might inactivate carbapenems and monobactams to some extent (Giske *et al.*, 2013).

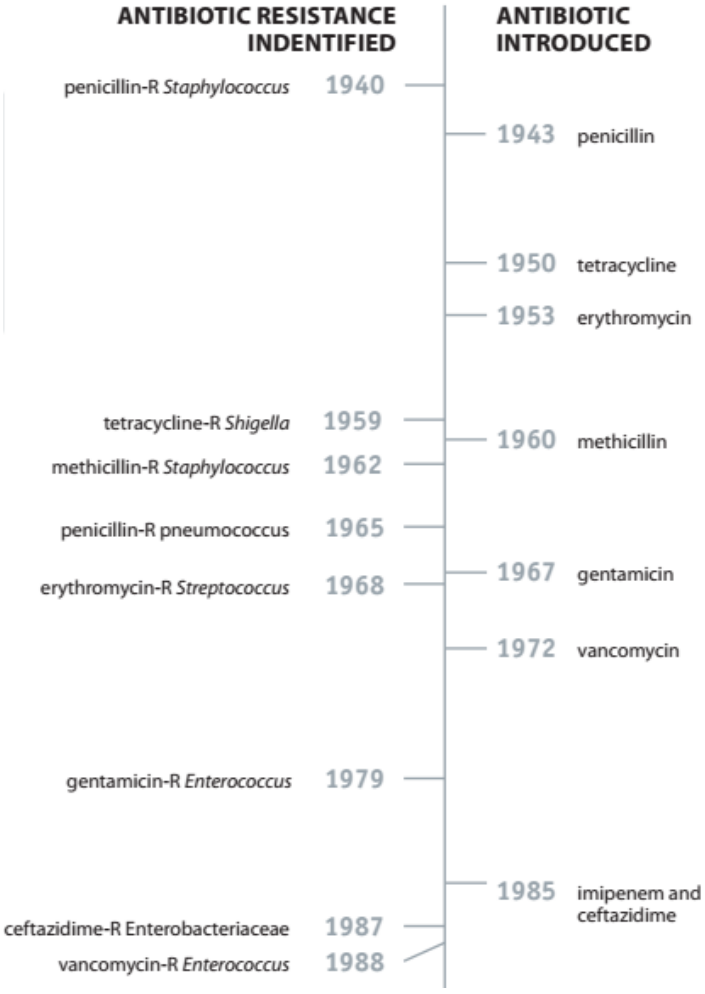

Figure 3-2 Antibiotic development and appearance of key resistance factors over the time (CDC, 2013)

Owing to their genetic plasticity and their short doubling-time, bacteria have the possibility to rapidly “test” distinct mutations in order to increase their ability to deal with the challenging conditions (Hancock, 1997). This and also the possibility of a quite rapid gene transfer between bacterial cells have a high impact on the widely spread antibiotic resistance. A number of resistance genes that are clinically relevant are considered to evolve from nonpathogenic bacteria and are believed to have been transferred to virulent

bacteria mainly through the horizontal gene transfer (HGT), a mechanism induced though damages in DNA (Kohanski, DePristo and Collins, 2010; Von Wintersdorff *et al.*, 2016).

The alarming evidence of fast development of such resistance in *E.coli* and *Klebsiella pneumoniae* to third generation cephalosporins and fluoroquinolones was reported annually by the European Centre for Disease and Prevention (ecdc.europa.eu). the leatest report from 2015 showed even an increase in aminoglycoside resistance ECDC, 2015). An example of *E. coli* resistance increase to aminopenicillins and fluoroquinolones in developed countries such as Austria Figure 3-3.

Sub-lethal concentrations of antibiotics in the environment help the formation of antibiotic resistance and multi drug resistance. These come as a result of the widespread of antibiotic use in humans and in promoting the growth of food animals (Gaskins *et al.*, 2002).

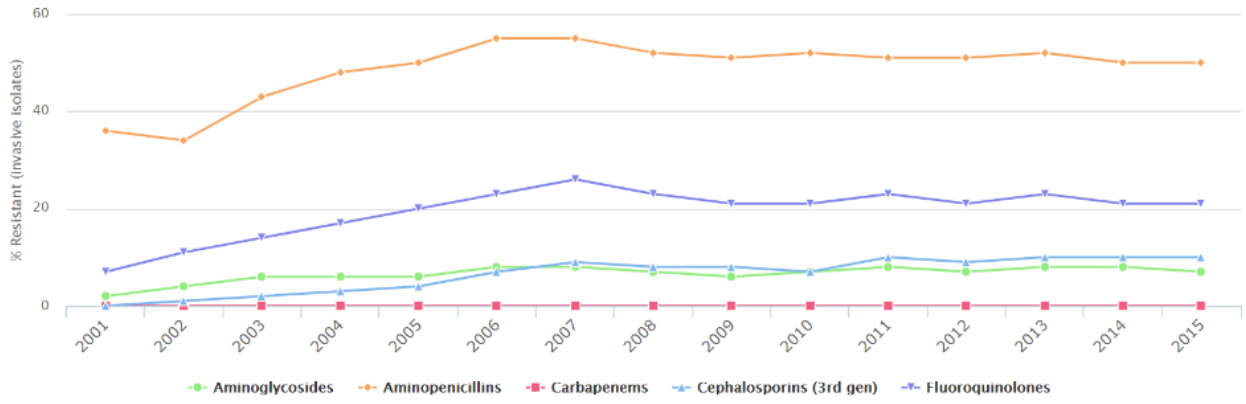

Figure 3-3 E. coli resistance development in Austria. Source: https://resistancemap.cddep.org/AntibioticResistance.php

Bacteria develop resistance by acquiring the genes as a consequence of improper use or excessive usage of antibiotics, this is the case, for example, for intestinal microorganisms: *E.coli*, *Clostridium* spp., *Bacteroides* spp. etc.(Fernandes *et al.*, 2017). Sensitive bacteria undergo various molecular and physiological reactions when confronting with subinhibitory concentrations of antibiotics, for example, gene expression, cell shape, growth, biofilm formation and predation (Chait *et al.*, 2015). Antibiotic concentrations

below MIC is the central driving effort that leads to selection of antibiotic resistance genes (Bottery, Wood and Brockhurst, 2016).

3.2 Resistance development and expansion

Antibiotic resistance is arising in bacteria through different factors. The wide use and misuse of antibiotics have helped the bacteria strains to acquire resistance to most commonly used antibiotics (Bottery, Wood and Brockhurst, 2016). The predominance of transferable resistance in bacterial pathogens is rising along growing antibiotic usage (Davies, 1997). A number of antibiotics lead to appearance of reactive oxygen species, generate misbalance in the nucleotide metabolism or operate on DNA triggering directly (Rodríguez-Rojas *et al.*, 2013) A short summary of this kind of resistance is gained by bacteria is presented in Figure 3-4.

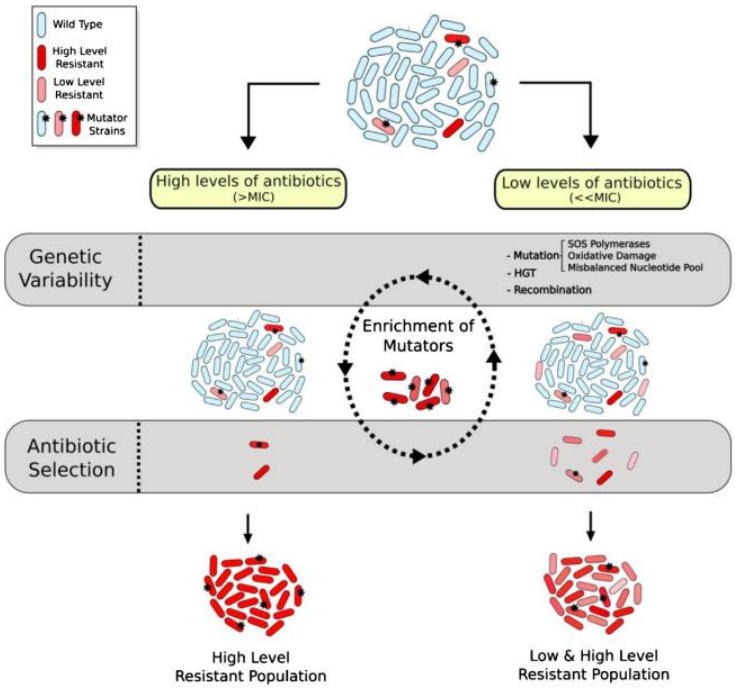

Figure 3-4 A simplified schematic overview on how bacterial population may gain their antibiotic resistance if they face high antibiotic concentration and below MIC.(Rodríguez-Rojas *et al.*, 2013)

In order to ensure the proper treatment with antibiotics, information about the resistance of microbes is necessary. Since nowadays the multidrug-resistant bacteria are quite

spread, there is not much possibility of the selection of antibiotics for the therapy (Saga and Yamaguchi, 2009). Undoubtedly, knowledge about the spread of microbes is an important issue for the right choice of antibiotic treatment (Mehl *et al.*, 2017). The potential development of resistance is a limiting factor of any antibiotic agent. This factor might be also important when treating viral, fungal or parasitic infections (Davies and Davies, 2010).

As mentioned in the previously, the main reason that resistance develops comes as a consequence of antibiotic usage at subinhibitory levels. Improper antibiotic usage at suboptimum levels has the effect of selection pressure and leads to mutations in bacterial cells which is used from the bacteria to stepwise increase the resistance to that and other antibiotics (Laxminarayan *et al.*, 2013). This may be seen also on the correlation of antibiotic usage and the development of resistance during and as a consequence of selected mutations while antibiotic application (Guillemot, 1999). An overview of various fields of antibiotics usage and the relations to resistance may be seen in Figure 3-5.

Antibioticc resistance may appear over mutation or DNA transfer. (Nigam, Gupta and Sharma, 2014). The resistance genes are the major source of clinical resistance (Bottery, Wood and Brockhurst, 2016). The extent of sublethal antibiotic concentrations impact in bacteria was shown in the case of *E. coli* as Long and colleagues did on 2012. They applied whole genome sequencing on *E. coli* and found out “thousands of mutational events, including point mutations, indels, prophage deletions and large duplications” were happening in the bacteria (Long *et al.*, 2016).

Antibiotics, as mentioned before, are present in nature also as a result of their production by microbes in order to protect themselves, gain advantage or even as interaction molecules. But their concentrations are believed to be much lower than in clinical area (Chait *et al.*, 2015). Nevertheless antibiotics and other antimicrobial agents are used in (Davies and Davies, 2010):

i) prophylactic, therapeutic and growth promotion in animals,
ii) household pets and aquaculture,
iii) for plants and agriculture as pest control and cloning,
iv) in toiletries as biocides and household cleaning and

v) hand care products,

vi) in research and industry for culture sterility, selection and cloning.

Usage of antibiotics in agriculture creates environments with widely propagated drugs at low concentrations (Chait *et al.*, 2015). This leads to increased resistant strains in the intestinal flora of the animals that are provided with antimicrobial growth promotants (Marshall and Levy, 2011). A simplified schematic overview of antibiotic usage in different areas and possible resistance propagation ways are presents in Figure 3-5.

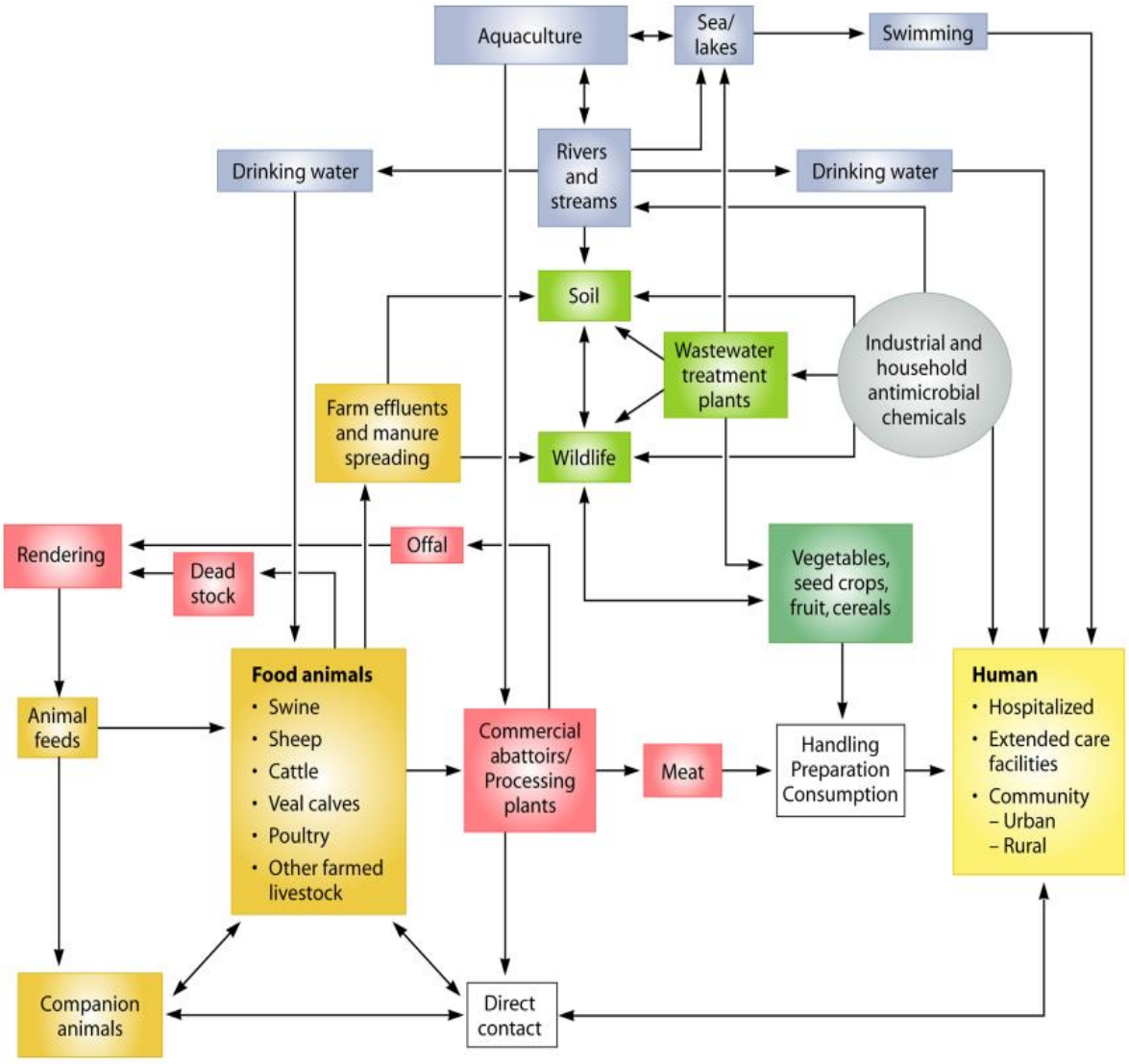

Figure 3-5 Antibiotic usage circulation and antibiotic resistance. Usage in humans, agriculture, and many other fields (Davies and Davies, 2010)

The medical and agricultural driven rise of antibiotic concentrations in the environment is remarkable and has led to a misbalance between microbes and antimicrobials (Von Wintersdorff *et al.*, 2016). A partial explanation of bacteria that may be antibiotic resistant which have been found to appear highly frequent in pristine environments might be the presence of sub-MIC antibiotic concentrations (Rodríguez-Rojas *et al.*, 2013). This might actually also be the case in clinical antibiotic use where commensal microbes might get in contact with bacteria at sub-MIC concentrations as a consequence of periodic antibiotic use and different allocation in the human body (Chait *et al.*, 2015).

Bacterial resistance expansion

Bacteria may gain their resistance against antibiotics using different mechanisms. These include horizontal gene transfer, biological mechanisms such as efflux and changes by mutations of targets. Gene transfer is one of the most impacting processes in resistance acquisition from other bacteria, distribution and transfer. Horizontal gene transfer is broadly acknowledged to be the mechanism that is important for the distribution of genes responsible for antibiotic resistance, for biodegrative pathways and determinants of pathogeny (de la Cruz and Davies, 2017). Using these mechanisms some species are able to receive and include in their own genes, DNA from dead bacteria. This results in gene modification and resistance acquisition, for example, pneumococci acquired resistance to penicillin (Nigam, Gupta and Sharma, 2014).

Horizontal gene transfer may occur through (Von Wintersdorff *et al.*, 2016):

- Conjugation
- Transformation
- Transduction
- Gene transfer agents.

Conjugation, shown in Figure 3-6 A, is the process of DNA transfer that demands cell to cell contact and which is encoded either by plasmid genes or in chromosomes encoded by conjugative elements. Transformation is the uptake of naked DNA and its

recombination from a bacteria that has to be in a state of competence. The limitation to DNA from similar bacteria might come from the homologous recombination and the process of DNA-repair (Thomas and Nielsen, 2005). Schematic overview of transformation is presented in Figure 3-6 B.

During transduction, bacteria acquire their antibiotic resistance genes through phages. Phage encoded genes are enriched as e consequence of antibiotic treatment. The phageome has been shown to be enriched in stress-related conditions and the interaction between phages and bacteria appear to expand under antibiotic treatment (Modi *et al.*, 2013). Phage-related fragments and bacteriophages have been highlighted as resistance transferring mobile genetic elements (MGEs) (Brown-Jaque, Calero-Cáceres and Muniesa, 2015). Phage involvement in the spread of antibacterial resistance genes (ARGs) seems to play a strong role toward bacterial resistance (Anand *et al.*, 2016). Transduction process is presented in part C of Figure 3-6.

Gene transfer agents (GTAs) are fragments produced in the host-cell. They look like structures of bacteriophages and are capable of transferring genetic content (Von Wintersdorff *et al.*, 2016). Resistance transfer through GTAs is presented in Figure 3-6 part D.

Antibiotic resistance and associated factors of pathogeny that are plasmid-mediated have been shown to have serious implications. Resistance transfer among various strains of *B. fragilis*, within the *B. fragilis* group and between *E. coli* and species of *B. fragilis* group has been shown for erlythromycine, clindamycine, tetracycline and chloramphenicol (Rotimi, Duerden and Hafiz, 1996). The drug resistance transfer may also follow from eubacterial R factors to *Myxococcus* which extends the number of Gram-negative microorganisms that allow plasmid transfer (Parish, 1975). Plasmid transferred antibiotic resistance is very well studied and it is known that the rapid spread of it is quite easy once the resistance genes have been established in plasmids (Von Wintersdorff *et al.*, 2016).

Transposons that are transferred through the genetic material propagation may often assist the progression of multiple resistance genes incorporation into the genome of susceptible bacteria (Tenover, 2006). Various drug resistance genes may be localized on

the same plasmid allowing the relative easy expansion of MDR (multi drug resistance) (Von Wintersdorff *et al.*, 2016). Their pandemic spread is a serious threat for human health (Laxminarayan *et al.*, 2013). Anyhow, resistance to antibiotics may occur as a consequence of mutations in one or more genes located in chromosomes of sensitive strains (Parker, 2013).

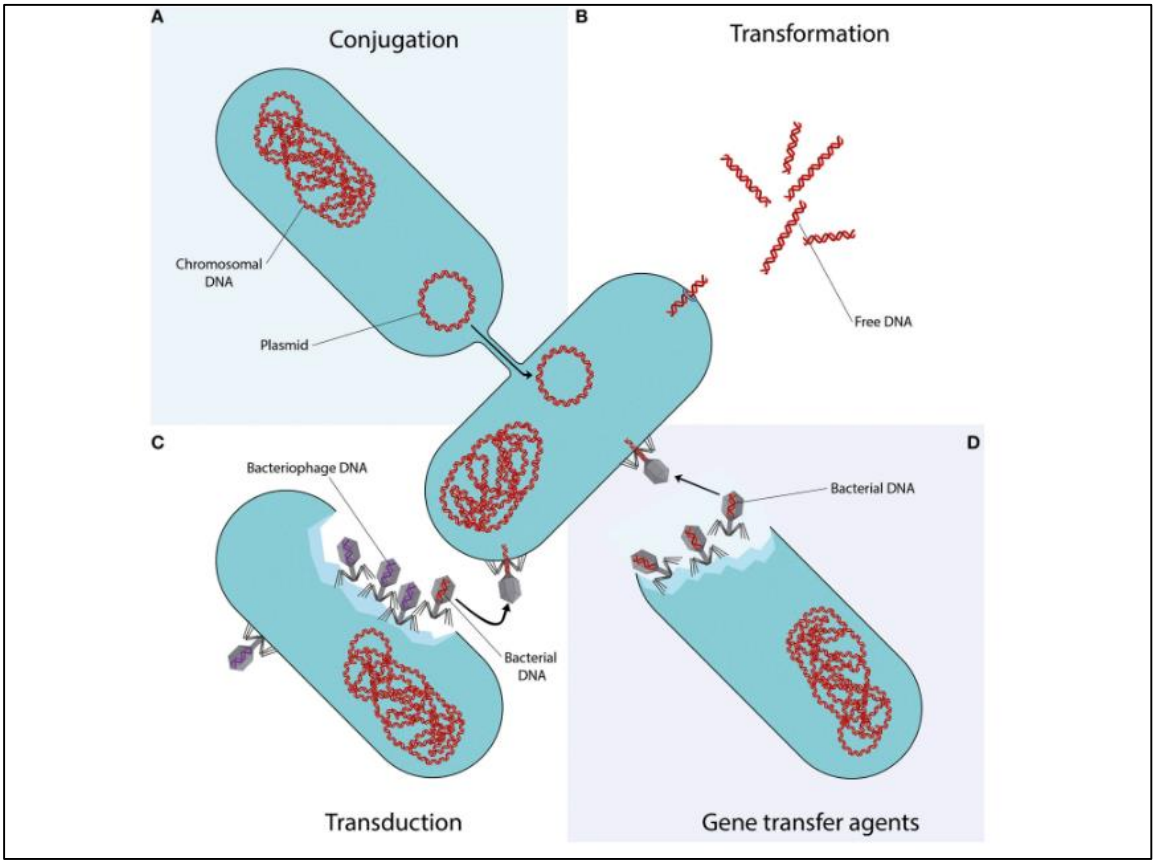

Figure 3-6 Horizontal gene transfer mechanisms. A) Conjugation: DNA transfer from donor cell to recipient cell through celsl to cell contact (pilli or adhesive), B) Transformation: extracellular naked DNA is uptake and integrated, C) Transduction: bacterial DNA is transferred through bacteriophages, D) Gene transfer agents (GTAs): fragments that are like bacteriophage particles carrying pieces of the genome of the producing cell.(Von Wintersdorff *et al.*, 2016)

The resistance acquisition through conjugation, transduction or transformation includes the incorporation of genetic material that is transferred from resistant strains to susceptible bacteria. Apart from these genetic mechanisms of resistance acquisition, there are also some biological mechanisms of bacterial resistance. They include mainly (Alanis, 2005):

- Antibiotic destruction or transformation
- Antibiotic active efflux
- Receptor modification

Producing different enzymes might help the bacteria destroy the drugs and defend itself from antibiotics. The most prominent is the production of β-lactamases and inactivation of β-lactam ring of β-lactams (Alanis, 2005; Watkins and Bonomo, 2016). Some bacteria might use their ability to develop active mechanisms that are able to pump the antibiotic outside the cell. Furthermore, modification of receptors is another quite effective method of bacteria to avoid antibiotics attacks. Bacteria change the receptor in order to decrease binding efficiency and therefore their antibacterial effect (Alanis, 2005).

Since multiresistant bacteria is expanding in pandemic manner and the development of new antibiotics is not really complying, the safety of affected patients is worldwide at risk (Laxminarayan *et al.*, 2013).

Multiple drug resistance (MDR)

Possibilities are (Denyer, Hodges and Gorman, 2004):

- R-Factor
- Mobile gene cassettes and integrons
- Chromosomal multiple-antibiotic resistance (Mar) locus
- Multidrug efflux pumps

R-Factor

R-Factors in *Enterobacteriaceae* have been published in 1972, and have been presented as extrachromosomal units of DNA that are able to autonomously replicate Cohen 1972.

R-Factors allow the bacteria to become resistant to multiple agents which might be chemically very distinct (Denyer, Hodges and Gorman, 2004). The possibility of resistance transfer through the R-factor has been shown even between far related bacteria (Kruse and Srum, 1994). Many R-factors encoding antibiotic resistance to different drugs in *P.*

aeruginosa and other Gram-negative bacteria have been identified and characterized, for example resistance to ampicillin, tetracycline, kanamycine and neomycine (Denyer, Hodges and Gorman, 2004).

Mobile genes cassettes and integrons

Integrons are one or more in integrated gene cassetes in chromosomes. Gene cassettes are the location of many resistance genes in Gram-negative bacteria (Denyer, Hodges and Gorman, 2004). There are more than 60 cassettes known, every one of which consists of a single gene that is promotor-less (normally resistance to antibiotics) and an element composed of 59 bases (Denyer, Hodges and Gorman, 2004; Partridge *et al.*, 2009).

Integrons are able as genetic elements to identify and catch various mobile gene cassettes which have to correctly be orientated in order to supply the promotor-less gene with and upstream promotor (Denyer, Hodges and Gorman, 2004). These gene-capturing elements have an important function in the resistance gene spread (Cury *et al.*, 2016). They incorporate new genes and add them to genetic material of the cell making possible for bacteria to possess a naturally cloning machine (Hall, 2012).

Gene cassetes may occur temporarily free circular mode and usually they do not possess own movements functions (Partridge *et al.*, 2009). They may not be regularly joined to an integrin (Hall, 2012).

Chromosomal multiple-antibiotic resistance (Mar) locus

The chromosomal multiple-antibiotic resistance (mar) locus has been found in *Escherichia coli* for the first time but has been now identified also in many other enteric bacteria. It is composed from two subunits: marC and marRAB (Denyer, Hodges and Gorman, 2004). Mar operons might be activated. Like other MDR mechanisms during bacterial exposure to sub-lethal concentration which, for example, might appear in a biofilm and they also help bacteria protect themselves if the concentration increase (Maira-Litrán, Allison and Gilbert, 2000).

The Mar phenotypes, coming from overexpression of marRAB or marC inactivation, have shown increased resistance to antibiotics that are structurally not related, to oxidative stress as well as to organic solvents and chemical disinfectants (Maira-Litrán, Allison and Gilbert, 2000; Denyer, Hodges and Gorman, 2004). Mar locus regulates the chromosomal genes expression which are implicated in the intrinsic MDR and susceptibility in *E.coli* and other *Enterobacteriaceae* (White *et al.*, 1997).

Multidrug efflux pumps

Multidrug efflux pumps actions are energy-dependent. They pump out a wide range of compounds without changing or degrading the compound and that are chemically and structurally not related (Kumar and Schweizer, 2005; Riou *et al.*, 2016). Although a common attribute might be structures with a hydrophobic part, therefore aminoglycosides antibiotics are not pumped out by this system since they are hidrophylic (Denyer, Hodges and Gorman, 2004).

Antibiotic resistance achieved through efflux pumps is identified in Gram-positive as well as Gram-negative bacteria. The resistance in Gram-negative bacteria is much delicate though (Kumar and Schweizer, 2005). Gram-positive bacteria possess efflux pump systems (called QacA) that pump the compounds across the bacterial membrane, while in Gram-negative bacteria that pump the substrate across plasmatic membrane and across outer membranes (Denyer, Hodges and Gorman, 2004).

3.3 Specific antibiotic resistances

It is crucial to identify antibiotic subgroups in order to apply a tailored treatment (Mehl *et al.*, 2017). Furthermore it is very important to have detailed knowledge about various biological and non-biological factors which might have an impact on the rate and extent of development. Detailed knowledge makes it possible to improve and find rational approaches on handling this problematic resistance development (Andersson and Hughes, 2012).

Bacterial resistance to β-lactams

The main mechanisms according to Watkins and colleagues are (Watkins and Bonomo, 2016):

- penetration failure through bacterial membrane
- passage of antibiotics through porins modification
- development of specific pumping mechanisms that leads to antibiotic efflux from periplasmic space
- intrinsic resistance: PBP alterations that lead to decreased binding effectiveness of β-lactams
- inactivation of β-lactam ring through hydrolysis using bacteria β-lactamase enzymes.

The mechanism of inactivating the antibiotic is the most usual resistance mechanism used by Gram-negative bacteria (Kohira *et al.*, 2016; Watkins and Bonomo, 2016). Nevertheless β-lactams are the most valuable antibiotics group when looking in term of total use.

In some species such as *Streptococcus* and *Neisseria*, the resistance to β-lactams appears by homologous recombination of DNA which is gained by transformation from related species (Hughes and Andersson, 2017).

Gram-negative bacteria such as *E. coli* and *K. pneumonia*e are a major health threat because of the increasing antibiotic resistance to carbapenems and to extended-spectrum cephalosporins. β-Lactam resistance is conferred in such bacteria particularly through the spread of genes encoding ESBLs, pAmpCs - AmpC-lactamases that are plasmid-mediated and carbapenemases (Agyekum *et al.*, 2016). Further mechanisms known for *Enterobacteriaceae* are target modifications and efflux pumps (Kohira *et al.*, 2016). The *Enterococcus E. facealis* shows a high-level antibiotic resistance to cephalosporins. It produces low-binding PBPs (PBP5) (Depardieu *et al.*, 2007). Moreover it has been shown that the resistanse to carbaopenems is rapidly increasing (Livermore, 2004).

An increased resistance to third class cephalosporins, which is usage associated, has been observed in surgical patients who developed VRE infections (Guillemot, 1999). Theit unnecessary application on angina, bronchitis and simple sinutis forms – disorders which might be treated with penicillins or tetracyclines – increases the risk of resistance devlopment for this antibiotic groups (Lüllmann *et al.*, 2016)The increasing resistance to carbapenems which have been used in the treatment of infections with ESBLs or multidrug-resistant strains, is due to carbapenemases (Agyekum *et al.*, 2016). These enzymes are acquired. They are encoded on plasmid located transposable genes (Giske *et al.*, 2013). There is evidence that phages play an important role in the transfer and spread of genes encoding for bacterial resistance to β-lactams, including ESBLs (Anand *et al.*, 2016). Anyhow, monobactams might be the choice since metallo-β-lactamases may not hydrolyze them (Giske *et al.*, 2013).

Bacterial resistance to aminoglycosides

Methylation for RNA is one of the possibilities bacteria has in order to be resistant to aminoclygosides. However, this is not the case for bacteria that have been susceptible to aminoglycosides. Production of enzymes that might modify the structure of aminoglycosides so that they may no longer interact with their target, rRNA, is the main mechanism of clinical resistance (Denyer, Hodges and Gorman, 2004). Such modifications might be phosphorylation, acetlyation or adenylylation of aminoglycosides (Wright, 2003).

Aminoglycosides are known to have a lower risk on developing antimicrobial resistance than cephalosporins (Mehl *et al.*, 2017). Nevertheless there are three enzyme classes that are responsible for aminoglycoside resistance. These are aminoglycoside phosphatases, aminoglycoside acyltransferases and aminoglycoside nucleotidylases (Denyer, Hodges and Gorman, 2004). Bacteria have been shown to acquire aminoglycoside resistance by up taking R-factors (Forth, Henschler and Rummel, 2009; Lüllmann *et al.*, 2016).

The resistance development to aminoglycosides has been shown to be slower than to many other antibiotic classes. Anyhow, their usage is limited and this might be for two

reasons. Firstly they are potentially ototoxic and nephrotoxic and secondly their usage requires measurement of aminoglycosides concentration in serum, that presumes experience and knowledge in obtaining the results (Mehl *et al.*, 2017). Their usage is anyhow increasing due to their broad psctrum effectiveness, mostly in the traéatment of infections Gram-negative bacteria, for example, Pseudomonas (Talaska, Schacht and Fischel-Ghodsian, 2006).

Resistance to tetracyclines

Mechanisms of tetracycline resistance in bacteria are mostly efflux and ribosomal protection (Denyer, Hodges and Gorman, 2004). Staphylopcocci strains have been shown to express genes that encode efflux pumps that help them against tetracyclines (Marquez, 2005). Resistance to tetracyclines appear to have spread in bacteria that are Gram-negative as well as Gram-positive. The spread appear to happen mainly by conjugation over plasmids or/and transposons. (Chopra and Roberts, 2001)

It has been further shown that for tetracyclines phages are involved in transferring and spreading the resistance encoding genes (Anand *et al.*, 2016). Tetracyclines are among other antibiotics which target the cell wall, that induce the genes expression leading to increase of resistance of bacteria to them and to other antibiotics(Heinemann, Ankenbauer and Amábile-Cuevas, 2000), Furthermore tetracyclines appear to alleviate the gene transfer in bacteria such as Enterococcus facealis and Bacteroides which have conjugative transposons (Rodríguez-Rojas *et al.*, 2013).

Resistance to macrolides

Mechanisms of macrolide resistance are intrinsic to Gram-negative bacteria because of their outer membrane and its permeability barrier. Nevertheless many mechanisms of resistans have been found in Gram-bacteria: target modification, efflux and ribosomal mutation (Denyer, Hodges and Gorman, 2004). Chemical modification is another possibility bacteria have developed to protect themselves from macrolide antibiotics such as erythromycin (Wright, 2011) Target modification prevents the active bounding of macrolides in the ribosomal peptide tunnel (Wright, 2003). Bacteria resistance to

macrolides is crucial for patients with cystic fibrosis and its treatment, since it appears to rise with the application of the drug (Thornton *et al.*, 2015). A decline in macrolide clinical use as a result of the macrolide resistance appearance (Hansen *et al.*, 2002).

Resistance to fluoroquinolones

The resistance of bacteria against fluoroquinolones might be manifested in two possible ways. Firstly, bacteria might establish gyrase genes mutations and secondly they might use efflux pumps to eliminate drugs from their cell by removing them (Piddock, 2006). Very important are gyrA mutations which comprise a hydroxyl group substitution with a hydrophobic group. This leads to conformational alterations and to the inability of fluoroquinolones to bind to the target (Denyer, Hodges and Gorman, 2004).

Resistance development to this class of antibiotics in bacteria is more complicated than many others. This process involves various mutations that affect drug target, regulators of drug efflux, but also genes acquired by horizontal gene transfer (Hughes and Andersson, 2017). Fluoroquinolone resistance has been associated with changes in the permeability of outer-membrane only in Gram-negative bacteria. The contribution of efflux system to resistance appears to be important for Gram-positive as well as Gram-negative bacteria (Denyer, Hodges and Gorman, 2004). On the other hand fluoroquinoliónes such as norfloxacin are believed to increase reactive oxygen species (ROS) concentrations in the bacterial cells that might lead to alleviation of mutation rates of their genome and to following acquirement of MDR (Long *et al.*, 2016).

Resistance to glycopeptides

Enterococci might develop resistance to glycopeptides by substituting D-alanine of the terminus D-alanyl-D-alanine (D-Ala-D-Ala) with lactic acid or serine which means change of the target of glycopeptid antibiotics (Depardieu *et al.*, 2007; Forth, Henschler and Rummel, 2009; Lüllmann *et al.*, 2016). The resistance is mainly mediated by plasmids where the ligases VanA, VanB and VanD are encoded that lead to substitution of D-alanine in D-Ala-D-Ala with lactic acid, resulting in D-Ala-D-Lac. While for serine the ligases VanC, VanF and VanG are also plasmid-encoded and lead to D-Ala-D-Ser

(Depardieu *et al.*, 2007; Wright, 2011; Giske *et al.*, 2013). These changes results in reduced affinity of glycopeptides to their target and no productive binding to the target may be established (Wright, 2011).

Vancomycine resistant Enterococci (VRE) are *Enterococcus faecalis* or *E. faecium* that have developed resistance to vancomycin. They are known to circulate and persist in hospital areas and may colonize many patients or other individuals - although only a few might develop an infection, treating those might be very difficult, because of their low susceptibility to most antibiotic drugs (Wright, 2003; Giske *et al.*, 2013). A usage associated resistance of VRE to vancomycine appears to be a determinant for surgical patient which develop VRE infections (Guillemot, 1999).

Furthermore the antibiotic presence in the environment is one of the important promoting factors of acquiring antibiotic resistance. On the other hand, independent evolution of resistance components that are highly specific even in absence of native production of antibiotics, for example, vancomycine resistance development in *Streptomyces coelicolor*, *Rhodococcus* and *Paenibacillus* (D´Costa *et al.*, 2006).

Antimicrobial peptides

Since bacteria are naturally surrounded by peptide antibiotics, they have been developing intrinsic resistance mechanisms against them. This might happen over mechanisms that are passive or inducible (Andersson, Hughes and Kubicek-Sutherland, 2016). Some bacteria species such as *Proteus, Morganella, Serratia, Burkholderia* and *Providenca* have developed passive resistance mechanisms. The alteration of lipid A to a more positively charged component has been presented to decrease the antimicrobial peptide effectiveness (Denyer, Hodges and Gorman, 2004; Andersson, Hughes and Kubicek-Sutherland, 2016).

Other intrinsic resistance mechanisms which have been reviewed very well in Andersson et al 2016, are, besides modification of membrane in Gram-positive and Gram-negative bacteria, also decreasing the effect of antimicrobial action of antimicrobial peptides

through efflux pumps and proteolytic degradation (Andersson, Hughes and Kubicek-Sutherland, 2016).

3.4 Risk of antibiotics resistance and the effect on future health care

Antibiotic resistance develops mostly as a result of antibiotic use and misuse. This usage is not only in human infectious diseases but also in other areas such as applications in agriculture and aquaculture. Additionally, the incorrect waste of antibiotic disposal leads to beneficiary conditions for resistance development (Davies and Davies, 2010; Liu, Steele and Meng, 2017).

The World Health Organization (WHO) stated on their Report on 2015: “Antimicrobial resistance will affect everybody, regardless of where they live, their health, economic circumstances, lifestyle or behavior. Therefore, everybody – in all sectors and disciplines – should be engaged in the implementation of an action plan, and in particular in efforts to preserve the effectiveness of antimicrobial medicines through conservation and stewardship programs.” (WHO, 2001).

An example of aminopenicillins resistance spread around the world is shown in Figure 3-7. Resistance spread will affect sectors beyond human health, such as animal health, agriculture, food security and economic development. The elevation of antibiotic levels in nature, resulting from medical as well as agricultural request and application has disturbed to a natural equilibrium between microbes and antimicrobials (Von Wintersdorff *et al.*, 2016). Furthermore the antibiotic levels are predominantly variable and depend on the environment, for example hospital sewage outlets or/and polluting pharmaceutical industries where the concentration levels might hit mg/ml levels, while those from aquatic environment or soil may be very low (Rodríguez-Rojas *et al.*, 2013).

Microbial resistance against antibiotics is a global issue. This may affect global health and food security and anyone of any age and from any country. It may appear naturally or be induced by antibiotic use or misuse in many fields. Antibiotic resistance leads to difficultness of treating the increasing infectious disease number such as pneumonia, tuberculosis and gonorroea. Pneumonia becoming very hard to treat. In the US, for

example, one third of the hospitalized patients with pneumonia die (Guillamet *et al.*, 2016). Multi-drug resistant tuberculosis (MDR/TB), for example, is not any more located in any region or only to HIV patients, it has emerged in diverse areas among Eastern Europe, Africa and Asia and among population as well health care workers (WHO, 2000). All this has as a consequence that hospital stays are prolonged; medical costs and mortality increase (WHO, 2016). It is therefore clear that antibiotics are distinct to other drugs since their usage and its impact are over and above individual use (Laxminarayan *et al.*, 2013).

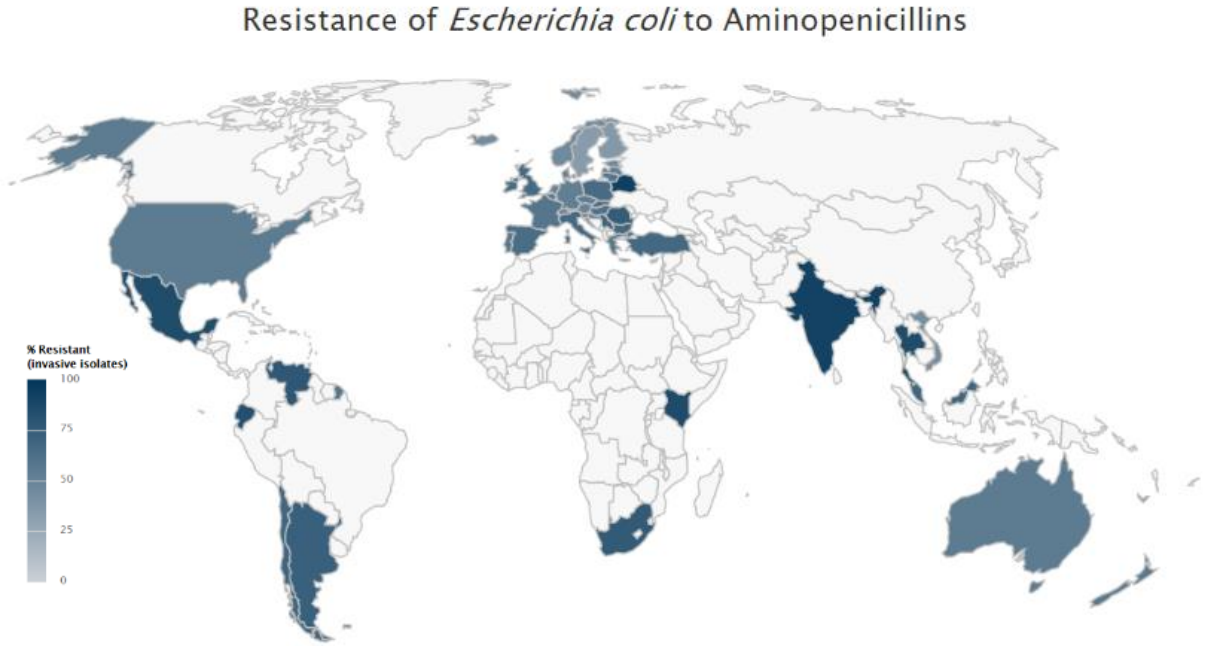

Figure 3-7 Distribution of *E. coli* resistance to aminopenicilline around the world. In most of the countries that have provided the data the resistance is 50% and higher (blue colored map part). The white part of the map are countries that have no data on this issue. (https://resistancemap.cddep.org/AntibioticResistance.php)

3.5 Possibilities of avoiding and resistance arrest

Since the resistance problem has been investigated and recognized, the scientific research and the knowledge about it has been expanding. Development of many new antibiotics over the time has been triggered by the need of tricking the bacterial resistance

mechanisms against antibiotics developed at that time. It is clear that no antimicrobial drug was able to evade from bacterial resistance (Rodríguez-Rojas *et al.*, 2013).

There have been some initiatives to raise awareness to this problem and also to present ideas on how to bear with it. For example, the WHO released on 2015 a plan for global action that might help for a successful administration of this issue in the future (Merrett, Bloom and Wilkinson, 2016). Since not even halt of the produced antibiotics are applied in the usage for human therapies (Davies and Davies, 2010), the attempt to approach to this issue in a system perspective was surely one of the best ideas (Merrett, Bloom and Wilkinson, 2016).

Addressing the antibiotic and -in general- antimicrobial resistance should be from multidisciplinary aspects since it is a complex medical problem and therefore, subjects such as Medicine and Micribiology along with Epidemiology, Genetics and Sociology are important disciplines that help to focus on this important issue (Laxminarayan *et al.*, 2013; Rodríguez-Rojas *et al.*, 2013). Additionally the involvement of all stakeholders and markets are crucial for effective governance of antibiotic usage, which should also include political engagement on national level and other institutions (Merrett, Bloom and Wilkinson, 2016).

However, the question of how the resistance development to antibiotics might be governed is still one of the emerging issues worldwide. This also for the fact that the usage of some antibiotics has already become obsolete, not only in Europe (Davies, 2013). The confusion in diagnosing if there is a bacterial or viral infection has the consequence that clinicians are not able to unambiguously prescribe the appropriate antibiotic drug (Laxminarayan *et al.*, 2013). Possibilities to avoid the development on resistance such as alternating therapy or drug combinations, are quite promising, although there is a strong need for further investigations until they may be put into action in daily basis (ter Kuile, Kraupner and Brul, 2016). Another important aspect is the implementation of stricter regulations and control of nonhospital application, but also improving the gene tracking of antibiotic resistance (Davies, 2013).

In order to really understand and develop approaches to control antibiotic resistance, it is needed to involve human resources, pharmaceutical, as well as food, agriculture sectors and information systems, areas that should be tightly connected to science and practice (Merrett, Bloom and Wilkinson, 2016). Creating and planning appropriate approaches to inform the patients and the possible affected people in tailored to individual needs. Additionally, along with appropriate use of antibiotics, the role of education should be recognized as of high importance, firstly to health professionals and public workers and to lead to a reorientation of social norms (Laxminarayan *et al.*, 2013). Information about drug selection possibilities, administration, and common resistance levels to particular drugs, drug combinations and the importance of matching half-lives, proper drug usage and combined drug economics (Merrett, Bloom and Wilkinson, 2016). Anyhow the usage of antibiotics should also stick to the definition after WHO on 1985: "Patients receive medications appropriate to their clinical needs, in doses that meet their own individual requirements, for an adequate period of time, and at the lowest cost to them and their community." (WHO, 1985)

4 Antibiotics prescription

4.1 Regulations on antibiotic prescription

Antibiotic prescription from a doctor is mandatory in Austria and most European countries. A sale over-the-counter is not allowed in pharmacies and is punished in most European countries.

In general sales over-the-counter and main regulations regarding the system of Austrian pharmacies are defined in many acts and regulations, the most important ones are:

- Pharmacy act,
- Regulation on Operation of Pharmacies,
- Medicines law, in which the production and marketing of medical products is regulated in Austria.

The Pharmacy Act is the major legal basis on preconditions on establishment of new and on operation of existing pharmacies. It includes their organization, management, operating hours, standby duty, staff employment and other regulations (Steindl, 2006).

Antibiotics belong to the pharmaceutical products that require a prescription in order to be purchased in a pharmacy store. The way how it should be done is determined in the Austrian regulations for pharmaceutical products (§ 3 Rezeptpflichtgesetz) and monitored by the Austrian Federal Office for Safety in Health Care (BASG).

The European Union has drafted guidelines on antimicrobial prescribing and stewardship which include the following points:

- Arrangement of tools and guidelines: this should guarantee the implementation of stewardship agenda that should cover community, care facilities and hospitals. Examples: in Germany: “Strategies to enhance rational use of antibiotics in hospitals”, in the Netherlands: “Practical guide for antimicrobial stewardship”; in the United Kingdom: “`Start smart then focus´ and TARGET antibiotic toolkit”.

- Experts in antimicrobial stewardship: sufficient number of specialists in the field of infectious diseases and in clinical microbiology should be established.
- Correct use of antimicrobials should be monitored and audited: this includes quality indicators and monitoring systems for them and also assure that feedback is kept regularly from prescribers results.
- Introduce electronic antimicrobial prescribing: which should be able to link indication, microbiological and consumption information.
- Microbiology services and diagnostics to guarantee rapid and proper diagnostic tests
- Incentive systems for correct prescribing
- National awareness and educational trainings on antimicrobial use: particularly for health care professionals but also for general public (including all age groups starting from elderly to children)
- Stimulate behavioral interventions in order to minimize inappropriate prescribing (ECDC, 2017)

Furthermore the European Union works constantly on regulations and orders that help minimize the overuse and misuse of antimicrobials, for example, the regulation on the evaluation and authorization of food additives in veterinary use (EUR-Lex, 2016).

Recognizing the increasing risk of antimicrobial resistance, the Austrian Federal Ministry of Health is addressing this issue in many important projects, two of them are:

  - Austrian national initiative on tackling antimicrobial resistance (NI-AMR), and
  - Austria's National Action Plan on Antimicrobial Resistance (NAP-AMR)

The first one includes the work of five expert groups (practitioners and academics): Diagnosis of Infectious, Diseases Surveillance, 'Antimicrobial Stewardship', Hygiene and Infection Prevention and Reporting and Information. The second project is based on European and international aims. It consists of concerns from major areas where antimicrobials are used: human medicine, veterinary applications, animal husbandry as

well as the environment and the food chain (BMG). After the prohibition of antibiotic usage as promoters of growth in Europe, a progress in antibiotic resistance has been noticed, for example in England and Denmark, where a decrease of vancomycine resistance has been recognized or the decrease of peniciline resistance in the UK in pneumococci bacteria (Livermore, 2004).

4.2 Antibiotic extensive and ungoverned usage in Southern Europe especially in Kosovo

Antibiotics are one of the most sold and used medications in developing countries (Cagri Buke *et al.*, 2003). In many countries in Southern Europe (e.g. Albania, Kosovo, Serbia, Bosnia, FYR Macedonia) there is no regulation or national guidelines about antibiotic usage that is followed by the pharmacists or health care staff (Cizman *et al.*, 2004). In Albania, for example, although there is regulation on paper, about 90% of pharmacies admit to sell antibiotics over the counter without any prescription (Hoxha *et al.*, 2016)

Easy prescription, unregulated, sale over the counter without prescription are problems with which many countries in the Balkans have to deal with. Noteworthy is the high usage of broad spectrum penicillins in many countries in the Balkans, as may be seen on Figure 4-1 from the data taken from the homepage of Center for Disease Dynamics, Economics and Policy (CDDEP) (http://www.cddep.org/). There was no data available for Kosovo and many other countries of the region, but there is evidence that the usage of broad spectrum penicillins in Kosovo is even higher than in Serbia, Croatia and Bosnia and Herzegovina (Versporten *et al.*, 2014). In dentistry, for example, the usage of broad spectrum penicillins is very common and very high in Kosovo (Haliti *et al.*, 2015). In Figure 4-2 may be seen a map of the antibiotic usage in many countries worldwide presented in percentage.

Self-medication with antibiotics

Kosovo appears to have a higher rate on antibiotic consumption than many other countries. This might be for the reason that the country still lacks not only rules and monitoring procedures for antibiotics in human health care but also on guidelines on treating any kind of infections in any area of health care (Ibraimi *et al.*, 2015; Zajmi *et al.*, 2017). These, combined with the ignorance about antibiotic effectiveness, are the main

reasons that sale over the counter and the number of antibiotics bought without any prescription continues to be very high. The consumption among the population appears to be mostly for treating flu and sore throat but very often also for cold. Some of the tested persons even think that antibiotics might help to treat pain (Zajmi *et al.*, 2017).

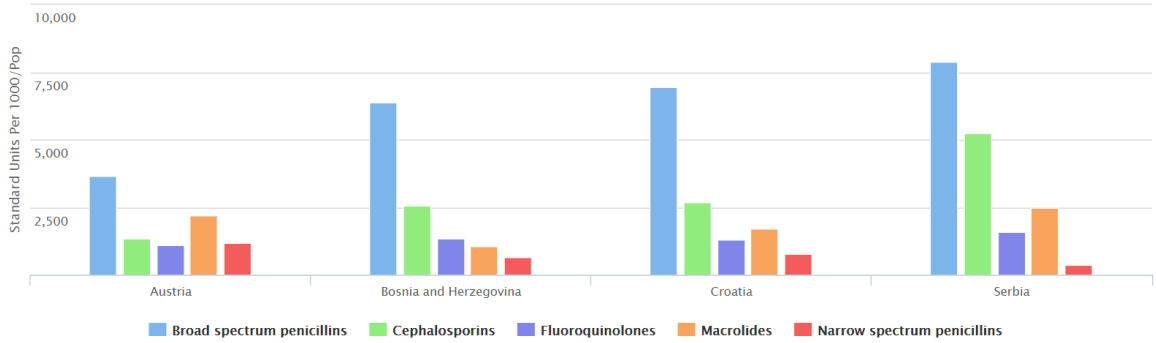

Figure 4-1 Antibiotic use, in 2014, in some countries of the Balkans (Bosnia and Herzegovina, Croatia, Serbia, Slovenia) in comparison to Austria.
https://resistancemap.cddep.org/AntibioticUse.php

The neighbor country, Albania has the same problem with antibiotics medication and it might be even more distinctive than in Kosovo. This is visible from the study published in 2014 where it was investigated that in Albania antibiotics appear to be widely misused by young adults for disorders such as runny nose, nasal congestion, sore throat, cough, fever, aches and pains, vomiting and even diarrhea (Jorgji *et al.*, 2014). The purchase without prescription in the pharmacy is very easy. Shabani and Redican published on 2017 alarming findings from patients that bought their antibiotics in pharmacies in 91% cases over the counter without any prescription or recommendation from health staff (Shabani and Redican, 2017). This is a very concerning increase from 2014 where a study claimed that over 75% of young adults bought antibiotics over the counter, without any prescription (Jorgji *et al.*, 2014). Although the current legislation does not allow pharmacists to sell antibiotics over the counter without prescription, they obviously do not follow these regulations most of the time (Hoxha *et al.*, 2016). This outcomes are incomparable with the developed European countries where in average 93% of

Europeans obtain their antibiotics through prescription from a medical practitioner (Figure 4-3).

The increase in self-medication with antibiotics is strongly connected to the lack of a proper law regulation, poor control from the responsible institutions and sanctioning the regulation disobey. The patients have an easy access to the drugs and need no prescription to get any of the antibiotics from the pharmacies. And the latter ones fear no consequences on selling them over the counter (Veseli, 2015). The project called "Capacity building to implement state of the art surveillance systems for antibiotic consumption and resistance in Kosovo", which was launched in cooperation with the European Union, confirmed the previously mentioned antibiotic overuse, the sale without prescription and the self-medication problem, as well (Raka *et al.*, 2015).

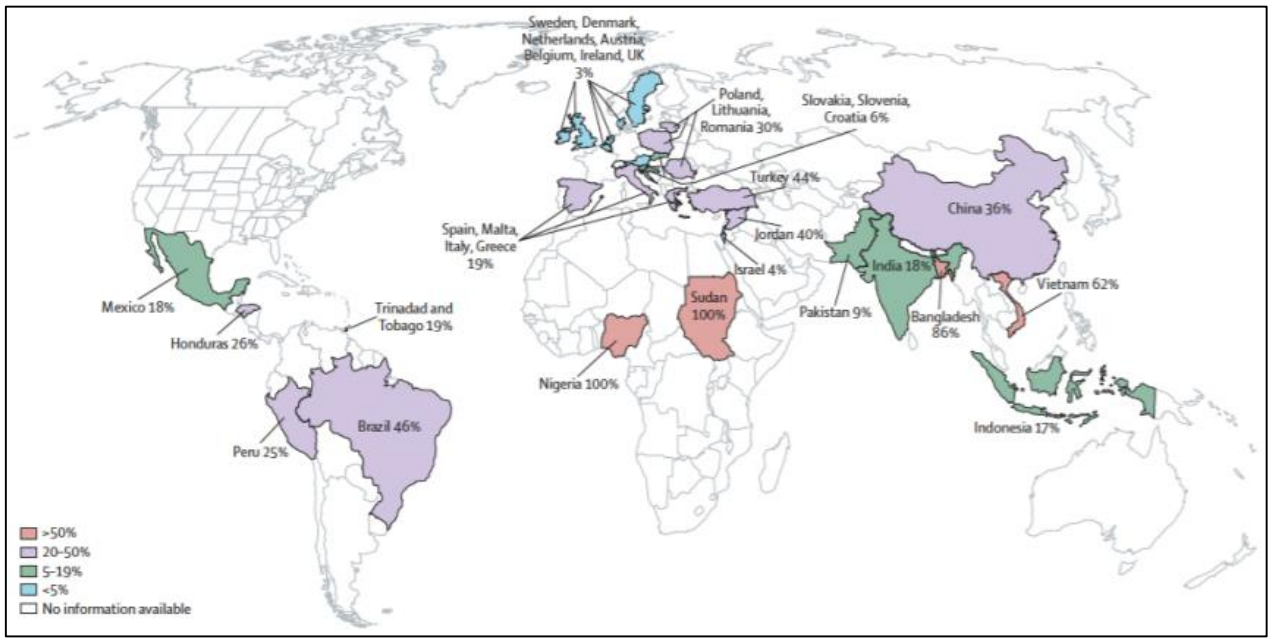

Figure 4-2 Antimicrobial use without prescription in some of the countries around the world (Gelband and Delahoy, 2014).

The issue of the improper use of antibiotics seems to be a general problem in populations with lower education, which do not seem to be aware of the consequences. This was also shown in a study conducted in Northern/Western and Eastern Europe in 2007 (Grigoryan *et al.*, 2007). In developing countries there is a general misinformation which leads to

misuse of antibiotics. The majority of the population believes that antibiotics are suitable for treating fever but also that antibiotics help overcome fatigue and malaise. A high number of people in these countries also think that antibiotics help as a pain killer (Cagri Buke *et al.*, 2003).

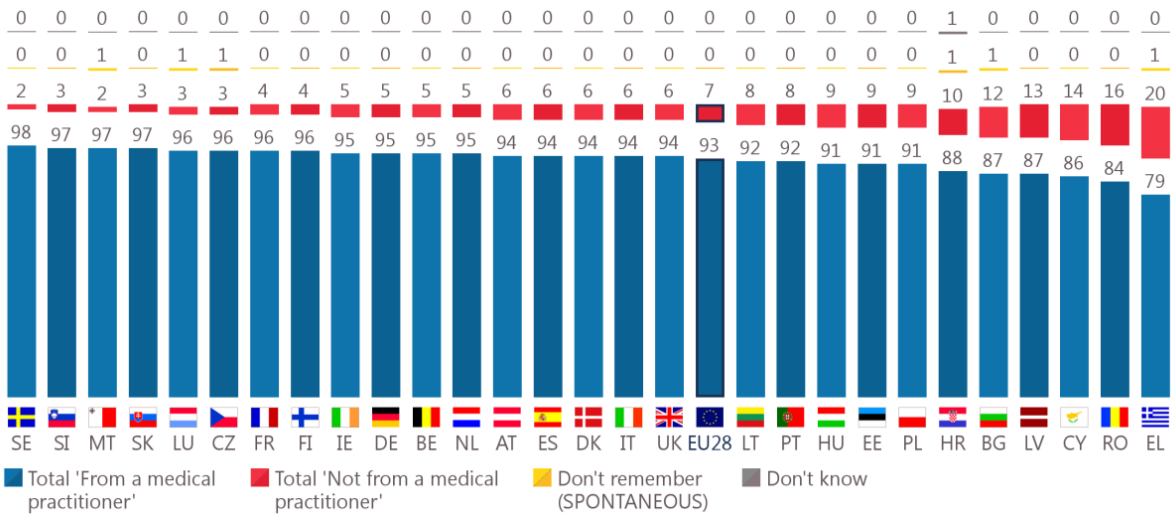

Figure 4-3 Antibiotic purchase in the European countries in percentage. In average 93% of Europeans obtain their antibiotics through prescription from a medical practitioner. Blue bars present total `from a medical practitioner, red bars the total `Not from a medical practitioner, yellow bars Don´t remember, grey bars Don´t know (EU-Report, 2016)

Ignorance about antibiotic usage and the lack of awareness of consequences of misuse is an important factor. This ignorance appears to be high not only in low educated patients but even in health care workers such as nursing or pharmacy students in Kosovo (Fejza *et al.*, 2016). Thus patients are misinformed or uninformed and the consequence is that they do not keep the usual treatment rules on completing the therapy, they just stop to take the antibiotics after they feel better (Veseli, 2015).

Antibiotic usage appears to be high also in Kosovo´s hospitalized patients, adults and children. Antibiotics are seemingly mostly used in cases with infections of respiratory tract, such as pneumonia and bronchitis (Raka *et al.*, 2016). In the biggest hospital in Kosovo, the University Clinical Center of Kosovo, β-lactams seem to be the antibiotics that are by

far the most prescribed drugs (Veseli, 2015). This appears to be a problem in the region of South Europe even for children and neonates (babies that are younger than 30 days) as Versporten and his collaborators published on 2016, which may be seen in Figure 4-4 (Versporten *et al.*, 2016).

All these factors assist strongly to the appearance and increase of antibiotic resistance. In this manner, Kosovo seems to be the leading country, with exception of vancomycine resistant Enterococci. The resistance rates in Kosovo seem to be 2 to 5 times higher than the ones presented by European Antimicrobial Resistance Surveillance Network (EARS-Net) (Zajmi *et al.*, 2017).

Alarming is the fact that cephalosporine and aminoglycoside resistance in *E.coli* appears to be very high in Kosovo and its neighbor country Former Yugoslavian Republic of Macedonia, as presented in the figure below (Figure 4-5). The data are taken from the hompage of CDDEP (https://resistancemap.cddep.org/AntibioticResistance.php). The reason for the high usage of aminoglycosides in developing countries is their low cost and their easy availability (Talaska, Schacht and Fischel-Ghodsian, 2006). Meanwhile in industrialized countries they are used usually in life threatening sepsis in children, intra-abdominal infections, urinary tract infections, or as alternative drugs in cases of infections with resistant bacteria, for example, infections with fluoroquinolone resistant bacteria (Xie, Talaska and Schacht, 2011).

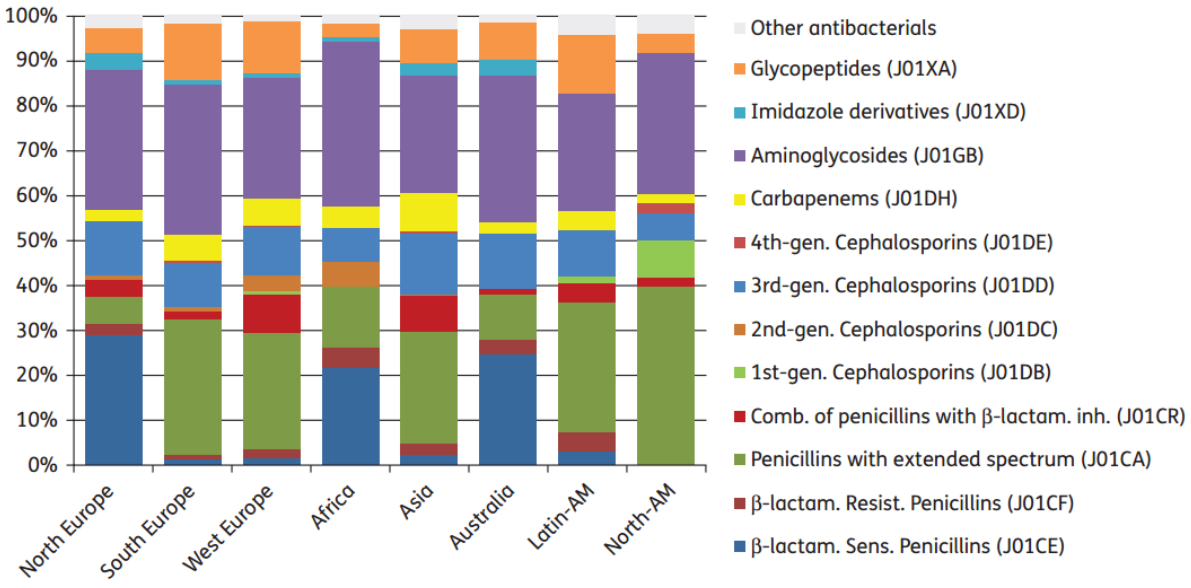

Figure 4-4 Antibiotic usage in hospitalized neonates (babies younger than 30 days). Kosovo is included in South Europe, where seemingly the highest usage of broad spectrum penicillins might be observed (green bars) (Versporten *et al.*, 2016).

Antibiotic usage seems not very different in the neighbor countries. As may be seen from Figure 4-2 the usage of broad spectrum penicillins in Serbia, Croatia and Bosnia and Herzegovina is quite high. The resistance data showed also a similar trend, the resistance to 3rd class cephalosporines was lower in Croatia and Slovenia, but also in Serbia, compared to Kosovo and Macedonia (Figure 4-5). Penicillins with extended spectrum appear to be mostly used in Albania as well, followed by cephalosporins (Hoxha, Malaj and Malaj, 2015). In Albania the usage of antibiotics in hospitalized patients seems to be also quite high compared to the European countries. The prescriptions are mostly in cases with urinary tract infections, surgery patients and pneumonia (Faria *et al.*, 2007).

Antibiotic usage in animals is an important point to consider, which appears to be high in Kosovo, especially the use of β-lactams. Even more alarming is the fact that farmers – according to Ibrahimi and his colleagues - appear to apply the antibiotics without following any treatment protocol (Ibraimi *et al.*, 2015). The inappropriate use results also in residues of β- lactams which have been found in cattle milk in many regions of Kosovo (Rama *et al.*, 2016). In Albania there have been extremely high resistance levels in *E.coli* and

Salmonella as a result of high antibiotic usage in poultry farming (Alcaine *et al.*, 2016). Unfortunately there is no systematic monitoring in Serbia where no data exist on pathogenis *E. coli* or data on antibiotics application in farming (Knezevic and Petrovic, 2008).

The high usage of antibiotics in all these fields and all the mentioned factors are highly connected with the resistance increase. In particular developing countries, where antibiotics are one of the most sold and used medications, have a high potential on developing resistances (Cagri Buke *et al.*, 2003). Lack of information and knowledge about antibiotic usage and about the importance of finishing the therapy might be one of the reasons why many people stop taking the antibiotics as soon as the symptoms disappear. This pushes resistance development forward and the risk of infections with resistant strains becomes very high.

The ungoverned usage of antibiotics in Kosovo and its neighbor countries has the consequence of resistance development, which is very high compared to Austria. In particular the resistance of *E.coli* to the 3rd generation of cephalosporins must not be ignored (Figure 4-5).

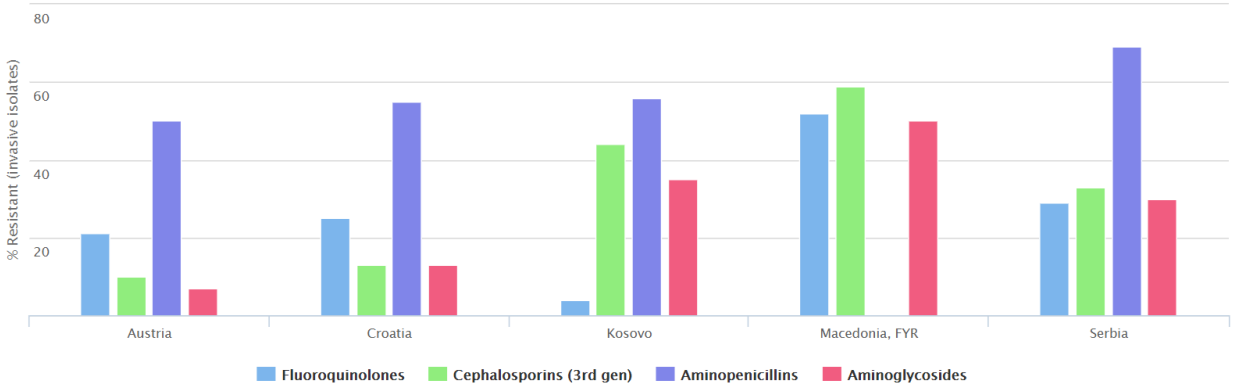

Figure 4-5 Antibiotic resistance of *E.coli* in Austria, in comparison to Kosovo, Macedonia, Serbia and Croatia (https://resistancemap.cddep.org/AntibioticResistance.php)

Resistant *E.coli* were found in animal farming in Serbia, where *E.coli* from animal intestines appear to have high resistance to ampicillin, amoxicillin combined to clavulanic acid and aminoglycosides among others (Knezevic and Petrovic, 2008).

It is shocking that even in developed countries such as Austria and Germany the presence of antibiotic resistant microorganisms was recently found in meat and milk, respectively. In Austria antibiotic resistant microorganisms have been found in meat that was meant to be consumed and was purchased directly from the food store. In the latest Greenpeace test, 3 out of 6 samples that were taken from food stores in Austria had antibiotic resistant microorganisms such ESBLs *E. coli* and one sample even with MRSA *Staphylococcus aureus* (Greenpeace, 2017). In the neighbor country, Germany, Schwendener and colleagues isolated antibiotic resistant *Micrococccus casealytis* strains from bovine and canine sources (Schwendener, Cotting and Perreten, 2017). These are only two examples that have been published some weeks ago in the Austrian newspaper, Der Standard.

5 Discussion

Knowledge and information about antibiotic use and effectiveness are the key to lower antibiotic resistance and better treatment chances

During the research for this thesis it became clear that there is a lot of knowledge and information on antibiotics effectiveness, how they should be used, in what indications and to what extent. The many published studies on antibiotic resistance made obvious that resistance development is very rapid and might affect all antibiotics. This has been recognized already as one of the biggest problems in healthcare.

Dealing with antibiotic resistance is one of the major problems that health institutions need to face. Therefore a proper effective governance is necessary in order to allow a sustainable access and in order to impede the rapid resistance development. New regulations or at least the reformation of existing ones might be a starting point. It might include regulations that are effective in all markets, all actors - not only at national level (Merrett, Bloom and Wilkinson, 2016).

There is a lot of work to be done in raising the awareness to this problem so that the necessary arrangements might be organized to help dealing with this issue. It has been mentioned during this thesis that the resistance development is not a simple problem. On the contrary, it is a very complex system that needs to be managed and controlled from different aspects and areas which have to be connected in this issue. This should include health care specialists, microbiologists, policy makers, ecologists, legislative bodies, educationalists, as well as pharmaceutical and agricultural industry (Aminov, 2010). It is important to build a successful relationship between all these areas in order to have a proper antibiotic use governance. This collaboration should go over national borders and achieve global levels (Merrett, Bloom and Wilkinson, 2016).

The rapid resistance development to current antibiotics increases the necessity of fast and more effective new drugs. When looking for new antibiotics one should always also consider the possibility of resistances that may come from the targeted bacteria.

Nevertheless the discovery of new antibiotics that might have new action mechanisms is a scientific bottleneck (Laxminarayan *et al.*, 2013).

The best method to avoid resistance development is to avoid the need for antibiotics. A very important starting point to work on is the body immune system. This would mean to do more in order to strengthen the body immune system by healthy, organic and vitamin rich food, healthy living and avoiding consumption of beverages that might harm or weaken the immune system. Furthermore the strengthened immune system would protect the body also from viral infections and other diseases, which would mean that the need for mistakenly prescribed antibiotics for viral infection would also diminish. The WHO report from 2015 states: “Every infection prevented is one that needs no treatment” (ECDC, 2015). Thus the need for antimicrobial treatment declines which leads to less usage of antibiotics which again leads to decreased antibiotic resistance development.

Another point that should be considered is the infection prevention through hygiene and good sanitation. These factors and other infection prevention scopes might impede the expansion of infections that might be hard to treat and infections with antibiotics resistant strains (ECDC, 2015).

It should not be forgotten that antibiotic therapy also has also numerous undesirable side effects in the human body and also in the intestinal microbiota of the host. It may lead to a decline of the number of beneficial microorganisms and to the development of microorganisms that might be pathogenic (Fernandes *et al.*, 2017).

One of the measures that is needed to help preventing antibiotic resistance should be the reduction or even arrest of antibiotic use in areas where it is not absolutely essential or where they might be replaced by alternatives. This might be the case in animal growth promotion and in routine prevention (Laxminarayan *et al.*, 2013). Antibiotic and in general antimicrobial usage in animals is an important factor that influences the resistance development and should be condensed to help resistance prevention. However, since the usage in animals is also necessary proper strategies to minimize the effect in the resistance development should be thought of and should be included in any systemic antibiotic usage and surveillance plan (Landers *et al.*, 2012).

Thinking of all the mentioned factors and areas that are involved in antibiotic usage and are assisting in the increase of bacterial tolerance to antibiotics, it is more than obvious that a global outstanding strategy is necessary to improve the antibiotic use and to avoid misuse. As WHO proposes in 2015, there is a need for national action plans on this important issue in every country. It is clear that this takes time and would be a long-term investment. It will also need a lot of resources in many fields such as laboratories, surveillance, human and animal health structures, operational research, professional education and regulatory capacities, and furthermore it will need the international collaboration and political commitment (ECDC, 2015).

- A helpful first move is probably the assessment of the appropriate usage of antibiotics by the scientific community which should include: firstly, parameter definition on effectiveness of antimicrobials and in which areas they need to be applied, secondly it should be a process that is evidence based normative and reflected from former antimicrobial programs this achievement should be deliberately inspected and updated depending on the antibiotic usage and resistance developing (Merrett, Bloom and Wilkinson, 2016).

The plan of the World Health Organization (WHO) published two years ago and called “Global action plan in antibiotic resistance”, defines five interesting objectives that should be implemented globally:

1st objective points out that the awareness and recognition of resistance to antibiotics should be improved by education, communication and training.

2nd objective makes clear that the knowledge and the evidence should be intensified using surveillance and research.

3rd objective is about prevention and declining of the infection rates by arrangements such as effective hygiene and sanitation.

4th objective is about antimicrobial drugs and its optimization in human and animal treatments.

5th objective examines the financial part, where it is proposed to build an economic case for all countries that want to do sustainable investments and it also proposes investment increase in, for example, diagnostic tools, vaccines, new medicines (ECDC, 2015).

Nevertheless, all countries should survey and collect the data of antibiotic usage in all fields especially in animal health, prophylaxis as well as growth promotion (Laxminarayan *et al.*, 2013). A successful example is Sweden that has developed a task force to inspect the national efforts, which has significantly impacted the antimicrobial usage limitations (Merrett, Bloom and Wilkinson, 2016). This is very important since the nontherapeutic use in animals and agriculture helps increasing the number of antimicrobial resistant strains, as it has been proven that vancomycin resistant strains were increased as a consequence of using related drugs in pig farming (Davies, 2013).

The increase and development of antibiotic resistance seems to be unstoppable (Davies and Davies, 2010). It is alarming that many of the mostly used and sold antibiotics are about to become obsolete in the world for the reason of the resistance invasion (Nicolaou *et al.*, 1999). The most selling antibiotics after Nicolau and colleagues are presented in Figure 5-1 which has been slightly modified.

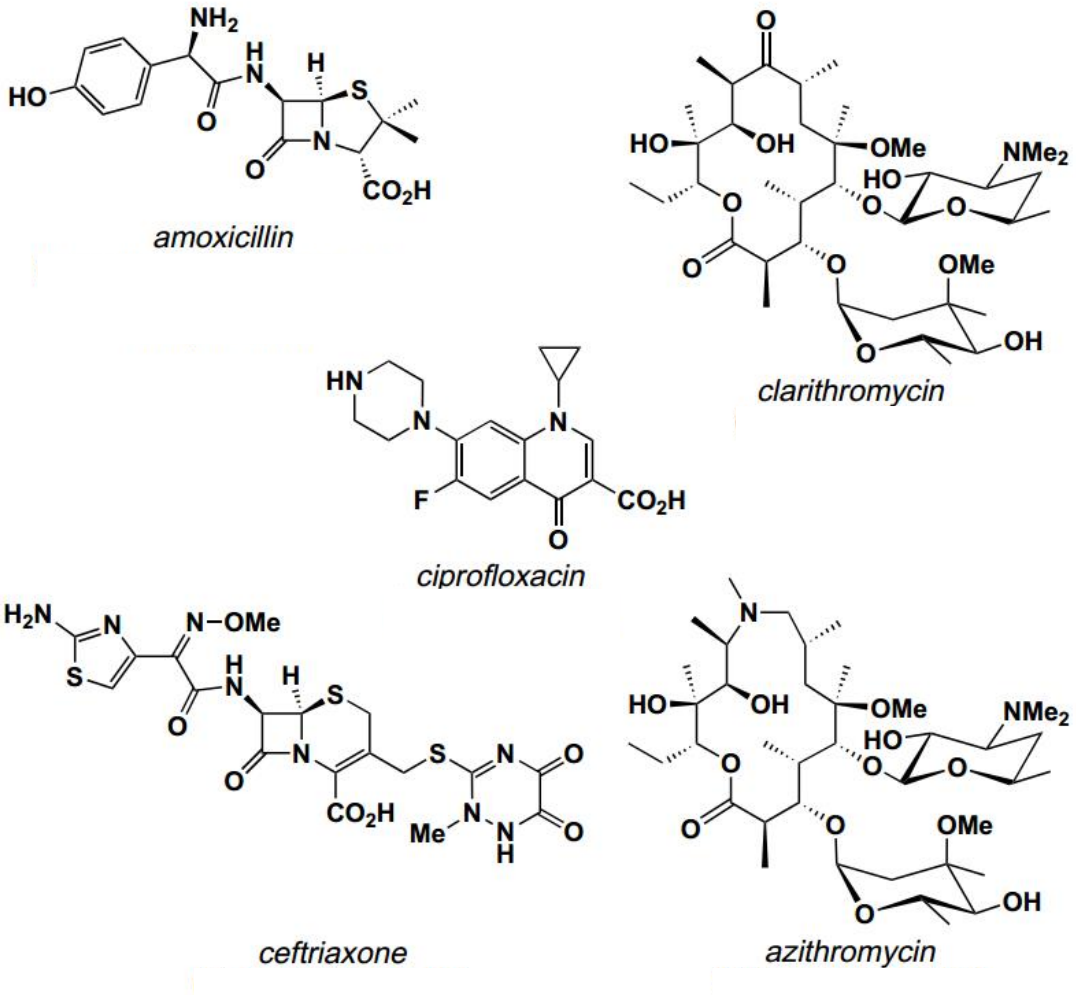

Figure 5-1 Most selling antibiotics after Nicolau and colleagues in 1997 (Nicolaou *et al.*, 1999)(modified)

Avoiding antibiotic usage *prevents* resistance development

As mentioned previously, antibiotics are one of the most used drugs worldwide and especially in developing countries. The possibilities to avoid or substitute antibiotic drugs usage are very scarce. Nevertheless there have been many efforts on reducing antimicrobial usage.

On this manner, there have been many attempts on using other medicinal sources on treating diverse infectious diseases. An example is the investigation and the benefit from

the Traditional Chinese Medicine (TCM). An example that was quite popular is the usage of the artemisin (qinghanousu) drug for its potent efficacy against malaria. Furthermore there is a range of herbal drugs that appear to be of antimicrobial exertion (Aminov, 2010).

Other alternatives on avoiding antibiotic use have been presented by Nigam and colleagues in 2014. They introduce a summary of alternative classes promoted also by the scientific community. These classes are:

1. phages, a different set of antibacterial effective viruses
2. bacteriocins, bacterial peptides that interact and modify bacterial membrane which results in bacterial killing
3. killing factors, which are conferred to be used to kill other bacteria in the starvation phases
4. non-antibiotic drugs with antimicrobial activities, although it is not clarified how exactly their antimicrobial mechanism is working, it has been shown that some drugs such as barbiturates, diuretic drugs, antihistamines etc. display antibiotic activity
5. quorum sensing, bacteria appear to communicate through messenger molecules which may be used as a therapeutic starting point (Nigam, Gupta and Sharma, 2014).

Resistance development should be our all concern, no matter where it appears in the world

Antibiotic usage survey is possible if proper standardized and feasible methods such as ESAC-Net (European Surveillance of Antimicrobial Consumption) of the European Centre for Disease Prevention and Control (ECDC) are developed. The establishment of a worldwide network which oversees developed as well as developing countries conquering the irrational use of antibiotics, should be a top priority objective of health institutions (Cagri Buke *et al.*, 2003).

Furthermore, the lack of databases about antibiotic consumption that are validated and comprehensive should be managed at European level. These data should consist of evidence of antibiotic use, misuse and overuse as the most important parameters and resistance inducing factors (Elseviers *et al.*, 2007). Since resistance appearance is different in countries worldwide partially mirroring the capacity of that country concerning infections and prescribing control (Livermore and Pearson, 2007).

Antibiotic resistance issue needs to be approached by each country with its particular method. This approach relies on the particular requirements and concerns of that country. Nevertheless it should be mandatory for each country to establish regulations on prescription, usage and distribution of antibiotics and also to make sure that they are followed, in human and veterinary medical fields but also very importantly in agriculture. This would help to diminish and maybe abolish the antibiotic purchase governed by cultural and economic reasons (Aminov, 2010).

This concerns mostly developing countries and also some of the countries in Southern Europe as has been shown in the previous chapter. The scarce variety of publications and the missing data should also be improved in the future. More studies in this field are needed.

A major aspect is the necessity of strictly controlling the implementation of the mandatory regulations over antibiotic prescription. This should happen in health institutions, pharmacies, farms, agricultural areas and all other fields where antibiotics are or might be used. In Albania, for example, antibiotic sale without prescription is alarmingly high although there are regulations in the existing legislations that prohibit pharmacists to sell antibiotics without prescription (Hoxha, Malaj and Malaj, 2015). Therefore the implementation of these regulations should be guarded and any violation should be sanctioned. Only this strictly followed rules enable the control and management of correct antibiotic use which will also diminish the big variation between the countries.

The lack of regulations and poor institutional management are noticeable also in other southern European countries such as Kosovo, Serbia etc. The developed European countries might be a crucial factor to help these countries on their way to develop their

legislation and also to build their controlling and managing institutions. This is very important for the whole region, but also for Europe, since, as mentioned in the previous chapters, antibiotic resistance might expand very quickly. The assistance developing this kind of survey mechanisms combined with the proper education of the personnel that is involved in antibiotic prescription and dispense should help to improve the growing antibiotic resistance issue and support its prevention.

6 Conclusion

Antibiotics as most used drugs are also connected with a range of using issues and also the rapid development of resistance in bacteria against them.

Many efforts have been started from different organizations in order to better manage these issues and to diminish the resistance development. However, there is still a lot of work to do:

- Education of health staff especially on antibiotic effectiveness and correct use in different areas: human medicine, veterinary applications, animal husbandry as well as the environment and the food chain
- Better information of people through various projects, in urban as well as in rural areas even in developed European countries such as Austria, Germany, France etc. where more than half of the population - on average – think that antibiotics kill viruses (EU-Report, 2016)
- Developing countries should be helped or instructed on establishing own regulations and legislations in accordance to European legislations
- Countries of southern Europe especially Kosovo, Serbia, Albania, Bosnia, and Macedonia, are in emergent need for cooperation in managing, controlling and creating new efforts on undergoing their health system issues and in particular on antibiotic usage and dispersion

Working on all these areas and developing new innovative strategies on managing and reducing antibiotic resistance development should be of very important manner at national and on global level. It is pleasing to know that a lot of work has been done and many efforts have started on this issue. Yet, there is still a lot to accomplish in order to have the desired results.

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