**Diplomarbeit**

# *Mechanisms of antibiotic resistance in gram-negative bacteria and their impact on health care in low-income countries*


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*Vorwort*

*Meinen engen Freunden und meiner Familie möchte ich großen Dank aussprechen. Sie haben mir ermöglicht, eine sorgenlose, lehrreiche und wunderbare Studienzeit in Graz zu verbringen.*
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**Table of abbreviations**

| |
|---|
| ABC... adenosine tri phosphate binding cassette |
| acetyl-CoA... acetyl coenzyme A |
| AMR... antimicrobial resistance |
| ATP... adenosine tri phosphate |
| DNA... desoxyribonucleic acid |
| EDTA... ethylenediamintetraacetate |
| EHEC... enterohemorrhagic Escherichia coli |
| EPI... efflux pump imhibitor |
| GIM... German imipenemase |
| GlcNAc... N-actetylglucoseamine |
| GTP...guanosine tri phosphate |
| IMP... imipenem hydrolyzing metallo beta lactamase |
| MATE and multidrug and toxic compound extrusion () family |
| MDR... multi drug resistant |
| MFS… major facilitator superfamily |
| MIC... minimal inhibitory concentration |
| MurNAc... N-acetylmuramic acid |
| $NAD^+$... nicotinamide adenine dinucleotide |
| OECD… Organization for Economic Cooperation and Development |
| PBP... penicillin binding proteins |
| pH... potentia hydrogenii |
| PDR... pan drug resistant |
| QRDR... quinolone resistance determining region |
| RND... resistance nodulation division () family |
| RRDR... rifamicin resistance determining region |
| rRNA... ribonucleic acid |
| SMR... small multidrug resistance () family |
| SPM-1... São Paulo metallo beta lactamase |
| STEC... shiga toxin producing *Echerichia coli* |
| UDP glucose... uridine diphosphate glucose |
| VIM... Verona integron-encoded metallo beta lactamase |
| WHO... World Health Organization |
| XDR... extensively drug resistant |

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**Zusammenfassung**

Eine der wichtigsten Errungenschaften der Medizin ist zweifellos die Entdeckung und Weiterentwicklung von antimikrobiellen Substanzen. Jedoch hat die Entstehung und Verbreitung von Antibiotikaresistenzen in den letzten Jahren ein noch nie da gewesenes Ausmaß erreicht und es wird geschätzt, dass innerhalb der nächsten dreißig Jahre die Folgen dieser Resistenzen die häufigste Todesursache sein werden und somit Krebs überholen. Die heute beobachtbaren klinischen Folgen und die, die wir für die Zukunft erwarten, umfassen weit mehr als nur die eingeschränkte Therapie von Infektionen. Vielmehr sind wichtige medizinische und sozio-ökomische Bereiche gefährdet, die wir durch die Verwendung von potenten Antibiotika als selbstverständlich wahrnehmen. Häufige chirurgische und diagnostische Eingriffe sowie die antineoplastische Chemotherapie werden durch die Ausbreitung von Antibiotikaresistenzen gefährdet. Eine Zunahme von durch Antibiotikaresistenzen bedingten disability-adjusted life years und ein starker Kostenanstieg in Gesundheitssystemen werden bereits weltweit beobachtet. Eine weitere Steigerung wird für die Zukunft erwartet. In dieser Diplomarbeit wird die Struktur und Funktion von wichtigen molekularen Resistenzmechanismen von gram-negativen Bakterien zusammengefasst und das Zusammenspiel von Bakterium, Mensch und Umwelt betrachtet.
Die speziellen Probleme von Ländern mit geringem und mittlerem Einkommen in Bezug auf Antibiotikaresistenzen werden beleuchtet, da man davon ausgeht, dass ebendiese Länder besonders hart von den Folgen von Antibiotikaresistenzen getroffen werden und gleichzeitig auch über weniger Ressourcen und Möglichkeiten verfügen, deren Auswirkungen zu mildern. Anschließend werden potenzielle Lösungsansätze diskutiert, die dabei helfen können, die globale Entstehung und Ausbreitung von Antibiotikaresistenzen einzudämmen.

**Abstract**

One of the most important achievements in medicine has been the discovery and development of antimicrobial drugs. However the rise of antibiotic resistance has reached an exceptional magnitude over the last few decades and is predicted to surpass cancer as the worldwide leading cause of death within the next thirty years.

The clinical consequences of antibiotic resistance that we can see today and those we expect for the future encompass more than limited treatment of infection. They include a variety of health care and socio-economic associated problems that arise if global antibiotic resistance increases. The endangerment of common medical procedures and treatments such as caesarean sections and chemotherapy as well as an increase in disability-adjusted life years and exploding health care costs are among the list of consequences.

To get a better understanding of the emergence and spread of antibiotic resistance the molecular resistance mechanisms must be examined and brought into context with the anthropogenic and environmental factors that influence antibiotic resistance.

This thesis aims to summarize the structure and function of important molecular resistance mechanisms of gram-negative bacteria. Additionally it shines a light on the complex interplay between bacteria, humans and the environment.

With low- and middle-income countries being especially endangered because of socio-economic characteristics that disadvantage the mitigation of antibiotic resistance the specific problems of these regions are discussed and possible starting points to better the situation are elaborated.

# 1 Introduction

## 1.1 Definition of antibiotic resistance

Antibiotic resistance describes a structural or metabolic characteristic of bacteria to be unaffected by antibiotic agents. A strain of bacteria is called resistant against a chemical agent if the minimum inhibitory concentration is higher than the admitted maximum dosage. Therefore the chemical agent cannot be used to treat the bacterial infection properly (1).

Resistance to chemical drugs cannot solely be found in bacteria but in almost all microorganisms, that cause infections in humans. Especially multi-drug resistant *Mycobacterium tuberculosis* emerged as one of the most severe threats to modern medicine in terms of effective treatment of infections. Other microorganisms like *Plasmodium falciparum* have acquired resistance against commonly used agents to an extent that treating infected patients in many countries where malaria is endemic is a severe challenge (2).

This acquired or secondary resistance contrasts with innate resistance. Innate resistance to a certain drug results from pre-existing and genetically defined characteristics of bacteria, which render the treatment ineffective. Causes of this type of resistance might be the absence of target structures for the chemical agent or attributes of the bacterial cell membrane that prevent antibiotics to work in the first place (1).

Microorganisms that show acquired resistance can be classified according to the number of antibiotic agents and classes they are resistant to. Multi-drug resistance (MDR), extensively-drug resistance (XDR) and pan-drug resistance (PDR) are the common definitions to ensure comparability in resistance levels of bacteria between studies from different institutions and countries. Multi-drug resistant microorganisms are resistant to at least one agent in three or more antimicrobial classes. Extensively-drug resistant microorganisms are resistant to at least one agent in all but two or fewer antimicrobial classes and pan-drug resistant microorganisms show no susceptibility to any agents of any antimicrobial classes (3).

## 1.2 Selection pressure and acquisition of resistance genes

The very first natural antibacterial agent was isolated in the early 1920ies from the microorganism *Penicillium notatum*- a mould of the *Penicillium chrysogenum* group by Alexander Fleming (4). A multitude of natural antimicrobial compounds have been discovered ever since including chloramphenicol and streptomycin. Some of the antibiotic drugs that are produced synthetically today actually derive from plants. For example, fluorochinolones are based on a chemical structure found within the alkaloid chinine. During the purification of chloroquine of the "fever tree" *Cinchona succirubra* in South America the antibiotic properties of a side product were found and eventually the chinolones we know today were developed (5).

The synthesis of antibacterial agents by plants and other microorganisms simultaneously requires the coexistence of resistance mechanisms of the same microorganism to protect itself from its own biochemical products. Therefore, resistance mechanisms *per se* are not a new phenomenon triggered by the administration of antibiotics, but rather have been a necessity for antimicrobial producing microorganisms (6,7).

Metagenomic analyses of authenticated ancient DNA showed that bacterial resistance mechanisms against antimicrobial agents occur naturally and existed long before selective pressure of clinical antibiotic use was exerted (8).

Although resistance mechanisms seem to have existed for several thousands of years, the exposure of bacteria in the human gut to antibiotic agents over the last decades has led to an increase in environmental pressure and therefore rendered bacteria more resistant than in pre-antibiotic times (8,9).

Bacteria, like any other form of life, must adapt themselves to their environment over time to survive as the modern theory of evolution suggests. Natural selection is the main mechanism of adaptive evolution and it is based on the following principles:

a. Reproductive success: selection is an evolutionary process to ensure the survival of the genome of species. Adaption to the environment favours reproductive success to guarantee further existence of genetic elements (10).

b. Environment: the process of natural selection depends on the environment. Phenotypes that are beneficial in a certain environment will be favoured (10).
c. Variation: selection acts on existing differences among individuals regarding a specific heritable trait (10). The original source of gene variants that produce new heritable traits (= genetic variability) is random mutation (11). But in the case of bacteria, they do not solely rely on mutations to acquire new genetic material, but perform another technique to exchange DNA, which is called horizontal gene transfer (12).

### 1.2.1 Spontaneous mutation

The genome of bacteria is represented by two types of structures: the bacterial chromosome and the extrachromosomal genetic elements, called plasmids or episomes (13,14).
While the bacterial chromosome contains genetic information, which is vital to the basic function of the bacteria, the extrachromosomal elements host a vast variety of additional nucleic sequences to accommodate for characteristics like the ability to perform horizontal gene transfer through f-pili or for resistance genes. The quick exchange of genetic elements through the extrachromosomal genetic material enables bacteria to acquire an enormous variability within their DNA over a relatively short period of time (13).
Spontaneous chromosomal mutations add to this variability of the bacterial genome. Mechanisms of spontaneous mutation include DNA replication errors, homologous recombination and mobilization of insertion sequences (15).
The random alteration of chromosomal sequences during mitosis combined with insufficient DNA repair mechanisms leads to genetic variation- a new allele. This spontaneous mutation will directly be passed down to the offspring. If the phenotype of this mutated gene benefits the bacteria it will prevail due to natural selection (16).
The mutations happen randomly and undirected. Therefore, mutations which turn out to be useful for resisting antimicrobial agents are present before the exposure of antimicrobial agents (11).

Certain accidental mutations have led to the creation of resistance genes whose translational products offered the bacteria a selective advantage when exposed to a distinct group of antibiotic agents. A mechanism of *Pseudomonas aeruginosa* that led to resistance to carbapenems originates from the mutation of the *oprD* (outer membrane porin protein) gene by interruption of the coding sequence by an insertion sequence. By inactivating the *oprD* gene the porins will not be expressed properly which leads to a lack of the entrance of carbapenems and therefore renders them ineffective (17,18). Resistance resulting from the inactivation of the *oprD* gene via interruption by an insertion sequence is also seen in *Klebsiella pneumoniae* (19).

Additional to spontaneous or accidental mutations, genomic instability in bacteria can occur, if bacteria are exposed to environmental stressors like a change in pH or the administration of antibiotics. Growth-limiting stress conditions like starvation, hypoxia or antibiotics induce mutagenesis to adapt to the altered environment. These stress-induced mutations occasionally generate fitter mutants and potentially accelerate adaptive evolution (20). Therefore, despite antibiotics being the main weapon to cure bacterial infections, they are also one of the main factors driving the emergence of multi-drug resistant bacteria (21).

There are several molecular mechanisms governing stress-induced mutagenesis in bacteria. One important mechanism is the classical SOS response, which is activated upon bacterial DNA damage. RecA protein attaches to the ssDNA and together they facilitate the cleavage of a transcriptional repressor which leads to an upregulation of over 40 genes involved in processes regarding DNA repair (20).

Despite the variety of inducible and spontaneous mutagenesis, the spread of the currently existing resistance genes cannot be explained solely by the phenomenon of mutation. It has only been about six decades since the discovery and introduction of antibiotics as commonly used drugs. Hence, mutations during this time would not be enough to explain the current variety of resistance genes (22). On the other hand, the mechanism of horizontal gene transfer explains the pace of spreading new genetic variants in bacteria well (12).

### 1.2.2 Horizontal gene transfer

By absorption of external DNA, bacteria can perform a highly effective strategy to acquire a large number of new genes over a relatively short period of time. Bacteria can obtain new virulence factors, enzymes that expand the nutritional ability of the cell or resistance genes through horizontal gene transfer (23,24). Conjugation, transduction and transformation are the three known mechanisms that facilitate this genetic exchange:

*Conjugation* is the process of intercellular DNA transfer by physical contact of bacterial cells. Conjugative plasmids regulate the translation of enzymes which are necessary to perform this exchange (25,26). Many resistance plasmids are conjugative plasmids and therefore encode functions necessary to promote their own cell to cell transfer (12).

The coupling of two bacterial cells is mediated by the expression of a DNA sequence called F-factor. It encodes surface structures, named pili, for adhesion of the donor and the recipient. The F-factor can be present on conjugative plasmids or integrated into the bacterial chromosome. Strains of bacteria which hold a plasmid containing the F-factor are called $F^+$- strains and strains in which such plasmids are absent are called $F^-$ -strains (26,27).

DNA sequences which code for components of resistance mechanisms are called R-factors. Primordially originating from seldom spontaneous mutations, these resistance factors cannot only be exchanged between bacteria of the same species but also between bacteria of different species (28).

Multiple resistance genes are not tied to the DNA of plasmids or the chromosome but rather can move between DNA molecules. These mobile genes, referred to as transposons, are flanked by nucleotide sequences that mark the start and the end of the gene (26).

If mobile antibiotic resistance genes and plasmids become associated with strains that have high epidemic potential, the clones become high risk (29).

*Transduction* is the transfer of nucleotide sequences from a donor to a recipient cell *via* a virus particle, a so-called bacteriophage. The viral genome is integrated into the host DNA and during replication and emergence of the viral capsid parts of the original viral genetic material are replaced by fragments of the bacterial DNA (30). Depending on the quality of the transferred genetic material two mechanisms

of transduction can be discriminated. Generalized transduction is the transfer of any part of the bacterial chromosome. If only specific parts can be transferred and insertion of the genome is limited to particular regions on the host chromosome it is called specialized transduction (31).

Antibiotic resistance genes are much more frequently transferred by generalized than by specialized transduction. The reason being is that antibiotic resistance genes are not likely to be located in core genome regions, which are the common sites for prophage integration into the chromosome (31). The closer two genes are located on the chromosome the greater the probability for them to be transferred together by a bacteriophage (30). Phage mediated transduction of antibiotic resistance genes varies between bacterial species and strain leaving some bacteria more prone to it than others. *Enterococci* are gram positive bacteria in which resistance genes acquired by transduction are well documented. Resistance against tetracycline (*tetM* genes) and gentamicin (*ant2-I* genes) has been identified to be transferred via a series of phages (32). Phages even enhance persistence of resistance genes, which was shown by a study testing phages and bacteria after aggressive inactivating treatments in wastewater (33).

Interestingly, antibiotic agents themselves can induce the expression of prophage gene products. In the case of shiga-toxin producing *Escherichia coli* (STEC) it was found that fluorochinolones, trimethoprime/sulfamethoxazole and a variety of beta-lactams, can rapidly induce an increase in the expression of shiga-toxin genes. This discovery might explain why antimicrobial therapy of disease caused by STEC is seen as a risk factor for progression to haemolytic-uremic syndrome (34,35). Similar outcomes have been documented for the administration of norfloxacin in patients with infection by enterohaemorrhagic *Escherichia coli* (EHEC) (36).

*Transformation* describes the process of exogenous DNA uptake, integration and expression without having direct contact with the donor cell. Bacteria, which are able to alter their genome in this way, are called naturally competent. Although natural transformation is thought to be a less significant mechanism in horizontal gene transfer regarding antibiotic resistance genes, research concerning this hypothesis is still ongoing. Recently, it has been discovered that *Acinetobacter baumannii* is able to acquire foreign DNA by transformation and this led to the

suggestion that this mechanism might have an influence in the establishment of antibiotic resistance within this strain (37).

# 2 Material and methods

This thesis is organised into the introduction, where the definition of antibiotic resistance and basic bacterial genetics are described. In the second section literature covering molecular mechanisms of resistance as well as contributing environmental and anthropogenic factors is summarized. Lastly, consequences of increasing resistance against first-line antibiotics in low- and middle-income countries are specified and the resulting challenges in health care are debated. For the aforementioned chapters a thorough literature search has been conducted and the main data bases from which studies and systematic reviews were extracted included PubMed and Google Scholar. Latest data provided by the World Health Organisation and the World Bank was retrieved from their official websites and the infectiology textbook "Medizinische Mikrobiologie und Infektiologie. Springer-Verlag Berlin Heidelberg 2012" was used for identifying basic resistance definitions. The following table outlines the search terms used on their own or in combination for each chapter:

| **Chapter** | **Search terms** |
|---|---|
| Selection pressure and acquisition of resistance genes | *"selection pressure", "antibiotic resistance", "antimicrobial resistance", "resistance genes", "horizontal gene transfer", "resistance plasmids", "natural selection", "adaptive evolution", "spontaneous mutation"* |
| Molecular mechanisms of antibiotic resistance | *"molecular resistance mechanisms in bacteria", "hydrolysis resistance mechanism", "beta lactamase genes", "macrolide esterase", "penicillin binding protein", "antibiotic influx", "bacterial porins", "biofilm formation"* |
| Environmental factors and | *„environment", "drug residues in* |

| | |
|---|---|
| anthropogenic sources of antibiotic resistance | *wastewater", "antibiotics sewage water", "livestock farming", "aquacultures", "antibiotic growth promotion", „prescription practice", „imprudent use of antibiotics", "overprescribing", "inadequate drug prescription", "international travel", "spread of resistance genes", "spread of resistant bacteria"* |
| Impact of resistant gram-negative bacteria on infection treatment | *"impact of antibiotic resistance", "clinical", "economic", "burden", "consequences of antimicrobial resistance", "developing countries", "infection control in low- and middle-incomecountries", "attributed mortality for antibiotic resistance"* |

Literature published until February 2020 that was considered relevant for this thesis was included, preferring studies from the last ten years. If containing vital information, older studies and systematic reviews were taken into consideration as well.

# 3 Results

## 3.1 Molecular mechanisms of antibiotic resistance

The following table summarizes the most important molecular mechanisms that facilitate antibiotic resistance in gram negative bacteria.

| *Molecular mechanisms of antibiotic resistance* |
|---|
| **Production of drug-modifying enzymes**<br>Hydrolysing enzymes<br>Beta-lactamases<br>Esterases<br>Epoxidases<br>Group transferases<br>Redox enzymes |
| **Modification of target molecules**<br>Alterations of penicillin-binding proteins<br>Alterations of type-II topoisomerases<br>Alterations of DNA-dependent RNA-polymerase |
| **Modification of antibiotic influx** |
| **Release of chemical agents** |
| **Biofilm formation** |

*Table 1: Overview of bacterial molecular resistance mechanisms*

### 3.1.1 Production of drug-modifying enzymes

Enzymatic strategies of antibiotic inactivation have enabled multiple species of bacteria to become resistant. The main chemical reactions of molecular inactivation are hydrolysis, group transfer and redox reactions. All of these affect antibiotic compounds and consequently decrease drug concentration actively . (38)

#### 3.1.1.1 Hydrolysing enzymes

Hydrolysis is a chemical reaction where bonds are cleaved with water as co-substrate. Enzymes that facilitate this type of reaction can be excreted by microorganisms to their local environment and therefore antibiotic compounds could be inactivated before coming into contact with the microorganism. The group

of hydrolysing enzymes encompasses beta-lactamases, esterases and epoxidases. Beta-lactamases inactivate most antibiotics belonging to the beta-lactam class. Esterases contribute to macrolide resistance and epoxidases are associated with fosfomycin resistance (38).

#### *3.1.1.1.1 Beta-lactamases*

Beta-lactamases were the first enzymes that were discovered referring to the development of antibiotic resistance and are still the most well-known. The expression of a penicillinase was first documented in *Escherichia coli* and since then has led to the discovery of a variety of bacteria being capable of producing beta-lactamases (39). The well-established classification system of Bush et al. differentiates four different groups of beta-lactamases according to their nucleic sequence. Group A (penicillinases), C (cephalosporinases) and D (oxacillinases) are similar in structure consisting of an active site with serine as central acting molecule. Otherwise, group B (metallo-beta-lactamases) is known to contain a zinc-ion in the centre of the active site (40). In recent years, an updated classification system of beta-lactamases, that takes the enzymatic function, and less their structure, into account, has been established. Arranging the enzymes according to their function has turned out to be practical in everyday clinical use. The updated functional classification of beta-lactamases consists of three groups. The first one includes cephalosporinases, the second group encompasses penicillinases as well as oxacillinases and the third group is equivalent to the metallo-beta-lactamases (41).
Beta-lactamases that comprise a serine at their active site are called serine-beta-lactamases and perform the ring-opening by a nucleophilic attack on the lactam-ring. This first step is followed by hydrolytic cleavage of the covalent enzyme intermediate (38). Interestingly, this mechanism is similar to the action of bacterial transpeptidases, which are responsible for catalysing reactions of cell wall synthesis adding peptides to the growing peptidoglycan (42). Theses bacterial transpeptidases act as penicillin binding proteins and are target structures for beta lactam antibiotics (43). A structural analysis of a group 2 beta-lactamase and a bacterial D-alanyl-D-alanine-carboxypeptidase/-transpeptidase showed homologous three-dimensional structures and it seems that these two enzymes

follow similar catalytic strategies. Therefore, it is likely, that certain beta-lactamases are derived from an enzyme with D-alanyl-D-alanine-activity (42).
The second group of lactamases are metallo-beta-lactamases, which are a diverse protein family that require one or two zinc ions for activity. Based on sequence alignments three subclasses B1, B2 and B3 can be distinguished (44). According to phylogenetic analyses these enzymes are ancient. The function of subclasses B1 and B2 traces back to almost 1 billion years ago. Subclass B3 evolved even before, supposedly 2 billion years ago (45). Most of the beta-lactam antibiotic classes can be degraded by metallo-beta-lactamases and especially carbapenems can be inactivated efficiently. However, monobactams are not known to be degraded by metallo-beta-lactamases. With carbapenems being the most potent class of beta-lactam antibiotics, resistance mechanisms against them are especially worrisome. Furthermore, no susceptibility to beta-lactamase inhibitors, like clavulanic acid, sulbactam or tazobactam, is described in metallo-beta-lactamases. The prevalence of metallo-beta-lactamases is facilitated by their genes being carried on mobile genetic elements and by genes being present in environmental species, constituting reservoirs of beta-lactam resistance genes (44).
Enzymes of the subclass B1 comprise a key zinc ion coordinating the residues of three histidines and one cysteine and encompass the metallo-beta-lactamases IMP, VIM, GIM and SPM-1. Subclass B2 enzymes have similar zinc-binding sites with the first position being asparagine instead of histidine. The tetramer L1 represents the types of enzymes belonging to the subclass B3 (46).
The subclasses B1 and B3 act on a broad spectrum and hydrolyse most of the beta-lactams including carbapenems whereas the subclass B2 consists solely of carbapenemases. Another possibility to categorize the metallo-beta-lactamases is to differentiate their genetic organization. While the genetic sequence of some enzymes is encoded by the chromosome, such as *BcII* from B. cereus, others are located on plasmids, which can be easily transferred through horizontal gene transfer because of their extrachromosomal origin (44). Metallo-beta-lactamases of subgroup B1 and B2 that are encoded by plasmids include the IMP (imipenemase) group and the VIM (Verona imipenemase) group. (45). The transferable metallo-beta-lactamases are commonly associated with integrons (44). Integrons are genetic elements that consist of an integrase gene (*intI1*, *intI2*,

*intI3* and *intI4*) and a recombination site (attI). According to the type of integrase, classes 1-4 can be distinguished. Integrons are capable of integrating and exchanging specific DNA sequences, so-called gene cassettes (47). Gene cassettes which carry resistance genes are able to move from one integron to another but depend on other genetic elements, such as plasmids or transposons, to be transferred between bacteria (46). The huge capacity of integrons to exchange and integrate gene cassettes enables them to express a wide range of antibiotic resistance genes. This facilitates rapid adaptation to selective pressure applied by administration of antibiotics (48).

Inhibitors of beta-lactamases have been well established over the last decades and helped to compensate for the evolving resistance against beta-lactam antibiotics. Compounds like clavulanic acid or sulbactam can be administered together with antibiotics and render them effective even in the presence of serine-beta-lactamases but remain inefficient towards metallo-beta-lactamases. Only some metal chelators, like EDTA, inactivate all metallo-beta-lactamases but they cannot be used therapeutically in humans and are therefore clinically insignificant (44).

Metallo-beta-lactamases are expressed in a variety of clinically important gram-negative bacteria like *Pseudomonas aeruginosa, Acinetobacter baumanii, Klebsiella spp., Escherichia coli* and *Enterobacter spp.* With resistance alleles being present on mobile genetic elements in above-mentioned bacteria, the spread of metallo-beta-lactamase resistance genes represents one of the most important challenges regarding the emergence of antibiotic resistance.(46)

#### *3.1.1.1.2 Macrolide esterases*

Macrolide esterases hydrolyse the ester bond of a cyclic lactone present in macrolides. The consecutive linearization of the molecule prohibits the binding to the ribosomal subunit rendering the antibiotic ineffective. Because the first macrolide esterase was an erythromycin esterase discovered in *E. coli*, the enzyme family is named after this erythromycin esterase. *EreA*, *EreB*, *EreA2*, *EreC* and *EreD* are the known genes that encode esterases that belong to this group although only *EreD* is chromosomally encoded. The other enzymes are encoded on mobile genetic elements, and are therefore easily transferable and

found not only in clinical but also in environmental isolates. *EreA2* especially seems to be clinically important because of its presence in a multitude of gram-negative bacteria like *Pseudomonas aeruginosa, Klebsiella pneumoniae, Escherichia coli, Salmonella enterica* and *Vibrio cholerae* (49).

#### *3.1.1.2 Group transferases*

Transferases are a class of enzymes that transfer functional groups from a donor molecule to an acceptor and thus are able to alter the function of a molecule dramatically. According to the functional group that is transmitted, different subgroups of this enzyme class can be distinguished. Acetyl-, Phospho-, Nucleotidyl-, Glycosyl-, ADP-ribosyl- and S-transferases are the enzyme subgroups which play an important role in modifying antibiotic drugs (50). These enzymes require co-substrates for activity (ATP, acetyl-CoA, $NAD^+$, UDP glucose or glutathione) and thus are only active in the cytosol (38).
In the case of aminoglycoside resistance, multiple transferases are responsible for the resistance mechanism. Aminoglycosides interact with the 16S rRNA, impairing the codon-anticodon decoding mechanism. The accuracy of translation is lost, which restricts cell function and eventually leads to cell death. If hydroxyl- and amino groups that are essential for binding to the 16S rRNA are altered, aminoglycosides cannot bind to their target and lose their efficacy. Enzymes that facilitate such alterations ultimately confer resistance to aminoglycosides. (38). N-acetyltransferases, O-phosphotransferases and O-adenylyltransferases mediate the modification of aminoglycoside molecules and render them ineffective. N-acetyltransferases use acetyl-CoA as a cofactor and ATP or GTP are used for donations of phosphate groups and adenine in the phospho- and adenylyltransferases (50). Aminoglycoside phosphotransferases, or aminoglycoside kinases, can be found in a variety of pathogenic bacteria. The responsible genes are localized on multidrug resistance plasmids, transposons and integrons, explaining their wide spread within the bacterial kingdom (38).

#### 3.1.1.3 Redox enzymes

Oxidation is commonly used in mammals to facilitate excretion of xenobiotics. In humans, membrane-bound cytochrome P-450 enzymes are responsible for these

redox reactions. Although these are common chemical reactions important for the function of cells, compared to hydrolysation and the transfer of functional groups, redox reactions are of minor importance in terms of antibiotic resistance. Nevertheless, some redox enzymes that accommodate for resistance mechanisms have been described (38). TetX is a FAD-dependent monooxygenase that is associated with tetracycline resistance. It catalyses monohydroxylation of the C-atom at position 11a of the tetracycline molecule which is followed by intramolecular cyclization and a subsequent non-enzymatic breakdown of the molecule (51). Monooxygenases also facilitate resistance against rifamycin antibiotics. The so-called Rox (rifamycin monoogygenases) incorporate a hydroxyl group at the second position of the naphtyl group in rifamycin antibiotics, which leads to linearization of the molecule and loss of function of the antibiotic (52).

### 3.1.2 Modification of target molecules

Bacteria are able to alter the molecular structures to which antibiotic drugs bind. This leads to decreased or total loss of antibiotic function. The binding of antibiotics to their target structures is very specific, even small changes in the bacterial molecular structure can lead to a deprivation of the ability to bind. Common bacterial target molecules for antibiotic drugs are penicillin-binding proteins, type II topoisomerases and DNA-dependent RNA-polymerases. These structural and/or functional proteins hold essential roles in the metabolism of bacteria like maintaining cell shape and DNA helices or facilitating transcription (53).

#### 3.1.2.1 Alterations of penicillin-binding proteins

Penicillin-binding proteins (PBPs) are located in the cytoplasmatic membrane or in the cytosol. These proteins play an important role in the synthesis of peptidoglycans of the cell wall. PBPs mediate the glycosyltransfer, meaning the assembly of MurNAc and GlcNAc chains and the transpeptidation, cross-linking of stem peptides. Interestingly, each bacterial species expresses one or two essential types of PBP (54). Beta-lactam antibiotics use PBPs as primary target molecules, mimicking a moiety of the stem peptide leading to a blockage of transpeptidation. The beta-lactam ring is a structural analogue of the D-Ala-D-Ala

dipeptide and inhibits PBPs competitively. Consequently, the cell wall synthesis is impaired which leads to death of bacteria. The mechanisms of bacteria to alter PBP include mutations in native PBPs, overexpression of low-affinity PBPs and synthesis of new entities of PBPs that cannot be affected by beta-lactams (50). Altered PBPs are well established as a resistance mechanism in gram positive bacteria like *Streptococcus spp.* and *Staphylococcus spp.*. Resistance of *Staphylococcus aureus* to methicillin and other penicillin-derivates is mediated by production of an additional type of PBP, PBP2a. This enzyme shows low-affinity to beta-lactams and can still work while being exposed to beta-lactams (50). Although modified PBPs are common in some gram-positive bacteria, they are rarely seen in gram-negatives. *Enterococci* are an exception, as they are naturally resistant to beta-lactams because of expression of PBP5fm, which shows low affinity to penicillin and its analogues (55).

#### 3.1.2.2 Alterations of type-II topoisomerases

DNA-gyrase and topoisomerase-IV are bacterial type-II topoisomerases that facilitate chromosomal supercoiling required for processes like DNA replication and protein biosynthesis. Both are vital for regulating the bacterial cell cycle progression. The enzymes are heterotetramers with two pairs of subunits, respectively, responsible for the formation of DNA complexes (GyrA and ParC) and for supplying energy for the catalytic processes (GyrB and ParE) (50). The DNA-gyrase applies super-helical density that allows chromosome condensation, it also releases torsional stress in pre-transcriptional and pre-replicational chromosome segments and promotes transcription initiation by the RNA-polymerase. Topoisomerase-IV supports DNA-gyrase in its function and obtains similar catalytic properties (56). It binds to distal DNA segments and captures intra- and intermolecular DNA crossovers. This way, topoisomerase-IV can relax chromosomal tension and detangle nucleotide sequences efficiently (57).
The antibiotic class of chinolones and their derivates, fluorochinolones, is characterized by their binding to the active site of type-II topoisomerases, prohibiting them from moving alongside the nucleotide strand and stopping the replication fork. Fluorochinolones form a complex together with the type II-topoisomerases and the binding site of the enzyme is called chinolone-binding pocket (50,56). The induced double-strand breaks of DNA trigger the intracellular

bacterial SOS-stress response and lead to overexpression of DNA repair enzymes (described in chapter “1.2.1 Spontaneous mutations”) (56).
At low antibiotic concentrations, fluorochinolones lead to bacteriostasis but with increasing doses, their administration can also be lethal to bacteria. The bactericidal effect of fluorochinolones increases when the induction of the SOS-stress response in bacteria is prevented (58).
If chromosomal mutations occur within the gene regions of type-II topoisomerases that code for the chinolone-binding pocket, called QRDR (quinolone resistance determining region), a decrease in drug affinity leads to resistance (56). Relevant alterations in the nucleotide sequence are located mostly in the N-terminal part of the GyrA or ParC subunit. Depending on the number of substituted amino acids and the position of alteration, resistance levels vary. In *E. coli,* minimal inhibitory concentrations (MIC) of fluorochinolones can be increased up to 32 times due to QRDR-mutations compared to the wildtype (50). Changes in the QRDR have also been found in *Enterococci* and are made responsible for high resistance rates against fluorochinolones. A Japanese study of *Enterococcus faecium* clinical isolates showed that an increase in the number of amino acid substitutions of the QRDR is positively correlated with an increase of the MIC. Therefore, mutations in the GyrA and ParC genes are highly suggestive of mediating the fluorochinolone resistance in the studied *E. faecium* (59).

#### 3.1.2.3 Alterations of DNA-dependent RNA-polymerase

DNA-dependent RNA-polymerase is the key enzyme in transcription and the target of rifamycins, a class of antibiotics vital for the treatment of tuberculosis and leprosy. The enzyme consists of five subunits: two α-, β-, β’- and σ-subunits. The first four subunits form the so-called apoenzyme, which performs all steps necessary for transcription. If the σ-subunit binds to the apoenzyme, the holoenzyme is formed, which is responsible for transcription initiation and recognition of bacterial gene promotors (50). Rifamycin resistance is commonly associated with mutations in the *rpoB* gene encoding the β-subunit. The so-called rifamycin resistance determining region (RRDR) is 81bp long and the main locus for resistance inducing mutations (60).

### 3.1.3 Modification of antibiotic influx

The envelope of gram-negative bacteria consists of several different layers and is more complex than the structure of gram-positive prokaryotes. In gram-positive bacteria the cytoplasmatic membrane is bordered by a thick layer of peptidoglycan whereas gram-negative bacteria have a thin oligomolecular mureine layer and a complex outer membrane that is unique to gram-negative organisms. There is a periplasmatic space between the cytoplasmatic membrane and the outer membrane (13).

The outer membrane of gram-negative bacteria is asymmetric with an inner and an outer leaflet. The inner leaflet consists of phospholipids and is similar to the structure of the cytoplasmatic membrane. The outer leaflet consists of phospholipids and lipopolysaccharides. Lipopolysaccharides can be further divided into three regions: the O-antigen, the core region and the lipid A (13).

The **O-antigen** being the most outer layer of the asymmetric outer membrane, differing highly from species to species, consists of hexose molecules and is responsible for triggering the production of specific antibodies in the host. The long hydrophilic side chains also protect bacteria from immunologic effectors of the host like the complement system. Inwards, the **core lipopolysaccharide** consists of galactose, N-acetyl-glucosamine and ketodesoxyoctonate. Disorders of the core lipopolysaccharide result in death of the bacterial cell. **Lipid A** borders the outer phospholipid layer and secures the lipopolysaccharide complex to the outer membrane. It is responsible for a multitude of pathophysiological effects of bacteria and is recognized by toll-like receptor IV and thus can trigger activation of lymphocytes. All layers of lipopolysaccharides together are called endotoxins, whereas only Lipid A is responsible for the toxic properties. If the bacterial cell is damaged, components of the lipopolysaccharide layer are exposed and trigger a pathophysiological reaction in the host organism that is specific to the bacteria.

The unique structure of the outer membrane of gram-negative bacteria limits permeability for a wide variety of molecules, leaving gram-negative bacteria intrinsically more resistant than gram-positive prokaryotes (13).

The outer membrane is traversed by channel proteins, so-called porins. Most outer membrane proteins have a β-barrel architecture and allow selective permeability (61). β-barrels are proteins that often function as transmembrane channels and

consist of multiple β-strands which are secondary protein structure elements. Interestingly, some microbial toxins contain β-barrel structures as well, like the α-haemolysin from *Staphylococcus aureus* (62).

The outer membrane channel proteins are water-filled and mediate transport of hydrophilic molecules into the cell. In contrast, hydrophobic compounds can diffuse directly through the lipid bilayer. Small nutritional molecules as well as hydrophilic antibiotics like quinolones and beta-lactams, can enter and metabolite products can exit the cell via integrated membrane proteins (61).

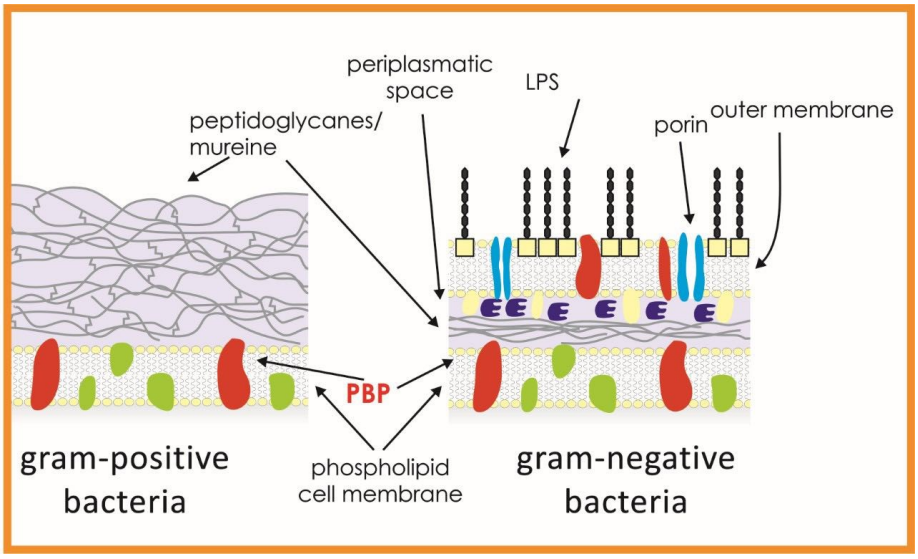


*Figure 1: Structure of cell envelope in gram positive and gram negative bacteria*

Porin channels show specific restrictions for the type of molecules that can pass. Depending on the type of outer membrane protein channel, size, hydrophobicity and charge of the molecules decide, if permeation is possible or not. Although the diameter of most porins is quite small, e.g. the OmpF porin of *E. coli* comprises 7 x 14 Å (1 Å = $10^{-10}$m), molecules with relatively high molecular weight can still pass through. Long flexible molecules with small cross sections may pass such narrow parts although with a much slower pace than smaller compounds. Hydrophobicity prevents molecules to pass through quickly and *vice versa*, hydrophilic compounds are preferred by these channels. This is caused by the orientation of water molecules and residues of amino acids within the porins. Based on the acidic or basic properties of the amino acids incorporated in the channels, positively or negatively charged molecules are preferred (63).

Porins differ in their preferred substrates and consecutively non-specific porins can be discriminated from specific porins. The three trimeric outer membrane porins of *Escherichia coli*, OmpF, OmpC and PhoE, so-called classical porins, are non-specific. Non- specific porins show poor substrate-selectivity and can be found in an open conformation at most times (64). The majority of gram-negative outer membrane porins belong to either the OmpF or the OmpC subfamilies with the exceptions of species-specific porins of, for example, *Pseudomonas aeruginosa* and *Acinetobacter baumannii* (64). The major outer membrane protein of *P. aeruginosa* is the specific channel protein OprF, which allows slower diffusion of molecules and shows only 8% of membrane permeability of *E. coli* (61,65). Since the term “porin” is broadly used to describe non-specific protein channels (like OmpC and OmpF) in *E. coli*, other terminology for these aqueous channels, like “protein channel”, should be used for prokaryotes that do not express general porins.

The outer membrane makes gram-negative bacteria naturally less susceptible to antibiotics than gram-positive bacteria and thus can be seen as a type of innate resistance mechanism (66). Moreover, when administering subtherapeutic doses of antibiotics to patients, changes in porin structure and/or number can occur, making bacteria more adaptable and resistant to drugs. Since beta-lactam antibiotics and fluoroquinolones enter the cell through porins, alterations in these transport proteins can diminish their antimicrobial effects. Modifications of porin function include the exchange of porin type, a decreased number of expressed porins and the expression of mutated porins. If the wild type protein is replaced by a mutated porin with a smaller channel size, it can strongly influence the diffusion rates of antibiotics, resulting in a much higher minimum inhibitory concentration than before the channel-protein replacement. A decreased porin-protein expression can be found as response to antibiotic therapy, like shown for *Enterobacter aerogenes* and *Enterobacter cloacae*. In *E. cloacae* the regulatory pathway that induces downregulation of porin synthesis and simultaneously enhances the expression of efflux pump components is associated with the activator marA (64).

Inside the beta-barrel of most porin molecules, a so-called eyelet or constriction zone determines the channel size and ion selectivity. This internal loop 3 creates a strong electrostatic field that influences influx through the porin. Because of its vital

role in modifying channel width and therefore permeation, mutations in the eyelet-encoding region of the chromosome can result in antibiotic resistance (64,67).
In addition to these porin changes, porin blocking molecules produced by bacteria have been identified. It could be shown, that polyamines like spermine, spermidine, cadaverine and putrescine can reduce general porin permeability. With the exception of spermine, all three substances can be produced by prokaryotes. A study of the OmpC and OmpF porins of *E. coli* revealed that these polyamines promote closure and reduce the frequency of openings of porins (68). The synthesis of polyamines may be enhanced by the conditions that can be found within the intestinal tract and therefore be used as a weapon of enterobacteria to reduce susceptibility to toxic molecules like bile acids and certain antibiotics such as beta-lactams (64).
*P. aeruginosa* expresses a multitude of different outer membrane porins such as OprF, OprB, OprB2, OprG, OprH, OprP, OprO and Occ. All of the mentioned protein channels are substrate-specific and are either responsible for structural and signalling functions (OprF) or for diffusion of nutritional molecules like glucose phosphate (OprB, OprB2, OprP, OprO) (65). Amongst other mechanisms, changes in outer membrane protein expression of *Pseudomonas aeruginosa* contributes to antibiotic resistance. Loss of protein channels belonging to the Occ group are associated with imipenem resistance and overexpression of OprH is known to protect the lipopolysaccharide layer from aminoglycosides and colistin (69).
Pathogens within the intestinal tract, that are able to adapt their porin expression quickly and in response to antibiotic administration, can outgrow the commensal microflora which lacks comparable resistance mechanisms (64). The previously described alterations in influx of antibacterial molecules often exist simultaneously with efflux pumps. Together, these two bacterial resistance strategies render bacteria fitter against toxic compounds and complicate antibiotic treatment (70).

### 3.1.4 Release of chemical agents

Crucial elements that determine the membrane permeability in gram-negative bacteria are efflux pumps. They are highly conserved structural membrane compounds that do not only exist in bacteria. These multi protein complexes

facilitate transmembrane transport of molecules that accumulate within the cell as well as in the periplasmatic space. The release of specific intracellular “signal-molecules” is obligatory for biofilm production and the establishment of multitude virulence factors as well as some basic physiological functions of bacteria. For example, the release of quorum sensing signal molecules via efflux pumps allow the bacteria to communicate among each other as well as with their host cells. Furthermore, antimicrobial molecules, that are produced by host cells and targeted at bacteria, such as bile salts or fatty acids, can be transported outside the cell via transmembrane pumps (71). For example, in *E. coli* constitutively expressed efflux pumps protect from bile salts that are naturally occurring in the intestinal tract of higher animals (63).

But efflux pumps also allow to lower concentrations of administered antibiotics, thus leaving bacteria either resistant or less susceptible (72). Efflux complexes that solely facilitate the transport of antibiotics tend to be encoded by mobile genetic elements. In contrast, efflux pumps that mediate transport of a broad variety of molecules, including different classes of antimicrobials also released from the host cell, tend to be encoded by the bacterial chromosome (73). For example, the multi drug efflux pump AcrB of *E. coli* does not solely transport tetracycline, chloramphenicol, β-lactams, novobiocin and macrolides, but also other compounds like fusidic acid, ethidium bromide and bile salts were accepted. This shows the big impact of efflux pumps on the permeability of multiple classes of molecules (63). These multi drug resistance efflux pumps constitute a much bigger challenge in antibiotic resistance than pumps with a narrower substrate specificity (73).

Efflux pumps can facilitate intrinsic resistance to antibiotics when being expressed constitutively on a low level. On the other hand, their overexpression can be induced by the presence of an effector, so-called phenotypic resistance, or by the selection of mutant bacterial cells. If mutations take place in regulatory genes of efflux pumps, it can lead to an alteration of the expression frequency and therefore to an increase in the number of molecules that can be produced per time unit. This inducible increase in expression of efflux pumps contributes severely to acquired resistance in gram-negative bacteria (71). In *A. baumannii* overproduction of three efflux pump systems with broad substrate specificity provide the bacteria with intrinsic resistance as well as acquired resistance to a wide range of antibiotics.

Mutated *A. baumannii* isolates that overexpress the efflux pumps AdeABC, AdeFGH and AdeIJK were compared to the corresponding wildtype. Analysis of the resulting antibiotic resistance of the *in vitro* mutants revealed the extent to which they contribute to resistance development. Interestingly, proteome analysis of the mutants and the wild type showed not only the induced overexpression of the efflux pumps, but also alterations in the production of membrane proteins. Proteins of *A. baumannii* that are necessary for biofilm production and cell adhesion to epithelial cells were expressed at a lower level in mutants than in the wild type which may lead to the conclusion that overexpression of efflux pumps may alter cell competence (74).

In contrast to porins, efflux pumps require energy to mediate transportation. The biochemical reaction that provides the necessary energy differs according to the type of transporter that is integrated into the efflux pump system. Primary transporters use hydrolysis of ATP molecules as source of energy. Therefore, these proteins can be assigned to the superfamily of ATP-binding cassette (ABC) transporters. Energy generated through the electrochemical membrane potential, the so-called proton motive force, is used by the other four groups of transporters that exist in efflux pumps of gram-negative bacteria: major facilitator superfamily (MFS), small multidrug resistance (SMR) family, resistance nodulation division (RND) family and multidrug and toxic compound extrusion (MATE) family (72). Transporter of the resistance nodulation division family (RND) exhibit the major clinically relevance and have been described in *Escherichia coli* (AcrB), *Pseudomonas aeruginosa* (MexB), *Campylobacter jejuni* (CmeB), *Acinetobacter baumannii* (AdeB) and *Neisseria gonorrhoeae* (MtrD). Comparative genomics of RND transporters show high levels of homology and evolutionarily well conserved structures (72).

Most efflux pumps of gram-negative bacteria consist of three components and hence, they are called tripartite transport systems. Spanning the inner and outer membrane as well as the periplasmatic space, the pump complexes consist of an active transporter found in the inner membrane, a periplasmatic fusion protein and an outer membrane channel/ factor (70). A complex interplay takes part between the transporters and the outer membrane factors. The permeability of the outer membrane determines the concentration of antibiotic drugs within the periplasmatic space. That can be a key factor in the function of transporters. If

transporters of the inner membrane show weak substrate specificity or quick saturation the amount of antibiotic molecules that enter the periplasmatic space and can immediately be removed by the active transporters is very limited. In this case, a lot of antibiotics can accumulate within the periplasmatic space and harm the bacterial cell. But if the permeation of the outer bacterial membrane is decreased, the antibiotic concentration within the periplasmatic space can be kept low and consecutively the active transporter is able to eliminate most antibiotic molecules quickly because the transporter is not saturated. This way the antibiotic concentration can be kept low within the periplasmatic space and the antibiotic compounds have less detrimental impact on the bacterial cell (70).

Transporters of the RND family and some of the ABC, MATE and MFS families show a tripartite structure and are more effective than single-component pumps. Once a molecule is transported through the inner membrane, the periplasm and the outer membrane, the same molecule cannot re-enter the cytoplasm unless it makes its way through the outer membrane again. Therefore, the synergism between the permeability of the outer membrane and the efflux pump is vital for the bacteria to enable sufficient resistance. Therefore, distortion of the outer membrane and increasing its permeability can be almost as effective as inactivating the efflux pump itself (75).

Singlet pumps are located in the inner membrane and their transporters operate on their own. They are less capable of lowering the intracellular antibiotic concentration, because molecules that have been excreted into the periplasm can make their way back into the cytosol by diffusion. Due to the fact, that the inner membrane consists of a phospholipid layer, lipophilic compounds can easily diffuse through (75).

In recent years, the discovery of natural compounds that inhibit bacterial efflux pumps sparked hope for new ways to combat resistance. (5′-MHC) in *Berberis vulgaris* and methoxychalcone in *Dalea versicolor* are examples of efflux pump inhibitors (EPIs) produced by plants. Their discovery has led to the attempt to synthesise potent EPIs but so far, only a few compounds reached the phase of clinical trial and all of them were discontinued due to either poor pharmacokinetics, low in-vivo efficacy or toxic side effects (76).

### 3.1.5 Biofilm formation

Bacteria do not solely live individually (planktonic), rather they are able to establish organized communities and build cell aggregates, so-called biofilms. Structurally, biofilms consist of a cluster of bacteria, either single species or multi-species, which is surrounded by a protective layer of extracellular polymers (77). The bacteria produce complex polysaccharides, proteins, lipids and nucleic acids and together these compounds make up the extracellular matrix covering the bacterial community. Host molecules like fibrin, platelets and immunoglobins can be integrated additively into the extracellular matrix (78).

Although biofilms can aggregate within fluids as floating mats, they are most prominently associated with solid surfaces. Biofilm formation involves several stages, starting with the attachment (first stage) onto a solid surface. The adherence of bacteria to a surface is determined by the nature of the substratum as well as the cell surface itself. Rough and hydrophobic materials promote the formation of biofilms much better than smooth and hydrophilic ones. On the other hand, cell appendages like pili or fimbriae also influence the rate and quality of adherence (77). After surface attachment, the biofilm starts with the maturing process. Maturation involves increased cell division, the forming of microcolonies and changes in gene expression. During this stage, the production of compounds that build up the extracellular matrix is predominant. The extracellular polymeric substances form a heterogeneous layer with protein channels integrated to facilitate transportation of nutrients and oxygen to the bacterial cells inside the biofilm (77). The establishment of a biofilm is not simply the aggregation of bacteria but rather a complex species-specific development. In some bacteria, the stages of biofilm formation have been studied precisely and with the help of whole-cell protein analysis and microscopy, the structural changes during biofilm establishment could be described. For example, in *P. aeruginosa* five different stages of biofilm development can be distinguished involving striking changes in motility and quorum sensing properties (79).

By attaching to a multitude of biotic as well as abiotic surfaces, bacteria can form cell aggregates that are highly persistent and recalcitrant, making the therapy of biofilm-related infections more complex. With the rising number of implanted medical devices and prosthetic implants per year, the prevalence of implant-

related infection also increases consecutively. Joint prostheses, breast implants, mechanical heart valves, pacemakers and defibrillators as well as central venous catheters, ventricular shunts and ventricular assist devices are common implants, that initiate biofilm formation (80).

Bacteria that tend to participate in biofilms are *Staphylococcus epidermidis*, *Staphylococcus aureus, Pseudomonas aeruginosa* and *Enterobacteriaceae* (81). Therefore, infections with the aforementioned pathogens remain difficult to treat due to the weak diffusion of antibiotic compounds into the inner layer of biofilms. Over the last few decades, the importance of biofilm formation in acute and chronic wounds has become more acknowledged, accompanied by intensive research in this field of microbiology. Extensive studying of biofilms has figured out the enormous advantages for bacteria to form these protective films and which obstacles has to be overcome by antibiotics to remain effective within this environment (80).

This communal organisation of bacteria comprises a multitude of virulence factors and survival advantages for these simple organisms. Antibiotic susceptibility of biofilm forming bacteria is extensively decreased. It could be shown, that in the case of biofilm-associated *E. coli*, more than 500 times of the minimum inhibitory concentration of ampicillin was required to provide a 3 log reduction in bacterial growth (77). Responsible factors for this decrease in susceptibility involve the slow and/or absent permeation of antibiotics through the biofilm. Bacteria in outer layers of the biofilm may be reached by the antibiotic compound, but bacterial cells that reside in the centre remain unimpeded by the drug. This results not only from the slower diffusion of antibiotics to the centre but also from the fact, that bacterial cells in deep layers of the biofilm are less active metabolically and thus less vulnerable to the administered antibiotics (78). In addition to this biofilms contain the so-called persister cells. These are phenotypic variants of bacterial cells that grow slowly and therefore remain below the threshold of antibiotic damage. Due to the heterogeneity of the extracellular matrix of biofilms, a gradient is established with levels of nutrients and oxygen getting lower in the centre of the biofilm formation (82). This depletion of nutrients preserves the cells in the centre of the biofilm in an inactive state with low metabolic activity. Thus, the persister cells can survive relatively high concentrations of antibiotics as the majority of antibiotics rely upon cell proliferation or active metabolism to exert their effects. When most

of the surrounding cells are damaged or killed, persister cells remain alive- although they do not proliferate during antibiotic administration. This does not imply that the majority of biofilm cells are more resistant to antibiotics *per se*, as experiments with dispersed biofilms showed, that if bacterial cells lack the protection of the biofilm matrix, the susceptibility to antibiotics is nearly the same as in planktonic cells. In contrast, persister cells can survive and they are preserved even in presence of antibiotic drugs that inhibit their growth (80,83). Even if most of the bacterial cells forming a biofilm are killed, when the course of antibiotics is finished, a small number of persister cells perseveres and can lead to reestablishment of the infection. This phenomenon results in the treatment consensus to apply prolonged combination antibiotic therapy (80). It is common to administer one class of antibiotic compound which targets the cells in the outer layer of the biofilm like ciprofloxacin, tobramycin or beta-lactams. Additionally an antibiotic compound which is effective against bacteria that lie dormant in the core of the biofilm and are metabolically less active is used such as colistin (84). Exposition of bacteria to sub-inhibitory concentrations of antibiotics can induce the expression of additional resistance mechanisms. Intrinsic mutations of bacterial genes were found to be increased within biofilms. Due to high population density, horizontal gene transfer is promoted in biofilm microcolonies which leads to an increase in exchange of resistance genes. In contrast to planktonic bacterial cells, bacteria in biofilms are physically protected from the immune system. The extracellular matrix covers potential bacterial antigens and therefore immune cells cannot initiate an adequate response (83). The cultivation of biofilm forming bacteria is usually very difficult and can result in delayed or incorrect diagnoses. This results in an increased complexity of infection treatment. Often bacteria do not dissolve from the biofilm when a sample is taken, which complicates the assessment if the biofilm is still present. A negative blood sample or culture of the sample of a potentially colonized implanted medical device do not imply that the infection is eliminated or the bacteria is erased. A more reliable technique to ensure getting an adequate sample is the use of mechanical forces like sonication or vortexing (77). Another diagnostic challenge of biofilm associated infections is that minimal inhibitory concentrations for bacteria that are dispersed, are often not coherent with the biofilm forming species. That leaves antibiotics that are usually sufficient therapeutic options, without effect (77,80). A few classes of antibiotics

appear to be more effective than others in treating biofilm microcolonies. Antibiotic compounds that can successfully kill non-growing bacteria work well in treating biofilm infections. Rifampicin, daptomycin, linezolid and tigecycline have been proven to act against biofilm bacteria, with respect to being more efficient against gram-positive biofilms. Fluorochinolones and colistin seem also work well against biofilms of gram-negative bacteria such as *P. aeruginosa* (80).

## 3.2 Environmental factors and anthropogenic sources of antibiotic resistance

Different aspects must be considered when talking about the origin and development of antibiotic resistance. The handling of antibiotics by health professionals, involving prescription practices and ongoing education about current local resistance patterns, as well as the interactions of antimicrobials with the environment are important factors for the emergence of antibiotic resistance. The use of antibiotics in animals, progressing globalization and international travel also affect this resistance development (85).
In *Table 2* the most important categories that enable the emergence and spread of antibiotic resistance are listed.

| *Environmental factors and anthropogenic sources of antibiotic resistance* |
|---|
| Imprudent administration of antibiotic drugs in humans |
| Extensive use of antibiotics in livestock farming and aquacultures |
| Dumping of antibiotics and their metabolites into the environment |
| International travel as vector for the spread of antibiotic resistance |

*Table 1: Overview of environmental factors and anthropogenic sources of antibiotic resistance*

Spread of resistant bacteria results in the use of stronger more potent antimicrobials, which then select for bacteria that also become resistant to last resort antibiotics. For example, the spread of Extended-Spectrum Beta-Lactamase (ESBL) expressing bacteria has led to an extensive use of carbapenems which

induces a further selection of gram-negative bacteria that harbour plasmid-mediated carbapenemases. Due to this shift towards the common use of more potent antibiotics, we will slowly but surely run out of sufficient drugs to treat infections in the future because of the lack of new antibiotic drugs in the pharmaceutical pipeline (86).

### 3.2.1 Imprudent administration of antibiotic drugs in humans

Effective antimicrobial drugs are undoubtedly a necessity in modern medicine. With the possibility to treat and control infections by various microorganisms, the development of potent antibiotics has enabled to perform medical procedures that would have been impossible in former times (87).

For example the accomplishment of total hip replacement surgery that is common today has been successful partly because of the availability of effective antibiotic prophylaxis for post-surgery infections. Without these potent antibiotic drugs it is estimated that both the rate of post-operative infection as well as the mortality from it will increase severely (88). Cesarian sections, regardless of elective or non-elective surgery have become a lot safer since the introduction of prophylactic antibiotic treatment which reduces the incidence of wound infections, endometritis and maternal infectious complications (89).

Furthermore, infectious diseases that resulted in severe illness and often death are mostly well treatable nowadays. During the 19$^{th}$ century most people died from diseases like pneumonia, diarrhea and diphteria because no effective treatment was available. With the increased discovery of antibiotic compounds and microbiological research the mortality of these infectious diseases decreased and infections that formerly presented a death sentence became well treatable (90).

Although in some diagnoses antibiotic administration is recommended without a doubt, in other cases the use of antibiotics depends on specific patient's characteristics like severity of illness and comorbidities. Medical conditions in which the decision to prescribe an antibiotic is not obvious often lead to mistakes in use of antibiotics (91).

Unfortunately, a multitude of studies have revealed that antibiotic drugs are often prescribed imprudently which consecutively contributes to the development of antibiotic resistance (92,93). Unnecessary exposition of humans to antibiotics: (a)

triggers colonizing bacteria to adapt to their new environment and express resistance, that can be transferred to potential pathogens and make them resistant as well; (b) can have severe side effects including impairing the natural and useful microbiome as well as interactions with other prescription medications; (c) creates additional health care costs; (d) contributes to the levels of antibiotics released into the environment when antibiotic compounds and their metabolites go into sewage systems and consequently into ground water and soil which then lead to an increased pool of environmental resistance genes (85,93).

Considering the above, it is important to explore and understand the factors of unwary administration of antibiotics in humans. Patients commonly acquire resistance after being prescribed an antibiotic in primary care for a respiratory or urinary tract infection. Resistance against the administered compound may persist up to 12 months but is strongest during the first month after prescription. This powerful link of prescribing antibiotics in primary health care to antimicrobial resistance in bacteria should be considered in the development of strategies to combat emergence of resistance (86).

Antibiotic prescribing predominantly takes place in general practice with a share of over 70% of total antibiotic prescribing within the whole health care system. European studies and surveys regarding the accuracy of antibiotic prescriptions showed, that the prescription practices of many general practitioners do weakly align with current guidelines and recommendations of expert clinicians. Hence, it is not surprising that antibiotics are commonly overprescribed (92). Research about the suitability of antibiotic therapy according to current English guidelines was conducted. During a period of two years, data from a British primary care data base was collected and the conditions of patients were categorized into three domains: conditions, where antibiotic treatment is i) always, ii) sometimes or iii) never appropriate. Most antibiotic prescriptions (91.4%) were made for conditions that only sometimes require antibiotic treatment like acute cough, lower respiratory infection and acute sore throat. For that reason it is essential to vigorously implement clear guidelines for antibiotic prescription and motivate physicians to act accordingly. This way unnecessary prescriptions of antibiotics could be minimized which helps contain antibiotic resistance (91).

Overprescribing of antibiotics occurs frequently in patients with symptoms of infections of the upper and lower respiratory tract. A study conducted in English

primary care facilities demonstrated that, for example, in cases of acute cough, acute bronchitis, acute sinusitis, acute otitis media and influenza like illness the frequency of prescribed antibiotics was higher than expert clinicians would have suggested (92).

Overprescribing of antibiotic drugs varies between practitioners and the underlying reasons vary as well. General practitioners declared their concern that patients with respiratory tract infection-related symptoms are often expecting to get an antibiotic therapy. Therefore, general practitioners feel pressured into prescribing although they would know better (94). Patient's expectations of therapy and the assessment of such expectations by the treating physician contribute to the doctor's decision whether an antibiotic prescription is necessary or not. During a study that examined the patient's influence in such decision making it was found that if a patient expected to get a prescription, he or she was nearly three times more likely to actually receive it. And if the treating physician thought, that the patient was expecting a prescription, the patient was ten times more likely to end up with one. Therefore, the physician's opinion of the patient's expectations was found to be one of the strongest determinants for prescription (95).

To further explore this phenomenon, a Berlin' study investigated such patient expectations and the factors of influence regarding the prescription of antibiotic drugs. In this study of 1778 participants, only 10.5% expected an antibiotic prescription for the common cold. Furthermore, this expectation was found to correlate with the level of the patient's education on the correct indications of antibiotic use and antibiotic resistance. In addition, among those that were expecting an antibiotic prescription, over 70% reported, that they trust their physician and his or her decision to administer or to refrain from antibiotics (94). These results imply that the number of patients irrationally expecting the prescription is lower than physicians may think. The study also gives the incentive to inform patients thoroughly about the use of antibiotics in respiratory tract infections and to explain the necessity of accurate and prudent use of antimicrobials (94).

Lack of separation of prescribing and dispensing drugs also contributes to an increase in drug prescription. Doctors owning a dispensing general practice may have financial incentives to prescribe more (antibiotic) drugs than doctors of non-dispensing practices. In many countries it has been prohibited to prescribe and

dispense simultaneously (Italy, Germany, Scandinavian countries) but in some it remains legal (e.g. Austria, Switzerland) (96).

In addition to overprescribing the unwary use of antibiotics also includes unnecessary use of broad-spectrum antibiotics. The frequent use of broad-spectrum antibiotics has enabled the successful treatment of infections without the knowledge of the causative bacterial species. Broad-spectrum antibiotics can select for resistant bacteria in both, the targeted pathogen as well as in non-pathogenic bacteria of the microbiome. While occurrence of resistance in the targeted pathogen may lead to treatment complications, selection of resistant gut bacteria can establish a pool of resistance genes in the human gastrointestinal tract. Opportunistic bacteria can draw genes from this pool *via* horizontal gene transfer and acquire resistance (97).

Prescribing of fluorochinolones for the initial treatment of acute cough in otherwise healthy adults is not recommended by any guidelines yet but this antibiotic class seems to be prescribed by general practitioners for that indication. It was found, that in addition to the patient's condition the education and overall antibiotic prescription rate of the physician determined the use of fluorochinolones for the initial treatment of acute cough. Doctors may perceive fluorochinolones as particularly potent antibiotics and therefore are tempted to use them more often (93).

The limited diagnostic possibilities in combination with insufficient guidelines for antibiotic administrations could be responsible for the careless prescription of antibiotics in general practices. Simultaneously, the research team of Smith represents approaches to improve adequacy of prescriptions (91). Rapid and accurate diagnostic tools would help physicians to identify bacterial pathogens in order to prescribe narrow-spectrum antibiotics rather than broad-spectrum drugs. The use of nucleic acid-based amplification technologies (polymerase chain reaction, next generations sequencing) and mass spectrometry based diagnostic tools allow the precise identification of microorganisms in clinical settings. Due to certain drawbacks of DNA based technologies and prices of new diagnostic tools, further research and developments need to be made to improve bacterial diagnostics and make them more available for primary care physicians (97).

Taking part in antibiotic stewardship programmes can help hospitals as well as primary care physicians to improve their quality of antibiotic use and to slow down

the emergence of antibiotic resistance. Measures of antibiotic stewardship programmes like adhering to guidelines for when and how to use antibiotics and de-escalation therapy show improvements in clinical outcomes such as a relative risk reduction for mortality of 35% and 56%, respectively. Switching antibiotic treatment from intravenous to oral as well as drug monitoring might also have positive effects on various outcomes and should be considered when administering antibiotics (98).

### 3.2.2 Extensive use of antibiotics in livestock farming and aquacultures

Antibiotics are not only limited to use in human medicine but also used in livestock farming and aquacultures worldwide. With the world population continuously increasing and low- and middle-income countries gaining higher living standards, the demand for animal source protein has reached an unprecedented level (99–101). To meet this demand, animal farming has intensified globally (102). Brazil, Russia, India, China and South Africa are countries which introduced more intensive livestock production systems in order to increase supply for animal protein (100). The main concern for many livestock farmers is productivity and profitability. Intensive livestock farming requires the use of cheap feeds and antimicrobials to prevent animal disease and promote growth. With extensive use of antimicrobials, antibiotic resistance is promoted because of increased selection pressure that is added on bacteria (102).

Progress of antibiotic resistance in farm animals is especially problematic because the biomass of livestock, fish and molluscs of aquacultures surpasses the biomass of humans. Therefore, the impact on antimicrobial use in farm animals is huge (103).

Antibiotics are used in livestock farming and aquacultures for many reasons: i) to promote growth, ii) to prevent disease in farm animals, iii) to reduce animal mortality rates, iv) to increase farm profitability and v) to compensate for poor hygiene standards are the most important ones (102,103). Since the use of antibiotics for growth promotion has now been banned from many high-income countries and although some middle- and low-income countries have followed, this practice is believed to still be used widely (85,102). Many small and mid-scale livestock production systems and aquaculture farms, for example in South-East

Asia, struggle to stay in business and consequently they are seizing every possible strategy to help keep production and profitability levels high. Low cost of antibiotic drugs in these countries help to further use antibiotics imprudently in farming. Regions in which antibiotics are used for intensive farming are countries that have unprecise or non-existing guidelines, insufficient law enforcement, weak compliance with existing guidelines, low levels of antimicrobial resistance (AMR) awareness or inadequate commitment to antibiotic stewardship programs.

The importance of more restrictive legislative has been recognized by countries like Thailand, Vietnam and Indonesia and they introduced bans for the use of antimicrobials for growth promotion in livestock (Thailand in 2015; Vietnam and Indonesia in 2018). Although restricting legislation for aquaculture is lacking worldwide, Thailand acted exemplarily and introduced comparative bans for fish farming (102). With China being the largest consumer of antimicrobials in veterinary medicine worldwide, implementations made by its government could function as an example for other Asian countries to do the same and act collectively (103).

Farmers may be difficult to convince to adapt new farming techniques without the extensive use of antimicrobials. Further studies are needed because of lack and quality of antimicrobial surveillance systems and thus proving data, in countries where antibiotics are used most often in livestock production. According to farmers in Vietnam and Thailand, veterinarians, the government and pharmaceutical companies should be responsible for monitoring the sales and administration of antibiotics rather than the farmers themselves (102).

Livestock sectors of monogastric animals, like pigs and poultry, as well as cattle are exposed to antimicrobial use. On average, pigs require the highest consumption of antimicrobials in livestock in OECD countries (100,102). Animals produced in aquacultures (shrimp, pangasius catfish) frequently receive antibiotics as well (102). A study that was conducted in Chile, the second largest producer of farmed salmon, showed that during the fattening stage of fish farming antibiotic drugs are administered frequently with the most used compounds being florfenicol and oxytetracycline (104).

Data of pharmaceutical and food industry regarding antibiotic sales and consumption are scarce for most countries. This results in difficulties to assess the scale and class of antibiotics used and prohibits detailed analysis of animal-related

antibiotic resistance (102). Nevertheless, it is estimated that animal-related antibiotic consumption exceeds use in human medicine and is believed to have been more than twice as much in 2010 (100).
Across pig production systems in Vietnam and Thailand penicillins, tetracyclines and aminoglycosides were the most used classes of antibiotics. Many commercially mixed feeds do not come with detailed labels on the packaging and therefore it is sometimes difficult or impossible to know which antimicrobials have been mixed with the feed (102). Antibiotics are sold without requirement of prescription in many countries worldwide. Consequently, use of antimicrobials in farming is often not monitored because farmers can buy antibiotics directly from pharmaceutical companies or pharmacies and administer them without surveillance. (102) It is estimated that the use of subtherapeutic concentrations of antibiotics will drastically increase globally because many middle-income countries are starting to shift their production practices to larger farming operations with bigger consumption of antibiotic drugs. Based on current consumption data and expert opinion, it was suggested that by 2030 the use of antimicrobials for animal production will increase by 67% (100).

***What are the consequences of this increased use of antimicrobials in farming?***

The increase in worldwide use of antibiotics in animals compromises the effectiveness of vital drugs and risks human medicine (105). In a European study the correlation between antimicrobial use in livestock farming and the prevalence of resistant commensal bacteria of cattle, poultry and pigs was conducted. The results indicate strong correlation between the use of antimicrobials and resistance of *E. coli* isolates in all seven examined countries. For fluorochinolones, third-generation cephalosporins and amphenicols it was found that even a small increase in use results in exponential increase in resistance (106).
Antibiotics used for growth promotion and disease prevention are administered in subtherapeutic concentrations which drastically promotes the emergence and spread of resistant bacteria (100). Small doses of antibiotics just kill a fraction of commensal as well as pathogenic bacteria in the gut of farm animals. On the rest of bacteria, selection pressure is exerted and promotes the formation and

proliferation of resistant phenotypes. The emerged resistance genes can then not only be spread within the same species but also to other animals or humans (102). Extensive use of antimicrobials in livestock farming and aquaculture consequently leads to antibiotic drugs and metabolites being released into the environment, which can induct selection for resistance genes in environmental bacteria in addition to the commensal bacteria of farm animals. In the gut of farmed animals, commensal resistant bacteria are also selected. The gut can act as a reservoir for resistance genes whereby bacteria entering the intestinal tract can exchange resistance genes with the commensal resistant bacteria. The resulting multi-resistant bacteria will also be released into the environment *via* faeces which again increases the size of the environmental resistance gene pool (104,107). At a farm setting, resistance genes can also be transferred *via* manure and flies (107).
There already have been multiple findings of multi-resistant gram-negative bacteria in pigs, cattle, poultry, fish and molluscs. Carbapenemase-producing *E. coli* and *Salmonella spp*. are examples of multi-resistant organisms found at livestock farms in Germany. Carbapenemase-resistant *E. coli* were also found at pig farms in Italy with one of the isolates even carrying gene, encoding colistin-resistance and aminoglycoside-resistance. Similar findings of resistant bacteria related to intensive pig farming have been made in the US, Southern China and India (107). The *mcr-1* colistin resistance gene is likely to have originated from Chinese pig farms where colistin was used for growth promotion for many years. With colistin being one of the last resort antibiotics that are administered if all other classes are ineffective, resistance against this compound is especially concerning. Recently pan-resistant *mcr-1* carrying strains of *E. coli* have been detected during a Chinese study that investigated raw meat, food animals and hospitalized patients. This emphasises the importance of critical use of antibiotics in farm animals (108).
Furthermore, carbapenemase-carrying gram-negative bacteria were isolated from cattle productions in France, Germany, the US, China and Algeria among other countries. Resistant strains of *E. coli, Salmonella spp., Klebsiella pneumoniae* and *Acinetobacter spp*. were also found in countries with extensive poultry production, especially broiler meat. (107). Particularly in India, the use of antimicrobials for animal production is especially alarming. In a country where 95% of adults carry β-lactam-resistant bacteria and no restrictions for antimicrobial use in the production

of cattle, chicken or pigs for domestic consumption are implemented, bacterial resistance is predicted to increase rapidly (100).

***What can be done to counteract this increase in antimicrobial use?***

In order to prohibit excessive use of antimicrobials more restrictive regulations must be made. The world health organisation already introduced first steps to minimise global antibiotic use and preserve the efficacy of these vital drugs. Experts issued a list of critically important antibiotics for humans whose compounds should be handled precautious in order to keep infections treatable. Guidelines concerning antimicrobial use in farm animals were released and suggest the following four recommendations:

(1) “overall reduction of use of all classes of medically important antimicrobials in food-producing animals”; (2) “complete restriction of use of all classes of medically important antimicrobials in food-producing animals for growth promotion” and (3) “for prevention of infectious diseases”. Furthermore, it is suggested, that (4) “highest-priority critically important antimicrobials for human medicine should not be used for treatment of food-producing animals” (105).

However, just implementing stronger regulations will not be the solution to this problem. Prohibiting the use of antibiotics in livestock farming and aquacultures can result in dramatic decreases in the livelihoods of many farmers worldwide. Especially in less developed countries where antimicrobial use in animal production is necessary to compensate for poor hygienic conditions. Improving hygienic standards and introducing surveillance systems to track antimicrobials are important steps that need to be taken accordingly (103,105). Introducing a threshold for the maximum dosage of antimicrobials used in animal farming would drastically reduce antimicrobial consumption if administered by OECD countries and China (103).

Therefore, sustainable ways to systematically reduce antimicrobial use in animals must be developed in order to minimise the use of antimicrobials while preserving the integrity of farmers (102).

In addition to enforcing more restrictive legislation, promoting low-animal protein diets has also been suggested to combat the problem. If fewer animals are used for food production, antimicrobial use could decrease respectively (103).

### 3.2.3 Dumping of antibiotics and their metabolites into the environment

Antibiotic compounds and their metabolites are frequently released into the environment where they exert selection pressure to environmental bacteria in water and soil (109,110).

Antibiotics enter sewage systems as a consequence of human waste and waste of pharmaceutical production. Although sewage water is often processed in wastewater plants, not all components of the wastewater effluent can be eliminated, and drug residues end up in the environment. Antibiotic compounds and metabolites as well as bacteria and antibiotic resistance genes can be transferred to water and soil and consequently an environmental pool of resistance genes and resistant bacteria can be established (110).

In multiple analyses of sewage water, resistant bacteria were isolated. Examples include ESBL-producing *Klebsiella pneumoniae* of a hospital sewage system in Brazil, β-lactam and aminoglycoside resistance genes of sludge in Germany and Portugal as well as ESBL-producing *Enterobacterioceae* in Spain and Ireland.

Countries that have little legislation on the disposal of pharmaceutical waste impose great risk for environmental contamination with highly potent chemical drugs. Especially parts of India and China are lacking according laws and their enforcement (85). The extent of contamination of soil, ground and drinking water with pharmaceuticals as a result of inadequate disposal was shown in an investigation conducted in India. Water of a treatment plant near Hyderabad that processes the waste of a large-scale pharmaceutic production area was tested and the concentrations of multiple different drugs that were found are alarming. Among metoprolol (beta blocker) and cetirizine (anti histamine), high concentrations of fluorochinolones were reported in the wastewater of the treatment plant. Drug concentrations in two lakes that are located in close proximity to the treatment plant were examined additionally. Fluorochinolone (2.5 mg/L for ciprofloxacin) and antihistamine (20 μg/L for cetirizine) concentrations exceeded the human therapeutic blood plasma concentrations. Furthermore, multiple wells in the area surrounding the treatment plant were also contaminated with a concerning volume of drugs (110).

To investigate the impact that wastewater of Chinese pharmaceutical production might have, a penicillin production wastewater treatment plant was examined.

Concentrations of the produced antibiotics were measured directly in the wastewater as well as upstream and downstream of the plant. Most of the antibiotics from the wastewater were eliminated and the downstream water only contained about 1% of the original concentration in the wastewater effluent. But ratios of resistant strains were highly elevated in the downstream river compared to the upstream river (wastewater effluent 63.6%; downstream river 47.7%; upstream river 11.8%). This concludes that even post-processing, antibiotic resistant strains of pharmaceutical wastewater effluent can be discharged into the environment (111).

These studies demonstrate, that industrial waste, which is not handled properly, can contain concerning volumes of drugs that are released into the environment. This does not only endanger the surrounding population whose drinking quality is dramatically decreased but it also extensively promotes selection pressure on environmental bacteria (110).

The use of veterinary pharmaceuticals for animal farming and aquaculture also increases environmental antibiotic levels. Veterinary drugs are either released directly into the environment (in case of aquacultures) or enter the ecosystem after being excreted by livestock. Furthermore, animal manure is often used as a fertilizer in agriculture. This way antibiotic residues and resistance genes (*via* bacteria) can spread widely to the soil of agricultural fields. Once antibiotics and their metabolites have entered the soil, they can be dispensed in the groundwater and recirculate multiple times. Environmental bacteria that contain resistance genes can come into contact with wildlife and consequently will be spread as the wildlife travels. Especially wild birds are seen as major contributors to the global spread of resistance genes. Studies have been conducted to investigate if proximity to human populations is sufficient to elevate the amount of resistance genes in wildlife. The results suggest this to be likely. That means that selection pressure of antibiotics need not be applied directly to wildlife. The findings of similar resistance genes in different settings propose the transmission between wildlife, livestock and the human population especially in clinical settings. Antibiotic levels found in the environment are believed to be high enough to exert selection pressure on environmental bacteria. If wild types are inhibited in growth, resistant bacteria prevail and are able to multiply. Consequently, the prevalence of resistance increases. Especially manure, farm soil, swine faeces, lagoon and river

sediments present hotspots for the selection of resistance genes (109). Furthermore, such nutrient-rich environments provide ideal conditions to facilitate horizontal gene transfer between bacteria (85).
Minimal inhibitory concentration distributions are used to investigate the potential effect of antibiotics on environmental bacteria and how antibiotic pollution influences resistance. The aim of such interdisciplinary studies is to explore the complex interplays between pharmaceuticals released into the environment and potential conclusions that can be drawn in order to create awareness and improve current regulations on disposal of pharmaceutical waste (109).

### 3.2.4 International travel as vector for the spread of antibiotic resistance

International travel has increased tremendously over the last years as it has gotten more affordable and more attainable to people worldwide. Short-term tourists, businessmen and women as well as members of the military are frequently travelling by plane covering great distances in short periods of time. In addition to the environmental and climate impacts of increased air travel, the spread of resistant bacteria and resistance genes is also accelerated for this reason (108). By coming into contact with the environment, people, animals and food of a foreign country, travelling alters the microbiome in the human gastrointestinal tract and thus also reduce colonization resistance for various bacteria (112). Visiting some regions where prevalence of resistant bacteria is especially high, for example in countries in Southeast Asia, China and India, imposes the risk of being colonized with pathogens and potentially infecting surrounding people back home. ESBL carrying *Enterobacterioceae* are endemic in some tropical and subtropical countries as the healthy population frequently carry these resistant bacteria. India, China, Southeast Asia, the Middle East, Northern Africa and Central as well as South America show high prevalence of these pathogens and travelling to those countries can be associated with acquiring multi-resistant bacteria (108).
During a study conducted in the Netherlands, colonization with ESBL-producing *Enterobacterioceae* of international travellers was investigated. Interestingly, 34.3% of people who had been ESBL negative before, acquired the resistant bacteria especially when travel destinations included South Asia. In addition to the country that was travelled to, risk factors for acquisition of ESBL-producing

*Enterobacterioceae* were use of antibiotics during travel (especially use of chinolones), suffering from traveller's diarrhoea and frequent consumption of food from street food vendors. After returning home colonization lasted for 30 days on average and living with a person who is colonized with ESBL-producing Enterobactericeae can lead to transmission of the bacteria to other people within the household with a probability of 12% (113).

To demonstrate the abundance of pathogens and resistance genes that are transferred *via* travellers, toilet waste from 18 international airplanes at Copenhagen airport was tested. Generally, Asian samples contained a higher abundance of resistance genes than samples from North America. For example, resistance genes in *Salmonella spp.* mostly were of South Asian origin (114). Travellers returning home to London from countries like Pakistan, India and Bangladesh were diagnosed with infections of either *Salmonella typhi* or *S. paratyphi A* that were resistant to ciprofloxacin in 80% and 88% respectively (112). Due to international travel increasing the risk of non-typhoidal salmonella infections that are frequently resistant to ampicillin and fluorochinolones, travel-associated salmonellosis is getting harder to treat (108).

Although colonization of pathogens may be transient and mostly limited in duration to a few months it is important, that after returning home, people living in close proximity to the infected traveller can also receive resistant bacteria. In contrast to wide-spread notions that acquiring resistant bacteria is only possible when exposed to inadequate hygienic standards, a study revealed, that travellers that enjoyed middle-class restaurants and upper-class living in Vietnam still acquired colistin-resistant bacteria. Furthermore, stool from returning travellers can contain highly resistant bacteria. In a study conducted in the Netherlands, colistin-resistant bacteria were isolated from stool samples of travellers that visited India previously. To minimise the risk of colonization with resistant strains, pre-travel preparations should include getting the necessary vaccinations as they reduce transmission of bacteria and antibiotic consumption (108). Travellers should seek counselling from medical professionals prior to departure regarding the self-treatment of illnesses during their travel. For example, in cases of mild traveller's diarrhoea, the administration of loperamide instead of antibiotics, is not associated with an increased risk of colonization with ESBL-producing *Enterobacterioceae* (113).

Avoiding certain foods while travelling can help minimise risk of colonization. Resistant bacteria, for example ESBL-producing *Enterobacterioceae*, have been isolated from various meat varieties as well as raw milk, seafood and vegetables. The well-known advice “Cook it, peel it or leave it” can help make smart food choices while visiting countries with high probability of colonization with resistant pathogens (112).

Effective hygiene practices and availability of clean drinking water when possible helps prevent infections. Safe sexual practices, like the use of condoms, also reduces risk of infections (108).

Introducing pathogen and antibiotic resistance surveillance systems at airports could improve the knowledge about the spread of resistance by international travel. By analysing the human waste of airplanes, a lot of data could be acquired about the type and origin of pathogens transported by air travel. Reliable and globally available data helps to predict possible resistance trends and may enable to enforce global resistance measures (114).

## 3.3 Impact of resistant gram-negative bacteria on infection treatment

### 3.3.1 General consequences of infections with resistant bacteria in health care

#### *3.3.1.1 Clinical impacts*

In recent reports conducted by the British government it is estimated that by 2050, antimicrobial resistance will be responsible for 10 million deaths per year worldwide and that annual health care costs related to antimicrobial resistance will increase dramatically. Consequently, infections due to resistant microorganisms would be the number one global health burden surpassing cancer. However, it is important to emphasise, that predicting the future impacts of antimicrobial resistance on a global scale is difficult because of the complexity of this matter as well as the lack of reliable data from multiple countries (87). Nevertheless, the negative consequences of antibiotic resistance are likely to be underestimated because only a fraction of the treated infections are diagnosed microbiologically. Therefore the real number of resistant pathogens responsible for infections is believed to be even higher (115).

To evaluate the consequences of resistance to a certain antimicrobial compound, the following factors must be considered: i) type and prevalence of the pathogen that exhibits resistance, ii) transmission properties of the pathogen and its resistance gene, iii) the type of infection caused by this pathogen, iv) the health burden of the infection and if v) alternative treatments are available (87).
Infections with resistant bacteria can result in adverse clinical and economic outcomes (115). Medicine and surgery as we know it today depend on the availability of effective antibiotics. Caesarian sections, open heart surgery and stem cell transplantations are just a few examples of procedures that were initially associated with high mortality partially because of the lack of effective infection control and treatment. Antibiotics have advanced modern medicine and we have gotten used to treating most infections successfully (87). In the face of infections becoming untreatable, successful medical procedures, surgeries and treatments of severe infections are at risk (116).
The health burden of infections with antibiotic resistant pathogens within the European economic area was evaluated in a recent study funded by the European Centre for Disease Prevention and Control. According to this literature, over 670 000 infections during 2015 were caused by antibiotic resistant bacteria. Approximately 33 110 deaths and over 874 000 disability-associated life years (DALY) were accounted for by resistant pathogens. Especially in infants with an age less than 1 year and in adults older than 65 years the burden of disease was especially high. The burden of disease was measured as “the number of cases of all types of infections with antibiotic-resistant bacteria, the number of deaths attributable to these infections and the resulting number of DALYs”. Antibiotic resistant-bacteria with an exceptionally high burden of disease were third-generation cephalosporin-resistant *E. coli*, *MRSA,* carbapenem-resistant *P. aeruginosa* and third-generation cephalosporin-resistant *K. pneumoniae*. This investigation reflects the importance of gram-negative bacteria in disease burden associated with antibiotic resistance (117).
Antibiotic prophylaxis enabled the mitigation of post-procedural infections and their consequences. As the efficacy of antibiotic prophylaxis declines, multiple invasive diagnostics, procedures and surgeries are associated with a higher rate of adverse effects (87). Furthermore, lack of effective antibiotics diminishes the safety of immunosuppressive therapy (115).

Infections with resistant bacteria commonly lead to a delay until effective treatment is administered. If the resistant pathogen initially is not susceptible to the calculated therapy, many hours and even days can pass until the appropriate antibiotic compound is given to the patient. Delays of effective antibiotic treatment do not only set the patients at risk but also the surrounding people (115).
The mortality of patients suffering from resistant bacteria is increased compared to infections with susceptible bacteria. However, it is important to consider, that patients infected with resistant bacteria often are ill with multiple comorbidities which worsen the outcome of infection treatment additionally (115). In patients with severe illnesses like sepsis, association with multi-resistant gram-negative bacteria is likely to worsen the outcome. In a Brazilian study, sepsis due to carbapenemase-producing *K. pneumoniae* was attributed a 30 day-mortality of 64.8% and ESBL-producing *enterobacteria* of 60.7%, whereas the 30 day-mortality of other pathogens was much lower (118).
Antibiotic resistance also results in the overuse of second-line and last-line antibiotics which consequently induces resistance levels against these potent antibiotics. This development of increasing resistance against multiple last-resort antibiotics is concerning as severe infections with multi-resistant pathogens are becoming untreatable. Furthermore, infections with resistant pathogens do not only replace infections with susceptible ones but add to the total number of infections (115).

#### *3.3.1.2 Economic impacts*

In addition to the clinical consequences, antibiotic resistance imposes challenges to the global economy. Health care costs are predicted to explode worldwide and have been declared similar to the estimated economic burden of climate change (2°C rise of surface level temperature) (87). Prolonged hospital stays, increasing stays at intensive care units and foregoing medical procedures in attempt to sufficiently treat the infection contribute to the predicted increase in health care costs (115,116).
Nosocomial outbreaks of resistant bacteria require infection control measures that can affect the closing and/or isolation of a whole ward which is especially expensive (115).

Furthermore, the impact of antibiotic resistance on the labour market has been recognized recently. With increased mortality and prolonged sickness, patients suffering from multi-drug resistant pathogens are absent from work for a longer period of time than when infected with pathogens sensitive to antibiotics (115). Considering the intertwined relationship between antibiotic resistance and economic aspect, it is clear that the challenge of ineffective treatment of infectious diseases is not nearly just a problem concerning the health sector but will ultimately affect every aspect of life on a global scale. And similar to climate change, the worst impacts are not distributed equally and low income countries would be hit hardest (87).

### 3.3.2 Consequences of antibiotic resistance in health care of low- and middle-income countries

The World Bank classifies economies by different categories such as geographic regions and income to enable adequate comparison of statistical data between different countries (119).

When comparing countries regarding antibiotic resistance and the availability of antibiotic drugs as well as the prescription practices, categorisation by income is often used. The gross national income per capita in US dollars determines the affiliation of each country to one of four different groups: low-income, lower-middle income, upper-middle income and high-income (120). According to the recent revision of the World Bank's classification of countries the current affiliation of each country to an income-category can be seen in *table 3*. Low-income economies are defined as countries which acquire a gross domestic product of 1035 USD or less (120). Healthcare in resource-low countries works vastly different than in well-developed and high-income countries (121). Low-income countries tend to facilitate less government funded health care and show a higher percentage of out-of-pocket payments by patients for health care services than in most high-income countries. Without or with little subsidization of costs for the poorest people, adequate health care service can heavily burden the household budget (121). Another interesting difference is that health care facilities in low-income countries are often owned and/or operated by private companies. In high-income economies the state takes part in financing and operating health care services which makes regulations and interventions by the state easier (121).

| **Low-income countries (GDP ≤ 1 035 USD)** | | | |
|---|---|---|---|
| *Afghanistan* | *Burkina Faso* | *Burundi* | *Central African Republic* |
| *Chad* | *Congo, Dem. Rep.* | *Eritrea* | *Ethiopia* |
| *Gambia, the* | *Guinea* | *Guinea-Bissau* | *Haiti* |
| *Korea, Dem. People's Rep.* | *Liberia* | *Madagascar* | *Malawi* |
| *Mali* | *Mozambique* | *Niger* | *Rwanda* |
| *Sierra Leone* | *Somalia* | *South Sudan* | *Sudan* |
| *Syrian Arab Republic* | *Tajikistan* | *Togo* | *Uganda* |
| *Yemen, Rep.* | | | |
| **Lower-middle income countries (GDP 1 035 USD – 4 045 USD)** | | | |
| *Angola* | *Algeria* | *Bangladesh* | *Benin* |
| *Bhutan* | *Bolivia* | *Cabo Verde* | *Cambodia* |
| *Cameroon* | *Comoros* | *Congo, Rep.* | *Côte d'Ivoire* |
| *Djibouti* | *Egypt, Arab Rep.* | *El Salvador* | *Eswatini* |
| *Ghana* | *Honduras* | *India* | *Kenya* |
| *Kiribati* | *Kyrgyz Republic* | *Lao PDR* | *Lesotho* |
| *Mauritania* | *Micronesia, Fed. Sts.* | *Moldova* | *Mongolia* |
| *Morocco* | *Myanmar* | *Nepal* | *Nicaragua* |
| *Nigeria* | *Pakistan* | *Papua New Guinea* | *Philippines* |
| *São Tomé and Principe* | *Senegal* | *Solomon Islands* | *Sri Lanka* |
| *Tanzania* | *Timor-Leste* | *Tunisia* | *Ukraine* |
| *Uzbekistan* | *Vanuatu* | *Vietnam* | *West Bank and Gaza* |
| *Zambia* | *Zimbabwe* | | |
| **Upper-middle income countries (GDP 4 046 USD – 12 535 USD)** | | | |
| *Albania* | *American Samoa* | *Argentina* | *Armenia* |
| *Azerbaijan* | *Belarus* | *Belize* | *Bosnia and Herzegowina* |
| *Botswana* | *Brazil* | *Bulgaria* | *China* |
| *Colombia* | *Costa Rica* | *Cuba* | *Dominica* |
| *Dominican Republic* | *Equatorial Guinea* | *Ecuador* | *Fiji* |
| *Gabon* | *Georgia* | *Grenada* | *Guatemala* |
| *Guyana* | *Indonesia* | *Iran, Islamic Rep.* | *Iraq* |
| *Jamaica* | *Jordan* | *Kazakhstan* | *Kosovo* |
| *Lebanon* | *Libya* | *Malaysia* | *Maledives* |
| *Marshall Islands* | *Mexico* | *Montenegro* | *Namibia* |
| *North Macedonia* | *Paraguay* | *Peru* | *Russian Federation* |

| | | | |
|---|---|---|---|
| *Samoa* | *Serbia* | *South Africa* | *St. Lucia* |
| *St. Vincent and the Grenadines* | *Suriname* | *Thailand* | *Tonga* |
| *Turkey* | *Turkmenistan* | *Tuvalu* | *Venezuela, RB* |
| **High income countries (GDP ≥ 12 536 USD)** | | | |
| *Andorra* | *Antigua and Barbuda* | *Aruba* | *Australia* |
| *Austria* | *Bahamas, The* | *Bahrain* | *Barbados* |
| *Belgium* | *Bermuda* | *British Virgin Islands* | *Brunei Darussalam* |
| *Canada* | *Cayman Islands* | *Channel Islands* | *Chile* |
| *Croatia* | *Curaçao* | *Cyprus* | *Czech Republic* |
| *Denmark* | *Estonia* | *Faroe Islands* | *Finland* |
| *France* | *French Polynesia* | *Germany* | *Gibraltar* |
| *Greece* | *Greenland* | *Guam* | *Hong Kong SAR, China* |
| *Hungary* | *Iceland* | *Ireland* | *Isle of Man* |
| *Israel* | *Italy* | *Japan* | *Korea, Rep.* |
| *Kuwait* | *Latvia* | *Liechtenstein* | *Lithuania* |
| *Luxembourg* | *Macao SAR, China* | *Malta* | *Mauritius* |
| *Monaco* | *Nauru* | *Netherlands* | *New Caledonia* |
| *New Zealand* | *Northern Mariana Islands* | *Norway* | *Oman* |
| *Palau* | *Panama* | *Poland* | *Portugal* |
| *Puerto Rico* | *Romania* | *Qatar* | *San Marino* |
| *Saudi Arabia* | *Seychelles* | *Singapore* | *Sint Maarten (Dutch part)* |
| *Slovak Republic* | *Slovenia* | *Spain* | *St. Kitts and Nevis* |
| *St. Martin (French part)* | *Sweden* | *Switzerland* | *Taiwan, China* |
| *Trinidad and Tobago* | *Turks and Caicos Islands* | *United Arab Emirates* | *United Kingdom* |
| *United States* | *Uruguay* | *Virgin Islands (U.S.)* | |

*Table 3: The 2020 World Bank Country classification by income*

In the following, countries within the lower-middle income and upper-middle income groups will be regarded as one group - middle-income countries. This term results from an extensive number of medical studies and health care reports in which the two middle categories are joined together as one (121–124).

In high-income countries, access to antibiotics has been taken for granted over the last decades whereas in many countries worldwide, sufficient access to antibiotic

compounds is still not ensured. Low- and middle-income countries face an especially difficult discrepancy: in mostly rural areas antibiotics are often either delivered with delay or not available at all whereas in cities and tertiary hospitals rising resistance rates make infection treatment difficult. Globally, more deaths are caused by the lack of access to antibiotics than by antibiotic resistance. Nevertheless, antibiotic resistance is a growing challenge in resource-low health care settings. For health care systems of low- and middle-income countries it is especially difficult to counteract antibiotic resistance as the necessary health care infrastructure is often poor. The aim for these countries will be to increase access to vital antibiotics while limiting the emergence of antibiotic resistance (125).
In many low-income countries the lack of epidemiologic research of antibiotic resistance and little reliable extensive and standardized microbiological data make it difficult to detect resistance and to examine its consequences. Resistance testing is essential for the adequate treatment of infections. The effectiveness of first-line drugs for common bacterial infections can be evaluated and the treatment can be adjusted accordingly. Lack of sufficient microbiologic data contributes to a higher morbidity and mortality of infected patients (126,127). To gain insight into the quality of microbiological testing in countries, where frequent quality assessment of laboratories is not implemented, the Regional Office for Africa of the World Health Organisation conducted two extensive surveys. In the first one microbiologic data of 78 participating African laboratories from 48 WHO member states between 2002 and 2009 was collected. The second survey involved 81 laboratories from 45 WHO member states and was conducted from 2011 until 2016. The aim was to fill in knowledge gaps of laboratories and provide a possible template to continue quality assessment of laboratories beyond the surveys to improve the availability of microbiological data. Proficiency of bacterial identification and antimicrobial susceptibility testing was analysed. It was found that laboratories struggled to perform adequate antimicrobial susceptibility testing and institutions were not able to correctly identify certain bacteria. Especially diagnostics of bacterial enteric disease (e.g. *Salmonella enteritidis, Salmonella typhi, Shigella flexneri)* and bacterial meningitis was examined and reported to be inadequate in most laboratories participating in the first survey from 2002 until 2009. Reasons for insufficient microbiological testing included use of wrong media for susceptibility testing, for example chocolate agar for *H. influenzae* instead of

EUCAST-recommended media, incorrect testing methods or wrong interpretation and reporting of microbiological test results (128,129).
Poor hygiene conditions, insufficient infection prevention and control measures and higher burden of bacterial illnesses in low- and middle-income countries additionally worsen the emergence and spread of antibiotic resistance (127,130). If first-line antibiotic treatments fail, alternative drugs are not always available in low- and middle-income countries or may be more expensive than in high-income countries (130). An investigation from Health Action International in cooperation with the World Health Organisation Regional Office Africa compared prices of a 7-day course of Ciprofloxacin 500mg across 93 countries. The average price a patient would have to pay for the originator brand of Ciprofloxacin was higher in African countries ($45,37) than in Europe ($43,63), the Eastern Mediterranean ($36,47) and South East Asia ($17,46). Although the average income varies a lot comparing African countries to European countries and Eastern Mediterranean countries the price of the originator brand varied little. Considering purchasing-power parity, the drug is very expensive for people in many low- and middle-income countries like Kyrgyzstan, Uganda, Colombia, Nigeria, Rwanda, Nicaragua and Kenia. Although prices of generic brands of Ciprofloxacin are lower and show more variability, they still can be considered as expensive in many countries (131). High prices of second-line drugs which are poorly adjusted to the country's economy make treating infections with multi-resistant pathogens in low resource countries difficult as little treatment options are available. A study from Hanoi revealed that 29.7% of neonates with hospital acquired infections carried gram negative bacteria that were resistant to 6 classes of antibiotics. If alternative treatments are not available or too expensive, neonates in low resource countries die from infections whereas in high income countries they could have been saved at lower costs. Infections with multi-resistant pathogens are associated with higher mortality and increased length of hospital stay. Therefore, accurate pricing of vital antibiotics should be established (132).
All the aforementioned factors contribute to low- and middle-income countries suffering from more additional deaths due to antibiotic resistance per capita than high income countries. For Thailand, for example, it is estimated that 19 000 deaths annually are attributed to multi-resistant bacteria, which is 3 to 5 times more than for the United States and the European Union (133).

Health professionals in India reported increased mortality in ICU as well as non-ICU patients due to antibiotic resistance, especially in gram negative bacteria (134). Furthermore, a study conducted in Indian hospitals also suggests that community-onset infections with multi-resistant pathogens are causing high mortality and longer duration of hospital stay (126).
Especially neonates' and children's mortality increases with higher levels of bacterial resistance. In low-income countries hospital-acquired neonatal infections occur more frequent than in high-income countries because often appropriate hygiene measures to prevent infection are not implemented due to lack of funds. In addition, antibiotic-enriched animal feed is used to prevent infections in animal breeding, which greatly increases the development of resistance and therefore these hospital-acquired neonatal infections account for more than 40% of neonatal deaths per year. Common neonatal infections caused by gram negative bacteria that are becoming progressively resistant include pneumonia and bloodstream infection (132). In this study the term "bloodstream infection" was defined according to an ECDC technical protocol. The requirements for a laboratory-confirmed bloodstream infection were

> *"one positive blood culture for a recognised pathogen*
> *or*
> *patient has at least one of the following signs or symptoms: fever (> 38°C), chills, or hypotension*
> *and*
> *two positive blood cultures for a common skin contaminant (from two separate blood samples, usually within 48 hours)"* (135).

Of all WHO regions, South East Asia is believed to be among the WHO regions with the highest risk of emergence and spread of antibiotic resistance worldwide and countries within this region can expect to suffer increasing clinical consequences due to resistance. In an extensive qualitative risk assessment conducted by the WHO Regional Office for South East Asia and the Institute Pasteur Paris potential factors of emergence and spread of resistant bacteria in the WHO South East Asia region were analysed. Contributing factors to the development and selection of resistant bacteria in humans and animals were

examined at a policy (e.g. antibiotic stewardship policy framework), system (enforcement of policies like wastewater management) and individual (e.g. poor prescribing practices among health professionals) level. Inadequate use of antibiotics in humans and animals is believed to be one of the most severe factors to drive antibiotic resistance in this WHO region. Because of the increasing consumption of antibiotics in humans, the unlimited access to antibiotics without prescription and the substandard quality of drugs, resistance levels will likely increase as well (136).

# 4 Discussion

Antibiotic resistance existed long before the clinical use of antibiotic drugs and will continue to accompany us in the foreseeable future .(8) To understand the clinical impacts of this global health challenge and examine possible solutions it makes sense to first identify bacterial resistance mechanisms on a molecular level and gain insight on the bacteria's evolving physiology. The variety of mechanisms to combat antibiotic drugs by bacteria is fascinating and once again displays the wiliness of nature.

Here, I listed the best-known groups of bacterial resistance mechanisms and summarized their function as well as gave examples of bacteria that are able to express these exact mechanisms. Antibiotic resistance cannot be completely understood only by examining bacteria on a molecular level but rather has to be put into context with humans and the environment (85).

Anthropogenic factors contribute largely to the emergence and spread of antibiotic resistant bacteria and are an important leverage point to implement measures to mitigate resistance. Prescribing practices of physicians, extensive use of antimicrobials in livestock farming and aquacultures, dumping of drugs in the environment as well as international travel are factors that contribute largely to antibiotic resistance.

Although bacteria and their resistance genes know no geographical borders, there are pressing leads that low- and middle-income countries are and will be affected most by the consequences of antibiotic resistance. In those economies it will be essential to solve the current discrepancy of little access to antibiotics in rural areas and the increasing resistance in more urban locations. The lack of quality

laboratory tests to identify bacterial strains as well as conducting accurate susceptibility testing puts some low- and middle-income countries in a less equipped position to combat antibiotic resistance. Poor hygienic conditions, insufficient infection prevention and control measures and higher burden of bacterial illness add to the aforementioned circumstances.

To mitigate the potentially catastrophic consequences of antibiotic resistance, awareness and joint global action are necessary. As extensive as the causes of antibiotic resistance are, as diverse are the steps that can be taken to tackle the problem. Implementing strict antibiotic stewardship programs to encourage better prescription practices, banning the use of antimicrobials in livestock farming and aquaculture, carefully disposing waste of pharmaceutical drug production to pollute the environment as little as possible and gathering more data on the distribution of resistant bacteria are vital steps which are already being carried out and have potential to be intensified.

With an estimate of over 10 million deaths per year by 2050 accounted for by antibiotic resistance it is clear that this issue has to be taken seriously, and global awareness and action must be increased (87).

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