β-Lactamase-producing bacteria
How can I resist you?

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Abstract

In 1945 Alexander Fleming won the Nobel Prize for his discovery of the first antibiotic, penicillin, which is a natural β-lactam antibiotic. In an interview with the New York Times in the same year, he warned that misuse of the drug could result in selection of resistant bacteria. Shortly after this prediction, a significant number of strains of bacteria had become resistant to penicillin. Since the discovery of penicillin, a lot of other antibiotics have been discovered. For decades bacterial infections were treated with antibiotics effectively, but bacterial antibiotic resistance became a worldwide public health problem that continues to grow. Antibiotic resistance can develop through spontaneous genetic mutations or by transfer of genes carrying antibiotic resistance markers. The most common type of resistance is caused by the bacterial production of β-lactamases that inactivate β-lactam antibiotics by hydrolyzing the β-lactam ring. Some bacteria are resistant to all approved antibiotics and can only be treated with experimental drugs. Multi-drug resistant bacteria have already caused many deaths. This urgently calls for measures to fight infections by alternative ways. The question I would like to answer in this thesis is: What can be done to reduce the rising risk of antibiotic resistance, especially of β-lactamase-producing bacteria?

Contents

Introduction

Antibiotics have been critical in the fight against infections caused by bacteria and other microbes. However, bacteria can become resistant to antibiotics. The most common and important mechanism of resistance in Gram-negative bacteria is production of β-lactamases. Enzymes like these are able to inactivate a particular class of antibiotics, called the β-lactams. Nowadays antibiotic resistance by bacteria has become an increasing major health problem. Bacteria may be intrinsically resistant to β-lactam antibiotics, or may acquire resistance by *de novo* mutation or via the acquisition of resistance genes from other bacteria. Antibiotic resistance is particularly rising because of inappropriate use of antibiotics in human medicine, but also because of practices in the agricultural industry. Pharmaceutical chemists tried to overcome this resistance by the introduction of new β-lactam antibiotics, nevertheless resistance arose to these antibiotics as well; by the occurrence of new types of β-lactamases. Another way to overcome β-lactamase-mediated resistance was by the introduction of β-lactamase inhibitors into clinical practice. Although β-lactamase inhibitors have a β-lactam ring that is characteristic for the structure of β-lactam antibiotics, they only possess little antibiotic activity of their own. However, when administrated together with a β-lactam antibiotic they can reduce resistance. Despite the seemingly powerful inhibition mechanism, some bacterial strains that are resistant to these inhibitors have emerged. Some bacteria are even resistant to all approved antibiotics and can only be treated with experimental drugs. Currently, pharmaceutical companies do not want to invest in development of new antibiotics anymore, because the development is too expensive, since new antibiotics will be used as little as possible to prevent the selection of bacteria that have developed resistance against these new antibiotics. The worldwide rise of antimicrobial resistance in bacteria, combined with the decreasing number of innovative antibacterial agents, can soon lead to failure of treating bacterial infections, both in humans and animals. Infections with β-lactamase-producing bacteria are associated with increased morbidity, mortality, and healthcare costs. This urgently calls for measures to fight infections by alternative ways. The question I would like to answer in this thesis is: What can be done to reduce the rising risk of antibiotic resistance, especially of β-lactamase-producing bacteria?

β-Lactam antibiotics and β-lactamase inhibitors

Bacteria often develop resistance to β-lactam antibiotics by synthesizing a β-lactamase that attacks the β-lactam ring of β-Lactam antibiotics. To overcome this resistance, β-lactam antibiotics are often given together with β-lactamase inhibitors such as clavulanic acid.

β-Lactam antibiotics

More than 80 years ago the first natural antibiotic penicillin was discovered at St Mary's Hospital in London by Alexander Fleming; *Penicillium rubens* (Fleming 1929). At first there was only little attention for his discovery, but when World War II began an urgent need for antibacterial drugs led to further development of penicillin for therapeutic use (Herrell & Keefer 1945). The penicillin could not be produced in quantities large enough to be able to treat infections in patients yet. There are many species of Penicillium, a hunt for a better source of penicillin followed, by Ernst Chain and Howard Florey (Chain et al. 1940). Eventually an employee at the Peoria laboratories, Illinois, named Mary Hunt found in 1943 a mold growing on a rotting cantaloupe. This new mold species appeared to be *Penicillium chrysogenum,* and produced 200 times as much penicillin as *Penicillium rubens*. Mutating the species by using X-rays and UV light, lead to a mutant that produces 1,000 times the amount of penicillin than Fleming's original culture (Raper et al. 1944; Veerapagu et al. 2008). This lead to the result that by D-Day, June 6, 1944, there was enough penicillin to treat every soldier that needed it. Because penicillin could now be used as a therapeutic agent for patients, the mortality rates of the United States due to bacterial infections reduced significantly (Ho 1999; Lerner 2004). For their work Alexander Fleming, Ernst Chain and Howard Florey were awarded the Nobel Prize in 1945 (Nobelprize.org 2013).

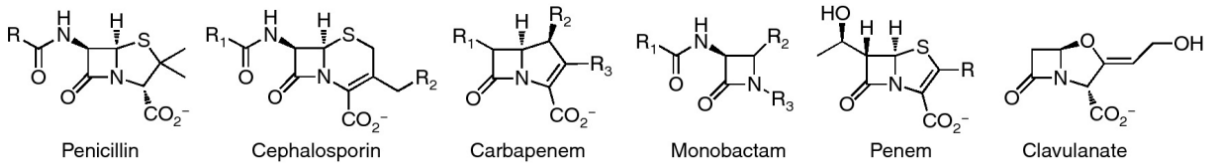

Figure 1. The β-lactam family and the β-lactamase inhibitor (Llarrull et al. 2010).

Table 1. List of the five types of β-lactams antibiotics and the β-lactamase inhibitors. (G = generation, V= veterinary, Ad. G = Advanced generation.)

Penicillins (Penams)		Cephalosporins/Cephamycins		Carbapenems	Monobactams	Penems	β-lactamase inhibitors
Benzylpenicillin	1st G	Cephalothin	1st G	Imipenem	Aztreonam	Faropenem	Clavulanic acid
Oxacillin	2nd G	Cefoxitin	2nd G	Meropenem			Sulbactam
Cloxacillin	2nd G	Cefotetan	2nd G				Tazobactam
Methicillin	2nd G	Cefmetazole	2nd G				
Ampicillin	3rd G	Cefotaxime	3rd G				
Amoxicillin	3rd G	Ceftazidime	3rd G				
Piperacillin	4th G	Ceftriaxone	3rd G				
		Ceftiofur	3rd G (V)				
		Cefepime	4th G				
		Cefquinome	4th G				
		Ceftobiprole	5th G				
		Ceftaroline fosamil	Ad. G				

In an interview with the New York Times, in the same year he won the Nobel Prize, Fleming warned for bacterial resistance against penicillin. Within four years after the widespread use of penicillin, resistance began to emerge. The first recorded case of resistance was in 1947 (Barber 1947). A lot of β-lactam antibiotics have been discovered since the discovery of the first antibiotic of this class, see table 1. Nearly all antibiotics in use today are compounds that were discovered during the 1940s to 1960s; the golden era of antibiotic discovery. A lot of these compounds were discovered by screening soil-derived Actinomycetes (Lewis 2013). Most of these antibiotics have lost their efficacy over time (Hogberg et al. 2010). Since the 1960s no new antibiotics have been discovered. Although several natural products or derivatives thereof have recently been developed and approved; these are all based on old discoveries (Lewis 2013).

The β-lactams are the most successful class of antibiotics developed so far (Lewis 2013). Among them, penicillins are the most frequently prescribed antibiotics, see figure 2 (Adriaenssens et al. 2011). β-Lactam antibiotics are antibiotics that contain a β-lactam ring; a four-sided structure, three carbon atoms and one nitrogen atom, see figure 1 (Nicola et al. 2010). The first synthetic β-lactam ring system was prepared by Hermann Staudinger in 1907 (Staudinger 1907). However this method did not get much attention until 1945, when the structure of penicillin was elucidated by Dorothy Crowfoot Hodgkin. She showed by X-ray crystallography analysis that penicillin is composed of a β-lactam structure (Crowfoot et al. 1949).

The β-lactams inhibit the formation of the bacterial cell wall. The bacterial cell wall is different from that of all other organisms due to the presence of peptidoglycan (Llarrull et al. 2010). Peptidoglycan is responsible for the rigidity of the bacterial cell wall and for the determination of cell shape. Peptidoglycan consists of poly-N-acetylglucosamine (NAG), N-acetylmuramic acid (NAM) and an oligopeptide in order to preserve the osmotic stability of the cell. The peptidoglycan layer is substantially thicker in Gram-positive bacteria (20 to 80 nanometer) than in Gram-negative bacteria (7 to 8 nanometer). A Gram-positive cell contains one membrane, with a big layer of peptidoglycan on the outside of the bacteria. A Gram-negative bacterium contains two membranes, with a small layer of peptidoglycan in between. The secondary membrane serves as a tough armor. Because of

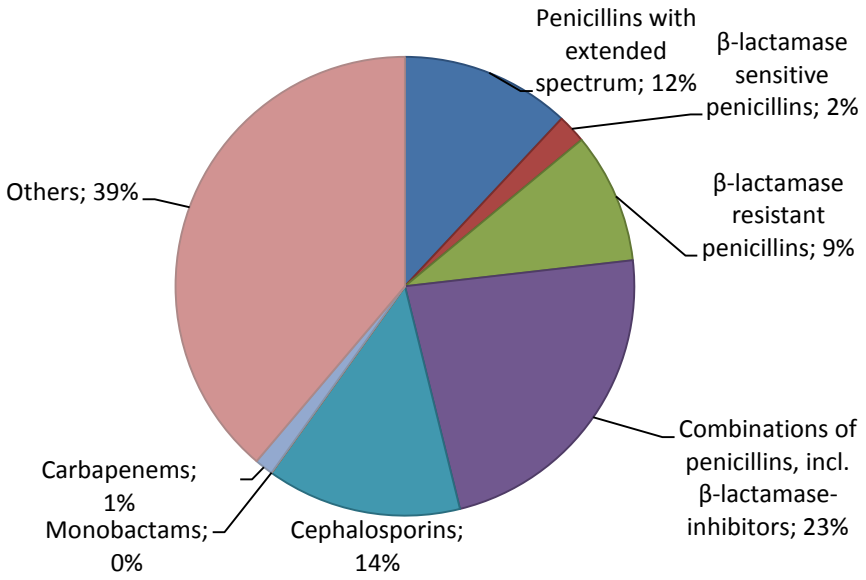

Figure 2. Distribution of the use of antibiotics for systemic use in hospitals in The Netherlands. 'Others' means non-β-lactams (Swab 2007).

the permeability barrier provided by the outer membrane (OM), Gram-negative bacteria are naturally resistant to many hydrophobic antibiotics. However to expand the arsenal of effective antibiotics against Gram-negative bacteria compounds that permeabilise the OM, hydrophobic substances have been developed (Savage 2001). β-Lactam antibiotics irreversibly bind to, and inactivate transpeptidase, also referred to as penicillin-binding protein (PBP). The function of these PBPs is cross-linking of the neighboring oligopeptides to form peptide bridges, resulting in rigid cell walls, see figure 4 (Nicola et al. 2010). The glycosidic units, NAM and NAG, are connected by transglycosidases. A pentapeptide is attached to each NAM unit, and the cross-linking of two D-alanine-D-alanine NAM pentapeptides is catalyzed by PBPs. The β-lactam ring is sterically similar to the D-alanine-D-alanine of the NAM pentapeptide, and PBPs `mistakenly' use the β-lactam as a `building block' during cell wall synthesis. This leads to acylation of the PBP, which will render the enzyme incapable to catalyze the transpeptidation reactions (Drawz & Bonomo 2010). When the cell wall synthesis has been inhibited by a β-lactam antibiotic, the peptides bridges cannot be formed anymore and the bacterial cell wall will rupture because of high internal osmotic pressure. Complex formation between the PBP and the antibiotic stimulates the release of autolysins, which are capable of digesting the bacterial cell wall. Autolysins are naturally produced by peptidoglycan containing bacteria in low levels; they are needed because the peptidoglycan layer is very rigid. These enzymes break the peptidoglycan layer down into small sections so that growth and cell division can occur, autolysins do this by hydrolyzing the bond between NAM and NAG molecules (Kitano & Tomasz 1979). In combination with β-lactam antibiotics, autolysins help to break down the bacterial cell wall, and thereby kill the bacteria. Whereas other antibiotics can kill non-growing cells, β-lactams are only active against growing bacteria (Lewis 2013).

The β-Lactam antibiotics discovered after penicillin, are: cephalosporins, carbapenems, monobactams, penems and β-lactamase inhibitors (table 1 and figure 1). Structural differences among these antibiotics confer differences in susceptibility to β-lactamases. To some antibiotics oxyimino groups were added by pharmaceutical chemists, in order to protect the β-lactams by shielding the β-lactam ring (see figure 3). Monobactams are β-lactam compounds wherein the β-lactam ring is not fused to another ring, in contrast to most other β-lactams. The only commercially available monobactam antibiotic is aztreonam (Breuer et al. 1985; Hauser 2012). The carbapenems are similar in structure to the penicillins (penams) and penems, however the sulfur atom of the structure has been replaced with a carbon atom, and there is a trans configuration of the hydroxyethyl group. This trans configuration in carbapenems increases their potency compared to penicillins and cephalosporins (Jones et al. 2005; Moellering, Jr. et al. 1989; Mushtaq et al. 2004; Norrby 1995; Papp-Wallace et al. 2011). The only antibiotics that are still effective against most β-

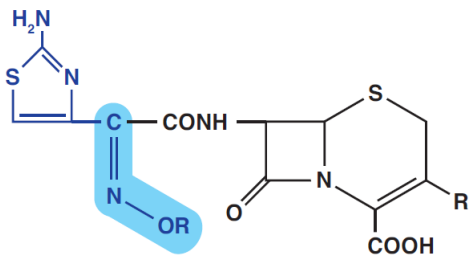

Figure 3. `β-Lactamase-stable' oxyimino cephalosporins. The oxyimino group is marked in light blue. The cephem nucleus is depicted in black (Cricco et al. 1999).

lactamase-producing bacteria are cephamycins (e.g. cefoxitin and cefotetan) and carbapenems (imipenem or meropenem) (Hamouda et al. 2011; Paterson 2000; Philippon et al. 1989). Cephamycins are a group of β-lactam antibiotics very similar to cephalosporins; sometimes cephamycins are classified as cephalosporins. Like cephalosporins, cephamycins are based upon the cephem nucleus (see figure 3). However, unlike most cephalosporins, cephamycins are very effective antibiotics against anaerobic microbes (e.g. in intra-abdominal sepsis, decubitus ulcers, and diabetic foot infections). The cephamycins (cefoxitin, cefotetan, and cefmetazole) are structurally different from the `true' cephalosporins and have enhanced stability to β-lactamases. Cephamycins contain a methoxyl side chain and are produced by *Streptomyces* (prokaryotic filamentous bacteria), in contrast to the penicillins and cephalosporins which are produced by fungi (Liras & Demain 2009).

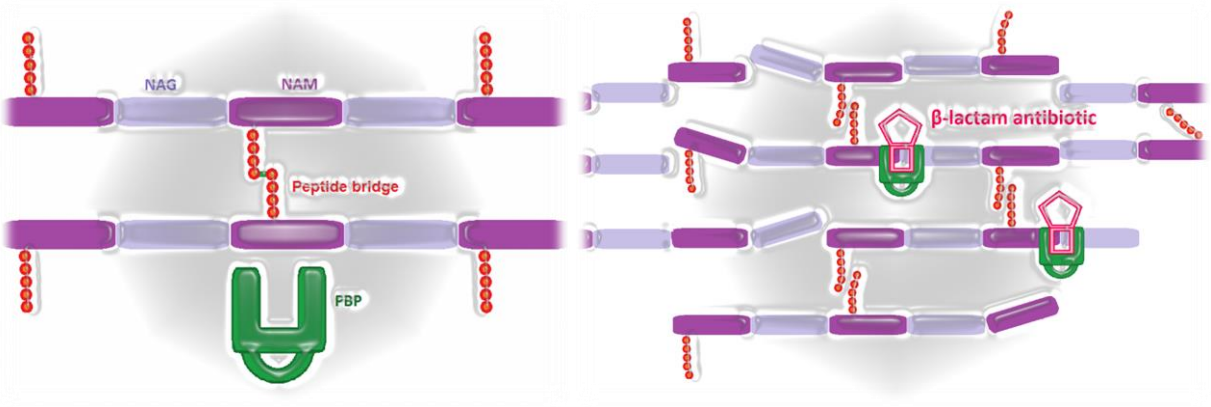

Figure 4. Left: Crosslinking of the peptidoglycan chains by the enzyme transpeptidase, which is a PBP (green). The NAG and NAM molecules are respectively depicted in light and dark purple. Right: The interference of β-lactam antibiotics (pink) with the PBP; there are no peptide bridges anymore and the NAG and NAM molecules are not aligned anymore, which results in disrupting the cell wall synthesis.

β-lactamase inhibitors

To limit the effectiveness of β-lactamases, β-lactamase inhibitors (clavulanic acid, sulbactam and tazobactam) are used in combination with β-lactams for the treatment of infections by β-lactamase-producing bacteria (Manickam & Alfa 2008; Sulton et al. 2005). All three β-lactamase inhibitors have a similar mechanism of action; they bind to β-lactamase and disable the enzymatic function (Kalp et al. 2009). In this way they allow the antibiotic to keep its lethal effect against β-lactamase-secreting bacteria. Just as β-lactam antibiotics, β-lactamase inhibitors also contain a β-lactam ring. Despite sharing the β-lactam ring, β-lactamase inhibitors do not display antimicrobial activity; its principal function is β-lactamase inhibition. The similarity in chemical structure between the antibiotic and the inhibitor allows the molecule to interact with β-lactamase. Clavulanic acid, sulbactam and tazobactam are all irreversible 'suicide inhibitors' which can permanently inactivate the β-lactamase, this may be more effective than reversible inhibition (Drawz & Bonomo 2010; Finlay et al. 2003). The mechanism of action of β-lactamase inhibitors is to form an irreversible link with β-lactamase by binding to a serine residue in the active site of the enzyme. This restructures the β-lactamase inhibitor molecule, generating a much more reactive species that is attacked by another amino acid in the active site, inactivating it permanently, and as a result inactivating the enzyme. This inhibition brings the antimicrobial activity of β-lactam antibiotics against β-lactamase-secreting bacteria back (Booth & McDonald 1991).

These inhibitors greatly improve the effectiveness of their partner β-lactams (amoxicillin, ampicillin, piperacillin, and ticarcillin) in the treatment of severe infections (Drawz & Bonomo 2010). A widely used multi-drug formulation is Augmentin, it is one of the most successful antibiotics currently on the market (Lewis 2013). Augmentin contains a combination of the antibiotic amoxicillin and the in 1976 discovered naturally produced β-lactamase inhibitor clavulanic acid isolated from *Streptomyces clavuligerus* (Brown et al. 1976; Salvo et al. 2007). When amoxicillin was combined with clavulanate, the minimum inhibitory concentration (MIC) was significantly lowered against *S. aureus*, *K. pneumoniae*, *Proteus mirabilis*, and *E. coli*. Other combinations are the antibiotic ampicillin together with the inhibitor sulbactam, and the antibiotic piperacillin together with the inhibitor tazobactam; these antibiotics are all extended spectrum penicillins. These three combinations of antibiotic and inhibitor were respectively introduced in clinical practice in 1981, 1986 and 1993 (Desai et al. 2008; Lamp & Vickers 1998; Nathani et al. 1998). In spite of this seemingly powerful inhibition mechanism, some bacterial strains that are resistant to even these inhibitors have emerged (Kim et al. 2009). Novexell and AstraZeneca just developed a new β-lactamase inhibitor, avibactam, which is active against most β-lactamases (AstraZeneca 2012).

β-Lactamases

Acquisition of resistant genes

The introduction of an antibiotic, acts as a selective pressure; only the bacteria carrying genes that confer resistance to the drug will survive and reproduce. When resistant bacteria reproduce, the resistant genes are passed on to their offspring; this is called vertical gene transfer. Resistant genes can also be acquired by horizontal gene transfer (Andersson & Hughes 2010). Horizontal gene transfer is any process in which an organism incorporates genetic material from another organism without being the offspring of that organism; genes pass from a resistant strain to a non-resistant strain, hereby conferring resistance. Horizontal gene transfer was described for the first time in 1959 by Japanese scientists who showed the transfer of antibiotic resistance between different bacteria species (Akiba et al. 1960). Horizontal gene transfer is even common amongst very distantly-related bacteria. This process is thought to be a significant cause of increased drug resistance (Barlow 2009; Hawkey & Jones 2009). Resistance against β-lactam antibiotics can be acquired by several routes: by transformation, conjugation, transduction and mutations (see figure 5). Bacteria can become resistant by spontaneous mutations; in bacterial populations mutations are continuously arising because of errors which occur during replication. When a mutation arises with a selective advantage, for instance antibiotic resistance, the mutant will rapidly become the main component of the population due to the fast growth rate of bacteria (Tenover et al. 1999; Tenover 2006). Transduction is the process by which DNA is transferred from one bacterium to another by a virus. When a bacterium with a gene for antibiotic resistance dies, the naked DNA is released in the surrounding environment. A bacterium can uptake this gene and transfer it from the naked DNA to its host DNA, this is called homologous transformation. The main method of transfer of genes from one bacterium to another is by conjugative plasmids. Conjugation is transmission of resistance genes via plasmid exchange with help of a pilus. This type of acquisition permits resistance to spread between a population of bacteria much quicker than mutation and vertical evolution would allow (Andersson & Hughes 2010). When a bacterium has taken up resistance genes, it can remodel its host DNA. Pathogens continuously develop resistance; as a result we are reaching the fourth generation of semi-synthetic β-lactams (Garza-Ramos et al. 2008; Higgins et al. 2010; Livermore & Woodford 2006).

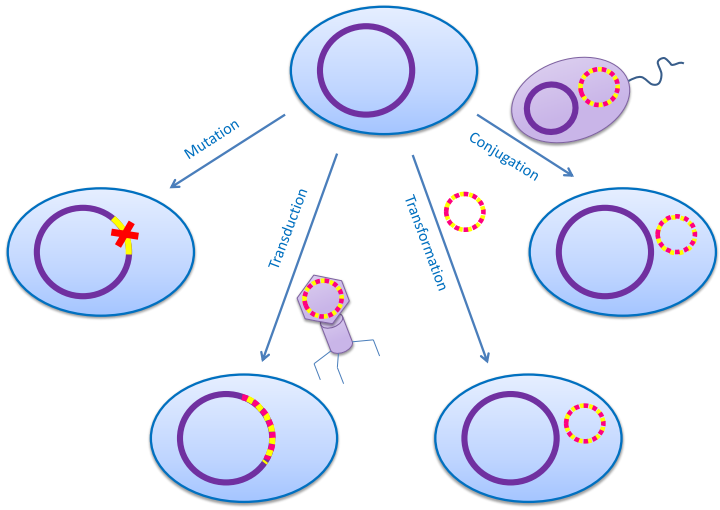

Figure 5. Schematic representation of bacteria acquiring resistant genes by horizontal gene transfer by transduction, transformation and conjugation. Resistance can also arise by novo mutation (Andersson & Hughes 2010).

Classification of β-lactamases

There are four ways how bacteria can become resistant against β-lactam antibiotics: by producing β-lactamase, by altered PBPs, by decreasing the expression of outer membrane proteins (OMPs) and by efflux pumps. The most common and important mechanism of resistance of bacteria to β-lactam antibiotics is the production of β-lactamases. These enzymes produced by some bacteria, provide resistance to β-lactam antibiotics. β-Lactamases can cleave the β-lactam ring and hydrolyze it, rendering the drug harmless, see figure 7.

There have been different ways proposed to classify β-lactamases. One of the first classification schemes was attempted by Sawai in 1968; it was founded on separation of penicillinases and cephalosporinases based on their response to antisera (Sawai et al. 1968). Built on the proposal of Richmond and Sykes in 1973, β-lactamases of Gram-negative bacteria were classified into 5 groups based on their substrate profile (Richmond & Sykes 1973). The first molecular structure classification scheme, based upon their amino acid sequences, was suggested by Ambler in 1980; the β-lactamases where classified in four classes (A, B, C and D), see figure 6 (Ambler 1980). Bush proposed an alternative classification in 1989 based on the action of the enzymes on the substrates (penicillin, oxacillin, carbenicillin, cephaloridine, expanded-spectrum cephalosporins, and imipenem) as well as susceptibility for inhibition by clavulanic acid. This classification was further updated in 1995 by Bush, Jacoby and Medeiros and revised again in 2009. However Ambler's molecular classification appears to be widely accepted instead of the Bush's phenotype classification, due to its simplicity and phylogenetic relationship among the enzymes. Class A and D include the classic and extended-spectrum β-lactamases (ESBLs) and are mostly composed of the TEM, SHV, CTX-M and OXA enzymes, class B comprises the metallo-β-lactamases and finally class C contains of the AmpC β-lactamases (Bush et al. 1995). Except for class B metalloenzymes, β-lactamases belong to the family of serine-reactive hydrolases (Ambler 1980; Rawlings et al. 2008).

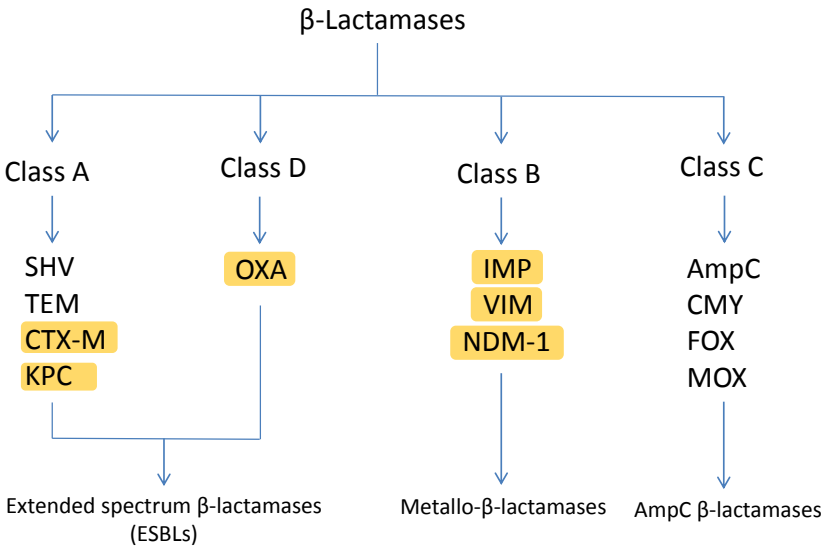

Figure 6. Classification scheme of a part of the β-lactamases. The β-lactamases marked in orange are carbapenemases (Ambler et al. 1991).

Types of β-lactamases

The β-lactamases have been subdivided in the AmpC-type β-lactamases, classic β-lactamases, extended-spectrum β-lactamases and metallo-β-lactamases.

AmpC-type β-lactamases

The first bacterial enzyme reported to destroy penicillin was the AmpC β-lactamase of *E. coli* (Abraham & Chain 1940). This was even before penicillin was used in medical practice. AmpC type β-lactamases are generally isolated from extended-spectrum cephalosporin-resistant Gram-negative bacteria. AmpC β-lactamases are characteristically encoded on the chromosome of several Gram-negative bacteria, such as: Citrobacter, Serratia and Enterobacter species (including *E. coli*). AmpC type β-lactamases can be carried on plasmids. AmpC β-lactamases, in contrast to ESBLs, hydrolyze cephamycins as well as oxyimino-β-lactams, and are not inhibited by β-lactamase inhibitors. Furthermore they can, in strains with the absence of outer membrane porins, be resistance to carbapenems (Philippon et al. 2002). It has been reported that β-lactamase-producing strains can also become resistant to cephamycins (cefoxitin and ceftazidime) due to the loss of the outer membrane porin Omp-K35 in the isolates of *Klebsiella pneumoniae* (*K. pneumoniae*) and *Escherichia coli* (*E. coli*). This porin forms a water filled channel that permits small hydrophilic solutes like β-lactam antibiotics to gain access to the cell interior (Ananthan & Subha 2005; Delcour 2009). Other AmpC-type β-lactamases have been named with inconsistency typical of β-lactamase nomenclature according to the resistance produced to cephamycins (CMY), cefoxitin (FOX) and moxalactam (MOX) (Philippon et al. 2002).

Classic β-lactamases

Many Gram-negative bacteria possess naturally occurring, chromosomally mediated ß-lactamases. These enzymes are thought to have evolved from penicillin-binding proteins, with which they show some sequence homology. This development was likely due to β-lactam-producing soil organisms found in the environment (Bradford 2001; Guysen 1991). However resistant genes spread more easily when plasmid-encoded. β-Lactamase genes are often located on plasmids, which may also carry virulence factors, that are transferable from strain to strain and between bacterial species (Da Silva & Mendonca 2012). The first plasmid-mediated β-lactamase isolated from a single strain of *E. coli* from a blood culture was the penicillinase TEM-1; reported in 1965 (Datta & Kontomichalou 1965). TEM is named after the first patient from whom the pathogen was isolated; a Greek girl named Temoniera. TEM-1 is the most common plasmid-mediated β-lactamase of organisms such as

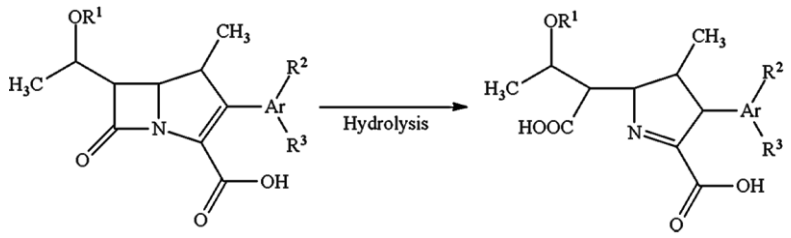

Figure 7. Hydrolysis of the β-lactam ring of the β-lactam antibiotic carbapenem by a β-lactamase (Pfaendler & Golz 2013).

E. coli (Paterson 2000). Being plasmid and transposon mediated, enabled the spread of TEM-1 to other bacteria species. This transposon is a genetic element which is capable of inserting into diverse plasmids of Gram-negative bacteria. Within a few years after the first isolation of TEM-1, the TEM β-lactamase spread all over the world and is now found in many different species of members of Enterobacteriaceae. Enterobacteriaceae are named for the organisms' predominant natural habitat, the intestines of animals (from Greek enteron, meaning `intestine'). It is a large family of Gram-negative bacteria that includes pathogens such as: Salmonella, *E. coli*, *Yersinia pestis*, Klebsiella and Shigella.

Another common plasmid-mediated ß-lactamase is the penicillinase SHV-1, which is chromosomally encoded in isolates of *K. pneumoniae* and usually plasmid mediated in *E. coli* (Pitout et al. 2005). SHV is named after the sulfhydryl variable active site, it was first mentioned in 1972 (Pitton 1972). Early β-lactam antibiotics, such as penicillins and first-generation cephalosporins, are efficiently hydrolyzed by β-lactamases such as TEM-1, TEM-2, or SHV-1. Till the end of the nineties SHV and TEM ESBLs were by far the most common and were strongly associated with care institutions (Matagne et al. 1999; Paterson 2000).

Extended-spectrum β-lactamases

Extended-spectrum cephalosporins, such as cefotaxime and ceftazidime, have been developed to get around the hydrolytic action of β-lactamases. These molecules escape most β-lactamases by the introduction of oxyimino side chains that make them less susceptible to β-lactamases. The oxyimino group was made by pharmaceutical chemists in order to protect the β-lactams by shielding the β-lactam ring (see figure 3). They confer stability to most serine β-lactamases (Nukaga et al. 1994). These β-lactams were increasingly efficient and resistant to β-lactamases (Delmas et al. 2010). However, with every new class of β-lactams that was used to treat patients, new β-lactamases appeared that caused resistance tot that class of drug (Bradford 2001). Mutations in the genes encoding TEM-1 or SHV-1 extend the spectrum of activity of the β-lactamase so that inactivation of third-generation cephalosporins and the monobactam aztreonam occurred (Paterson 2000). In 1983, within two years after the clinical introduction of cefotaxime and ceftazidime, a new group of enzymes was reported in Germany; the ESBLs (Knothe et al. 1983). These ESBLs were found among different enterobacterial isolates from inpatients at intensive care units (ICUs). ESBLs were derived from the classic β-lactamase genes by a few point mutations in the gene resulting in one or more amino acid substitutions around the active site that enabled the enzyme to bind and hydrolyze the new drugs (Manoharan et al. 2011). The first ESBL, capable of hydrolyzing the newer β-lactams, was a variant of SHV-1 with a change of a single amino acid, which was named SHV-2. SHV-2 was recognized because of its abnormal resistance to cefotaxime and ceftazidime, which was transferable by conjugation to *E. coli* (Knothe et al. 1983). ESBLs are β-lactamases which have the ability to hydrolyze the β-lactam ring of the extended-spectrum cephalosporin and monobactam antibiotics containing an oxyimino group (e.g. ceftazidime, ceftriaxone, cefotaxime and aztreonam), rendering the antibiotics useless (Paterson 2000; Pitout et al. 2005; Tenover et al. 1999). ESBLs can be inhibited by β-lactamase inhibitors (clavulanic acid, tazobactam, and sulbactam) (Manickam & Alfa 2008). ESBLs are commonly resistant to other antimicrobial agents, such as fluoroquinolones, aminoglycosides and trimethoprim-sulfmethoxazole because of mobile genetic elements encoding other antimicrobial resistance determinants and/or chromosomal mutations (Fennell et al. 2012).

The only antibiotics that are still effective against most ESBL-producing bacteria are cephamycins and carbapenems (Paterson 2000; Philippon et al. 1989). Very quickly after the detection of SHV-2, isolates with an identical phenotype were found in hospitals in France in 1984 (Jacoby et al. 1988; Quinn et al. 1989). In this case it was a variant of the TEM-2 β-lactamase which has two amino-acid substitutions, and was named TEM-3.

In 1990 a new ESBL family was isolated in Munich; CTX-M β-lactamases. They were named after the place of discovery and their greater activity against the oxyimino-β-lactam cefotaxime (a third generation cephalosporin) (Bauernfeind et al. 1990). Most of the enzymes from this family confer resistance mainly to cefotaxime rather than to ceftazidime (both are third generation cephalosporin) (Bauernfeind et al. 1990; Bauernfeind et al. 1992). CTX-M enzymes have a small active site, which can recognize the bulky extended-spectrum cephalosporins because of the flexibility of their active site and an extensive network of electrostatic interactions (Delmas et al. 2010). Instead of arising by mutation, like TEM- and SHV-type ESBLs, CTX-M β-lactamases are plasmid mediated, normally found on the chromosome of Kluyvera species. CTX-M enzymes are not very closely related to TEM or SHV β-lactamases; they show only approximately 40% identity with these two commonly isolated β-lactamases. Since 2000, *E. coli* producing CTX-M β-lactamases involved in community-onset urinary tract infections (UTIs) have emerged worldwide. CTX-M-15 is currently the most widely spread CTX-M enzyme, which was detected for the first time in *E. coli* from India in 2001 (Karim et al. 2001).

The earliest β-lactamases producing bacteria, primarily *K. pneumoniae*, were almost exclusively associated with nosocomial outbreaks, primarily in ICUs, and it was very uncommon for them to be associated with community acquired infections (Winokur et al. 2001). However, in the last few years this situation has radically changed by the arrival of CTX-M enzymes. These enzymes are not limited to hospital-acquired infections caused by Klebsiella species anymore, and are now mostly expressed in *E. coli* strains recovered from community patients. These patients mostly had UTIs and did not have a history of hospitalization or antimicrobial use (Calbo et al. 2006; Dubois et al. 2010; Friedmann et al. 2009; Valverde et al. 2004). The factors contributing to their successful spread remain unclear (Mnif et al. 2010). Clonal outbreaks of CTX-M-15-producing *E. coli* have been reported globally with the occurrence of a highly virulent *E. coli* clone. *E. coli* with CTX-M, especially CTX-M-15, most often leads to UTIs, but also to bacteremia (the presence of bacteria in the blood) and gastroenteritis (stomach flu). Furthermore the numbers of isolates expressing ESBLs have increased in non-ICU areas, such as nursing homes and healthcare associated facilities (Ben-Ami et al. 2006; Pitout et al. 2004; Rodriguez-Bano et al. 2006).

The first Klebsiella pneumoniae carbapenemase (KPC)-producing *K. pneumoniae* isolate was reported in North Carolina in 2001 (Yigit et al. 2001). Carbapenemase possess a prominent carbapenem-hydrolyzing activity. KPC-producing bacteria are a group of emerging highly drug-resistant Gram-negative bacilli causing infections associated with significant morbidity and mortality (Arnold et al. 2011). The carbapenemase bla_{KPC} gene that is rapidly spreading worldwide is located on a Tn3-based transposon (Tn4401). This is an active transposon capable of mobilizing bla_{KPC} genes at high frequency (Cuzon et al. 2011). In 2010 the KPC-producing Enterobacteriaceae are the most common carbapenemase-producing Enterobacteriaceae in the UK, see figure 8.

Another growing family of ESBLs are the OXA-type enzymes (oxacillinases). They were discovered in 1991 in Turkey and are frequently detected in *Pseudomonas aeruginosa (P. aeruginosa),* while most

ESBLs are found in *Enterobacteriacea*e (Bush et al. 1995; Queenan & Bush 2007). OXA β-lactamases are characterized by their high hydrolytic activity against oxacillin and cloxacillin (both penicillins). When the OXA-type ESBLs are cloned into *E. coli*, it provides weak resistance to oxyimino-cephalosporins, but they provide a fairly high-level of resistance in *P. aeruginosa* transconjugants (Hall et al. 1993). The fact that carbapenems are the treatment of choice for serious infections caused by ESBLs, has led to an increased reliance on carbapenems in clinical practice (Rhomberg & Jones 2009). OXA-48 is one of the few members of the ESBLs that produces carbapenemase (Moquet et al. 2011). OXA-48 had first been identified from a clinical *K. pneumoniae* isolate recovered in Istanbul, Turkey, in 2001 (Poirel et al. 2004). The carbapenemases are able to recognize almost all hydrolysable β-lactams, which makes it the most versatile family of the β-lactamases with a range of spectrum unrivalled by other β-lactamases. Most carbapenemases are unable to be inhibited by the commercially available β-lactamase inhibitors (Livermore & Woodford 2006; Nordmann & Poirel 2002; Walther-Rasmussen & Hoiby 2006).

Ever since the eighties a steady increase of strains producing ESBLs have been reported worldwide (Paterson et al. 2004). Since the late 1990s, ESBL-producing *E. coli* have been detected in retail meat and food producing animals in Europe and the United States (Aarestrup et al. 2006; Blanc et al. 2006; Jouini et al. 2007; Kojima et al. 2005; Zhao et al. 2001). A recent large survey from 1997 to 2000 of 1610 *E. coli* and 785 *K. pneumoniae* isolates from 31 centers in 10 European countries found that the prevalence of ESBL in these organisms ranged from as low as 1.5% in Germany to as high as 39–47% in Russia, Poland, and Turkey (Goossens 2001; Winokur et al. 2001). Until now more than 600 ESBLs variants are known; over 100 CTX-M-type β-lactamases, over 200 TEM-type enzymes, almost 200 SHV genes, around 10 OXA-type enzymes and some other ESBLs (www.lahey.org/Studies 2013). ESBLs have continued to increase in variety and are now a global health concern (Fennell et al. 2012).

Metallo-β-lactamases

In 1995, there was an worrying report from Japan about a plasmid-mediated gene recovered from a *Serriatia* isolate that encoded for an enzyme called Imipenem-hydrolyzing β-lactamase 1 (IMP-1), which was capable of hydrolyzing carbapenems (Imipenem) (Ito et al. 1995). This was the first detected carbapenemase, belonging to the metallo-β-lactamases. Metallo-β-lactamases have a structure that renders them highly resistant to most β-lactams. ESBLs have a serine-based hydrolytic mechanism, while metallo-β-lactamases contain zinc in the active site and are inhibited by EDTA (Queenan & Bush 2007). Metallo-β-lactamases have been detected primarily in *P. aeruginosa*, however, there are increasing numbers of reports worldwide of this group of β-lactamases in the *Enterobacteriaceae* (Queenan & Bush 2007).

Another member of the metallo-β-lactamases is the Verona integron-encoded metallo-β-lactamases (VIM) family, which was first reported in 1997 in a *P. aeruginosa* isolate from Italy (Lauretti et al. 1999). VIM is just as the other metallo-β-lactamases capable in hydrolyzing carbapenems.

A third growing family of metallo-β-lactamases is the plasmid encoding New Delhi Metallo-β-lactamase 1 (NDM-1) gene. This Metallo-β-lactamase, for which we do not have an inhibitor, has recently emerged and was named after New Delhi, where it was first described in 2009 (Kumarasamy et al. 2010; Yong et al. 2009; Yum et al. 2002). A Swedish national, originally from India, travelled to New Delhi (India) and acquired there a urinary tract infection. The infection was unsuccessfully treated in a New Delhi hospital, and, after the patient's return to Sweden, a carbapenem-resistant *K. pneumoniae* strain bearing the novel gene was identified (Yong et al. 2009). Examples of bacteria resistant to all existing antibiotics are the *Neisseria gonorrhoeae* strain H041 and *Enterobacteriaceae* expressing the NDM-1 (Kruttgen et al. 2011; Ohnishi et al. 2011). Carbapenemase-producing Enterobacteriaceae are spreading fast in the UK, as is seen in figure 8. In 2009 NDM had become the commonest acquired carbapenemase among isolates according to the Health Protection Agency (HPA) Antibiotic Resistance Monitoring and Reference Laboratory. The HPA is an independent body that protects the health and well-being of the population. Around half the patients originated from the UK have a history of medical exposure in India or Pakistan, where the enzyme is circulating widely. The nature of the exposure ranged from cosmetic surgery to renal transplantation. The worldwide increase in the use of carbapenems would be expected to drive resistance and this has begun to occur in the USA for KPC carbapenemase and in India for NDM metallo-carbapenemases (Hammerum et al. 2010; Hawkey & Jones 2009). Most carbapenemase-producing bacteria are very multi-resistant to all β-lactams, ciprofloxacin and most, sometimes all, aminoglycosides (Hawkey et al. 2011).

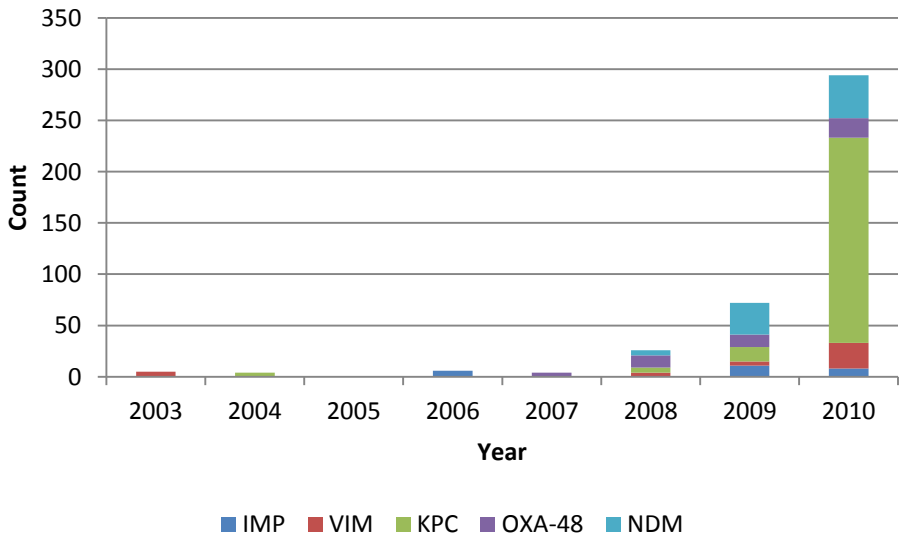

Figure 8. Carbapenemase-producing Enterobacteriaceae in the UK referred to the Antibiotic Resistance Monitoring and Reference Laboratory (ARMRL) (Livermore 2012).

The selection and spread of β-lactamase-producing bacteria

The selection pressure applied by the antibiotics that are used in clinical and agricultural settings has promoted the evolution and spread of genes that confer resistance. Because of the multiple levels of transmission it is very complicated to elucidate the epidemiology of the β-lactamase-producing Enterobacteriaceae. The sources and movement of antibiotic resistance are shown in a scheme in figure 9.

Selection of resistant bacteria by antibiotics in nature

Antibiotic resistance genes already existed before we begun using antibiotics. This fact offer direct evidence that antibiotic resistance is an ancient, naturally occurring phenomenon widely spread in the environment (D'Costa et al. 2011). Most antibiotics are produced by strains of fungi and bacteria that occur naturally in all environments, including soil. And most antibiotic-producing strains carry genes encoding resistance to the antibiotic they produce, as a self-protecting process (Allen et al. 2010). Determinants of antibiotic resistance exist naturally and were probably subjected to horizontal transfer long before the extreme selection pressure that was imposed in the `antibiotic era'. This predisposition for the genetic exchange of resistance elements is certain to have facilitated the rapid spread of antibiotic resistance in pathogenic bacteria.

Selection of resistant bacteria by medicine

Besides the presents of antibiotics in nature, millions of kilograms of antimicrobials are used each year in the treatment of people, animals and in agriculture (Mellon et al. 2001). The excessive use of prescribed antibiotics is the major factor in the increasing rates of bacterial resistance (Pechere 2001). A single dose of antibiotics leads to a bigger risk of occurrence of resistant organisms in a person for up to a year to that particular antibiotic (Costelloe et al. 2010). A large number of people

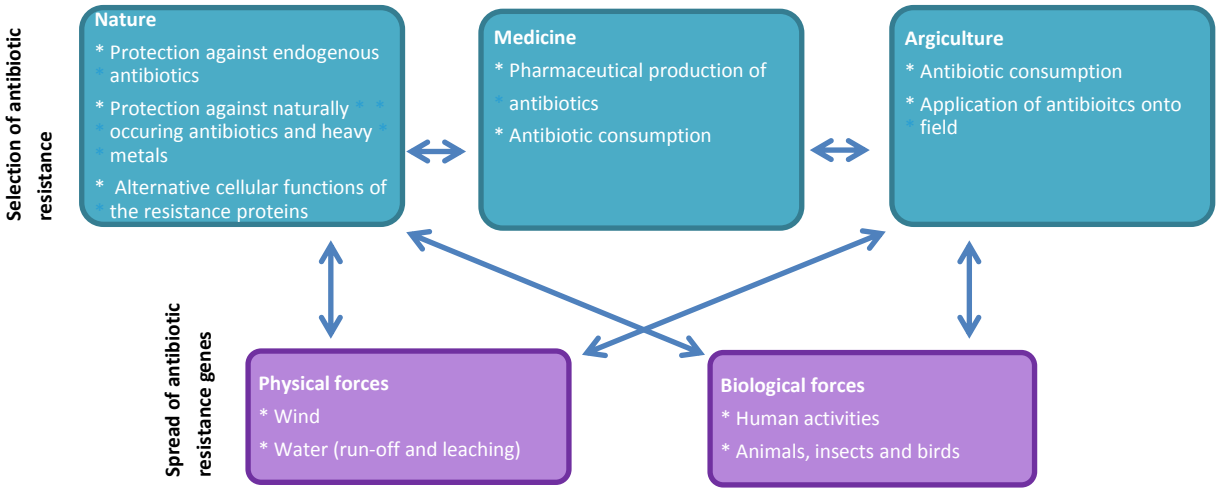

Figure 9. Sources and movement of antibiotic resistance genes in the environment. Resistance genes are selected by Nature, use of medicine and when AGPs are used in agriculture. Physical and biological forces cause widespread dissemination of resistance genes throughout many environments (Allen et al. 2010).

do not finish a course of antibiotics (varying from 10% to 44%, depending on the country) (Pechere et al. 2007). Inappropriate prescribing of antibiotics, for example physicians who prescribe antibiotics when they are not necessary, and physicians who do not know which antibiotics to prescribe, caused selection of resistant bacteria. A third of the people worldwide believe that antibiotics are effective for the common cold (McNulty et al. 2007). The common cold is the most popular reason antibiotics are prescribed even though antibiotics do not work against viruses (Arroll et al. 2002; Eccles & Weber 2009). Furthermore, antibiotics are for a large part unnecessarily prescribed for ear infections, bronchitis, sore throats and sinusitis. Even when prescribed correctly for bacterial infections, a patients failure to complete the full course of medicine can induce antibiotic-resistance (Kardas et al. 2005). U.S. Food & Drug Administration (FDA) data showed that 16.4 million kg of antibiotics are being produced each year, out of which 20% is used in human medicine and 80% of all antibiotics in the U.S. are sold for use in animal agriculture, of which are most identical or nearly so to human drugs (Center for a Livable Future. 2010).

Selection of resistant bacteria in agriculture

Shortly after the introduction of the therapeutic use of antibiotics, the growth-promoting effect of antibiotics in chickens was discovered. Experiments with germ-free chickens seemed to indicate that the action of the AGPs is mediated by their antibacterial effect. In these experiments, chicks with or without the administration of antibiotics were raised either in an environment with reduced hygiene to create chronic immune stress or in a clean environment. The chicks raised in the unhygienic environment without administration of antibiotics, significantly grew slower, had less feed consumption, and higher levels of plasma interleukin-1, in comparison to chicks raised in the clean environment or chicks raised in the unhygienic environment with administration of antibiotics (Roura et al. 1992). So, this effect of antibiotics is not obtained in environments in which bacteria are absent (Jukes & WILLIAMS 1953).

The vast majority of antibiotics used in animal agriculture (72%) are given to groups of animals in their feed or water often for nontherapeutic use, and 8% is for therapeutic use in agriculture (U.S.Food and Drug Administration. 2010; U.S.Food and Drug Administration. 2011). Antimicrobials are used for timely mass treatment of animals to eliminate or minimize an expected outbreak of a disease (called metaphylaxis) or routinely put in the food and water of healthy livestock at very low doses for growth promotion, particularly in pig, poultry and cattle production. Use of AGPs has become an important part of intense animal agriculture. The addition at low levels of certain antibiotics to the diet increases the rate of growth of young animals and the efficiency with which they metabolize food as measured by the ratio between food intake and increase in body weight. The average growth improvement was estimated to be between 4 and 8%, and feed utilization was improved by 2 to 5% (Ewing & Cole 1994). According to a study by the United States Department of Agriculture, 30% of feed costs among young swine are saved when antibiotics are administrated, however these savings disappear when the pigs get older (Mason & Mendoza 2009). A number of mechanisms of action were attributed to AGPs, but no clear understanding has been accomplished (Graham et al. 2007). However, several hypotheses has been proposed to explain the actions of the AGPs: nutrients may be protected against bacterial destruction, absorption of nutrients may improve because of a thinning of the small intestinal barrier, the antibiotics may decrease the

production of toxins by intestinal bacteria, and there may be a reduction in the incidence of subclinical intestinal infections (Feighner & Dashkevicz 1987).

β-lactam antibiotics, including third generation cephalosporins, such as ceftiofur, are used increasingly as growth promoter, these antibiotics are known to cause co-selection and to select for cross-resistance in bacteria common to man and animals. The use of antibiotics in non-therapeutic levels has been shown to select for antibiotic resistance in both commensal and pathogenic bacteria, in animals, animal-based food products and water, air and soil samples collected around large-scale animal feeding locations (Sapkota et al. 2007). Meat is routinely contaminated with antibiotic resistant bacteria. According to the Netherlands National Institute for Public Health and the Environment (RIVM) anno 2010, 88% of all broilers in the Netherlands were contaminated with antibiotics, while this was only 15% in 2006 (Leverstein 2010). Some antimicrobials used as AGPs (e.g. glycopeptides and streptogramins) are crucial drugs for the treatment of serious, possibly life-threatening, bacterial diseases in humans, such as infections with *Staphylococcus* or *Enterococcus*. Using antibiotics in animal agriculture leads to antibiotic-resistant infections in humans. A panel convened by the Institute of Medicine (IOM) and the World Health Organization (WHO) concluded antibiotic use in animal agriculture has been linked to the establishment and spread of drug-resistant infections in humans (Institute of Medicine. 2010; WHO 2002a).

Spread of antibiotic resistance genes by physical forces

Physical forces, such as those created by wind and watershed, are important drivers of the spread of antibiotic resistance genes (Allen et al. 2010). Antibiotics and their resistance genes have been widely distributed in the environment since before the introduction of antibiotic chemotherapies, but human activities have probably increased the prevalence of resistant bacteria in the air and water (Gandara et al. 2006; Rosas et al. 2006). Marine and freshwater ecosystems comprise bacteria from many sources, including antibiotic-resistant bacteria because of anthropogenic causes (Baquero et al. 2008). Due to the movement of antibiotics and resistance genes on the wind and on feathers, it is unlikely that any environment can be considered truly pure. Even bacteria from environments that are thought to be immobile can be moved by the forces of nature, an example is the global spread of bacteria on desert dust (Kellogg & Griffin 2006).

Direct contamination of watercourses can occur via animals in fields and spreading of fertilizer on land. Veterinary antibiotics are often excreted unchanged. For example, up to 75% of the broad-spectrum antibiotic tetracycline administered to swine was excreted unaltered (Chee-Sanford et al. 2001). The excreted antibiotics can persist in the environment, which creates an opportunity for selection of resistant bacteria. It has been estimated that approximately 70 million tons of animal fertilizer are spread onto agricultural land per year in the UK (Hutchison et al. 2004). Multidrug-resistant bacteria have been detected in subsurface water flow several months after pig slurry was applied to agricultural soils, illustrating persistence and dissemination to water catchments (Byrne-Bailey et al. 2011).

Spread of antibiotic resistance genes by biological forces

Wild animals offer a biological mechanism for the spread of antibiotic resistance genes. For example, 90% of the bacterial isolates from mice and voles from the countryside of England were resistant to β-lactam antibiotics (Gilliver et al. 1999). However, almost no resistance was found in the fecal enterobacteria of wild elk, deer and voles in Finland (Osterblad et al. 2001). Because Finland is considerably less densely populated than England, these results suggest that human activities affect antibiotic resistance in bacterial populations in wild animals (Allen et al. 2010).

ESBL-producing bacteria are most commonly found in the gastro-intestinal tract of colonized patients (carry the bacteria without having symptoms) and fecal carriage is thought to be the most important factor for the spread of ESBL-producing bacteria in the community (Kluytmans et al. 2013). Both human and animal feces contain large numbers of ESBL-producing coliforms (rod-shaped Gram-negative non-spore forming bacteria), which can cause cross-colonization to other individuals. This route is clearly very important in food-producing animals and extensive rapid spread through whole herds of animals has been seen (Garcia-Alvarez et al. 2012; Liebana et al. 2006; Teale et al. 2005).

Reports have also raised concerns about the spread of ESBL-producing *E. coli* in healthy food producing animals or in food products like meat, fish and raw milk, in several countries in Europe and Asia (Cortes et al. 2010; Duan et al. 2006; Goncalves et al. 2010; Hammad et al. 2008; Jensen et al. 2006; Jouini et al. 2007; Meunier et al. 2006; Tian et al. 2009). Recently, ESBL-producers were described for the first time in healthy dairy cattle and retail meat in the USA (Doi et al. 2010; Wittum et al. 2010). Furthermore, significant genetic similarities according to mobile resistance elements and virulence genes were found between ESBL-producing *E. coli* isolates from retail chicken meat and humans in the southern part of the Netherlands isolated during 2008 and 2009. This shows that a large part of the ESBL genes from the intestinal *E. coli* population from human, living in the southern part of the Netherlands, are derived from chicken meat. Therefore chicken meat is a likely contributor to the recent emergence of ESBL *E. coli* infections in humans (Kluytmans et al. 2013). However in a study performed in Switzerland these similarities between ESBL carriage in animals and human have not been found (Geser et al. 2012). Nevertheless, the findings in the Netherlands raise serious food safety questions regarding the abundant presence of ESBL-producing *E. coli* in chicken meat. Food has been identified as an important source of *E. coli* in the human gut. Food provides a continuous contribution of diverse *E. coli* (Bettelheim et al. 1977; Corpet 1988).

Evidence exists that antibiotic-resistant bacteria travel far. Large quantities of antibiotic resistant bacteria are now found all over the world; even in the arctic wildlife an unexpectedly high presence of drug-resistant *E. coli* was documented. These resistant bacteria may have been transported to the Arctic by migratory birds (Rosenblatt-Farrell 2009; Sjolund et al. 2008). In another study, 472 bacterial isolates were taken from vertebrates from the coastal waters of the north-eastern United States, including marine mammals, sharks, and birds. From these isolates, 58% showed resistance to at least one antibiotic, from which 43% were multidrug-resistant (Rose et al. 2009). A lot of farms use waste lagoons, which are responsible for an alternative route for the spread of antibiotic-resistant bacteria by birds and insects (Rosenblatt-Farrell 2009). Bacteria from flies collected from the areas surrounding a poultry production facility can demonstrate resistance consistent with the types of antibiotics being used there. Foreign travel may be a major risk factor for developing community-onset ESBL-producing *E. coli* infections (Hsieh et al. 2010). An example of international travel is the spread of the NDM-1 gene, as described above.

The treatment of infection with β-lactamase-producing bacteria

A study performed in a University Hospital in Switzerland identified three major risk factors for infection with the ESBL-producing bacteria: mechanical ventilation, onset of symptoms and/or start of treatment abroad, but mainly prior and repeated antibiotic use (Kuster et al. 2010). Every year approximately 25,000 people in the European Union (EU) die, because of infections with antibiotic resistant bacteria, most commonly acquired in hospitals (WHO 2002b).

To treat an infection caused by a resistant bacteria it would help if the determination of the exact bacterium could be done quickly (Forssten 2009). A bacterium is defined as resistant by the National Committee for Clinical Laboratory Standards (NCCLS) interpretive guidelines (NCCLS 2000). Resistance of microorganisms to antimicrobial agents is confirmed by measuring the MIC; the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation. The MIC is also used to monitor the activity of new antimicrobial agents. The double-disk approximation method is one of the first tests for the detection of ESBLs; nowadays it is still a reliable method. In this test, the bacteria are applied onto an agar plate. A disk with amoxicillin-clavulanate together with disks containing one of the oxyimino-β-lactam antibiotics are placed on the plate. A positive result is obtained by enhancement of the zone of inhibition of the oxyimino-β-lactam caused by the combined effect of the clavulanate in the amoxicillin-clavulanate disk (Jarlier et al. 1988). Several different methods for the detection of ESBLs in clinical isolates have been suggested. While each of the tests has value, none of the tests is able to detect all of the ESBLs. Currently new techniques are being developed to have a faster determination of ESBL-producing bacteria. One of them is able to indicate antibiotic resistance within 10 to 12 minutes, while other methods took hours. This technique makes use of bacteriophages which are completely nonthreatening to humans and combined with specific antibodies can produce a color change in a sample (Guntupalli et al. 2013).

Frequently β-lactamase-producing bacteria possess co-resistance to other agents besides β-lactam antimicrobials, which reduces the antimicrobial treatment options available (Fennell et al. 2012). Cefepime (a fourth generation cephalosporin), β-lactam/β-lactamase inhibitor combinations, can be used when a first-line therapy has failed (Paterson 2000). Because there is almost no antibiotic effective against infections caused by bacteria producing ESBLs, the treatment is problematic. Nevertheless determination of the bacteria still takes a lot of time or it is too expensive, so sometimes just a broad spectrum antibiotic is given to the patient. This can lead to resistance and nosocomial infections with more and more virulent organisms. This way the patient will only get sicker (Forssten 2009).

An example of a case that went wrong is that of the IC of the Maasstad Hospital in Rotterdam, the Netherlands. From the middle of 2009 until July 2011 there was in the Maasstad Hospital an outbreak of the multi resistant OXA-48 producing Klebsiella bacteria. OXA-48 is resistant to almost all antibiotics and is one of the few members of the Klebsiella family to possess a prominent carbapenem-hydrolyzing activity because they produce carbapenemase (Moquet et al. 2011). *K. pneumoniae* can cause serious infection of the lungs. The Maasstad Hospital initially kept quiet about the *K. pneumoniae* infection and did not take adequate measures to address the situation until after the health inspectorate forced it to do so. They made a notification of the outbreak on the 31st of

May 2011. 118 people were shown to be a carrier of the Klebsiella bacteria; 28 of them died of which 3 most certainly due to the infection with the bacteria. When a bacterium is resistant to carbapenem and all other approved antibiotics the doctors can only use Colistin or Tigecycline. Colistin (polymyxin E) was discovered more than 50 years ago and is now hardly used anymore, because of its severe adverse effects; mainly kidney damage (Li et al. 2006). Another drug that can be used is Tigecycline. Which was developed in response to the growing prevalence of antibiotic resistance in bacteria, it was approved in 2005. However it does not always work very efficient against Oxa-48 producing *Klebsiella*.

Another example of wrong treatment is shown in figure 10. This figure shows the use of antibiotics in 12 European countries from 1997 till 2009. In total 27 European countries were able to deliver seasonal data of antibiotic use, 15 countries were missing more than 1 year of data and are not shown in figure 10. For these 27 countries an average increase of 30% of total outpatient antibiotic use was found in the winter period (first and fourth dot in graph) in comparison to the summer period (second and third dot in graph). This increase of antibiotic use fluctuated from 11% in Cyprus to more than 50% in Lithuania and Hungary. These data can help to reduce antibiotic resistance by using it for public health strategies and to optimize the prescription of antibiotics (Adriaenssens et al. 2011).

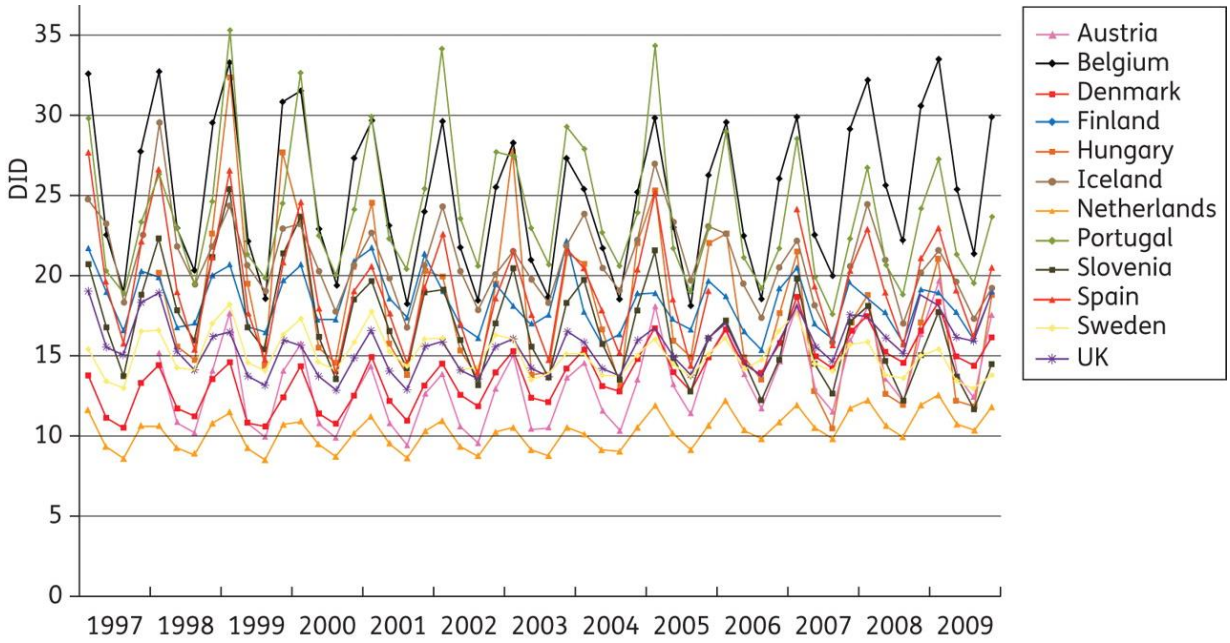

Figure 10. Antibiotic prescriptions in 12 European countries measured every season from 1997 till 2009. DID = defined daily dose (DDD) per 1000 inhabitants per day (Adriaenssens et al. 2011)

What is already happening against antibiotic resistance?

Bans for the use of antibiotic growth promoters in agriculture

Infections in humans, caused by antibiotic resistant bacteria, are highly related to agricultural overuse of antibiotics, including food poisoning caused by resistant Salmonella and Campylobacter (Swartz 2002). Growing concern over foodborne infections led the government of the United Kingdom to form an independent advisory committee in 1968, chaired by Professor Michael Swann (Swann 1969). The principal recommendations of the Swann Committee on AGPs were permission to supply and use an antibiotic without prescription for adding to animal feed should be restricted to the antibiotics which are of economic value in livestock production under United Kingdom farming conditions, have little or no application as therapeutic agents in man or animals and will not impair the efficacy of a prescribed therapeutic antibiotic or antibiotics through the development of resistant strains of organisms (European Environment Agency 2001). Against the Swann recommendations some antibiotics were accepted by the EU in 1975. However, the first ban on farm use of antibiotic growth promoters (AGPs) was enacted in 1986 in Sweden (Cogliani et al. 2011). Sweden claimed that reduced antibiotic use in food-producing animals has resulted in long-term benefits from a reduction of the prevalence of antibiotic resistance in animal bacteria (Commonwealth of Australia 1999). To follow the impact of removing the use of AGPs, Denmark created in 1995 'DANMAP', a system to monitor antibiotic resistance in farm animals (Cogliani et al. 2011). The Danish swine producers voluntarily decided to discontinue the use of all AGP in diets for pigs heavier than 35 kg and to use antibiotics only when animals are sick (Bager et al. 2000). Five years after the ban of AGPs in food animals in Denmark, resistance rates of bacteria to these antibiotics reduced significantly. The farmers in Denmark thought they could not produce pigs as efficiently as before, but that has been proven wrong. In general, animal food production continued to grow in the countries were AGPs were banned; to ensure the health and safety of the animals, proper adjustments in practices were made (Cogliani et al. 2011). Since the ban, the Danish pork industry even grew with 43%, making Denmark one of the top producers of pork in the world. Based on the Danish research reports on antibiotic resistance caused by AGPs, it was decided in the EU to evaluate the risk of using AGPs in

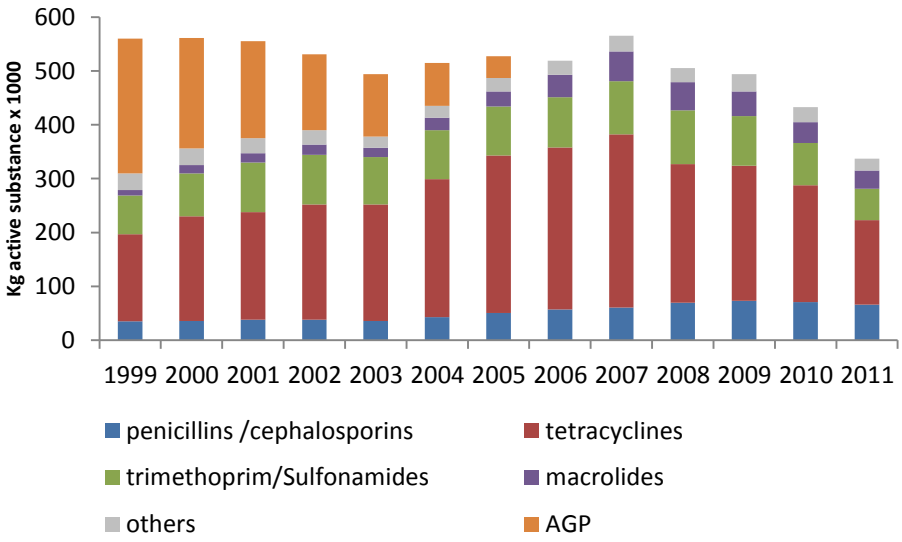

Figure 11. Therapeutic veterinary antibiotic sales in The Netherlands 1999-2011 (LEI 2011). The use of β-lactams (penicillins and cephalosporins) are depicted in blue, and the use of AGPs in orange.

animal feed. This work led to the conclusion that the use of any AGP belonging to the same class of antimicrobials that is also used for therapy in humans is regarded as irresponsible (Aarestrup & Wegener 1999). The EU-legislators followed the recommendation from the committee, and a ban on a part of the AGPs was introduced in all EU countries in 1999. The EU has implemented a comprehensive ban on the use of all antibiotics for growth promotion since 2006 (Macmillan Publishers Limited 2012). However the use of antibiotics grew from this moment till 2008; instead of using AGPs, farmers asked for more therapeutic antibiotics from veterinarians. Luckily this growth did not continue, the use of antibiotics in livestock reduced from 2007 till 2011, see figure 11. Other developed nations also applied similar procedures; however in many developing countries antibiotic use is rather uncontrolled. The reason that not all farmers in the world stopped using AGPs is because of increases of the costs, since there will be more death animals and the animals grow slower. When no AGPs are used, costs will rise only $0,05 cents per pound of pork, brought to market in the U.S. Particularly considering the export market, the demand for antibiotic-free pork is expected to become more prevalent (Holt et al. 2010). This perspective has stimulated nutritionists and feed manufacturers to search for new and safe alternatives to keep livestock healthy and grow fast.

Compared to the rest of Europe, the Netherlands has the highest antibiotic consumption and antibiotic resistance rates in the agricultural sector (Grave et al. 2010). However the government of The Netherlands planned to reduce the antibiotic use across the livestock sector by 50% by 2013. Dutch sales of antibiotics for use in livestock fell 51% in the first half of 2012 compared with the same period in 2009, amid a government push to cut veterinary use of antimicrobial drugs. This means that the policy objective for 2013 was already accomplished in 2012 (Nethmap 2013).

Last year the FDA banned cephalosporins from being used in animals at unapproved dose levels, frequencies, durations, or routes of administration. Furthermore the FDA also banned cephalosporins intended for human use in animals, the use of cephalosporins in companion animals, injecting the antibiotic into chicken eggs, and use of cephalosporins in food-producing animals to prevent disease instead of treating it. This is an important step in protecting the effectiveness of this class of antimicrobials which is needed to protect the health of both humans and animals (Voelker 2012).

Campaigns

Besides bans on the use of AGPs in agriculture, campaigns in order to reduce antibiotic resistance were started by companies and institutions.

In 2003, together with its broad-based coalition of organizations (e.g. the Environmental Defense), McDonald's presented its international policy for antibiotic use in food animals. This policy helps to phase out the use of AGPs that are approved for use in one or more countries for human medicine. A partner of the coalition is the American food service company Bon Appétit, which announced later that year a ban of livestock routinely fed with medically important antibiotics (Firkins 2003).

Another example of action against antibiotic resistance is the Dutch foundation ‘Wakker Dier’ which began a protest against broilers fed with AGPs in 2012. Supermarket ‘Deen’ stopped selling broilers fed with AGPs. Large companies such as Applegate Farms, Chipotle Mexican Grill, McDonald’s, and

Bon Appétit Management Company have also taken steps to reduce antibiotic use in animal agriculture by their producers (www.aboutmcdonalds.com 2003; www.bamco.com 2013).

The Centers for Disease Control and Prevention (CDC), along with hospitals and leading medical organizations, are implementing extensive programs to educate both patients and physicians about reducing antibiotic overuse (www.cdc.gov 2010).

Since hygiene is very important against the spread of antibiotic resistance bacteria, major resources have been used in public and healthcare-setting campaigns to improve hygiene and antibiotic use (Earnshaw & Gait 1998; Earnshaw et al. 2009; Goossens et al. 2006). In 2012 an 'Antibiotic Resistance Week' was organized, here the National Prescription Service (NPS) highlighted the importance of good hygiene habits. According to the NPS, in order to decrease our need of antibiotics, people need to focus on preventing the spread of infection (www.nps.org.au 2013).

Is resistance reversible?

In theory resistance can disappear in the same way it developed. Bacteria that develop mutations whereby they lose their resistance can occur. When antibiotics are not present in their environment, the bacteria might lose their resistance, because of the lack of selective pressure (Wiley 2004). Figure 12 shows the trends in tylosin (a macrolide) use for growth promotion and erythromycin resistance among *Enterococcus faecalis* and *Enterococcus faecium* isolated from pigs at slaughter from 1995 to 2001 (WHO 2002b). Appropriate use of existing antibiotics can limit the spread of antibiotic resistance, preserving antibiotics for the future (WHO 2002b). However, in some cases, resistance will not disappear because there is no evolutionary disadvantage in being resistant once adaptation has taken place (Gillespie 2001). A few studies have been performed to test whether this reversibility of antibiotic resistance is also feasible in real-life. The main findings of these studies were that reversibility in clinical settings is expected to be slow or non-existent (Andersson & Hughes 2010). In one study it was shown that although adaptations against cefotaxime result in a loss of resistance against a first generation drug with a β-lactamase inhibitor, reverse evolution back to the starting allele is generally not possible (Tan et al. 2012). When a bacterial gene is mutated and the bacterium has become resistant against an antibiotic it normally leads to a loss in fitness and it will grow slower. Because of this observation it was assumed that when antibiotics will be removed from the environment of the resistant bacteria, non-resistant bacteria will gain the upper hand. However when bacteria obtain resistant genes, they also often acquire compensatory mutations which results in growth just as fast as or even faster than the non-resistant bacteria. So, unfortunately this theory of reversal of resistance has been shown to be ineffective (Yim 2013).

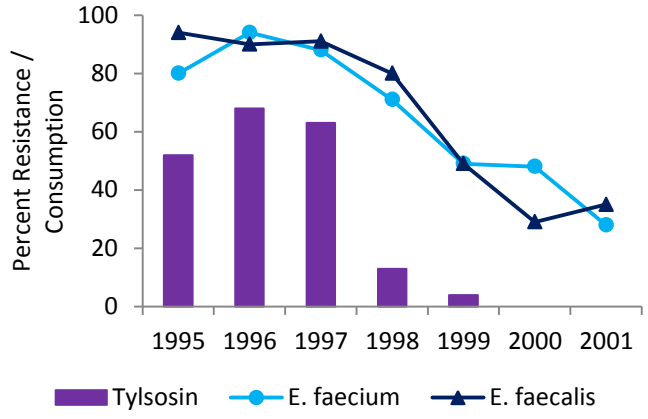

Figure 12. Decrease in bacterial resistance in Denmark after most feed uses of antibiotics were banned in 1998, data from the World Health Organization. Trends in erythromycin resistance in enterococci from pigs and tylosin use for growth promotion in Denmark (World Health Organization 2002).

Alternatives

Resistance to any antibiotic will eventually develop, limiting its useful lifetime (Rosenblatt-Farrell 2009). Pharmaceutical companies have lost most of their interest, because of the prospect of very limited profits. Antibiotics are typically used for therapy for a short time; mostly only used for several days. In contrast, patients with a chronic disease require daily drug treatment often for the rest of their life. For example, the best-selling cholesterol-lowering drug Atorvastatin, should be taken daily and had an annual turnover of US$ 12 billion, whereas the best-selling antibiotic Levofloxacin (a broad spectrum fluoroquinolone) should be taken only for a few days, and has an annual turnover of US$ 2.5 billion (Lewis 2013). Only five major pharmaceutical companies, GlaxoSmithKline, Novartis, AstraZeneca, Merck and Pfizer, are still active in the discovery of antimicrobial drugs (Yanling et al. 2013). Although the current bad situation in antibiotic research, some new drugs have recently been approved by the FDA or are in late stages of the pipeline (Devasahayam et al. 2010).

The future may look unclear, as there are relatively few new agents on the horizon. New approaches to antimicrobial chemotherapy are needed to maintain or optimize our therapeutic armamentarium and a look at the future prospects for new therapeutic antibacterial agents (Zinner 2007). As resistance to available antibiotics continues to increase, it will become necessary to develop new agents with novel mechanisms of action. An improvement of this approach could be that weaker selective pressure will be executed by the new antimicrobials, because they inhibit virulence rather than growth (Clatworthy et al. 2007). Alternative medicines against bacterial infections that do not induce resistance, by rendering bacteria harmless instead of killing them, would be a solution against antibiotic resistance.

A lot of research is done these days to find new 'weapons' against antibiotic resistance. All of them can help in reducing the rising risk of antibiotic resistance, a few examples of antibiotic therapies are summed up here.

Acidification, probiotic organisms and prebiotic compounds

In the context of eliminating antibiotics for growth promotion and preventing against intestinal diseases in pigs within the EU, the primary alternatives for antibiotic use include, acidification of the feed by organic acids (carboxylic acids, and short chain fatty acids (SCFA)), feeding probiotic organisms and prebiotic compounds (Hajati & Rezaei 2010). SCFAs are commonly available for pig producers. Normally the addition of SCFAs lowers the pH and buffering capability of the diet, increases gastrointestinal acidity, proteolysis and nutrient digestibility, encourages valuable bacteria at the expense of pathogens and after absorption it controls many physiological processes. The following advantages are estimated: developed health and resistance to disease, more rapid growth, improved efficiency of diet consumption and better quality of meat (Mroz 2005).

Prebiotics are non-digestible foods that make their way through our digestive system and help good bacteria grow and do well. Prebiotics keep beneficial bacteria healthy. They alter the intestinal microbes and immune system to reduce colonization by pathogens (Hajati & Rezaei 2010). By adding prebiotics to poultry diets, producers can minimize the use of antibiotics and thereby drug resistance

to bacteria. It have been reported that prebiotic supplementation can improve the health status of the bird's gastrointestinal tract (Paterson et al. 2004; Patterson & Burkholder 2003).

Probiotics are live microbial feed supplements which beneficially affects the host animal by improving its intestinal microbial balance. They have a beneficial effect on the host by helping to build up the immune system (Fuller 1989).

Lysozyme

According to a study from 2012 pigs showed enhanced growth performance because of administration of lysozyme. In addition, the consumptions of lysozyme had improved the morphology of the small intestine and the pigs contained a lower rate of *Campylobacter* in the gastrointestinal tract. The researchers concluded that granulated lysozyme is a proper alternative to antibiotics in 10 day old pigs consuming liquid diets (May et al. 2012).

No antibiotics

In order to reduce the occurrence of β-lactamase-producing bacteria, alternative treatment of UTIs is an important objective, since β-Lactams are commonly used to treat them. Antibiotics should not be described too quickly. It appeared that more than one-third of women in the study with UTI symptoms said they were willing to wait a week to see if the infection would improve on its own before starting antibiotics. More than 70% of the women who didn't use antibiotics for a week showed improvements or had their symptoms disappear completely. None of the participating women developed kidney infection, according to the study (Knottnerus et al. 2013).

Cranberries

Another study has been performed where the effectiveness at preventing UTIs by antibiotics (TMP-SMX) was compared to that of cranberry extract (Cranmax®; Proprietary Nutritionals, USA) in a group of premenopausal woman, who were susceptible to get UTIs. A previous meta-analysis showed that cranberry supplements reduced UTI recurrences by 39% compared with placebo or no intervention (Jepson & Craig 2008). Unlike antibiotics, the cranberry extract does not kill bacteria. The fruit contains compounds that avoid the pathogenic bacteria from sticking to the bladder wall. However, TMP-SMX was proven to be more effective against the UTIs. After 12 months, the cranberry group experienced more symptomatic UTIs than the TMP-SMX group. On average there were 4.0 infections in the cranberry group compared to 1.8 in the antibiotic group. The cranberry group also had a higher proportion of women who had experienced at least one symptomatic UTI (78.2% versus 71.1% in the antibiotic group). The average time for the occurrence of the first symptomatic UTI was 4 months for the cranberry group and 8 months for the TMP-SMX group. However, the choice whether to use antibiotics or the cranberry extract should be weighed against the greater development of antibiotic resistance. Just one month after start of treatment, TMP-SMX-resistant *E. coli* isolates were identified in 86.3% of fecal samples from patients in the antibiotic group, versus 23.7% in the cranberry group. Also asymptomatic bacteriuria isolates were investigated, 90.5% TMP-SMX-resistant *E. coli* were found in the antibiotic group versus 28.1% in the cranberry group (Beerepoot et al. 2011).

Nisin

A candidate as a new type/class of antibiotics that shows promising characteristics is nisin. This peptide which is used as a preservative in the food industry has the ability to kill bacteria effectively by pore-formation in the plasma membrane. This happens via interaction with a high affinity to Lipid II, which is attached to the membrane (Breukink et al. 1999). Lipid II is the target for at least four classes of antibiotic, including the clinically important glycopeptide antibiotic vancomycin (Breukink & de 2006).

Rational drug design

Starting from crystal complexes computer assisted drug design is a powerful instrument to find more potent inhibitors of pathogenic bacteria. The combination of high throughput screening methods in combination with virtual modeling has allowed the discovery of some new molecules, which are active against clinically important pathogens like Methicillin-resistant *Staphylococcus aureus* (MRSA). To find new antibiotics, powerful instruments can be used like high throughput screening methods, crystallization and virtual modeling programs, to optimize `old structures' or to find new lead structures in the future, for example of PBPs. β-Lactamase is a good drug target because it is unique to the pathogen and can be inhibited by a small molecule (Zervosen et al. 2012). With 3D computer programs researchers are trying to fit in the functional group with an enzyme already existing or a new molecule. This is called rational drug design (Lewis 2013).

Breast-Milk Protein

An ingredient found in human milk may make surface infections by the resistant bacteria MRSA more sensitive to attack by antibiotics. Researchers from the University at Buffalo added a purified protein complex from human milk which was called 'Human Alpha-lactalbumin Made LEthal to Tumor cells' (HAMLET) to aggressive strains of antibiotic-resistant bacteria and added this to Petri dishes and to the insides of the noses of mice. It was concluded by the researchers that the bacteria were more responsive to antibiotics when they are used in combination with HAMLET. HAMLET can increase the activity of some antibiotics (e.g. methicillin, vancomycin, gentamicin and erythromycin) against multi-drug resistant *Staphylococcus aureus*. Hereby the bacteria become sensitive to those antibiotics, this was proven for planktonic and biofilm bacteria and in an *in vivo* model of nasopharyngeal colonization in mice. It is assumed that HAMLET attacks pumps located in the cell membrane of the bacteria. By addition of HAMLET to the antibiotics, bacteria get sensitive to these antibiotics that they used to be resistant to (Marks et al. 2012).

Phages

Bacteriophages were already discovered in 1915, by Frederick Twort and Felix d'Hérelle; phage therapy was immediately recognized (Shasha et al. 2004). Although extensively used and developed, treatment with phages is not approved in countries other than Russia and Georgia. Phages are currently being used therapeutically to treat bacterial infections that do not respond to conventional antibiotics. For example, phages for killing food poisoning bacteria (Listeria) are in use (Guenther et al. 2009). Phage therapy claims several advantages over traditional antibiotics (Aguita 2008). Yeast infection and diarrhea are frequent side effects of antibacterial therapy because the beneficial bacteria of the genital tract and intestines are also killed, disrupting the ecology and enabling other pathogens to grow and cause diseases (Pirisi 2000). Phage therapy is effective against multidrug-resistant pathogenic bacteria, because the mechanisms by which it induces bacteriolysis differ completely from those of antibiotics. Substituted microbism (infection with micro-organisms) does not occur because phages have a high specificity for target bacteria. Phages can respond rapidly to the appearance of phage-resistant mutants because the phages themselves are able to mutate; the cost of developing a phage system is less than that of developing a new antibiotic; and because phages do not affect eukaryotic cells, side effects from phages are uncommon (Matsuzaki et al. 2005).

VHHs

Small, soluble single-domain fragments derived from the unique variable region of camelid heavy-chain-only antibodies (VHHs) can be potent inhibitors of enzymes, and can inhibit bacterial and viral infections (Conrath et al. 2001; Forsman et al. 2008; Lauwereys et al. 1998). VHHs could combat antibiotic resistance where conventional antibodies fail. VHHs recognizing β-lactamase could inhibit enzyme function and increase bacterial sensitivity to β-lactam antibiotics. In a study of Conrath from 2001, VHHs were generated which specifically recognize and inhibit TEM-1 and BcII β-lactamases (BcII is a mettallo-β-lactamase from *Bacillus cereus*). Addition of the VHHs to the TEM-1 β-lactamase leads to a higher sensitivity of the bacteria against ampicillin. This inventive strategy could generate many potent inhibitors for all types of β-lactamases. These VHHs can be a rich source of the next generation drugs to combat bacterial antibiotic-resistance (Conrath et al. 2001).

Discussion and Conclusion

Since the discovery of penicillin, a β-lactam antibiotic which interferes with bacterial cell wall synthesis, many more β-lactam antibiotics and antibiotics with other mechanisms have been discovered. Within four years after the widespread use of penicillin, bacteria containing resistance against this drug began to emerge. These bacteria turned out to produce an enzyme, called penicillinase, a so-called β-lactamase, which is able to hydrolyze the β-lactam ring structure of penicillin and thereby render penicillin inactive. Penicillin is now considered to be a narrow spectrum antibiotic, because most bacteria have become resistant to penicillin. The development of bacterial resistance to antibiotics is inevitable. Sooner or later a bacterium will become resistant to an antibiotic and due to selection pressure of that antibiotic only this bacterium will survive and multiply. For many decades new kinds of antibiotics were being discovered and most bacterial infections could be treated successfully with these antibiotics. Nowadays, unfortunately, not many new antibiotics are being discovered and bacterial resistance is becoming a major problem. Pharmaceutical companies do not want to invest in development of new antibiotics anymore, because the development is too expensive, since new antibiotics will be used as little as possible to prevent the selection for antibiotic resistance to these new ones. One group of antibiotic resistant bacteria are the ESBL-producing bacteria. ESBLs are β-lactamases which have the ability to hydrolyze the β-lactam ring of the extended-spectrum cephalosporin and monobactam antibiotics containing an oxyimino group. ESBL-producing bacteria often also carry genes that confer resistance to other kinds of antibiotics and are thus often multi-drug resistant. The only antibiotics that are still effective against most ESBL-producing bacteria are cephamycins and carbapenems, since they have a structure that renders them highly resistant to most β-lactamases. More and more bacteria that are resistant to most or even all antibiotics that have been approved for use in humans are emerging. An example of bacteria resistant to all antibiotics are bacteria expressing the New Delhi metallo-β-lactamase 1 (NDM-1) gene, which is a carbapenemases. NDM-1 was first detected in 2008 in a Swedish patient from Indian origin and since then found in many more countries, including the United Kingdom, the United States, Canada and Japan. The gene for NDM-1 can easily spread from one strain of bacteria to another, like ESBLs can. In general it may be expected that, if no adequate measures are taken, soon many people will die of bacterial infections that can now still be treated successfully; like in the times before the discovery of antibiotics. To turn the tide, different measures have been taken over the years, but more are required. There is an urgent need for action. The fast development of resistant bacteria is mostly due to unnecessary and excessive use of antibiotics in humans but also in livestock, and the spread of these resistant bacteria is due to inadequate hygiene and biosecurity. Both the development and the spread of antibiotic resistant bacteria could be.

Decreasing the use of antibiotics in human medicine only will have a small effect on the current situation. Therefore substantial efforts must be made to also decrease incorrect overuse in agriculture. The β-lactam antibiotics, mainly penicillins, have been excessively used as growth promoters in livestock, much more than as therapeutics in human. The resistant bacteria in animals can be transmitted to humans via three ways, usually through the consumption of food animals, but also through close or direct contact with animals, or through the environment. The transfer from animals to humans is particularly clear for ESBL-producing *E. coli* strains from broiler chickens to humans. The Netherlands is one of the countries where antibiotics to humans are prescribed as little

as possible, but on the other hand antibiotics are used in livestock excessively and the Netherlands is one of the countries that relatively use the most antibiotics in agriculture worldwide. The use of β-lactams antibiotics should be diminished dramatically in livestock in the Netherlands.

Antibiotics for treatment of human infections should only be available via a doctor. This way the consumer can be informed about the importance of finishing the course and has a lower chance of using antibiotics when they are not needed.

The prevention of the spread of antibiotic resistant bacteria from human to human should also be improved. Many infections caused by antibiotic resistant bacteria are still acquired in hospitals. A striking example of bad management is how the outbreak with a carbapenemase (OXA-48)-producing *K. pneumoniae* in the Maasstad Hospital in Rotterdam in 2011 was handled. The Maasstad hospital had not immediately reported to the health authorities that they encountered carbapenem resistant bacteria and did not immediately quarantined infected patients. As hospital-acquired infection is a major cause for antibiotic-resistance, stringent infection control procedures are essential in health and residential facilities to prevent the spread of resistant bacteria.

The laboratory detection of ESBLs can be complex and, at times, misleading. Fast and easy-to-use tests to determine the type of microorganism and the type of resistance, causing an infection, would be very effective in the battle against antibiotic resistant bacteria and research in this area should be stimulated. If the pathogen is properly identified, the best strategy to fight it can be chosen rationally. In case of viral infections or infections caused by ESBL producing bacteria, for example, using β-lactam antibiotics could be counterproductive.

The emergency of antibiotic resistance seems unavoidable; nevertheless there is still a need for the development of new antibiotics. Luckily a few promising antibacterial drugs with novel mechanisms of action are in development and new types of targets have emerged. However, measures must be taken to prevent or at least delay this process. National and supranational governments and non-governmental organizations should sponsor companies that develop new antibiotics or β-lactamase inhibitors and supply funds to stimulate academic research on antibiotics. If governments would establish subsidies and financial assistance to compensate costs of antibiotic research, more companies might be attracted to this area.

In order to prevent resistance against new antibiotics, the use of antibiotics should be reduced. In order to reduce the rising risk of antibiotic resistance, antibiotic growth promoters were banned in Sweden and Denmark. Because of this the Danish pork industry even grew with 43%, making Denmark one of the top producers of pork in the world. This Danish ban of growth promoters was later followed by a ban by the EU affecting all countries in the EU. Antibiotics that are considered as critical or very important for the treatment of infections in humans are also used for animals. These antibiotics should not be used in agriculture, to preserve the efficiency of these microbial agents for treatment of infections in humans. In my opinion governments should ban the use of antibiotics as a growth promoter for livestock all over the world. The human health is more important than the cost of producing meat and the price of it in the supermarket. Maybe the governments can even subsidize the ban of AGPs by farmers; when the antibiotics use for livestock is reduced, the medical costs for patients will also be reduced. There should be a fine for providing farmers with AGPs. Furthermore, because of reduction of antibiotic use bacteria might even lose their resistance genes.

Nowadays a lot of research is done in order to find good alternatives for the current antibiotics. An example of a potential alternative for current antibiotics could be lama heavy chain-only antibody fragments (VHHs) that block outer membrane proteins that are involved in bacterial pathogenesis or are essential for bacterial viability, or that block the β-lactamases. VHHs are much smaller than conventional antibodies and can most probably reach the outer membrane proteins, which are inaccessible for classical antibodies.

A worldwide ban on AGPs, quick measures against outbreaks of antibiotic-resistant bacteria and the production of alternative or new antibiotics will help reducing antibiotic-resistant bacteria.

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