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Aspects of Bacterial Resistance to Silver

SUSANNE SÜTTERLIN

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Abstract

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Bacterial resistance to antibiotics has increased rapidly within recent years, and it has become a serious threat to public health. Infections caused by multi-drug resistant bacteria entail higher morbidity, mortality, and a burden to health care systems. The use of biocides, including silver compounds, may affect the resistance to both biocides and antibiotics and, thereby, can be a driving factor in this development.

The aim of the following thesis was to investigate the frequency of silver resistance and the effects of silver exposure on bacterial populations being of clinical significance and from geographically different parts of the world. Furthermore, it explored the genetic background of silver resistance, and if silver could select directly or indirectly for antibiotic resistance.

By a range of methods, from culture in broth to whole genome sequencing, bacterial populations from humans, birds and from the environment were characterized.

The studies showed that *sil* genes, encoding silver resistance, occurred at a high frequency. *Sil* genes were found in 48 % of *Enterobacter spp.,* in 41 % of *Klebsiella spp.* and in 21 % of all human *Escherichia coli* isolates with production of certain types of extended-spectrum beta-lactamases (CTX-M-14 and CTX-M-15). In contrast, silver resistance was not found in bird isolates or in bacterial species, such as *Pseudomonas aeruginosa* and *Legionella spp*., with wet environments as their natural habitat. One silver-resistant *Enterobacter cloacae* strain was isolated from a chronic leg ulcer after only three weeks of treatment with silver-based dressings. The *in-vivo* effects of these dressings were limited, and they failed to eradicate both Gram-positive and Gram-negative bacteria. The activity of silver nitrate *in vitro* was bacteriostatic on Gram-positive species such as *S. aureus* and bactericidal on Gram-negative species. In *Enterobacteriaceae*, *sil* genes were associated with silver resistance phenotypes in all but one case. Using whole genome sequencing, single nucleotide polymorphisms in the *sil*S gene were discovered after silver exposure in isolates with expressed silver resistance. This resistance could co-select for resistance to beta-lactams, co-trimoxazole and gentamicin.

The findings of this thesis indicate that silver exposure may cause phenotypic silver resistance, and it may reduce the susceptibility to mainly beta-lactams and select for bacteria with resistance to clinically important antibiotics.

Keywords: Antimicrobial resistance, Silver resistance

Susanne Sütterlin, Department of Medical Sciences, Clinical Microbiology and Infectious Medicine, Akademiska sjukhuset, Uppsala University, SE-75185 Uppsala, Sweden.

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To my family

List of Papers

This thesis is based on the following papers which are referred to in the text by their Roman numerals.

I Sütterlin, S., Tano E., Bergsten, A., Tallberg, A.-B., Melhus, Å. (2012) **Effects of silver-based wound dressings on the bacterial flora in chronic leg ulcers and its susceptibility *in vitro* to silver.** Acta Dermato-Venerologica, 92: 34–39.

II Sütterlin, S., Edquist, P., Sandegren, L., Adler, M., Tängdén, T., Drobni, M., Olsen, B., Melhus, Å. (2014) **Silver resistance genes are overrepresented among *Escherichia coli* isolates with CTX-M production.** Applied and Environmental Microbiology, 80 (22): 6863–69.

III Sütterlin, S. and Yin, H., Zhang, X.-J., Li, L.-H., Sun, L.-W., Melhus, Å. (2015) **High carriage rate of CTX-M-producing *Escherichia coli* in Chinese preschool children.** *Submitted manuscript.*

IV Sütterlin, S., Dahlö, M., Tellgren-Roth, C., Melhus, Å. (2015) **High frequency of silver resistance in invasive isolates belonging to genera *Klebsiella* and *Enterobacter.*** *Manuscript.*

Contents

Abbreviations

ESBL	Extended spectrum beta-lactamase
MIC	Minimal inhibition concentration
MBC	Minimal bactericidal concentration
cfu	Colony forming unit
SNP	Single nucleotide polymorphism
AP-PCR	Arbitrarily-primed polymerase chain reaction
MLST	Multi locus sequence typing
ST	Sequence type
TEM	Temoniera beta-lactamase
SHV	Sulfhydryl variable beta-lactamase
CTX-M	Cefotaximase-Munich beta-lactamase
BURST	Based upon related sequence type
Omp	Outer membrane protein
MRSA	Methicillin-resistant *Staphylococcus aureus*

Preface

Among the most commonly prescribed drugs, antibiotics are undoubtedly the number one. We use them for a wide range of bacterial infections, from quite simple forms with high spontaneous recovery rates to life-threatening conditions with no chance of survival without antibiotic treatment. Antibiotics are a prerequisite to more advanced medicine, including transplantations and oncologic treatments. Their use, overuse and misuse have, however, brought us rapidly closer to the post-antibiotic era, with antibiotic resistance having become one of the greatest challenges in modern medicine.

Ever since their introduction, the incidence of bacterial resistance to antibiotics has continuously increased. The result of this evolution is a higher frequency of treatment failure, prolonged hospitalisation periods and higher morbidity and mortality rates. The decreased effectiveness of pre-operative antibiotic prophylaxis and an increased need for combination antimicrobial chemotherapy in advanced intensive care have also caused a cost explosion for health care systems.

Even though we have not yet reached the post-antibiotic era, we are just getting its first foretaste. About 25,000 patients die every year in Europe as a result of infections caused by resistant bacteria, and both national and international surveillance programs are facing an exponential growth in the incidence of multi-drug resistance in most human bacterial pathogens.

As a consequence of the development described above, local, national and global initiatives have been taken to combat antibiotic resistance. Two main targets have been identified: Lowering the consumption of antibiotics and reduction of the dissemination of resistant bacteria by providing better infection control. There is broad approach to this with a One Health perspective, with humans, animals and the environment thereby being considered in the same way. An example of how this can work is that both human and veterinary care personnel should be encouraged to use antibiotics under stricter and more rational aspects, and that access to antibiotics by non-health care professionals must be restricted.

In Europe, the Scandinavian countries play a pioneering and leading role. In Sweden, intervention studies have shown that it is possible to drastically reduce antibiotic usage. However, despite a decline (or at least no further increase) in the number of antibiotic prescriptions, what is disappointing, is that the rate of bacterial resistance in clinical isolates is not on the wane.

Furthermore, it has been extremely difficult to stop the spread of multi-drug resistant bacteria in the community. It is, therefore, possible that some factors other than the selective pressure from just antibiotics may contribute to the current situation.

A report published by the European Commission in 2009 identifies biocides as a potential risk factor for the development and spread of antibiotic resistance. Biocides are chemical substances with antimicrobial activity similar to antibiotics, and they are widely used in health care as disinfectants and preservatives. Although they might have the same effect as antibiotics, they are under less control, and, as the European Commission stated, there is a significant lack of knowledge in this field.

A popular biocide in the pre-antibiotic era was silver. With the introduction of antibiotics, silver was more or less forgotten with one exception: many Swedish citizens had their eyes treated with silver nitrate shortly after birth to prevent the dreaded gonococcal conjunctivitis, even in the 1960s.

Along with a growing number of antibiotic treatment failures, silver has experienced some revival. Silver is often presented as an alternative or a complement to antibiotics, and it has been suggested that resistance to silver does not occur. However, history tells us a different story, and the question is rather not if a bacterium will develop resistance to an antimicrobial substance but when it will do so. Starting to use silver in clothes, shoes, toothbrushes, pacifiers, etc. to combat bacteria without careful thought, might even aggravate a serious problem that already exists. The above considerations form the basis for the following thesis.

Introduction

Bacteria are considered to be the oldest form of life on earth, and they appeared more than 3.5 billion years ago. Already at the time of their appearance, the environment was extremely hostile. For their survival, they had to evolve certain strategies to manage the toxic natural elements and chemical compounds they found in their vicinity. In addition, it was vital to find appropriate ecological niches to avoid desiccation, high osmotic pressure, radiation, and extreme pH changes. Although microscopic, bacteria harbour a machinery that is impressively versatile in its simplicity. Meanwhile, they have adapted to most global conditions and can live in areas where only a very small number of other organisms could survive.

Bacteria of interest and the infections they can cause

The Enterobacteriaceae family

Several genera can be found in the *Enterobacteriaceae* family. These Gram-negative facultative anaerobic rods are widely distributed in soil, water, plants and intestines of animals and humans. For structure of the Gram-negative cell wall, see *Figure 1*.

The type species is *Escherichia coli.* It is the predominant facultative species in the bowel of humans, and it is present in the gut microbiota of nearly all vertebrates.[1, 2] If it is found in a water supply system, it indicates a continuing faecal contamination. There are several recognised categories of diarrheagenic *E. coli*: enterohemorrhagic (EHEC), enterotoxigenic (ETEC), enteropathogenic (EPEC), enteroinvasive (EIEC), and enteroaggregative *E. coli* (EAEC). To identify these categories PCR-methods are usually used. Apart from diarrhoea, *E. coli* is a leading cause of urinary tract infections, septicaemia, and neonatal meningitis.[3] *E. coli* represents a great reservoir for antibiotic resistance, and the emergence of ESBL-producing strains have increased the fatality rates.[4]

Other important members of the family are the genera *Klebsiella* and *Enterobacter*. *Klebsiella* and *Enterobacter* can be found as commensals in humans but have their natural inhabits in soils and on plants. Both genera can also cause infectious diseases, often in nosocomial contexts. *Klebsiella pneumoniae* and *Enterobacter cloacae* rank between fifth and ninth

among the causative agents of septicaemia, but more frequent are the urinary tract infections. *Klebsiella* and *Enterobacter* are more resistant to beta-lactams than *E. coli*, and, due to its ability to form biofilms, *K. pneumoniae* can be extremely difficult to eradicate from the lungs of patients with ventilator-associated pneumonia.[5, 6]

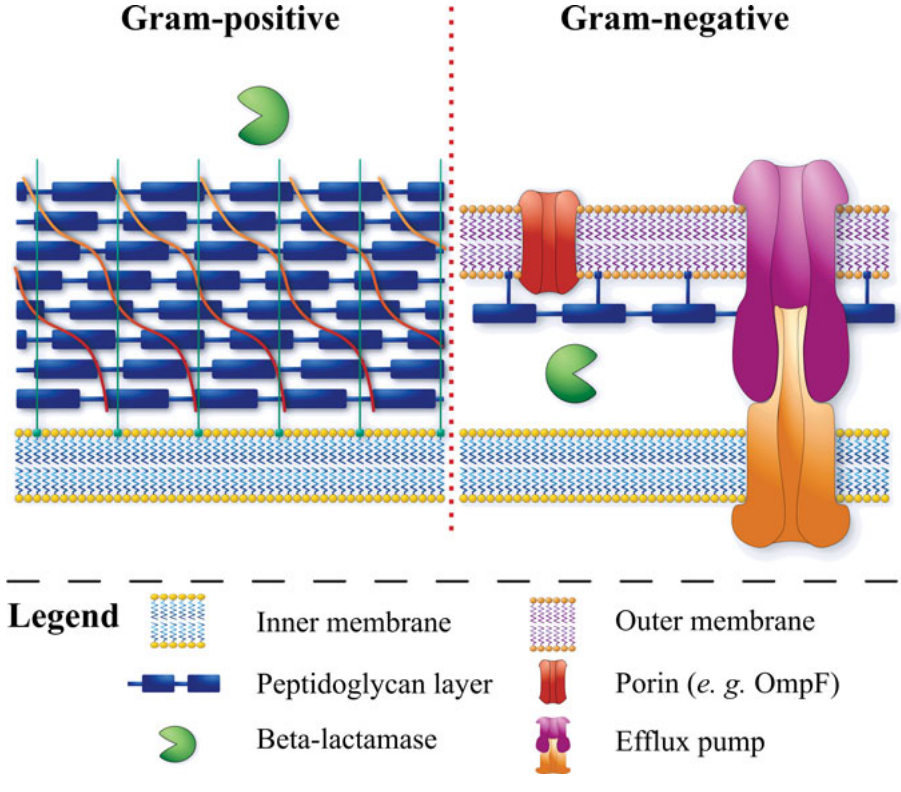

Figure 1. Schematic figure of cell wall of Gram-positive and Gram-negative bacteria. The Gram-positive cell wall consists mainly of several layers of cross-linked peptidoglycan, a unique material for bacteria, together with proteins, polysaccharides and teichoic acids. Gram-positive bacteria are usually rather resistant to drying but more permeable and susceptible to disinfectants and antiseptics than Gram-negative bacteria. In Gram-negative bacteria, there is a periplasmatic space between the inner and outer membranes. The outer membrane surrounds the single layer of peptidoglycan, and the cell communicates with the external environment through proteins (Omps) in this membrane. The Gram-negative cell wall consists essentially of lipopolysaccharides, proteins and phospholipids. It provides a good permeability barrier for hydrophobic and high molecular-weight hydrophilic substances, but it makes the bacterium vulnerable to desiccation.

Primary wound pathogens

Pyogenic streptococci and *Staphylococcus aureus* are Gram positive, facultative anaerobic bacteria with complex nutritional requirements. Together, they cause the majority of the skin and soft tissue infections in humans.

Streptococcus pyogenes, or the group A streptococcus, is the most virulent of the pyogenic streptococci. It is an exclusive human pathogen, and the mucosa of the upper respiratory tract and non-intact skin are preferred sites for colonisation and ports for entry. *S. pyogenes* is equipped with a large number of virulence factors, and can cause a broad spectrum of infections, including impetigo, acute otitis media, tonsillitis, erysipelas, lymphangitis, necrotizing fasciitis, toxic shock syndrome, and septicemia. It can hide intracellularly and form biofilm. It is still susceptible to penicillin, after more than 70 years of exposure.[6]

The main habitat of *S. aureus* is the skin of primates. The relationship with the host is usually benign, but when the epithelial barrier is destroyed and/or medical devices are implanted severe infections can be the result. *S. aureus* is the leading agent of post-operative infections and infections associated with foreign bodies. It is also an important cause of acute endocarditis, joint and bone infections, and community-acquired septicemia. Depending on the type of exotoxins it expresses, it can cause food poisoning, necrotizing pneumonia, toxic shock syndrome, and scalded skin syndrome.[6, 7] Its production of biofilms is a therapeutic problem, and in late years dissemination of MRSA in the community has become more frequent. Without full susceptibility to methicillin or similar drugs, the outcome is less certain.[8]

Environmental bacteria

Pseudomonas aeruginosa is Gram-negative rod with a strictly aerobic respiratory metabolism. It is a soil organism that can utilise a wide range of nutrients. Since its requirements are simple, it can grow in almost any moist environment, including sink drains, liquid soaps, eye-drops, humidifiers, and antiseptic solutions. The bacterium does not usually colonise healthy humans, but can cause severe pneumonia in patients with mechanical ventilation, neutropenia or cystic fibrosis. It is one of the leading causes of burn wound infections, and it is frequently found in chronic ulcers. It is the most studied of all biofilm formers.[9] *P. aeruginosa* is naturally resistant to several antibiotics and can rapidly develop resistance during antibiotic therapy. It is thereby difficult to treat.[6]

The *Legionellaceae* consist of the single genus *Legionella*. They are all strict aerobic and nutritionally fastidious Gram-negative rods. They are normally found in aqueous environments and form biofilms. *Legionella pneumophila* is the clinically most important species. It is the leading cause of Legionnaires' disease, a form of pneumonia. The infection can be mild to

life-threatening. In its natural environment, *L. pneumophila* is a facultative intracellular parasite of free-living amoebae. When infecting humans, it attacks primarily alveolar macrophages, which have many features in common with amoebae. When living intracellular, the bacterium is protected from biocides.[10]

Antimicrobial resistance

The underlying mechanisms for the development and spread of antibiotic resistance are complex.[11, 12] Most resistance mechanisms are pre-existent. In order to become clinically significant they need to become incorporated into a pathogen. This can be achieved by means of genetic exchange, by translation of pre-existing genes that may be activated by selection or induction, or when mutational events extend the substrate range of resistance-mediating enzymes.[12]

Antimicrobial resistance due to global response systems

Bacterial cells have wide spectrum of mechanisms to use when reacting acutely to sudden environmental changes. The resistance mechanisms at play on the cellular level are, however, divided into only four groups: 1) reduced permeability of the cell wall (porin loss, active efflux), 2) modification of the antimicrobial substance (beta-lactamases), 3) modification of the target protein (PBP-changes), and 4) altered metabolic route. For silver, the first two mechanisms, a loss of porins or an activation of efflux pumps, are of most interest.

Porins, or outer membrane proteins (Omps), are channels in the bacterial cell membrane of Gram-negatives that allow substances needed for the bacterial cell metabolism to penetrate into the cell (*Figure 1*). Some antimicrobial substances enter the cell through the same porins, and a transcriptional down-regulation of these Omps reduces the intracellular concentration of antimicrobial agents and thus, results in decreased susceptibility.[13]

Efflux pumps are transport proteins that often use active transport to clear the cell from antibiotics or other harmful substances (*Figure 1*). Multidrug efflux pumps have been described, *e.g.* like AcrB in *E. coli*, that rather unspecifically clears a wide range of substances.[14]

Sometimes bacteria combine these two resistance mechanisms. An example of this is the multiple antibiotic resistance (Mar) phenotype. It is characterised by decreased susceptibility to multiple antibiotics, caused by a combination of porin losses and increased efflux that is activated by the *mar* operon.[15] The Mar phenotype is not only induced by antibiotics,[16] it is also

induced by drugs like diazepam,[17] illustrating the complexity and cross-reactivity of bacterial response systems.

Other bacterial species, like *P. aeruginosa*, use the production of biofilms as a defence strategy. Several mechanisms contribute to a reduced susceptibility to antibiotics among biofilm producers: Firstly, a biofilm acts as a physical barrier which impedes the permeability of antibiotics into the cell.[18] Secondly, the majority of bacterial cells within the biofilm are in stationary growth phase. Antibiotics like the beta-lactams, that require a high bacterial division rate for their activity, become inefficient.[18, 19] Furthermore, biofilm facilitates horizontal gene transfer between bacteria,[20] and it increases the mutation frequency.[19]

Antimicrobial resistance due to genetic exchange

The bacterial genome is characterised by a remarkable plasticity that is caused by horizontal gene transfer, genome rearrangements and the activity of mobile DNA elements. A common differentiation is made between the ability of mobilising of genetic elements within a cell and between two different cells. Horizontal gene transfer means the transfer of genetic elements between bacteria by cell-to-cell contact through conjugation and transduction, or without cell-to-cell contact through transformation or phages.

Pre-existing antibiotic resistance genes may become mobilised from the chromosomes of bacterial species with limited clinical significance and get introduced into important human pathogens. For instance, two plasmid-mediated AmpC beta-lactamases have been mobilised from *Aeromonas spp*. and *Citrobacter freundii* and are now frequently isolated from clinical *E. coli* isolates.[21] The complexity of exchange of genetic material between different species is illustrated in *Figure 2*.

In order to accomplish horizontal gene transfer, genetic elements have to be mobilised. Most mobile elements, like transposons, integrons or genomic islands, can be integrated into other genetic elements, but not all are mobile by themselves. For instance, despite the fact that integrons can integrate themselves by an integrase, their mobilisation is achieved indirectly, often as parts of integron cassettes that are incorporated into transposons.[22, 23] Transposons are able to move vertically, between the chromosome and extra-chromosomal DNA, *i.e.* plasmids. Horizontal transfer of transposons is accomplished by conjugative plasmids or phages.[24]

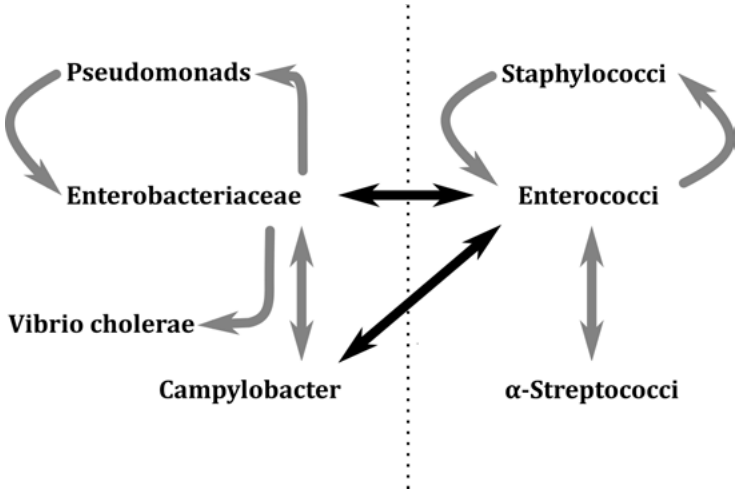

Figure 2. Illustration of intraspecies genetic exchange (adapted from Tenover et al.[12]).

While the vast majority of integrons are embedded in chromosomes, class 1 integrons are the most wide-spread variant in clinical isolates. It has been postulated that clinical class 1 integrons may have evolved from environmental class 1 integrons from *Betaproteobacteria* species.[22] Gene cassettes from clinical isolates frequently contain genes conferring resistance to quaternary ammonium compounds (*qacEΔ*), sulphonamide (*sul1*), trimethoprim (*dhfr*) and streptomycin (*aad*).[25] The mobilisation of class 1 integrons in Gram-negative bacteria is usually associated with transposons of *Tn*21 and *Tn*402 types.[22, 26]

Circulation of resistant bacterial clones

Bacterial clones are bacteria that share identical genetic and phenotypical properties resulting from a common origin.[27, 28] In a global perspective, a tool for the determination of clonality of an isolate is multilocus sequence typing (MLST). This technique uses genetic sequence variations, based on usually seven representative housekeeping genes.[29] Clonal relationships between sequence types are often determined using the BURST (based upon related sequence types) minimal spanning tree algorithm.[30, 31] There is at least one MLST-scheme for most human pathogens.

For *E. coli*, there are two highly virulent sequence types, ST131 and ST405. Other sequence types, like ST10, ST69 and ST23, have been associated with acquired resistance.[28] The most well-known *K. pneumoniae* clones are ST14 and ST15. They are both part of the largest eBURST group.[28] Among *P. aeruginosa*, the virulent clones ST235, ST111 and ST175 are globally spread and represent the majority of the multi-drug resistant *P. aeruginosa* strains worldwide.[32] However, as the MLST categorisation of

bacteria is based on seven housekeeping genes, little is known about the properties that distinguish the above mentioned successful clones from non-related sequence types.

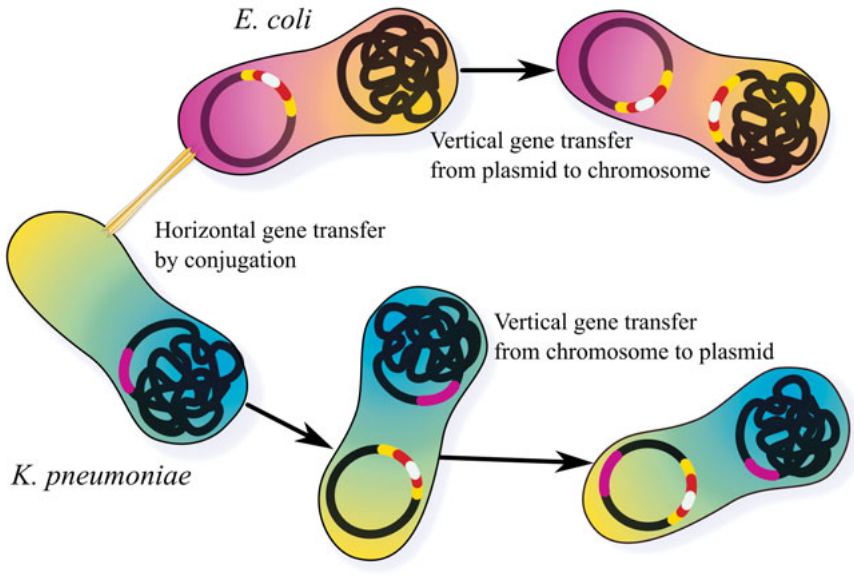

Figure 3. Illustration of the complexity of horizontal and vertical gene transfer between the two species E. coli and K. pneumoniae. By incorporating genes into the chromosome, antimicrobial resistance can become a permanent part of a successful clone. Furthermore, vertical gene transfer contribute to preserve the reservoir of antimicrobial resistance genes.

The above globally successful clones are likely to act as a reservoir and host for mobile genetic elements and thereby contribute to the dissemination of multiresistance[28] (*Figure 3*).

Lack of One Health perspective on antibiotic resistance

Several studies have attempted to reduce the rate of antibiotic resistance by restricting the prescriptions of antibiotics for humans. Unfortunately, these studies have not been successful; the resistance to specific antibiotics have remained the same, and not even a decrease of the overall resistance to antibiotics has been shown.[33, 34] Possible underlying mechanisms are the presence of gene cassettes coding for multi-drug resistance and the persistence of resistance genes once they have been acquired.[35]

Antibiotic resistance in human pathogens may be stabilised by a continuous low level of antibiotic exposure and give the pathogens the possibility to adapt to their hosts.[36] Under continuous selective antimicrobial pressure,

adaptive mutations allow the bacteria to regain their original fitness while maintaining their antibiotic resistance.[12, 36]

Antibiotics, even in low concentrations, exert a selective pressure and cause the formation of reservoirs of resistant bacteria where they are used.[37] An important reservoir for antibiotic resistance is livestock animals. They are often fed with antibiotics to prevent infections and maximise the meat production. It has been shown that these animals harbour human pathogens and antibiotic resistance determinants.[38, 39] Furthermore, water environments are a great reservoir for resistance and virulence genes,[40] and associations exist between bacteria from these environments and clinical strains.[41] Another effective reservoir that may contribute to the spread of antimicrobial resistance is the faecal flora of animals, where bacteria harbour resistance genes on mobile genetic elements and where successful bacterial clones frequently occur.[42, 43] Human behaviour itself also contributes to a spread of antibiotic resistance through bacterial exchange within the household,[44] by travelling[45] and through adoption[46] (*Figure 4*).

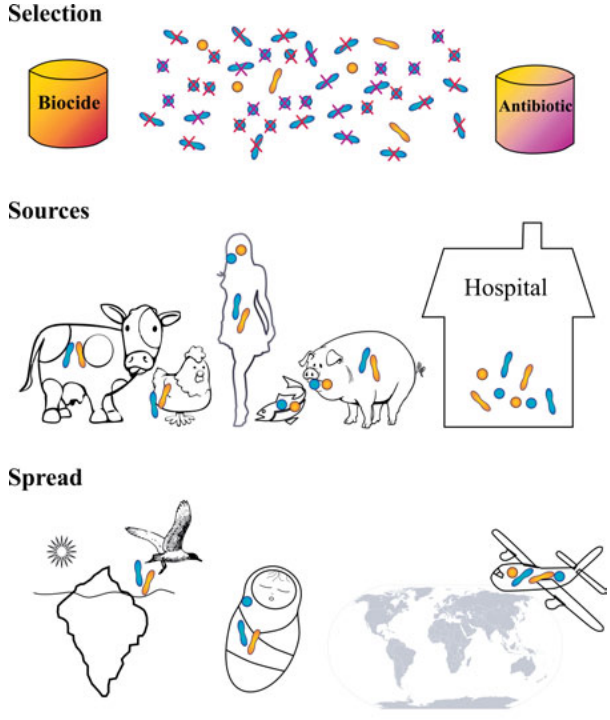

Figure 4. Illustration of the One Health perspective of antibiotic resistance. Selection: Biocides and antibiotics exert selective pressure that favours growth of resistant bacteria. Sources: Environments that are exposed to antimicrobials are a source and reservoir for resistant bacteria. Spread: Human and animal activities contribute to the spread of resistant bacteria.

The success story of CTX-M-producing *Enterobacteriaceae*

In Gram-negative bacteria, beta-lactamases with extended spectrum have emerged as a significant public health problem.[47] In the 1990s, the SHV and TEM were the predominant ESBL-types in a global perspective, but today, CTX-M enzymes stand for the majority of the ESBL-production.[47, 48]

The putative progenitors of the CTX-M family, are chromosomally encoded cefotaximases of *Kluyvera spp*. ($bla_{klu/CTX\text{-}M}$). Insertion sequences are frequently found upstream of the chromosomal cefotaximase, and they can be mobilised under stress conditions.[49] The evolution of this mobilisation process occurred independently in different geographical regions and has resulted in phylogenetically diverse CTX-M clusters.[50] The most disseminated CTX-types are currently CTX-M-14 and CTX-M-15, both frequently present in humans, animals and the environment in both densely populated but also in remote areas.[51]

Maintenance and dissemination of CTX-M genotypes occurs to a significant extent by plasmids of incompatibility group FII.[52] IncFII plasmids are well-adapted to members of the *Enterobacteriaceae* family,[53] and persistence and spread of resistance determinants like CTX-M-15 are facilitated after their incorporation into these plasmids. This might also explain why CTX-M enzymes are predominantly found in *Enterobacteriaceae* but not to the same extent in *P. aeruginosa*.

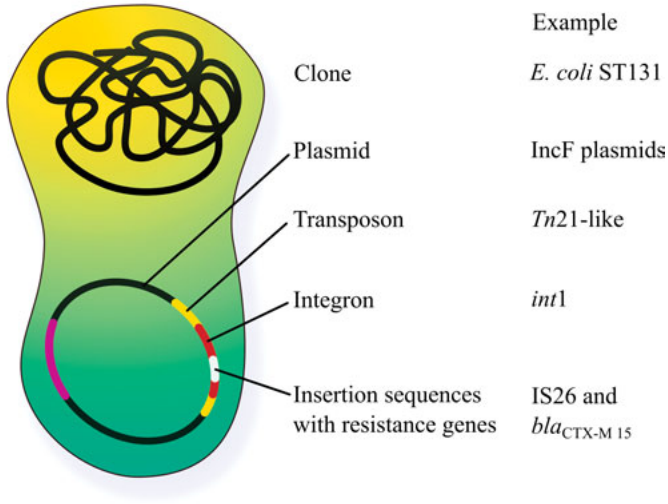

Figure 5. Hierarchy of genetic structures participating in gene transfer, maintenance and expression of resistance genes. The column on the right gives typical examples.

Several clones, especially of *E. coli* or *K. pneumoniae,* frequently express CTX-M enzymes. *E. coli* clone ST131 often produces CTX-M-15[54] and frequently carries IncFII plasmids. Other virulent clones that frequently express CTX-M are ST405 and ST69.[28] *K. pneumoniae* clone ST11, which is often isolated in Asia, harbours CTX-M-14 or CTX-M-15.[51] Despite the above statements, there is no strict link between CTX-M enzymes and certain clones. It is rather the local conditions that allow CTX-M-producing bacteria to emerge.[4, 28]

In the context of CTX-M producing species, the concept of 'genetic capitalism' is frequently mentioned. It describes the observation that several clones, once they had acquired a resistance mechanism, were more prone to accumulate additional resistance and had a greater likelihood to become multi-drug resistant.[51]

Silver

Role of silver in human medicine

The growing and serious threat of antibiotic resistance in modern medicine has renewed the interest in silver compounds. The antibacterial properties of silver nitrate have been known since at least the Middle Ages. Historically, silver nitrate has a long tradition in the treatment of chronic ulcers and other types of wounds. The hard form of silver nitrate was known as *lapis infernalis* or 'lunar caustic', referring to the pain associated with silver treatment and the use of silver as a metaphor for the moon.[55]

Figure 6. Examples of frequently used silver-based dressings.

Beside silver nitrate, silver has been used in other combinations like silver sulphadiazine, a substance frequently used in treatment of burns. Recent silver-products contain silver in the form of nanoparticles.[56, 57] The ancient tradition of silver treatment of wounds is thereby continued, and a great variety of silver-based dressings are available on the market.

Silver can now be found in a wide variety of medical devices, e. g. central venous catheters, endotracheal tubes and urinary catheters, to prevent nosocomial infections.[56] Furthermore, silver is widely used in consumer products in order to prevent unwanted microbial growth, although neither data on antimicrobial efficacy nor sufficient risk assessments are available.[56, 57]

Antibacterial activity of silver

The mode of action of silver is not known in detail. There are, however, indications that silver is bound to different cell wall structures[58-60] as well as DNA molecules and damage these.[58, 61] The cell membrane has recently been pointed out as one of the more important targets.[62, 63]

Bacterial resistance to silver

Since the reintroduction of silver products as treatment alternatives for burn wounds, there has been an increasing number of reports on bacterial resistance to silver.[64-66] Silver-resistant bacteria have mainly been isolated from patients in burn care centres,[59, 67-69] but some have also been isolated from the environment.[70]

There are indications that several bacterial species can accumulate the metal,[71, 72] thereby removing it from solutions. Electron microscopy studies have shown, that silver accumulates on cell surfaces.[72]

Efflux has been suggested as an important mechanism of bacterial resistance in Gram-negative species.[73, 74] In 1999, Gupta *et al.* described the *sil* operon, the genetic and molecular basis of silver resistance found in a *Salmonella typhimurium* isolate.[75] The operon codes for the silver binding proteins SilE and presumably SilF, two efflux pumps SilCBA and SilP, and the regulator proteins SilSR (*Figure 7*). The *sil* operon was found on plasmids that also harboured antibiotic resistance genes.[76, 77]

Data on the frequency of *sil* genes in different bacterial species are limited. Although silver resistance has been found in a variety of species, presence of the *sil* operon has mainly been reported in *E. cloacae* isolates: *sil* genes were found in 103 out of 164 clinical *E. cloacae* isolates from a German hospital.[78] Another study reported *sil* carriage in six out of ten wound isolates.[79]

In *E. coli*, the *sil* operon and the chromosomal copper/silver efflux system *cus* both contribute to silver resistance and also interact with each other.[80]

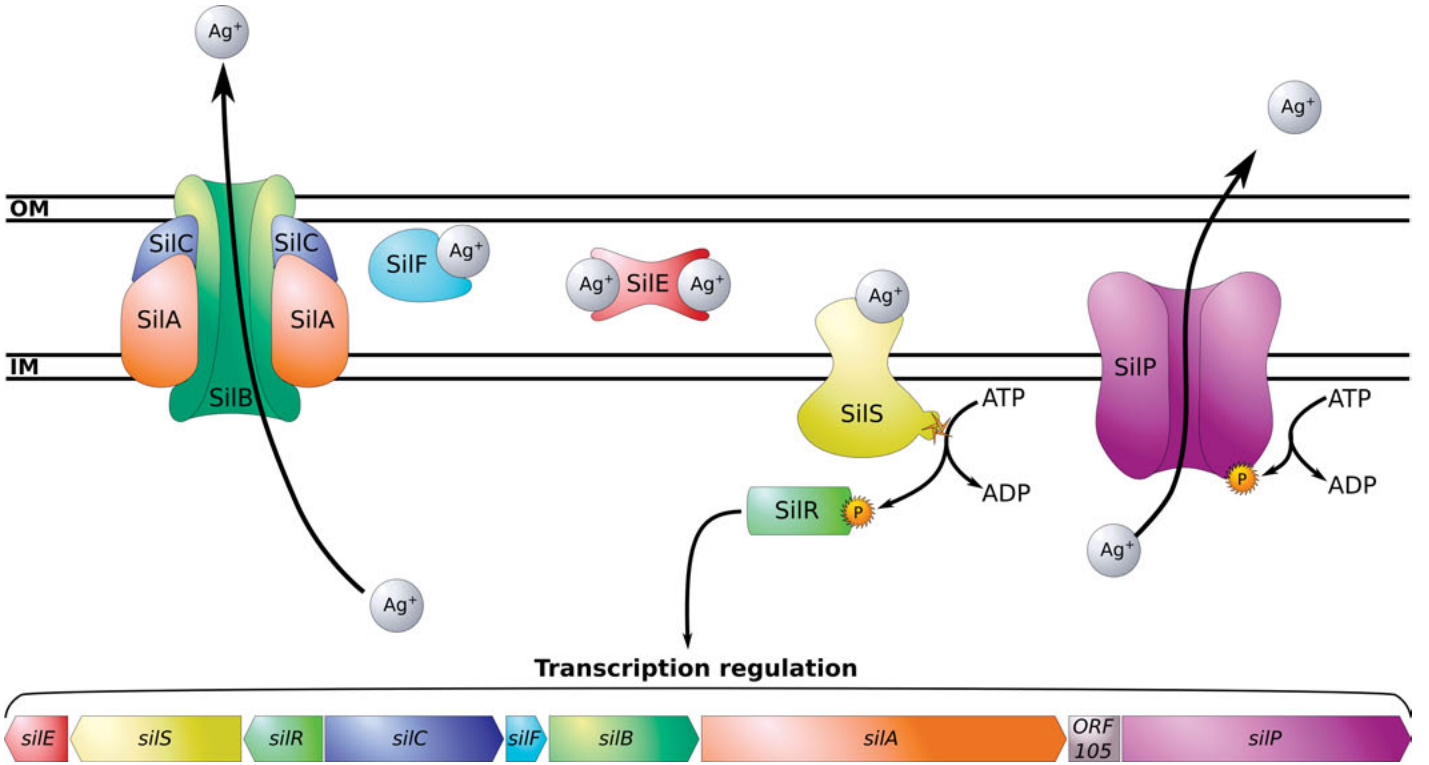

Figure 7. The sil operon and its proposed transcriptional products. Top: proposed function of genes from the *sil* operon (adapted from Gupta *et al.*[75], Randall *et al.*[80]). Bottom: the *sil* operon of plasmid pUUH239.2. OM – outer membrane, IM – inner membrane.

Apart from active efflux of silver, silver-resistant *E. coli* isolates are frequently porin-deficient.[73, 80]

In contrast to Gram-negative bacteria, little is known about silver resistance of Gram-positive bacteria. Genes from the *sil* operon have been described in three MRSA isolates, but none of them were phenotypically resistant.[81] Not even after *in-vitro* attempts did resistance to silver nitrate develop.[62]

Risks associated with the use of silver

The use of silver in products has almost exploded since the turn of the century. Now, words of warning have appeared from researchers that emphasise the risks of silver usage. Concern is raised regarding the toxicity of silver for humans, animals and the environment. Also potential links to antibiotic resistance are mentioned.

Frequent intake of silver leads to deposition of silver in tissues, particularly the skin and those rich in fat. In the former case, the combination of silver depositions and sunlight can cause argyria or argyrosis. Silver nanoparticles are known to convey cytotoxicity to a number of cells, including fibroblasts, hepatocytes, osteoblasts or bone-marrow cells.[57, 82, 83] In analogy with humans, silver exerts toxic effects on animals and the environment, and the exposure is mainly due to emissions from the industry.[84]

Constant exposure of bacteria to low concentrations of silver may not only cause silver resistance, but may also result in co-resistance to antibiotics.[64, 85]

Co-selection of antibiotic and heavy metal resistance

The mechanisms responsible for co-selection of antibiotic and metal resistance can be classified as follows:

Cross-resistance

Cross-resistance occurs when resistance to different compounds is mediated by the same structure, but only one of these compounds activates the mechanism. Classical examples are the multidrug efflux pumps that are found in many members of the *Enterobacteriaceae* or in *P. aeruginosa*. For instance, the resistance-nodulation-division (RND) efflux pumps play an important role in innate resistance[14] but also, when overexpressed, for the multi-drug resistance phenotype of clinical isolates.[86, 87] In *E. coli*, the AcrB efflux pump is a multidrug efflux pump with penicillins, fluoroquinolones, chloramphenicol, detergents and cationic dyes as substrates.[14] Furthermore,

E. coli expresses the copper(I)/silver(I) resistance efflux transporter CusCFBA that is the only known heavy metal specific RND transporter.[88] In contrast to AcrB, CusCFBA is far more specific: in addition to copper(I)/silver(I), cross-resistance was only found for the drugs dinitrobenzene, dinitrophenol and ethionamide.[89]

Another mechanism conferring cross-resistance is deficiency of outer membrane channels. Common ways to alter permeability are alteration of porin size and loss of porin proteins. In *P. aeruginosa*, resistance to carbapenems is mostly mediated by a combination of porin loss and active efflux.[90] Furthermore, deficiency of porins has been described as a cause of resistance to different beta-lactams in Gram-negative bacteria.[91-93] In an *in-vitro* study, silver-resistant *E. coli* isolates were porin-deficient and thus, resistant to cephalosporins.[73]

Co-resistance

The possibility of co-resistance is present when genes coding for resistances are located together on (mobile) genetic elements like plasmids, transposons and integrons. Due to this association, co-selection of resistance determinants might occur.

One of the best-documented systems for co-resistance is, maybe, that of mercury and antibiotics. The *mer* operon, determining mercury resistance, has been found on plasmids and in chromosomes, frequently in the context of *Tn*21 and *Tn*21-like transposons.[94] These transposons harbour *mer* genes and genes conferring resistance to spectinomycin-streptomycin *aad*A.[95] A primate study showed that mercury from dental amalgam fillings caused a selection for plasmids carrying mercury and antibiotic resistance genes.[96] Thus, it has been postulated that mercury may be a driving force for the selection of antibiotic resistance genes.[97, 98]

Another example, that is very illustrative of the necessity of a One Health perspective where antimicrobial resistance is concerned, is the co-resistance of copper and zinc. Copper, zinc and antibiotics are all used as growth promotors or disinfectants in commercial swine herds. There are several studies which suggest that there are links between these determinants. The usage of zinc in a pig nursery was associated with the selection of MRSA isolates.[99] In another study, copper as food supplement contributed to the selection of resistance to antibiotics.[100]

Although the silver resistance determinant *sil* has been described in mobile genetic elements like IncH plasmids[76] and can be found in plasmids from strains involved in hospital outbreaks[75, 77, 78] and in a mobile island from copper-resistant *E. coli* strains,[101] data on co-resistance between silver and antibiotics is lacking.

Co-regulation

Transcriptional and translational regulation systems can be activated in response to bacterial stress.[15, 86, 102, 103]

In *P. aeruginosa* the CzcRS system is a transcriptional regulator involved in the regulation of quorum sensing, the resistance to the metals zinc, cadmium and cobalt, and it also mediates antibiotic resistance.[104-106] Although the multiple antibiotic resistance regulator MarR of *E. coli* is regulated by copper,[107] cross-resistance of copper and antibiotics has not been documented so far.

Aims of this Doctoral Thesis

With the major problem of multiresistant bacteria and the increasing use of silver in health care and consumer products as a background, the overall intention of this thesis was to fill in knowledge gaps concerning the occurrence, the mechanisms and the possible collateral damage of silver resistance. In order to achieve this aim, we sought

- to investigate the antimicrobial effects of silver *in vivo* and *in vitro* on bacteria with Gram-positive or Gram-negative cell walls and different environmental niches.

- to investigate the distribution of genetic and phenotypic silver resistance in isolates from infected patients, human and avian carriers and from the environment.

- to investigate the genetic background to phenotypic silver resistance.

- to investigate if there are links between resistance to antibiotics and resistance to silver through co-selection.

Materials and Methods

Bacteria

The bacteria referred to in this thesis were clinical isolates from patients at Uppsala University Hospital and Changchun Children's Hospital. There was also a collection of isolates from wild birds. The main focus was put on human pathogens belonging to the *Enterobacteriaceae* family, *i.e. E. coli*, *Enterobacter spp*. and *Klebsiella spp*., with *P. aeruginosa* and Gram-positive bacteria like *S. aureus* or beta-hemolytic streptococci also having been included. An overview of the strain collection is given in Table 1.

Study I

Wound samples were collected from 14 patients with chronic leg ulcers. All patients had undergone wound treatment at Uppsala University Hospital from November 2006 to September 2007. The patients were categorized into two groups: Group 1 was treated with silver dressings for a period of 3–5 weeks, and Group 2 received treatment with silver dressings for at least 2 months. In addition, 14 *Enterobacteriaceae* and *P. aeruginosa* strains with different antibiotic resistance profiles, including multi-resistance, were chosen to evaluate their ability to develop resistance to silver.

Study II

The bacterial collection of this study consisted of human (n = 105) and avian (n = 111) *E. coli* isolates from faecal samples. The human as well as the avian study populations were composed of national (Swedish) and international isolates and included both producers and non-producers of ESBL.

Study III

Faecal samples were collected during a two-week period at Changchun Children's Hospital, China, in 2009 from forty children aged 0–3 years and admitted to a neonatology or a gastroenterology ward.

Study IV

The presence of silver resistance and genes encoding silver resistance was investigated in a total of 752 blood isolates collected at Uppsala University Hospital during the years 1990–2010. The species distribution was as follows: *E. coli* (n = 223), *Enterobacter spp.* (n = 165), *Klebsiella spp.*

(*n* = 208) and P. aeruginosa (n = 156). Furthermore, 87 Legionella isolates, mainly derived from the hospital water pipeline system, were included. Bacteria were identified to the species level with standard laboratory procedures, and, when needed, by VITEK 2 (Biomerieux, USA) or MALDI-TOF (Bruker Daltonics, Germany). All isolates were stored at -70 °C.

Table 1. *Overview over the study strains.*

Strains	**Properties**	**Study**
S. aureus (*n* = 14)	Clinical isolates derived from chronic leg ulcers.	I
E. coli (*n* = 4) *E. cloacae* (*n* = 5) *K. pneumonia* (*n* = 2) *P. aeruginosa* (*n* = 3)	Randomly chosen isolates with different antibiotic profiles used for further *in-vitro* investigation of silver exposure.	I
E. coli (*n* = 216)	Faecal *E. coli* isolates from the following defined populations: Human source: • Patients with diarrhoea, non-ESBL-producing isolates (*n* = 52). • ESBL-producing *E. coli*, Uppsala University Hospital screening routines (*n* = 34).[108] • ESBL-producing *E. coli*, ESBL-screening of healthy travellers outside Scandinavia (*n* = 19).[45] Avian source: • Herring gulls, Commander Islands, Bering Strait, Russia (non-ESBL-producing *E. coli n* = 25, ESBL-producing *E. coli n* = 1).[43] • Yellow-legged gulls, Southern France (non-ESBL producing *E. coli* (*n* = 25), ESBL-producing *E. coli* (*n* = 16).[109] • Mainly mallards, Uppsala (non-ESBL-producing *E. coli n* = 17) • Black headed gulls, Kalmar (non-ESBL-producing *E. coli n* = 25, ESBL-producing *E. coli n* = 2).[110]	II
E. coli (*n* = 27)	Faecal screening isolates from children admitted to Changchun Children's Hospital, China, ESBL-producing *E. coli*.	III
E. coli (*n* = 223) *K. pneumonia* (*n* = 129) *K. oxytoca* (*n* = 79) *E. cloacae* (*n* = 131) *E. aerogenes* (*n* = 32) *E. agglomerans* (*n* = 2) *P. aeruginosa* (*n* = 156)	Blood-stream isolates collected at Uppsala University Hospital during the years 1990–2010.	IV
Legionella spp. (*n* = 87)	Isolates from the water supply system of Uppsala University Hospital.	IV

Silver resistance

Susceptibility testing to silver nitrate

MIC and MBC of silver nitrate was carried out according to the guidelines of the Swedish Reference Group for Antibiotics. Bacteria were suspended in IsoSensitest broth (Oxoid Ltd., UK) containing silver nitrate at concentrations ranging from 4–512 mg/L with a final bacterial concentration of 10^5 cfu/mL. After 18–20 h of incubation, MIC was defined as the lowest concentration yielding no visible growth. A silver nitrate MIC of > 512 mg/L, classified the bacterium as silver-resistant. The lowest concentration of silver nitrate killing 99.9 % of a bacterial inoculum was termed the MBC.

Exposure of bacteria to silver *in vitro*

To induce silver-resistance, a stepwise selection procedure following MIC testing was performed. Ten µL of the bacterial suspension was inoculated into a series of tubes, each containing 1 mL of IsoSensitest broth supplemented with increasing concentrations of silver nitrate (4–512 mg/L). The tubes were incubated at 37 °C overnight, and from the tube with the highest silver nitrate concentration and still visible growth, the new inoculum was taken. The experiment was repeated until a MIC of silver nitrate > 512 mg/L was reached, or after 10 passages had been performed. If a strain developed resistance to silver at least five sub-cultivations on blood or CLED agar were performed. After each passage, ≥ 5 cfu were tested if they still grew in IsoSensitest broth containing silver nitrate at a concentration of 512 mg/L.

Growth curves

Growth curves were obtained using a BioscreenC reader (Labsystems, Finland). The bacteria were grown in IsoSensitest broth with or without silver nitrate (128 mg/L). The bacterial inocula (250 µL of each strain at a concentration of 5 x 10^3 cfu/mL) were suspended in a honeycomb plate and immediately placed in the BioscreenC at 37 °C for 24 h. The optical density was determined every 10 min at 600 nm, after shaking the plate for 10 s at maximum amplitude. Each growth curve represented the mean of two independent experiments in triplicate.

Detection of genes in the *sil* operon

DNA was prepared by boiling bacteria in PCR-water for at least 10 min, and amplification was carried out in a GeneAmp PCR system 9700 cycler (PE Applied Biosystems, USA) using Taqman Mastermix (Qiagen, Germany). Gene specific primers for *sil* genes and appropriate annealing temperatures were used as previously described.[78, 111] PCR-products were separated by gel electrophoresis and analysed visually.

Sanger sequencing of the *silS* gene

Sequencing of the *silS* amplicons was performed on an ABI 3730 XL Automated Sequencer (Applied Biosystems, USA). SNP calling was carried out with novoSNP.[112] As reference, the *silS* gene from pUUH239.2 (NC_016966) was used.

Next generation sequencing

DNA was prepared using QIAquick PCR Purification Kit (Qiagen, Germany). The DNA was thereafter sequenced in an IonTorrentTM with a read length of 400 bp, according to the manufacturer's instructions (LifeTechnologies, USA). The reads were assembled into a draft genome using the AssemblerSPAdes plugin in TorrentSuite 4.2 with LifeTechnologies' recommended settings. Databases were created for each of the assembled genomes.

Antibiotic resistance

Antibiotic susceptibility testing

Susceptibility testing was performed according to the recommendations of the Swedish Reference Group for Antibiotics or the European Committee on Antimicrobial Susceptibility Testing. Isolates were tested by disc diffusion, and, when indicated, by MIC-determination using Etest (AB Biodisk, Sweden).

Isolates with reduced susceptibility to cefpodoxime, ceftazidime and/or cefotaxime were tested for ESBL-production by a modified double disc diffussion synergy test.[113] The plates were incubated for 16–24 h at 35 °C in room atmosphere.

Amplification and characterisation of resistance genes

The DNA was prepared and amplified as described above for the *sil* genes. Investigated resistance genes were *merA* (mercury resistance),[114] $bla_{CTX\text{-}M}$, bla_{TEM} and bla_{SHV} (beta-lactamases),[108] and *qnr*[115] and *aac(6)-lb*[116] (plasmid-mediated quinolone resistance). Genes encoding beta-lactamases were further typed by sequencing using an ABI 3130 instrument (Applied Biosystems, USA). The sequences obtained were compared with published sequences, employing the NCBI Basic Local Alignment Search Tool (BLAST).[108, 117]

Outer membrane protein profiles

Outer membrane proteins were extracted from late logarithmic phase cultures at 37 °C in Mueller-Hinton broth. The bacterial cells were washed, lysed with lysozyme, and, after adding RNase and DNase, disrupted by five freeze-thaw cycles. Membrane pellets were received after ultracentrifugation, treated with N-lauroylsarcosine and resuspended in Laemmli sample buffer. The proteins were stained with bromophenol blue and subjected to polyacrylamide gel electrophoresis.

Epidemiological typing

PCR-based fingerprinting

Fingerprints of bacterial isolates were produced using AP-PCR. The primers used in the studies were ERIC-1R,[118] ERIC-2,[119] A70-9, 208 and 272.[120] Amplified products were analysed with gel electrophoresis and interpreted visually. Two isolates with identical band patterns were considered to be the same strain.

MLST

MLST was performed for *E. coli*, *K. pneumoniae* and *E. cloacae* isolates using established protocols.[121-123] For *E. coli*, the seven housekeeping genes *adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA,* and *recA* were amplified and sequenced. The analysis on the latter two species was carried out *in silico* using whole genome sequence data. Chromatograms were edited with the seqtrace software,[124] and ST analysis was performed using the MLST websites for *E. coli* (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli), *K. pneumonia* (http://bigsdb.web.pasteur.fr/klebsiella/klebsiella.html) and for *E. cloacae* (www.pubmlst.org).

PCR-detection of the O25b-ST131 clone

For detection of the *E. coli* O25B-ST131 clone, an allele-specific PCR amplifying the *pabB* gene was used. The PCR was carried out as described by Clermont *et al*.[125] with slight modifications.

Clonality with BURST

Genetic relationship was determined using the BURST (based upon related sequence type) algorithm as implemented in eBURST (version 3; http://eburst.mlst.net)[30] and in the goeBURST software.[31] The stringent group definition of clonal complexes (CCs) was used, i. e. only sequence types that shared identical allels at ≥ 6 of 7 loci were grouped. Population snapshots were based on group definitions with 0/7 identical alleles in eBURST, displaying related and unrelated sequence types.

Statistical analyses

Where appropriate, differences in the distributions were analysed with Fisher's exact test. A difference was considered statistically significant for $p \leq 0.05$.

Results

Antibacterial activity of silver

Antibacterial activity of silver *in vivo (I)*

Wound treatment with silver dressings was not able to eradicate the primary wound pathogens *S. aureus*, beta-hemolytic streptococci and *P. aeruginosa* (I). In 9 out of 14 ulcers, *S. aureus* continued to grow after at least three weeks of treatment. Likewise, cultures remained positive for *P. aeruginosa* (3/14) and beta-hemolytic streptococci (3/14) after treatment.

Genetic fingerprinting before and after treatment revealed that the isolates had identical DNA-patterns, indicating that treatment was not successful in eradicating bacteria from the chronic leg ulcers (*Figure 8*).

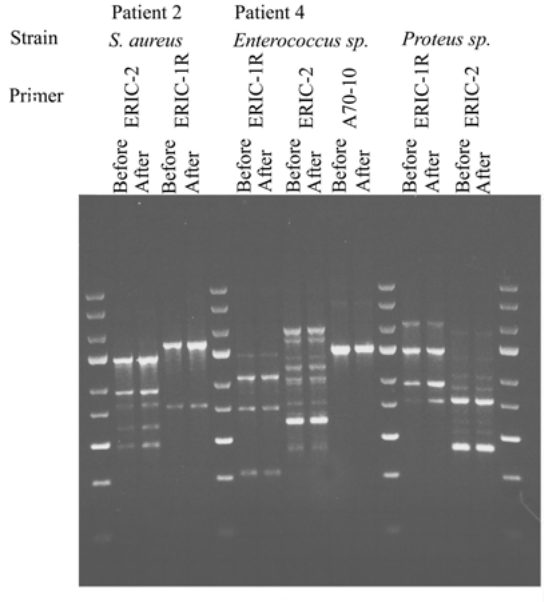

Figure 8. Representative gels after electrophoresis of AP-PCR products from isolates obtained before and after 3 weeks of treatment with silver-based dressings. Lanes 1, 6, 13 and 18: DNA size markers.

Silver nitrate MICs and MBCs

The reference strain *E. coli* ATCC 25922 yielded a MIC of 16 mg/L ± one dilution step at all times. Independent of cell wall structure, MIC-values for silver nitrate ranged from 8–32 mg/L for the majority of the tested strains (n = 464), indicating that this is the range for the wild type. For the MIC distribution, see (*Figure 9*).

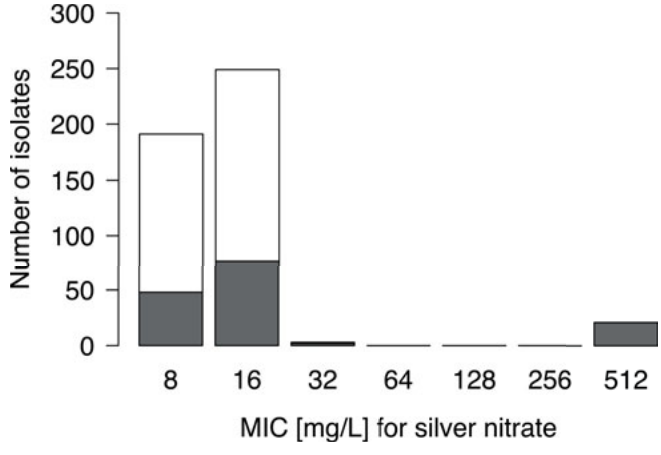

Figure 9. Distribution of silvernitrate MICs among the Enterobacteriaceae family in studies I, II and IV. Grey – *sil*-positive strains, white – *sil*-negative strains.

To determine the MIC was difficult for *Enterobacter spp.* They had a tendency to randomly jump over certain concentrations of silver nitrate. A representative example of this phenomenon is shown in (*Figure 10*). When cells from the tubes containing ≥ 64 mg/L silver nitrate were used for determining the MIC, they always grew in silver nitrate concentrations of > 512 mg/L.

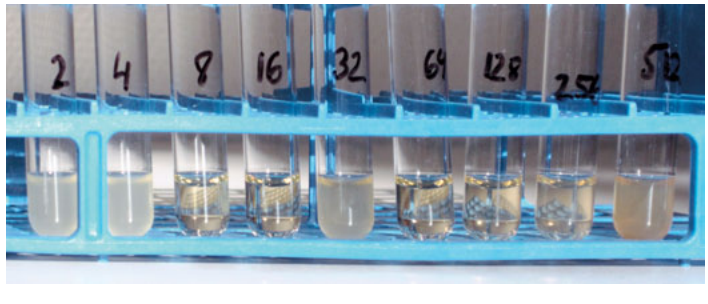

Figure 10. Result of the silver nitrate MIC determination for E. cloacae strain B8275034. The phenomenon of jumping concentrations in the dilution series can be observed in tube 5 (32 mg/L) and tubes 9 (512 mg/L). The concentrations of silver nitrate is 2–512 mg/L in increasing order from left to right.

The MBC determination showed that silver nitrate had a bactericidal effect on Gram-negative bacteria. The MBC-values for these bacteria were in the same range as the MIC-values (16–32 mg/L). In contrast, silver nitrate exhibited only a bacteriostatic effect on Gram-positive bacteria. All tested Gram-positive isolates had MBCs of ≥ 512 mg/L.

Frequency of silver resistance

Frequency of phenotypical resistance to silver nitrate

Phenotypical resistance to silver nitrate predominated in *E. cloacae* ($n = 16$) but was also found in *E. aerogenes* ($n = 2$), *K. pneumonia* ($n = 2$) and *K. oxytoca* ($n = 2$) (I, IV).

During the treatment of a chronic ulcer with a dressing containing silver (Aquacel Ag®), an *E. cloacae* isolate (SM0700965 II) resistant to silver nitrate was found (≥ 512 mg/L). Before treatment, no *E. cloacae* was isolated, and when this isolate was detected the wound was treated with silver-based dressings over a period of three weeks (I).

MIC-testing for silver nitrate on an extensive strain collection of *Enterobacteriacae* ($n = 443$), revealed elevated MIC-values (≥ 64 mg/L) to silver nitrate in *E. cloacae* (15/99, 15 %), *E. aerogenes* (2/29, 7 %), *K. pneumoniae* (2/95, 2 %) and *K. oxytoca* (2/59, 3 %) (IV). None of the tested *E. coli* isolates expressed resistance to silver nitrate without in-vitro exposure to the substance (I, II, IV).

Genetic resistance to silver

Frequency of *sil* genes in isolates from infected patients and carriers

Genes of the *sil* operon were only found in species belonging to the *Enterobacteriaceae* family. No *sil* genes were detected in *P. aeruginosa*, *Legionella spp.*, *Enterococcus spp*., beta-haemolysing *streptococci* or *S. aureus.*

The silver-resistant wound isolate SM0700695 II carried *sil* genes (I). Out of 839 blood-stream isolates, 176 (21 %) harboured *sil* genes. These genes were most frequent in *Enterobacter spp.* (80/165; 48 %) and *Klebsiella spp*. (86/208; 41 %). Of the investigated species, the highest frequency was found in *E. cloacae* (76/131; 58 %) and *K. oxytoca* (39/79; 49 %) (IV). No difference in frequency of *sil* genes in blood-stream isolates was noted during a time period of 20 years (*Figure 11*).

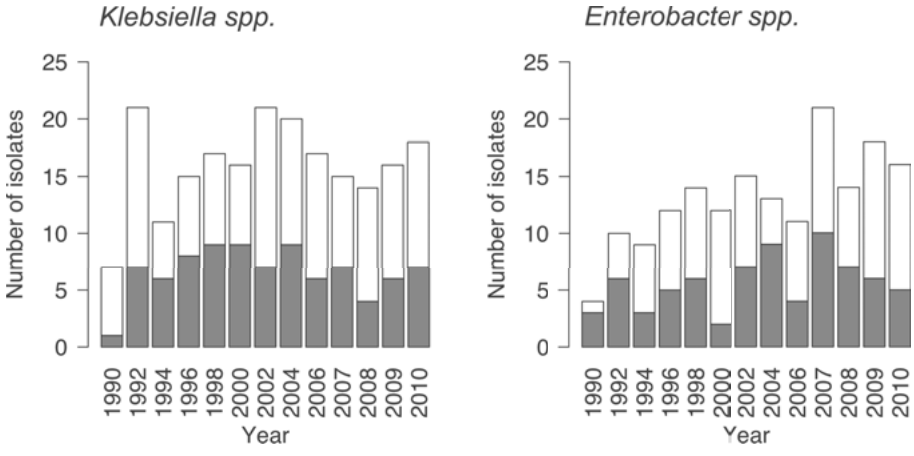

Figure 11. Sil-gene-positive isolates in relation to the total number of isolates tested over time for Klebsiella spp. and Enterobacter spp. Grey: *sil*-positive isolates, white: *sil*-negative isolates.

In *E. coli*, the presence of *sil* genes was comparatively rare with an overall frequency about 5 % (blood stream 5 % (10/223) (IV), and among faecal isolates 4 % (2/52) (II) and 6 % (13/216) (II).

Factors associated with *sil* gene carriage

For the blood-stream isolates (IV), information on age, gender and admitting ward was accessible. The carriage rate of sil genes increased with the age of the patients. Accordingly, the lowest carriage rate (24 %) was observed in the neonatology ward. Blood-stream isolates from patients admitted to the oncology and hematology wards had the highest carriage rate of sil genes (66 %). Although 65 % (244/377) of the blood-stream isolates belonging to the genera Enterobacter or Klebsiella were cultured from male patients, female patients were significantly more often sil-gene carriers than male patients (69/133 vs. 97/244, p = 0.03) (IV).

Sil genes were not detected in any avian *E. coli* isolate (II).

Clonal aspects on *E. coli* isolates carrying *sil* genes

Isolates carrying *sil* genes belonged to a variety of sequence types and clonal complexes. Although the limited number of sequence typed *sil*-positive strains, the majority belonged to the largest group at SLV level as calculated by the goeBURST analyses (*Figure 12*).

None of the isolates belonging to ST131 carried *sil* genes.

Table 2. *Summary of E. coli types carrying sil genes according to MLST.*

Strain	Sequence type	Specimen	ESBL production, CTX-M type if known	Study
S8	58	Faeces	CTX-M-14	II
S10	940	Faeces	CTX-M-14	II
S11	10	Faeces	CTX-M-14	II
P9	388	Faeces	CTX-M-15	II
P14	205	Faeces	CTX-M-15	II
P18	127	Faeces	CTX-M-15	II
P21	1312	Faeces	CTX-M-15	II
R7	424	Faeces	CTX-M-15	II
R9	940	Faeces	CTX-M-15	II
R20	155	Faeces	CTX-M-15	II
F32	10	Faeces	No ESBL production	II
F32	409	Faeces	No ESBL production	II
B0909531	540	Blood	No ESBL production	IV
B1011268	410	Blood	ESBL production	IV
B0804035	1011	Blood	No ESBL production	IV
B0607370	23	Blood	No ESBL production	IV
P12-1	Not typeable (clonal complex 10)	Faeces	CTX-M-14	III

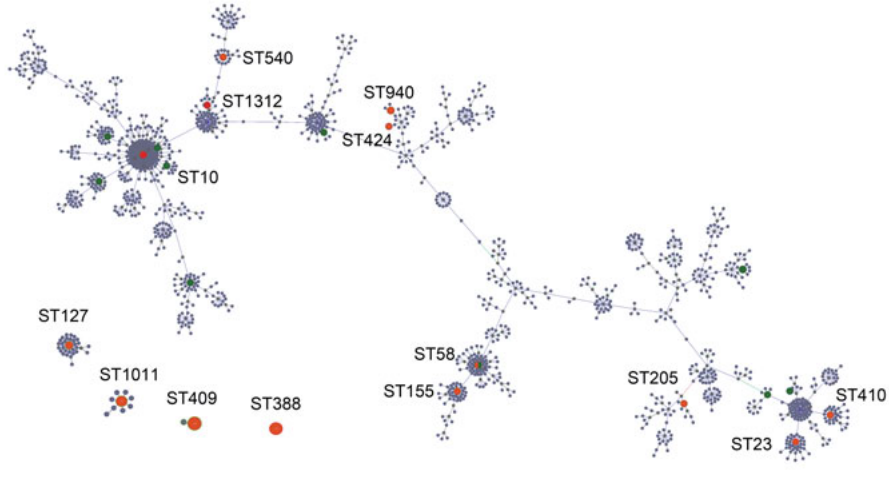

Figure 12. Minimal spanning tree illustration of sequence types of sil-positive E. coli isolates from the studies (calculated by goeBURST algoritm). Light red dots: *sil*-positive isolates.

The *sil* operon and phenotypic silver resistance

Sil genes and phenotypic resistance to silver nitrate

In all strains that showed phenotypic resistance to silver nitrate without a previous silver exposure, *sil* genes were detected (I, IV). Furthermore, during *in-vitro* exposure, all *sil* positive strains developed resistance to silver nitrate (IV). Carriage of *sil* genes was, however, not a prerequisite to silver resistance, since there was one strain lacking *sil* genes that developed resistance (I).

In-vitro resistance was unstable after at least five subcultivations for one isolate without *sil* genes (I), none out of 13 *sil*-positive isolates (II) and 4/17 *sil*-positive isolates (IV).

In whole genome-sequenced isolates, the *sil* operon was complete and all genes were in the same order compared with reference from plasmid pUUH239.2.

Fitness of silver-resistant strains

The development of silver resistance *in vivo* and *in vitro* had a fitness cost. The cost differed between the strains (*Figure 13*).

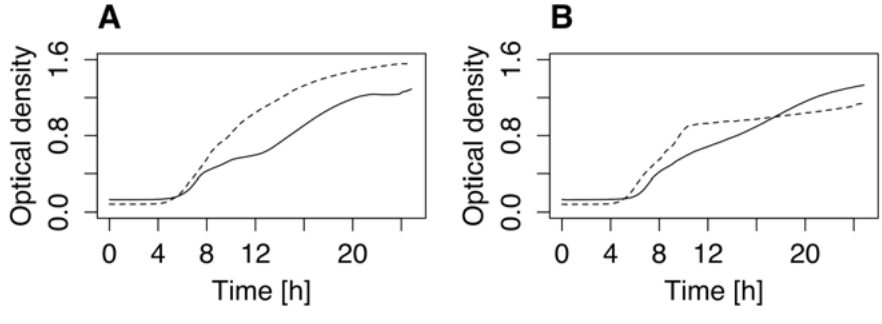

Figure 13. Growth curves of silver-resistant isolates after in-vivo selection. (A) *E. cloacae* B8275034 and (B) *K. pneumoniae* B0910808. Dashed line: after selection, solid line: during selective pressure of silver nitrate (128 mg/L).

SNPs in the *silS* gene

To explore the genetic events taking place in the *sil* operon in the two strains *E. cloacae* B09014770 and *K. pneumoniae* B1018747 during the exposure to silver, the whole genome sequencing was carried out before (AgS) and after (AgR) the exposure. Upon comparison of the genomes (AgS-AgR), SNPs in the *silS* gene were observed. In *E. cloacae* strain B09014770, there was a SNP at T965A, whereas *K. pneumoniae* strain B1018747 had one at G629A.

To examine how common these SNPs were, 17 additional, *silS* genes were sequenced before and after the emergence of silver resistance. SNPs were found in 12 out of 17 isolates. They were distributed over the whole length of the gene, but a certain accumulation of mutations was noted in two segments, 629–725 bp and 919–1054 bp (*Figure 14*).

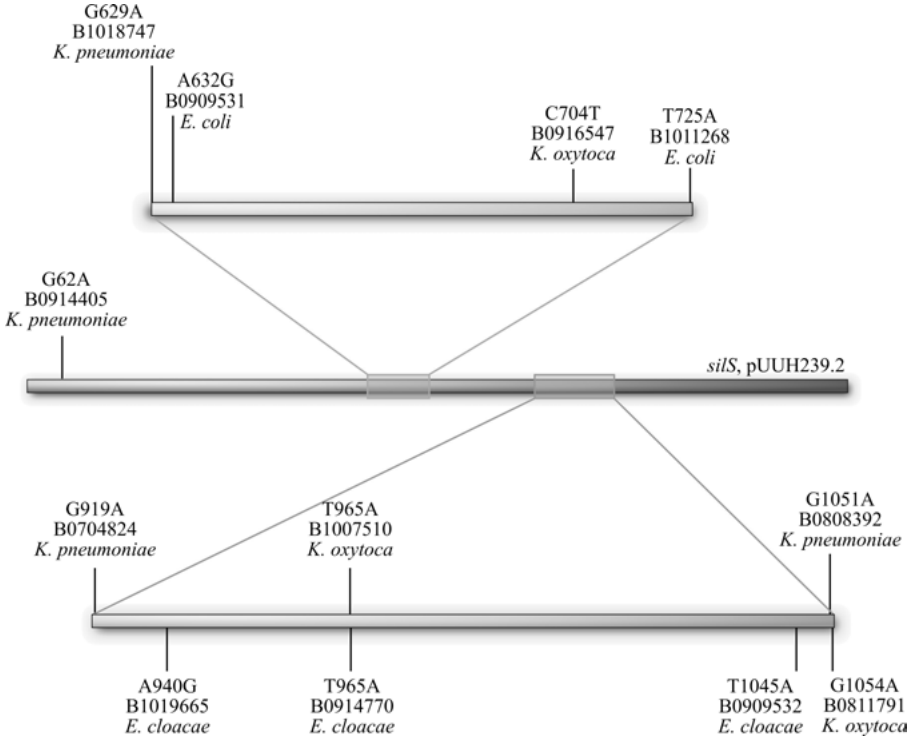

Figure 14. Localisation of SNPs in the silS gene after in-vivo and in-vitro selection. Sequence of *silS* from pUUH239.2 was used as reference.

Co-selection of antibiotics and silver

Co-selection in isolates without *in-vitro* exposure to silver

Half of the isolates (11/22) that expressed resistance to silver were co-resistant to antibiotics (I, IV). Decreased susceptibility to beta-lactams was frequently noted, and resistance to co-trimoxazole and ciprofloxacin was also found (Table 3).

Table 3. *Summary of antibiotic resistance in isolates with silver resistance without in-vitro exposure to silver (only strains with decreased susceptibility were listed).*

Strain	Species	Co-selection (SIR categrorisation)	Reference
SM0700694 II	*E. cloacae*	Cefotaxime I*	I
B1013331	*E. cloacae*	Cefotaxime R	IV
B0813794	*E. cloacae*	Cefotaxime R, Ceftazidime R, Aztreonam R, Piperacillin/Tazobactam R	IV
B0709348	*E. cloacae*	Co-trimoxazole R	IV
B0608132	*E. cloacae*	Ciprofloxacin I	IV
B8527023	*E. cloacae*	Cefotaxime R, Ceftazidime R, Aztreonam R, Piperacillin/Tazobactam R	IV
B8413030	*E. cloacae*	Cefotaxime R, Ceftazidime R, Piperacillin/Tazobactam R	IV
B8275034	*E. cloacae*	Cefotaxime R, Ceftazidime R, Aztreonam R, Piperacillin/Tazobactam R	IV
B0910808	*K. pneumoniae*	Cefotaxime I, Ceftazidime I, Ciprofloxacin R, Co-trimoxazol R	IV
B8195012	*K. pneumoniae*	Piperacillin/Tazobactam I	IV
B4121026	*K. oxytoca*	Piperacillin/Tazobactam I, Ciprofloxacin R	IV

* I (Indeterminate), R (Resistant)

Co-selection in isolates with *in-vitro* silver resistance

In-vitro exposure to silver nitrate affected the antibiotic susceptibility in some strains, and this susceptibility was either increased or decreased. The drugs most often involved in a change in the SIR categorisation were the beta-lactams, ciprofloxacin, gentamicin and co-trimoxazole (Table 4).

Table 4. *Summary of developed antibiotic resistance after exposure to silver nitrate in vitro.*

Strain	Species	Changes in susceptibility	Reference
S4279/06	*E. cloacae*	Imipenem S to R*	I
R07**	*E. coli*	Piperacillin/Tazobactam S to I	II
R09**	*E. coli*	Ceftibuten I to R	II
R20**	*E. coli*	Ceftibuten I to R	II
S11**	*E. coli*	Ciprofloxacin R to S	II
P03**	*E. coli*	Ceftibuten S to R, Ciprofloxaci*n* R to S, Piperacillin/Tazobactam S to I	II
P21**	*E. coli*	Ceftibuten I to R	II
P14**	*E. coli*	Piperacillin/Tazobactam S to R, Co-trimoxazol R to I	II
F23	*E. coli*	Piperacillin/Tazobactam S to R, Gentamicin S to R, Co-trimoxazol S to R	II

*S (Susceptible), I (Indeterminate), R (Resistant)
**ESBL-producer

Effect on outer membrane proteins

Outer membrane protein profiles were analysed on *E. coli* isolates before and after silver-exposure experiments. After silver exposure, two out of six isolates lost OmpC expression, whereas one isolate lost OmpF expression. Loss of OmpC porin was most common (II).

There was no obvious pattern matching the antibiotic susceptibility changes.

Association of *sil* genes and CTX–M production in *E. coli*

Sil genes were more often found in ESBL-producing *E. coli* than in non-ESBL-producing *E. coli* (II). The frequency of *sil* genes in CTX-M-producing *E. coli* varied depending on the source. While *sil* genes were relatively common in ESBL-producing *E. coli* from stool samples collected in Sweden (21 %, 11/53) (II), the frequency of *sil* genes in ESBL-producing *E. coli* from children admitted to Changchun Children´s Hospital (4 %, 1/27) (III) or birds (0/9) (II) was very low or zero (Table 5).

Table 5. *Overview over strain collections with CTX-M producing E. coli.*

Source of strain*	Host	Frequency of sil genes	Reference
Uppsala University Hospital screening routines (*n* = 34)	Mainly adults	24 % (*n* = 8)	II
ESBL screening of healthy travellers outside Scandinavia (*n* = 19)	Mainly adults	16 % (*n* = 3)	II
Yellow legged gulls, Southern France (*n* = 9)	Birds	0	II
Screening isolates from children admitted to Changchun Children's Hospital, China (*n* = 27)	Children	4 % (*n* = 1)	III

* All isolates originated from stool samples.

Out of all investigated CTX-M types, *sil* genes were only present in CTX-M-15 (31 %, 8/26) and CTX-M-14 (17 %, 4/23). None of the other strains with CTX-M types carried *sil* genes (55 (*n* = 17), 9 (*n* = 9), 27 (*n* = 2), 64 (*n* = 1), 101 (*n* = 1)) (II, III).

Association of *sil* genes and antibiotics

The association of *sil* genes and antibiotics was investigated in human feacal *E. coli* isolates with and without ESBL production. Isolates with *sil*E gene were more likely to be resistant to co-trimoxazole (92 % *vs.* 40 %, P = 0.0005) and gentamicin (46 % *vs.* 17 %, p = 0.022) than *sil*E-negative isolates (II).

Association of *sil* genes and *mer*A genes

In fecal *E. coli*, *mer*A gene was present in 22 out of 216 isolates (10 %), more *mer*A genes were detected in human *E. coli* (16 %, 17/105) compared with avian *E. coli* (5 %, 5/111) (p = 0.004) (II). In feacal samples from children admitted to Changchun Children's Hospital, *mer*A gene was found in two samples (III). Only one isolate had *sil* and *mer*A genes simultaneously, the isolate was derived from a patient in a surveillance-programme at Uppsala University Hospital (II).

In blood-stream isolates, *mer*A was most common in *Klebsiella spp*. (34/208 isolates; 16 %), followed by *E. coli* (26/223 isolates; 12 %), *P. aeruginosa* (17/156 isolates; 11 %), and *Enterobacter spp*. (8/165 isolates; 5 %).

Discussion

Antibacterial effects of silver

The first products that appeared on the market and used silver as a biocide were silver-based dressings for wound treatment. Therefore, we investigated the antibacterial effects of topical silver treatment on the bacterial wound flora. We found that treatment of 14 chronic leg ulcers with silver-based dressings for at least three weeks did not eradicate primary wound pathogens or prevent wound colonisation with secondary wound pathogens. Furthermore, after only three weeks of topical treatment, we isolated a silver-resistant *E. cloacae* strain from the wound of one patient.

These findings are worrying since the use of silver-based dressings on chronic leg ulcers has increased[62] to such an extent as to become very costly despite lacking clinical evidence of their efficacy.[126, 127] We noted that *S. aureus* and pyogenic streptococci had high silver nitrate MBCs. This suggests that silver exerts only a bacteriostatic effect on the primary wound pathogens. Similar findings have been described by Feng *et al.*[58] and Randall *et al.*[62] when they investigated the effects of silver on *S. aureus*.

To determine silver nitrate MICs or MBCs is not always easy, and some research groups, therefore, seem to have chosen some alternatives to standard laboratory procedures used for antibiotics. The preferred route is to use more unconventional definitions of bactericidal activity and to develop new tests that are optimised in one way or another to show bactericidal effects of silver *in vitro*.[60] The value of these tests in clinical settings can, however, be questioned. In contrast to the results of custom-made tests, we did not find a bactericidal effect of silver on *S. aureus* or pyogenic streptococci, and that was independent of the conditions (*in vitro* or *in vivo)*. Therefore, we believe that it is crucial to use established laboratory procedures and modify them as little as possible. This way, other groups can repeat the experiments, and results can be compared.

In contrast to the findings in Gram-positive bacteria, the silver nitrate MBCs were almost identical to the MICs in Gram-negative bacteria, i. e. Gram-negative bacteria are definitely killed by silver. This finding is interesting since it is usually the other way around for commonly used disinfectants and antiseptics. However, despite our *in-vitro* findings, members of the *Enterobacteriaceae* family and *P. aeruginosa* were not eradicated by topical silver treatment *in vivo*. Several reasons for this have been suggested, includ-

ing insufficient release concentrations of silver from topical dressings[64] and a high binding rate of silver ions to halides and proteins that may further reduce the fraction of free (active) silver ions in a complex wound environment.[56] This may contribute to the failure to eradicate wound pathogens and colonisers in the majority of the ulcers investigated.

The results of *in-vitro* killing kinetics for the different types of silver-based dressings available suggest that the bactericidal effect of silver on Gram-negative bacteria is achieved within 4 h.[128, 129] These findings indicate that there is only limited benefit of prolonged treatment, and, moreover, long-term treatment might increase the risk to select for resistant Gram-negative species. Thus, the necessity of long-term treatment with silver-based dressings is questionable.

Distribution of silver resistance

In the present thesis, we used a standard laboratory method to investigate the silver nitrate MIC distribution in a strain collection consisting of members of the *Enterobacteriaceae* family, *P. aeruginosa* and *Legionella spp*. Noteworthy was that phenotypical resistance, in terms of increased MICs without prior exposure to silver *in vitro*, was only found in bacteria belonging to the two genera *Enterobacter* and *Klebsiella*.

The species with the highest frequency of silver resistance was *E. cloacae,* a finding which is in accordance with other studies.[59, 67, 79] As many as 15 % of the invasive *E. cloacae* isolates were phenotypically silver-resistant.

In congruence with the phenotypical findings, genetic determinants of silver resistance, *i. e. sil* genes, were exclusively found in members of the *Enterobacteriaceae* family. Even in the genetic context, *sil* genes were most frequent in *Enterobacter spp.* (48 %) and *Klebsiella spp.* (41 %). For *E. cloacae,* we found a *sil* gene carriage rate of 58 %, a figure which is comparable with the 63 % found in a recent study of clinical isolates at a German hospital.[78]

Compared with *Enterobacter* and *Klebsiella,* few human *E. coli* isolates carried *sil* genes. However, in Swedish human *E. coli* isolates with production of CTX-M-14 and -15, we found an elevated frequency of *sil* genes (up to 21 %). If *sil*-positive *E. coli* isolates were few in Swedes, they were close to non-existent in other populations. *Sil* genes were not found in any avian *E. coli* isolate, although the isolates were collected from diverse geographical regions, and the birds lived in some cases in areas with a high human activity.[109, 110] Almost as surprising was, that despite a very high isolation frequency of CTX-M-producing *E. coli*, only a single Chinese child carried a strain with *sil* genes. It is possible that one of the most important sources of

CTX-M genes in China, *i.e.* chicken,[130] lacks exposure to silver, as wild birds seem to do.

Interestingly, *sil* genes were not detected in invasive *P. aeruginosa* isolates or in the *Legionella* isolates that were included in the study. These bacteria prefer wet environments and are both excellent biofilm formers. *Legionella spp*. can, in addition, hide inside eukaryotic cells. In their natural habitat they ought to come in contact with silver, albeit at low concentrations. Its toxic effects can probably be avoided by the strategies mentioned above. Carriage of *sil* genes is, therefore, not necessary.

Accumulation of resistance genes has been described as an adaptive mechanism to environmental requirements that is driven by selective pressure.[42] Silver is a rare but naturally occurring metal that can be detected at low concentrations in rivers and lakes, even in pristine unpolluted areas.[84] A recent investigation of Gullberg *et al.*[36] showed that very low concentrations of heavy metals were able to exert selective pressure. Nevertheless, silver ions are very reactive, and they bind rapidly to proteins and other molecules in the surrounding. The antibacterial effect in natural habitats might, therefore, be limited or short-lived.

Kremer *et al*. suggested another possibility; that silver resistance could be a potential fitness factor contributing to the successful establishment of an outbreak strain in the hospital environment.[78] We found *sil* genes more often in isolates from the hematology ward, a ward with intensive use of chemotherapeutics, disinfectants and antibiotics, and *sil* genes may represent a selective advantage in hospital environments.

Remarkably, there was a higher rate of silver resistance in bacteria from females than from males. Silver is used as preservative in cosmetics and as a disinfectant of vegetables and salads. It has also been offered as a food supplement.[56, 131] Although this is highly speculative, it is possible that the female lifestyle may expose women to silver to a higher extent than men.

The distribution of silver resistance in different bacterial populations and species raises more questions about the source(s) of *sil* genes and the driving forces involved in the acquisition. If additional bacterial populations are investigated in the future, it would be of value to change the primers used for the *sil* gene screening. Results of the whole genome sequencing showed that there were mismatches between the primers designed by Percival *et al.*[111] and some strains from our strain collection. With the primers used by Kremer *et al.,*[78] the number of isolates positive for different *sil* genes increased.

Genetic background to silver resistance

We found silver resistance in *Enterobacter* and *Klebsiella* without prior exposure to silver *in vitro*. All these isolates with phenotypical resistance had *sil* genes. Inversely, phenotypical resistance to silver nitrate developed easily after *in-vitro* silver exposure for all isolates that carried *sil* genes. The selected resistance phenotype was stable for the majority of *sil*-positive isolates. One isolate lacking *sil* genes developed silver resistance after provocation. This phenotype was instable after subcultivation.

We found that bacterial cells selected by the exposure experiments had mutations in the *sil*S gene. This gene encodes a sensor kinase that activates the response regulator *sil*R.[75, 80] Mutations in *sil*S gene occur in silver-exposed strains and result in an activation of the *sil*CFBA efflux pump.[80] Randall *et al.* show that derepression of *sil*CFBA alone was not sufficient to achieve a phenotype with a high level of resistance to silver. In addition to the active clearance of silver from the cytoplasm with the *sil*CFBA efflux pump, cells prevent increasing silver concentrations in the cytoplasm by loss of porins and an up-regulation of the periplasmatic silver-binding protein *sil*E.[80]

Randall *et al.* found that both OmpC and OmpF had to be absent in order to achieve a resistance phenotype.[80] According to our results, this is not a prerequisite to silver resistance in *sil*-positive *E. coli*. The loss of porins has, however, a fitness cost.

Co-selection of antibiotic and silver resistance

S*il* genes were overrepresented in CTX-M-producing *E. coli*, indicating a potential co-selecting mechanism. Interestingly, in our material, *sil* genes were solely found in *E. coli* that produced CTX-M-15 and CTX-M-14, the most frequent CTX-M types.[50, 51] This finding indicates that the *sil* genes may contribute to the dissemination of these CTX-M types or vice versa.

However, the present material has some limitations. Both CTX-M-15 and -14 were mainly isolated from adults in Sweden, whereas other CTX-M types were, with few exceptions, derived from birds from South France or Chinese children. As mentioned earlier, the rate of CTX-M-producing *E. coli* in Chinese children was quite high, but the most prevalent CTX-M type (CTX-M-55) lacked *sil* genes. Noteworthy was that the only Chinese *sil*-positive isolate was producing CTX-M-15. With the chicken as an important reservoir for CTX-M-producing isolates in China,[130] and it is likely that other factors than silver drives the selection of CTX-M-producing isolates in this country.[132, 133]

Silver resistance was associated with resistance to beta-lactams, irrespective of CXT-M enzymes, co-trimoxazole, gentamicin and quinolones. We found this pattern in all investigated isolates of the *Enterobacteriaceae* fami-

ly with phenotypic resistance, genotypic resistance, and after *in-vitro* selection of silver resistance. In addition, we could also show that porin losses were linked to silver resistance.[73, 80] Even if these losses were not solely responsible for a reduced susceptibility or a resistance to beta-lactams, they may contribute to beta-lactam resistance not caused by CTX-M production.

Although silver resistance was associated with CTX-M-producing isolates and resistance to certain clinically important antibiotics, no association was found between silver resistance and mercury resistance. This finding was unexpected, as dental amalgam, an alloy consisting of mercury and silver, is a well-documented source for both metals, is present in many human mouths all over the world, and has been found to select for mercury resistance.[96, 97, 134] A common selective source for mercury and silver resistance seems not that likely.

Conclusions

The antibacterial effect *in vitro* of silver on Gram-positive bacteria was bacteriostatic, whereas it was bactericidal on Gram-negative bacteria.

The *in-vivo* activity of silver-based wound dressings was limited, as they failed to eradicate both Gram-positive and Gram-negative bacteria.

Genetic and phenotypic silver resistance was only found in members of the *Enterobacteriaceae* family. Most frequent was silver resistance in *Enterobacter spp.* and *Klebsiella spp*.

Genetic silver resistance was most frequently found in human isolates. It was not observed in bacteria of avian origin or in species with wet environments as their natural habitat.

In *Enterobacteriaceae*, *sil* genes were associated with a silver-resistant phenotype in all cases but one. Selected mutants expressing silver resistance had SNPs in the *sil*S gene, which is part of the regulon of the silver efflux pump silCFBA.

Possible links for co-selection were found for beta-lactams, co-trimoxazole, gentamicin and silver. In contrast, no associations between silver and mercury resistance were found.

Sammanfattning på svenska

Multiresistenta bakterier är ett allvarligt och snabbt ökande problem i dagens sjukvård, och de är huvudsakligen ett resultat av en mångårig och hög konsumtion av antibiotika. Bakterier med antibiotikaresistens kan även selekteras av biocider, kemiska substanser med antibakteriell effekt. En biocid som har fått en renässans under senare år är den giftiga tungmetallen silver, som ofta marknadsförs som ett alternativ till antibiotika. Risken finns att en okontrollerad användning av silverprodukter bidrar till spridningen av antibiotikaresistens. Mekanismer som leder till bakteriell silverresistens är mångfaldiga, och en viktig genetisk markör för silverresistens är de *sil*-gener som ingår i *sil*-operonet, ett kluster av gener som kodar för en silverpump och funktionellt relaterade proteiner. Förekomsten av silverresistens hos bakterier och *sil*-genernas betydelse för denna resistens är sparsamt undersökta och vilar mest på fallrapporter.

I avhandlingen undersöktes förekomsten av silverresistens i ett antal bakteriepopulationer från människor, djur och olika miljöer. Vidare undersöktes silvrets roll som potentiell co-selektor för antibiotikaresistens. Speciell vikt lades på *sil*-operonet och dess roll vid silverresistens. Två av arbetena utfördes i samarbete med personal vid Sårcentrum, Akademiska sjukhuset, och vid Barnsjukhuset i Changchun City, Kina.

Resultaten i avhandlingen togs fram med hjälp av såväl gamla, klassiska som nyare molekylärbiologiska tekniker. Odlingsbaserade metoder användes för artidentifikation, resistensbestämning och beskrivning av egenskaper som fitness hos isolaten. En central metod i avhandlingen var resistensbestämning mot silvernitrat med buljonspädning samt ett protokoll för selektion av silverresistens. Molekylärbiologiska metoder omfattade målsekvensbaserad PCR, epidemiologiska typningsmetoder (bl. a. AP-PCR, MLST, integrontypning, CTX-M-typning) samt sekvensering enligt Sanger och helgenomsekvensering med IonTorrent. För att analysera sekvensdata tillämpades en omfattande arsenal bioinformatiska programvaror.

I en klinisk undersökning av sårfloran hos patienter med kroniska bensår som behandlades med silverförband, observerades en silverresistent *Enterobacter cloacae* (MIC-värde > 512 mg/L för silvernitrat) efter enbart tre veckors behandling. Detta blev den första gången en silverresistent bakterie dokumenterades i Norden. I samband med denna studie noterades att silvernitrat hade huvudsakligen en baktericid effekt på medlemmar i familjen

Enterobacteriaceae. Hos stafylokocker och streptokocker var silvrets effekt däremot bakteriostatisk, vilket kan förklara varför viktiga sårpatogener inte eradikerades under den lokala silverbehandlingen. Det noterades även en koppling mellan silverresistens och betalaktamaser av CTX–M-typ, enzymer som bryter ner några av de kliniskt viktigaste grupperna av antibiotika, samt mellan silverresistens och antibiotikakänslighet; vid silvernitratexponering reducerades känsligheten för såväl silver som för karbapenemer och piperacillin/tazobaktam.

I en stamkollektion som gick över artgränser, observerades silverresistens enbart hos isolat från människor men inte från fåglar. Dessutom var *sil*-generna ånyo överrespresenterade i CTX-M-producerande *Escherichia coli,* den tarmbakterie som orsakar flest urinvägsinfektioner. I och med att dessa multiresistenta bakterier är mycket vanliga i Kina, gjorde vi en studie på Changchuns barnsjukhus. Trots att frekvensen CTX-M-producerare låg runt 70 %, registrerades endast ett enda isolat som *sil*-positivt. Således betydligt lägre frekvens än i Sverige.

Vi gick därefter vidare och studerade ett flertal gramnegativa arter isolerade under en 20-års period (1990-2010) från 734 blododlingar. Vi noterade att andelen isolat med genetisk silverresistens var hög hos *Klebsiella*-arter (41 %) och *Enterobacter*-arter (48 %), och den fenotypiska silverresistensen var vanligare hos *Enterobacter cloacae* än resistensen mot kliniskt viktiga antibiotika som aminoglykosider, kinoloner och trimsulfa. Med hjälp av s.k. multilocus sequence typing (MLST) kunde vi påvisa att mer än hälften av isolaten som bar på *sil*-gener tillhörde högriskkloner av global betydelse. Helgenomsekvensering gav belägg för att hypermutabla regioner i den regulatoriska genen *silS* i *sil*-operonet sannolikt spelar en viktig roll vid utvecklingen av resistensfenotypen.

Sammanfattningsvis, avhandlingens resultat tyder på att silver kan bidra direkt och indirekt till antibiotikaresistens.

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