Dissemination of Multiresistant Bacteria

Their Selection, Transmission, Virulence and Resistance

GUSTAF STARLANDER
Abstract

Multiresistant bacteria are an emerging threat in modern medicine. Consumption of antimicrobial agents among humans, animals and in agriculture causes a selection of resistance genes. Dissemination of bacteria carrying resistance genes occurs both globally and locally, and hospital settings pose a special risk for spread when staff, environment and vulnerable patients interact. The overall aim of this thesis was to analyse underlying factors that facilitate the dissemination of multiresistant pathogenic bacteria in hospital settings.

Clusters of resistant bacteria from six occasions were investigated. Vancomycin-Resistant Enterococci (VRE), Methicillin-Resistant Staphylococcus pseudintermedius (MSRP), ESBL-producing Escherichia coli and Klebsiella pneumoniae, and carbapenemase-producing Pseudomonas aeruginosa were analysed by a range of methods, from cultures on broth to PCR and whole genome sequencing. Type of resistance, clonality, virulence factors, mobile genetic elements, epidemiology, survival in the environment, and patient history were examined variously depending on study.

The results showed that VRE resistance genes can be acquired during treatment with vancomycin. Furthermore, contamination of the hospital environment could quickly cause an outbreak, when patients are frequently relocated and exposition to contaminated rooms increase. Resistant bacteria emerging among companion animals, such as the dog-associated MRSP, can pose a zoonotic threat, when a virulent clone finds a new niche in humans.

The ability of Gram-negative bacteria to survive in a hospital environment is probably better than expected, given the right prerequisites; incorrect use of sinks enabled the spread of ESBL-producing K. pneumoniae and carbapenemase-producing P. aeruginosa. Additionally, the survival on hospital associated materials was longer for ESBL-producing E. coli than the AmpC-producing counterpart, which could tell part of why ESBL-producing E. coli is increasing. Hence, the survival in the environment calls for consideration when choosing materials and equipment for hospitals and nursing homes.

Exchange of bacteria occurs continuously between humans and our surroundings. Outbreaks of multiresistant bacteria are rare in Sweden but expose the weaknesses in healthcare when occurring. The organization, materials and equipment of hospitals facilitate the dissemination of resistant bacteria, as does animals and humans around us and even the genes in our own microbiota.

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It is not difficult to make microbes resistant to penicillin

*Sir Alexander Fleming, Nobel Lecture, December 11, 1945.*
List of Papers

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


III Starlander, G, Sütterlin, S., Tellgren-Roth, C., Melhus, Å. The Initial Epidemiology of a Major Clonal Outbreak Caused by VanB-Carrying Enterococcus faecium Clone ST192. Manuscript


V Fraenkel, C.J., Starlander, G., Tano, E., Klintstedt, M., Sütterlin, S., Melhus, Å. The first Swedish outbreak with VIM-2-producing Pseudomonas aeruginosa was prolonged and probably due to contaminated hospital sinks. Manuscript


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Attachment papers published by the author in the field of research cited in this thesis


VIII  Starlander, G., Wirén, M., Melhus, Å. (2011) First documented case of a Staphylococcus lugdunensis strain carrying the mecA gene in Northern Europe. *Infect Ecol Epidemiol*, 1
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<td>Arbitrarily Primed Polymerase Chain Reaction</td>
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<tr>
<td>CC</td>
<td>Clonal Complex</td>
</tr>
<tr>
<td>CoNS</td>
<td>Coagulase-Negative Staphylococci</td>
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<tr>
<td>DNA</td>
<td>DeoxyriboNucleic Acid</td>
</tr>
<tr>
<td>ESBL</td>
<td>Extended-Spectrum β-lactamase</td>
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<tr>
<td>EU</td>
<td>European Union</td>
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<td>EUCAST</td>
<td>European Committee on Antimicrobial Susceptibility Testing</td>
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<tr>
<td>IS</td>
<td>Insertion Sequence</td>
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<tr>
<td>MALDI-TOF</td>
<td>Matrix Assisted Laser Desorption/Ionization – Time of Flight mass spectrometry</td>
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<tr>
<td>MLST</td>
<td>Multi Locus Sequence Typing</td>
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<td>MRSA</td>
<td>Methicillin-Resistant <em>Staphylococcus aureus</em></td>
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<td>MRSP</td>
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<td>PBP</td>
<td>Penicillin-Binding Protein</td>
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<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<td>rep-PCR</td>
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<tr>
<td>RNA</td>
<td>RiboNucleic Acid</td>
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<tr>
<td>SNP</td>
<td>Single Nucleotide Polymorphism</td>
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<tr>
<td>SRGA</td>
<td>Swedish Reference Group for Antibiotics</td>
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<tr>
<td>ST</td>
<td>Sequence Type</td>
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<tr>
<td>Tn</td>
<td>Transposon</td>
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<tr>
<td>US</td>
<td>United States of America</td>
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<tr>
<td>UTI</td>
<td>Urinary Tract Infection</td>
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<tr>
<td>VRE</td>
<td>Vancomycin-Resistant Enterococci</td>
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<td>WGS</td>
<td>Whole Genome Sequencing</td>
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Introduction

Infectious diseases caused by bacteria have successfully been treated with antibacterial agents since the 1940’s, effectively reducing illness and death. Pneumonia and several other community-acquired infections that killed considerable parts of the population just a century ago, can now be treated at any primary care unit. The development of antibiotic drugs is truly a medical marvel that we today consider a matter of course, and we no longer remember when family members and friends were lost due to simple infections.

The development of potent antibiotics has been a prerequisite for the progress of modern advanced healthcare, such as neonatal care, chemotherapy, immunomodulation, organ transplantation, advanced surgery and intensive care. These fantastic drugs have, however, drawbacks. Their wide use in humans, animals and the food industry has successively selected for resistance genes. The resistance to commonly used antibiotics has subsequently transferred between bacteria and been introduced to clinically important species. The ensuing dissemination of multiresistant bacteria has thereby become a major threat to public health.

The American Center for Disease Control (CDC) estimates that approximately 2 million people in the United States (US) are infected with antibiotic resistant bacteria each year, causing approximately 23,000 deaths (1). The corresponding death figure in the European Union (EU) is 33,000 (2), and the extra cost for bacterial resistance in healthcare is approximately €1.5 billion, according to estimations made by the European Medicines Agency (3).

The isolation frequency of resistant bacteria varies widely between both continents and countries. On the Indian subcontinent the majority of the leading bacteria are multiresistant, whereas most bacterial isolates are still susceptible in Northern Europe. Antibiotic resistance is, however, on the rise worldwide, and this development must be checked if we want to keep the healthcare as we know it.

There is a well-established correlation between antibiotic consumption and resistance rates, but factors that affect the dissemination of resistant bacteria are less explored. Outbreaks are the most spectacular situations in which these factors can be studied, but we share and exchange continuously our microbiome with other humans, animals and the environment.
Clinically important bacteria

The cell wall

Based on the structure of the bacterial cell wall and how it stains, most bacteria can be divided into Gram-positives or Gram-negatives. Apart from its diagnostic importance, some key features of the cell wall have clinical implications.

Gram-positive bacteria have a thick, multi-layered, peptidoglycan cell wall. Their cell surface is almost glue-like, and they are more resistant to harsh physical conditions, including drying and osmotic pressure. Cleaning an area that is contaminated by Gram-positive bacteria can therefore be a challenge (4). In general, Gram-positives are relatively susceptible to antibiotics and disinfectants.

Gram-negative bacteria, on the other hand, have only a single layer of peptidoglycan. The other layers have been replaced by an outer membrane and a periplasmic space. Fimbriae plays an important role in the adherence to surfaces. As antibiotic drugs and disinfectants usually must penetrate the outer cell membrane to have an effect, Gram-negative bacteria are less susceptible to many antibacterial substances. On the other hand, the Gram-negative bacteria need humidity to survive in the environment, and they are more reliant on favourable physical conditions to thrive (5).

![Figure 1. Schematic picture of the differences between Gram-positive and Gram-negative bacteria](image-url)
Gram-positive bacteria

Enterococci

Enterococci are facultative anaerobes belonging to the phylum of Firmicutes (Latin “Firm Skin” - due to their thick cell wall). The cell wall gives them a high capacity to survive for long periods in any type of environment. Furthermore, they are intrinsically resistant to most of the commonly used antibiotics, including the cephalosporins (6). Their clinical relevance has increased during the last decades, and this is directly related to their ability to resist antibiotic drugs. Apart from their intrinsic resistance, they can quickly acquire resistance due to a remarkable genome plasticity (7).

Enterococci colonize the human intestinal tract and are associated with urinary tract infections (UTIs), but they can also cause endocarditis, septicaemia and intra-abdominal or wound infections. Patients with suppressed immune systems are most vulnerable, and prolonged hospitalization and previous use of antibiotics increase the risk of infection (8-11).

Two species are involved in most infections: Enterococcus faecalis and Enterococcus faecium. While E. faecalis is the more commonly isolated bacterium, E. faecium stands for the greatest clinical challenge, as it is more resistant to the first line drug ampicillin. The second choice is the expensive vancomycin. It requires an intravenous administration and has a narrow therapeutic range. The medical burden of E. faecium is thereby high.

Staphylococci

Staphylococcus is another genus of the phylum Firmicutes, and they have many features in common with the enterococci. They are facultative anaerobes, and are, depending on their production of the blood-clotting enzyme coagulase, divided into coagulase-positive or coagulase-negative (CoNS) staphylococci. The coagulase-positive Staphylococcus aureus is the clinically most important staphylococcus in human medicine. It is involved in a wide range of infections from simple skin infections to osteomyelitis, respiratory infections, endocarditis, meningitis and septicaemia (5).

CoNS are skin commensals that easily contaminate foreign bodies. They can thereby render problematic infections associated with prosthetic devices and infections in immunocompromised patients.

*Staphylococcus pseudintermedius*

In the late 1970s, it was for the first time suggested that coagulase-positive staphylococci isolated from animals were not necessarily S. aureus. Two new species, Staphylococcus intermedius and Staphylococcus hyicus, were therefore proposed, and since then several coagulase-positive species have been described. In 2005, *Staphylococcus pseudintermedius* was separated from the
S. intermedius group. This new species had been isolated from dogs, horses, cats and pigeons, and could at the time only be differentiated from S. interme-
diarius by genotypic methods (12). Later it was found that most isolates previ-
ously identified as S. intermediarius from dogs were most likely S. pseudinter-
mediarius (13-15).

S. pseudintermedius is a part of the normal skin and oral flora of healthy
dogs and cats. The bacterium can harbour virulence factors that closely resem-
ble those found in S. aureus (16). Thus, it can cause infections in animals with
clinical manifestations similar to those of S. aureus in humans. For example,
exfoliative skin infections such as canine/feline pyoderma, wound infections
and abscesses. Human infections are, however, very rare and in most cases
associated with bite wounds (17, 18).

Gram-negative bacteria

The Enterobacteriaceae family

Members of the Enterobacteriaceae family are rod-shaped, facultative anaer-
obic, sugar fermenting, and most of them reduce nitrate to nitrite. The majority
use flagella for their mobility. Two of the leading pathogens in this family are
Escherichia coli and Klebsiella pneumoniae (5).

Escherichia coli

E. coli is part of the natural flora in the lower intestines of most warm-blooded
organisms. As a commensal it benefits its host by producing vitamin K (19)
and in animal models it has shown to prevent infections by other microorgan-
isms (20). E. coli is; however, a very heterogeneous group of bacteria and four
main phylogenetic groups have been described, A, B1, B2 and D. While
groups A and B1 are most common in the commensal flora, group B2 is the
most virulent and the leading cause of infections (21). Furthermore, some
strains carry genes encoding toxins involved in gastrointestinal infections. E.
coli is currently the most common cause of Gram-negative infections (22) and
a leading cause of UTIs, peritonitis and sepsis. E. coli use mainly the faecal-
oral route for transmission, and it is a well-documented indicator of faecal
contamination in the environment (23).

Klebsiella pneumoniae

K. pneumoniae is too part of the human intestinal flora but can also be found
in the mouth and nasal cavity (5). It is an opportunistic pathogen that may after
aspiration from the oropharyngeal tract cause a severe form of pneumonia,
hence its name. Patients with compromised immune systems, e.g. diabetes,
chronic obstructive lung disease, alcoholism, liver and kidney failure, are es-
pecially at risk (5, 24, 25). This bacterium is often involved in different types
of healthcare-associated infections and is thereby a major problem in intensive care units, including neonatal units (26). Antibiotic therapy and invasive devices such as respiratory tubes and urinary catheters increase the risk of nosocomial *K. pneumoniae* infections.

*K. pneumoniae* can quickly acquire resistance genes and many clinically encountered strains are multiresistant (usually defined as resistance to three or more classes of antibiotics), some even to carbapenems (25).

**Pseudomonas aeruginosa**

*P. aeruginosa* is a rod-shaped, facultative anaerobic bacterium. It has an astonishing ability to colonize a vast range of environments, especially in moisture. It can cause folliculitis, lens associated keratitis and otitis externa among healthy individuals, but compared to Enterobacteriaceae, it is rarely a part of the commensal flora. On the other hand, it is a major problem as an opportunistic bacterium among immunocompromised patients. *P. aeruginosa* is among the leading causes of nosocomial infections, particularly in patients with burn-wounds, cystic fibrosis, neutropenia and patients in need of intensive care (27-29). Although less virulent than other major pathogens, it can colonize lungs, urinary tracts and soft tissues and endure through bio-film production.

Furthermore, it can spread through medical equipment, including sinks and invasive devices and hence cause hospital outbreaks (28, 30). It is a model organism in using several different resistance mechanisms, such as increased efflux pumps, decreased porin permeability and utilization of β-lactamases (both acquired and intrinsic), combined (29, 31-34). It has an astounding ability to adapt to the environment and quickly develop resistance to multiple antibiotics, often leaving carbapenems as first line treatment. Pandrug-resistant isolates are now found, leaving clinicians at a dead end in treatment options (35).

**Bacterial genomics**

**Genetic diversity in bacteria**

Bacteria are prokaryotic organisms and multiply by binary fission. Each generation is thereby genetically identical to their “parent”. However, like all organisms, bacteria may mutate during replication. These mutations result in a genetic diversity that can be beneficial or detrimental for the bacterium (36). In addition to sporadic mutations, some bacteria can increase their genetic diversity by three forms of horizontal gene transfer (between the same generation):
1. **Transduction:** The process when a bacteriophage (a virus that infects bacteria) brings genetic material from one bacterium to another.

2. **Transformation:** The process when a bacterium bind, take up and integrate foreign DNA through homologous recombination. As opposed to transduction, which is thought to be accidental, transformation is facilitated by recombination genes in the bacterial genome to increase bacterial genetic diversity.

3. **Conjugation:** The transfer of a genetic information (essentially a plasmid) from one cell to another, through direct mechanical contact between bacterial cells.

**Mobile genetic elements**

**Plasmids**

Unlike eukaryotic cells, bacteria lack a nucleus and a nuclear membrane. Instead, their single chromosome float around in the cytosol. In addition to the chromosome, some bacteria have one or more plasmids, i.e. extra-chromosomal genetic elements with independent replication systems.

Plasmids rely on both self-encoded and host-encoded genes. Since they need the bacterial host for encapsulation and protein production, they cannot be considered independent life. The replicons, or the genes responsible for encoding the replisome (the molecular machinery that replicates the DNA of the plasmid), can be identified with Polymerase chain reaction (PCR) (37).

Plasmids can be conjugative or non-conjugative. Conjugative plasmids can be transferred from one bacterial cell, which has the plasmid, to another bacterium which has not, by conjugation. Conjugation occurs when a donor cell attaches to a recipient cell by “sex-pili”, encoded by the plasmid. The pili are rod-shaped polymeric proteins that when connected to a recipient bacterial cell creates a channel through which a copy of the DNA of the plasmid is transferred and replicated (38).
Certain plasmids only exist in closely related bacteria, so-called narrow host-range plasmids. Broad host-range plasmids can, in contrast, be transferred between a wide range of bacteria. Plasmids can also be classified into incompatibility groups according to their ability to co-exist in the same bacterial cell (39). Similar replicon types exclude mutual existence, as this would lead to uncontrolled gene expression.

Plasmids do not contain genes essential for basic functions of the hosting bacterium, but they can contain resistance or virulence genes that give the bacterium advantages. Genetic information can be added to or subtracted from the plasmid by transposons and integrons. Consequently, plasmids can accumulate a large number of resistance genes that can be transferred to another bacterium. A bacterium never earlier exposed to antibiotic drugs can thereby harbour several resistance genes in a plasmid. To avoid selection of this bacterium is quite difficult.

The origin of resistance genes can often be found in non-pathogenic bacteria in the environment. During selective pressure, the genes are mobilized to a plasmid. The plasmid is thereafter transferred to a pathogen, and with a transposon the gene can be moved to another plasmid or to the chromosome.
Transposons

Transposons (Tns) are genetic elements that can move from one location within the DNA to another. The simplest version is an insertions sequence (IS) based on a central region encoding a transposase enzyme, flanked by two inverted repeats. The transposase enzyme works as a “cut-and-paste” enzyme and can insert the IS into a new region of the genome. In addition to the transposase enzyme, larger Tns can have additional genes included in the Tn and these genes can, together with the Tn, be moved to other places in the genome or to a plasmid. Tns can also encode conjugative enzymes that allow them to transfer between bacteria, sometimes in response to external antimicrobial stress (40).

Integrons

Integrons are genetic assembly platforms that contains a site-specific recombination system able to exchange, integrate and express specific DNA elements called a gene cassette. The integron is not movable by itself, but it can accumulate genes in its cassette. It can be situated on the chromosome, on plasmids or be linked to IS and Tns. It is based on three genes, the intI gene that encodes an integrase needed for site-specific recombination within the integron, a recombination site (attI) that is recognized by the integrase and the promoter (Pc) for transcription and expression of gene cassettes present in the integron (41).

Virulence factors

Virulence (Latin for poisonous) is the ability to cause disease in an organism. Virulence factors are the molecules that stand for the pathogenicity. Virulence factors can both influence the function of the host cell directly or allow the bacterium to thrive in a new environment. Typical virulence factors are molecules that enable the bacterium to attach to and invade host cells (e.g. adhesins and invasins), evade or suppress the immune system of the host, obtain nutrition (e.g. hemolysins and proteases), and influence the function of the host cell (toxins) (42). Some virulence factors are intrinsic and essential for the bacterium, such as the endotoxins and capsules. They are therefore chro-
mosomally encoded. Other virulence factors are acquired through transduction and transformation or from mobile elements. In the latter case it is possible that genes encoding virulence and resistance are co-selected.

Antibiotic drugs

Global antibiotic consumption

During the first decade of the new millennium, the global consumption of antibiotics increased from 50 billion standard units (SUs) to 72 billion SUs annually. That is an increase of 35%, and it is expected to rise even more. India, China and the US, in that order, had the highest consumption, and the increase in percentage was highest in the BRICS-countries (Brazil, Russia, India, China and South Africa) (43).

Broad-spectrum β-lactams accounted for 55% of all sales (43). In countries where antibiotic prescriptions are monitored, there is a clear correlation between drug daily dose (DDD), use and misuse of antibiotics, and resistance rates. Approximately 65,000 tons of antibiotics were used in food-animal production during 2010 (44). Since the meat consumption is expected to rise, so is the antibiotic consumption. Approximately 80% of all antibiotics in the US are consumed in the food-animal production (44). Animals and humans share many pathogenic bacteria, and both strains and resistance genes can be transferred between species.

β-lactams

The β-lactams is a large class of antibiotics and the most common type of antibiotic prescribed worldwide (43, 45). They all contain a β-lactam ring and depending on the side groups and acyl side-chains attached to the β-lactam core, they are grouped into penicillins, cephalosporins, monobactams and carbapenems.

![Figure 4. The β-lactam ring.](image)
The mode of action is to inhibit peptidoglycan cell wall synthesis, which is essential for bacterial growth. The final step of the peptidoglycan cell wall synthesis is facilitated by a transpeptidase called Penicillin-Binding-Protein (PBP). It crosslinks the terminal amino acid residues of the peptidoglycan precursors (NAM/NAG-peptides). β-lactams are structurally similar to the precursor and irreversibly bind to the active site of PBPs and prevents this final step. The accumulation of peptidoglycan precursors triggers autolytic hydrolases, already existing peptidoglycan is degraded and the bacterial cell undergoes lysis (46). Bacteria have slightly varying PBPs and different β-lactams have different affinity for certain PBPs.

β-lactams are indispensable in clinical practice; they are bacteriocidal (kill bacteria), have a low toxicity and low rates of adverse reactions, and are of the narrow- to the broad-spectrum type. They can be administered orally or intravenously and are widely distributed in body fluids, penetrating most extracellular compartments. β-lactams antibiotics are the first line choice when treating acute infections such as meningitis, sepsis, pneumonia, pyelonephritis, septic arthritis, extra-intestinal infections and skin- and soft tissue infections (46).

Penicillins

Sir Alexander Fleming (1881-1955) discovered in 1928 that a Penicillium mold had antimicrobial properties against staphylococci (47, 48). This discovery was successively refined in the 1940s to what came to be mass-produced penicillin, the first β-lactam to be used in clinical practice. Penicillins are usually divided into four groups: 1) Narrow-spectrum natural penicillins (e.g. penicillin V and penicillin G); 2) Narrow-spectrum penicillinase stable penicillins (e.g. flucloxacillin and cloxacillin); 3) Moderate-spectrum aminopenicillins (e.g. amoxicillin and ampicillin); and 4) Extended-spectrum penicillins (e.g. piperacillin). Just as aminopenicillins, piperacillin has a wider spectrum with the ability to penetrate the porins of Gram-negatives as well as Gram-positives but are susceptible to β-lactamases. Piperacillin is therefore only used in combination with the β-lactamase inhibitor tazobactam.

Cephalosporins

The cephalosporins are semi-synthetic β-lactams derived from the fungus Acremonium (previously known as Cephalosporium). Numerous cephalosporin derivatives have been developed and are grouped into five generations. These generations reflect their chronological order and to some extent the antimicrobial spectra, with a more Gram-negative spectrum in later generations (46, 49). Third generation cephalosporins (e.g. cefotaxime, ceftazidime and ceftriaxone) have activity against several members of the Enterobacteriaceae family and other clinically important bacteria. Ceftazidime is the drug with the most Gram-negative spectrum, including Pseudomonas aeruginosa. They are more resistant to β-lactamases than the previous generations and are often
the first choice when treating UTI, severe pneumonia, meningitis and septicemia.

**Monobactams**
Aztreonam is the only available monobactam and consists of a β-lactam ring alone. It is active against Gram-negative bacteria and seldom used.

**Carbapenems**
The carbapenems (e.g. imipenem/cilastatin, meropenem, ertapenem) were developed in the late 1980s, and have the broadest spectrum of all antibiotic drugs for systemic use in humans. Their activity includes both Gram-positive and Gram-negative aerobic and anaerobic bacteria. Their extremely broad spectrum is attributed to their high affinity to various PBPs, and they are stable against virtually all β-lactamases, but the carbapenemases. They are used to treat patients with septic shock, intra-abdominal infections, nosocomial infections, meningitis and infections caused by multiresistant bacteria (50).

**Glycopeptides**

**Vancomycin**
Vancomycin was isolated from the fungus *Streptomyces orientalis* found in soil samples from the jungles of Borneo, in 1957. It has activity against Gram-positive cocci and bacilli, and it is the first line treatment of MRSA and ampicillin-resistant *E. faecium*. It is not bacteriocidal against enterococci and needs to be combined with an aminoglycoside to reach bacteriocidal properties when treating patients with septicemia or endocarditis.

The mode of action of vancomycin is through inhibition of peptidoglycan cell wall synthesis. In comparison to β-lactams, which inhibits the enzyme in peptidoglycan synthesis, glycopeptides bind the D-ala-D-ala part of the NAM/NAG-peptides that are synthesised as subunits to peptidoglycan. By doing this, it prevents PBP from incorporating the subunits into new peptidoglycan, causing cell death.

Vancomycin is not absorbed orally (a feature that is used when treating *Clostridium difficile* colitis) and needs to be administered intravenously. Rapid infusions can cause side effects and the drug has both nephrotoxic and ototoxic effects. Serum levels need therefore to be closely monitored (51).
Resistance mechanisms

β-lactamases

There are several mechanisms that confer resistance to β-lactams. These include alterations in PBP s, loss of porins that enable the drugs to reach the targets and alterations in efflux pumps. However, the most important cause of resistance is the production of β-lactam hydrolysing enzymes, β-lactamases. Many Gram-negative bacteria possess chromosomally encoded β-lactamases, probably to compete within a niche with organisms producing β-lactams.

The first β-lactamase was identified in *E. coli* as early as in 1940 (52), and the first plasmid-mediated β-lactamase, TEM-1 was described in 1965 (53). Some years later, a second type of plasmid-mediated β-lactamase, SHV-1, was identified in *E. coli* and *K. pneumoniae* (54). Numerous β-lactamases have since then been documented. They exhibit a vast variety and differ in molecular characteristics, substrate choices, inhibition profiles, how they are expressed, etc. β-lactamases are commonly categorized according to Ambler class (55) depending on protein homology or according to Bush-Jacoby-
Medeieros functional group (56) depending on their substrate and inhibition profile.

**Extended-spectrum β-lactamases**

The increasing prevalence of TEM-1 and SHV-1 among clinically important bacteria led to the introduction of the more stable third-generation cephalosporins in the beginning of the 1980s. In 1983 came the first report of a plasmid encoded β-lactamase capable of hydrolysing the third generation cephalosporins (57). This enzyme came to be known as an extended spectrum β-lactamase (ESBL).

Initially the ESBL definition included only the clavulanic acid inhibited, molecular class A, functional class 2be β-lactamases with the capacity to hydrolyse third-generation cephalosporins, penicillins and monobactam at a rate of minimum 10% of that for penicillin G (58). This definition has since then been widened. In 2008, a new definition of ESBL was proposed to suit clinical practice. It included all plasmid-mediated β-lactamases that had the capacity to hydrolyse third generation cephalosporins and/or carbapenems (59). This definition is currently used in the Scandinavian countries, but it has not reached a global acceptance.

The CTX-M pandemic

During the 1990s, TEM and SHV type ESBLs were predominant worldwide. These enzymes were to a high degree associated with epidemic clones and *K. pneumoniae* was the main carrier. In 1989, a new β-lactamase with hydrolytic activity against cefotaxime was described in Germany (60). It was named CTX-M-1 (Cefotaxime-Munich). Ever since, the number of CTX-M β-lactamases has rapidly increased, as well as the organisms producing them.

Due to their close nucleotide homology, it has been suggested that the origin of the CTX-M enzymes can be found among chromosomal β-lactamases in *Kluyvera spp*. Different mobile elements have probably assisted the mobilization of the CTX-M genes to plasmids. In contrast to TEM and SHV type enzymes, which are carried by a few plasmids of different incompatibility groups, the CTX-M genes are carried by a vast number of plasmids, including both narrow-host and wide-host range plasmids. This fact has probably been important for the extensive dissemination of CTX-M type enzymes between bacterial clones and species (61-63). CTX-M-15 has become the predominant type in most countries, while CTX-M-14 is frequently isolated in countries in South-East Asia and Spain (64).

Since the turn of the century, CTX-M has been the most common ESBL type globally in both hospitals and other settings. Moreover, the faecal carriage has increased in the community (61), and bacteria carrying these enzymes have been found in farm animals, pets and sewage systems.
The E. coli clone O25:H4-ST131

More than 20% of all infections caused by E. coli producing CTX-M has the sequence type (ST) 131 (65). This clone belongs to serogroup O25:H4, which in turn belongs to the highly virulent phylogenetic group B2. Retrospective studies have found that the ST131 clone has been responsible for a large part of the CTX-M-15 pandemic (66, 67). The “success” of the ST131 clone is thought to be the result of selective advantages in the form of enhanced virulence and multi-drug resistance, due to a relatively recent acquisition of plasmids carrying mainly the CTX-M-15 gene (63). To identify this clone, multi-locus sequence typing (MLST) has been used. It is relatively expensive and time-consuming, why rapid substitute methods have been developed. These methods include PCR for the pabB allele (68) or ST131-associated single nucleotide polymorphisms in specific genes (69).

AmpC β-lactamases

E. coli hydrolysing β-lactams was reported in 1940 (52), and the AmpC sequence from E. coli was published in 1981 (70). AmpC occurs naturally in the Enterobacteriaceae family and in some other bacterial species such as P. aeruginosa. The genes are carried chromosomally, and β-lactam resistance is due to mutations causing overexpression of the enzyme, either by hyperinduction or by constitutive hyperproduction, depending on species (71). In E. coli the reason for the resistance is hyperproduction (72).

AmpC type enzymes hydrolyse all β-lactams, but with a preference for cephalosporins above penicillins. The affinity and hydrolyzation rate for carbapenems and fourth generation cephalosporins is very low (73), and the inhibitory effect of clavulanic acid or tazobactam is modest (74). In addition to AmpC, some bacterial isolates may exhibit porin loss and/or increased efflux pump activity. The combination can decrease the concentration of β-lactams in the periplasm and cause carbapenem resistance (75). Yet, carbapenems are the first-line choice when treating infections cause by AmpC-producing organisms. The main advantage of fourth generation cephalosporins against AmpC-producing species is the increased permeability into the periplasm.

Plasmid-mediated AmpC type enzymes were first observed in 1989 (76). Since then more than 180 types have been found. The CMY-2 type is the most common. Its prevalence has been somewhat higher in North America and Southeast Asia than in Europe (71), but it has never reached the magnitude of CTX-M type enzymes. The mobilization of AmpC-genes to plasmids is similar to that of CTX-M and can include new promotor regions causing overexpression (77). It is not possible to distinguish between chromosomal or plasmid-mediated AmpC phenotypically.
Carbapenem resistance in *P. aeruginosa*

The low permeability of the outer cell membrane of *P. aeruginosa* causes natural resistance to many antibiotics and carbapenems are dependent on the passage through the OprD porin. Low-level carbapenem resistance is often due to downregulation, mutation or loss of the OprD porins alone or in combination with AmpC production (29, 31, 78). Furthermore, efflux pumps such as the MexAB-OprM, MexCD-Opr, MexEF-OprN and MexXY-OprM confers resistance to many classes of antibiotics, including medium resistance to carbapenems, when upregulation is induced or mutations result in overexpression (29, 31, 78, 79).

**Carbapenemases**

Acquired resistance to carbapenems is caused by acquisition of carbapenemases, yielding high level resistance. Large varieties of carbapenemases have been identified, whereof the Metallo β-lactamases (MBL) are dominant among *P. aeruginosa*. As MBLs have zinc in their active site for hydrolyzation, chelators such as edetic acid (EDTA), dipicolinic acid (DPA) and mercaptopropionic acid (MPA) are used for detection. MBLs confer resistance to all β-lactams, except monobactams, and are slightly inhibited by clavulanate and tazobactam. Continuously expanding, so far, ten groups of MBL have been reported in *P. aeruginosa* (80-82). These enzymes include multiple allotypes and have various characteristics. Most are sporadic, but the IMP, VIM, GIM, NDM and SPM are reported continuously, of which VIM-2 is dominating worldwide (83).

VIM-2 was initially found in a *P. aeruginosa* isolate in France in 1996 (84). Subsequently, isolates of *P. aeruginosa* carrying the VIM-2 gene have been reported from many continents (83). Nosocomial outbreaks of *P. aeruginosa* carrying MBL genes are also dominated by the VIM-2 gene. (83, 85-92). Two high risk clones account for part of the dissemination, the ST 111 and ST235 (79, 93, 94). Most VIM-2 are located in class 1 integrons, often in combination with other resistance genes usually against aminoglycosides (92-95). Although integrons are not transferable themselves, they are linked to transposons (96) that facilitate the possible mobilization between plasmids and the chromosome (97, 98).

**Vancomycin-resistance in enterococci**

*E. faecium* isolates resistant to vancomycin were first reported from England and France in 1986 (99, 100). Later on, an increasing isolation frequency of Vancomycin-Resistant Enterococci (VRE) was seen in Europe, mainly in the community setting. This was likely due to transmission from animal strains, thought to have arisen from the use of avoparcin (a related glycopeptide) in
livestock (101). Conversely, in the US, VRE was isolated mainly in the hospital setting, thought to be due to widespread use of vancomycin in clinics (102). Since the emergence, VRE has now spread world-wide (103-106) and poses a major problem especially in hospitals.

The resistance to vancomycin in Enterococci is due to the acquisition of a Van operon (a set of genes with a combined function). The Van operon expresses a ligase that produce an alternative peptidoglycan precursor that vancomycin cannot bind. The alternative peptidoglycan precursor has the C-terminal of the D-Ala-D-Ala precursors exchanged for D-Serine or D-Lactate, yielding low affinity to vancomycin. Furthermore, the operon expresses other enzymes that eliminate the original precursors, thus completely removing the target for vancomycin.

Eight types of acquired vancomycin resistance in enterococci have been detected (VanA, VanB, VanD, VanE, VanG, VanL-VanN) and one type of intrinsic resistance (VanC) (107-109). These are divided by the sequence of the resistance ligase. VanA is responsible for the majority of VRE cases in humans globally and is best characterized. It is found on the transposon Tn1546 and is induced by the presence of vancomycin through a sensory-regulatory system. However, VanB is on the rise with increasing incidence in Europe (110-112) found on Tn1549/Tn5382.

VanD-type glycopeptide resistance was first reported in 1997 (113). It differs from the more common VanA and VanB-types by being constitutively expressed and located in the chromosome, instead of on a mobile element and therefore supposedly not transferrable by conjugation (114). Furthermore, the VanD-carrying bacteria also contain mutations in genome, eliminating the normal high-affinity pathway without obvious effect of the extra operon-included enzymes (114, 115). Ruminococcus spp. have been suggested to be a reservoir of VanD-genes in the intestinal flora (116).

Since the first report, VanD-type carrying E. faecium has only been isolated in a few samples in USA, Canada, Brazil and the first report from Europe was in 2005, in France (113, 117-120). One previous sample was isolated in Sweden in 2007 (121).

Methicillin-resistance in staphylococci

Methicillin was introduced in 1960 to take care of β-lactamase producing S. aureus. After just a year, resistance to this β-lactamase-stable penicillin was noticed (122). Methicillin-resistance in staphylococci is characterized by the acquisition of the mecA-gene (Attachment paper VIII), which encodes for an alternative PBP called PBP2a. PBP2a causes resistance to all β-lactams but ceftobiprole and ceftaroline, since it has a low affinity for β-lactams. The peptidoglycan production thereby continuous even in the presence of antibiotics that inhibit the original PBP. A homologous mec-gene, mecC, was recently discovered (123).
The mecA-gene is carried on a mobile genetic element called staphylococcal cassette chromosome mec (SCCmec). The mec-gene is located in a structure called a mec-complex, consisting of an IS (IS431), the mec-gene itself and regulatory mechanisms. Furthermore, additional structures may be included, conveying resistance to other classes of antibiotics. The SCCmec also carries cassette chromosome recombinases (ccr), which can excise and integrate the SCCmec into the staphylococcal chromosome (124).

SCCmec are classified by the combination of mec-complex and ccr and the combinations are traditionally designated roman numerals, but a new nomenclature is suggested (125). Characterization of SCCmec provides information about the evolutionary origin and spread of resistant staphylococci.

Risk factors for acquiring resistant bacteria

General risk factors for acquiring multiresistant bacteria are (8-11, 126-130):
- underlying medical conditions (such as diabetes, malignancies and organ failure)
- prior exposure to antibiotics (especially cephalosporins and quinolones)
- invasive medical devices (such as ventilation and catheters)
- length of stay
- patient characteristics (e.g. age and sex)

Certain risk factors are more associated to specific species. Staying at intensive care units is a typical risk factor for acquisition of multiresistant P. aeruginosa and, to some extent, K. pneumoniae. Lack of isolation and hospitalization in the same units as another VRE carriers is a risk factor (11) for colonization. The risk of becoming colonized with ESBL-producers during a travel outside Scandinavia appears to be much higher (%) than during a prolonged hospital stay (%) (131). The countries with the highest risks are India, other parts of Asia and the Middle East, in that order (131). Other factors that increase the risks during travels are traveller’s diarrhoea and use of antibiotics (131, 132). Prolonged carriage of ESBL-producing E. coli is associated with certain phylogroups (133).

People in close contact with animals are at risk of being colonized by animal-associated staphylococci (134). Pet owners and veterinary staff are at risk of being colonized by MRSP (13, 135), especially if the animals are sick (136). But infections are rare and colonization is transient (135).
Carriage of multiresistant bacteria in humans

The prevalence of human carriers of multiresistant bacteria is not known; healthy people are very seldom screened for carriage, and the sensitivity of faecal cultures is not always optimal. The figures we have are usually biased, since most cultures are obtained either from people displaying symptoms of infection or from patients taking part in screening programs in hospitals. They are all more likely to be carriers than the average population. Additionally, coherent numbers regarding isolation frequency of resistant bacteria is lacking from many parts of the world and knowledge is mainly based on isolated reports.

The European Centre for Disease Control and Prevention (ECDC) collects data from all European countries, regarding resistance rates of invasive clinical isolates. In general, countries in Northern Europe have lower isolation frequency of resistant bacteria than the southern and eastern countries. In Sweden 2015: 6.4% of *E. coli* produced ESBL (30-38.5% in Slovakia, Italy and Cyprus), and 2.5% were also resistant to aminoglycosides and quinolones. 3.4% of *K. pneumoniae* produced ESBL (France 26%, Italy 34%, Greece 51%, Slovakia 66.5). Less than 0.1% of Enterococci were VRE (Greece 20%, Romania 25% and Ireland 46%). The isolation frequency of *P. aeruginosa* resistant to carbapenems were slightly higher in Sweden with 8.5%, but far from the rates in Romania 68.5%, Slovakia 58.4% and Greece 43.9% (137).

In the Asian countries, the resistance rates vary too, but generally at a higher level. Among Chinese isolates from intra-abdominal infections during the period 2002-2013, 66.7% of *E. coli* isolates were ESBL-positive and 39%
of the *K. pneumoniae* isolates. In Thailand the numbers were 50% and 37%, respectively, and in Vietnam 48% and 30% (138). Among isolates causing UTIs the numbers were on the same level or even slightly higher (139), indicating a high carriage rate in the general population in these countries.

India has probably among the highest isolation frequencies of multiresistant bacteria in the world. Up to 80% of the clinical *E. coli* and *K. pneumoniae* isolates are ESBL-positive. Furthermore, carbapenem resistance is found among 5-11.5% of the *E. coli* isolates and 9-57% of the *K. pneumoniae* isolates (140-142). In a study from 2016, the faecal carriage of ESBL-producing *Enterobacteriaceae* was 63% among hospitalized patients and 34% in a healthy group (143). The estimated prevalence of faecal carriage of ESBL-producing *Enterobacteriaceae* in South East Asia by WHO in 2010 was 1.1 billion people. The corresponding figures are 280 million people in the Western Pacific region, 180 million people in the Eastern Mediterranean region, 110 million people in Africa, 48 million people in America and 35 million people in Europe (144).

**Dissemination of bacteria**

Multiresistant bacteria disseminate both locally and globally. Resistant bacteria spread both between people and between people and animals, as well as to and from the environment around us. High population density, poverty, and insufficient sanitation seem to facilitate the dissemination of multiresistant strains in the community. Healthcare-associated strains are found among most multiresistant bacteria, indicating that certain strains have found a niche in hospitals and nursing homes.

ESBL-producers can be found in both wastewater and fresh water all over the globe (145), indicating a massive genetic reservoir. As with any faecal-oral transmitted disease, the role of water pollution cannot be ignored. Furthermore, there is now evidence that the food industry can play a role in the dissemination of ESBL-producing bacteria (146, 147). The use of antibiotics in animal farming has resulted in resistant strains. These strains have been transmitted from animals to humans and their resistance genes to human strains (148). Apart from the transmission from food animals, we appear to share bacteria with our pets and family members (149-152). Even though little research has been done, companion animals (cats and dogs) from both Europe, America, Africa and Asia have shown to be faecal carriers of ESBL-producing *Enterobacteriaceae*, mainly of CTX-M type at rates from 0-22% (153) and examples of infections transmitted between species have been shown (154, 155).

Staphylococci have shown a much higher rate of interspecies transmission and some genetic lineages of MRSA seem to be able to adapt to hosts of different species (155). The isolation frequency of the pig associated Clonal Complex (CC) 398 of MRSA has during the first decade after the first reports
in 2005 (156, 157) almost four-folded in livestock. At the same time, the clinical and screening isolates in humans have increased. From initially being isolated only in people directly exposed to the livestock, it is now also isolated from non-exposed people. This indicates that the increasing animal reservoir has a spill over to the public (158). An increasing isolation frequency of MRSP has been noted among companion animals in both the US and in Europe (159, 160). In recent reports, the isolation frequency in clinical samples has been ranging from 9% up to 47% and stepwise accumulation of resistance mechanisms has facilitated the rapid dissemination (160, 161). Although MRSP colonization among pet owners is uncommon (135) and S. pseudintermedius is usually associated with bite-wounds (17), the isolation frequency in humans could be underestimated due to the close resemblance of the bacterium to S. aureus (18). Furthermore, MRSP can be found in the environment around colonized animals (162).

Figure 7. Possible routes of dissemination (144) Copyright © American Society for Microbiology. Reproduced with permission.
Among animals, VRE is isolated mainly in poultry and swine (163-165) and in the environment around them (166, 167). Certain CCs of VRE are isolated in infections from both in pigs and humans. These clones harbour the VanA-genes on similar plasmids and integrons indicating a possible transfer of genes between hosts (168). Furthermore, as Enterococci survive harsh environments, healthcare associated strains and VRE are found in large parts of the sewage systems (166), the soil of the city (169) and in fresh water (170).

On the contrary, multiresistant strains of \textit{P. aeruginosa} has not been widely associated to food productions, animals or farming, mainly since the bacterium is not commonly carried by healthy individuals (171), including animals. Resistant strains are still linked to hospital settings and acquisition occurs during admittance, but just as ESBL-producing Enterobacteriaceae and VRE, multiresistant strains of \textit{P. aeruginosa} is found in the waste water systems of hospitals (172).

**Outbreaks in hospital settings**

An outbreak has the following definition according to the WHO:

“A disease outbreak is the occurrence of cases of disease in excess of what would normally be expected in a defined community, geographical area or season. An outbreak may occur in a restricted geographical area or may extend over several countries. It may last for a few days or weeks, or for several years.”

In a hospital setting, transmission of bacteria can take place between patients, their visitors, the environment and the healthcare workers. Several outbreaks have been published (173), and intensive care units (ICU) with vulnerable patients and a large number of risk factors are often in focus. Transmissions can, however, occur in any unit, especially if basic hygiene rules are not applied. The fact that the hands of hospital staff can be contaminated by patient bacteria has been long known (174). Objects in a hospital environment, such as beds, floors, furniture, and medical devices are continuously contaminated by patients and their visitors. VRE-carrying patients are known to contaminate the hospital environment (175), and patients staying in an ICU room previously occupied by a patient with VRE or MRSA have a significantly higher risk of acquiring these bacteria, with an increased odds ratio of 1.4 (53, 176).
Figure 8. Modes of transmission of bacteria within healthcare settings.

The prerequisites for an outbreak are met when:

- There is a source of resistant bacteria – e.g. a colonized index patient (177, 178) or contaminated object (30, 179).
- The source is brought into an environment where a group of people with mentioned risk factors are assembled – e.g. a hospital or nursing home (180).
- There are means of transferring the bacteria – e.g. a lack of isolation or hygiene routines among healthcare workers, mobile patients, visitors and inappropriate cleaning measures (181).

Baseline endemic isolation frequency of various bacterial infections exist at most hospitals and outbreaks are suspected when a deviation is observed. The awareness of clinical or laboratory staff is often crucial to bring attention to a statistical deviation from the expected isolation frequency. With unique cases, such as the VanD-case (Paper I), only a single isolate is needed since none is expected, while more common pathogens, such as ESBL-producing *Enterobacteriaceae*, need a higher isolation frequency before a deviation is seen.

According to the Swedish Disease Act (2004:168) some infections with resistant bacteria are notifiable diseases, for example MRSA, VRE and ESBL-producing *Enterobacteriaceae*. In addition, MRSA, VRE and ESBL{\textsubscript{CARBA}} require tracing. Every region or county is required to have a Medical Officer responsible for reporting and tracing a suspected outbreak.

An outbreak investigation aims to remove the source and reveal the mode of transmission for the dissemination. Characterization of the suspected out-
break organism, the patients involved, places where they have been, procedures, devices used, and known risk factors are evaluated. The mode of spread varies between species and the investigation is adapted to the outbreak organism, hence knowledge of the ecology of different species is essential.

There is a clear interaction between hospitalized patients, visitors, the environment and healthcare workers. Colonized patients are an obvious reservoir, but sources in the hospital environment need to be considered when trying to end hospital associated outbreaks. Healthcare workers who are colonized play a crucial role in transmission of bacteria between patients (151). In a meta-analysis it was shown that compliance to antibiotic guidelines and hand hygiene rules among healthcare workers had the largest impact on solving outbreaks (126).

The aim of infection control measurements is to stop and prevent further spread. This includes enforcement of hygiene measurements (especially hand hygiene), active screening, contact precautions, isolation, antibiotic stewardship and environmental cleaning. However, the evidence for these measurements are moderate to weak or even insufficient (182). Most intervention programs involve many strategies at once, they are performed after an outbreak has occurred, often in a rush due to the need of urgent control. Consequently, there are few if any prospective, controlled randomized trials that can conclude which type of intervention that is effective (183).

Laboratory methods

Species identification

Species are identified at clinical laboratories according to set-up standards. Traditionally this includes Gram-staining, phenotypic characteristics and different types of biochemical reactions. When the conventional methods do not suffice, 16S rDNA sequencing is an option. The 70S ribosome of prokaryotic organisms is made up of two subunits, 50S and 30S. The smaller subunit consists of 16S rRNA, which is highly specific among species. By sequencing the amplified variable part of the 16S rDNA and compare it with a database, bacteria can be identified (184).

However, today most Swedish clinical laboratories use matrix assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF) to quickly identify bacterial species. Samples of bacteria from a colony are smeared on a target plate and a “matrix” of chemicals is added. The sample is put into a chamber and the “matrix” prevents decomposition of the sample and enables the laser to hit the sample, heat it and desorb it into ions. The ions go through a “flight tube” were light ions get through faster than heavier. A pattern of ions can be read and plotted in a mass spectrometer and the result is compared to a database (185).
Antibiotic susceptibility

The gold standard for antibiotic susceptibility is agar and broth dilution series to determine the minimum inhibitory concentration (MIC) of an antibiotic agent. The methods are time-consuming and usually only used by researchers. In recent years, commercial kits for common species and antibiotics have become available. Isolates can also be sent to the European Reference Laboratory for Antibiotic Testing in Växjö, Sweden.

In Sweden, disc diffusion is the standard method for antimicrobial susceptibility testing. Discs infused with antibiotic agents are applied to agar plates inoculated with bacterial suspension. After incubation, the inhibition zones are measured, and the isolate is classified as susceptible, intermediate or resistant according to the breakpoints recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST). Previously the Swedish standards were based on recommendations from the Swedish Reference Group for Antibiotics (SRGA), but now the reference groups in the EU are harmonized.

The E-test is a similar method, but instead of discs, a strip with a gradient of the antibiotic agent of interest is applied to the agar plate. After incubation, the MIC of the tested antibiotic can be determined, and it corresponds to the concentration at which the bacteria do not grow.

Polymerase chain reaction (PCR) and sequencing

PCR

PCR is a standard method in molecular biology to amplify DNA. It is based on a template consisting of DNA, two primers (sense and anti-sense) with a short DNA sequence complimentary to the DNA-target site, deoxynucleotide triphosphates (dNTPs, the building blocks of new DNA) and Taq-polymerase (an enzyme that can polymerize new DNA, which is heat stable and isolated from a thermophilic bacterium) mixed in a buffer solution. The reaction mix is thereafter heated to separate the DNA strands, cooled to allow the primers to anneal and then heated again to a temperature which allows the Taq-polymerase to elongate the primer by adding dNTPs and producing new DNA strands that are copies of the target DNA. By repeating these cycles, the original target-DNA is amplified exponentially. The result can be visualized by adding DNA-binding chemicals that fluorescence under UV-light after separation of the PCR-products in a gel electrophoresis.

Real-time (RT)-PCR is a further development of the amplicon detection step, using probes that fluorescence when the target DNA segment is amplified. Furthermore, as the fluorescence is proportional to the amount of amplified DNA, the amount of DNA can be semi-quantified.
PCR sequencing (Sanger Sequencing)

PCR-sequencing is a method for analysing the PCR product to see the specific genetic code. The method is based on the same theory as regular PCR but additionally a modified di-deoxynucleotidetriphosphate (ddNTP) is added to the PCR solution. The ddNTP prevents elongation of the DNA when incorporated instead of dNTP and is labelled by radioactivity or fluorescence. This causes the PCR to produce copies of the DNA target strands of different lengths with an end consisting of a specific (A, T, G, C) ddNTP that can be identified. By arranging the DNA strands according to their length (mass) the sequence can be read.

Whole genome sequencing (WGS)

Whole genome sequencing is the process were not only a specific gene is analysed by PCR sequencing, but the whole genome of an organism. This is done by fragmenting the template DNA into platform specific sizes (according to the manufacturer of the machine). The fragmented DNA is then labelled and analysed by PCR sequencing in parallel reactions yielding multiple DNA-sequences from different parts of the genome. This raw data of short sequences is called “reads” (including information on ID, nucleotide sequence, orientation and quality score). Through software algorithms these reads can be de-novo assembled into “contigs” according to where the reads overlap and scaffolded in order thus completing the whole genome. The contigs can also be mapped against a reference genome if known.

WGS is heavily dependent on software algorithms and computational power to analyse the results and bioinformatic knowledge to interpret the results. Specific genes are often identified against databases through online software.

DNA Microarrays

DNA Microarray is a platform that uses multiple (up to thousands) DNA-probes fixed to a matrix. The DNA-probes hybridizes to specific regions in the target sequence and is detected by fluorescence. The probes can target both short sequences and whole PCR products (up to 1000bp). DNA microarrays hereby allow multiple gene detections at once. It is suitable for pathogen identification by targeting conserved regions and subtyping by targeting variable regions.

Epidemiological typing methods

To find out whether isolated bacteria within a suspected outbreak come from the same strain or are genetically diverse, various molecular epidemiological
techniques can be used. Typing methods should be able to discriminate sub-types of strains from the majority. Methods differ in reproducibility, stability, discriminatory power, applicability in different species, complexity of interpretation of data, labour intensity and cost.

Plasmid profiling
Plasmid profiling is an old subtyping method based on the fact that different bacterial strains carry a variable number of plasmids. Isolates are lysed with an alkali, which disrupts chromosomal DNA, but not plasmids. The plasmid DNA is stained with fluorescent dye, separated by electrophoresis and visualized under UV light (186). This method is rarely used for epidemiology today, but characterization of plasmids is still of interest as it can tell the evolutionary origin of genes and/or strains.

Pulsed-Field Gel Electrophoresis (PFGE)
Pulsed-field gel electrophoresis has been the gold standard for bacterial molecular subtyping, since the 1980’s, due to its universal applicability, stability, high discriminatory power and good reproducibility. An electrophoresis is a method to separate charged molecules according to size in a gel by adding electricity. Smaller molecules move faster through the gel than larger thus creating a pattern. However, molecules larger than a certain size move all together creating a problem when analysing the DNA, as it consists of large molecules. This can be overcome by cutting the DNA with restriction enzyme and repeatedly changing the direction of the current through the electrophoresis as this separates even larger DNA molecules, creating an electrophoresis fingerprint of the genome (187). As the mutation rate of bacteria is high the PFGE will always differ slightly and a similar pattern of ≤6 bands is concluded to indicate a clone, according to criteria by Tenover et al. (188).

PCR-based methods
Arbitrarily primed-PCR (AP-PCR) and repetitive element-PCR (rep-PCR)
AP-PCR and rep-PCR are two similar DNA fingerprinting methods that can be used when analysing outbreaks with genetically stable species. It is performed in the same fashion as a regular PCR but instead of using specific primers for a gene, short primers that can attach to arbitrary places in the genome are used. In rep-PCR primers that attach to repetitive nucleotide sequences in the genome is used. This creates an electrophoresis pattern that is random every time the AP-/rep-PCR is run, but a similar pattern among clones
as the template DNA is the same. This can be used to quickly compare if isolates are from the same clone, but the reproducibility is low.

**PCR ribotyping**
The genome of most bacteria contains several alleles of rRNA operons with different lengths of the space between the 16S and 23S RNA genes. This intergenic spacer region varies between species and strains. PCR ribotyping uses primers that amplifies the intergenic spacer regions, which creates amplicons of various sizes, visualized after electrophoresis. Due to its moderate discriminatory power it has only gained acceptance subtyping *Clostridium difficile* (189).

**PCR-restriction fragment length polymorphism (PCR-RFLP)**
PCR-RFLP is based on PCR amplification of a specific gene, followed by digestion of the PCR product by a restriction enzyme and subsequent electrophoresis (190). By this method you can get information about the gene content without sequencing, but it’s species specific and mainly applicable in certain species.

**Multi locus variable number tandem repeat analysis (MLVA)**
Bacteria have genetic regions (loci) with repetitive sequences, where the amount of repetitions vary depending on strain. MLVA is a multiplex PCR technique using up to 10 specific primers, targeting these species-specific regions. The amplified products vary in size depending on repetitions and can be separated and read by electrophoresis. (191). This is mainly applicable in Enterobacteriaceae and *P. aeruginosa* but has some of the advantages of MLST (described next) at a lower cost.

**Sequencing methods**

**Multi locus sequence typing (MLST)**
Multi Locus Sequence Typing is a method used to identify differences in “house-keeping genes”. “House-keeping genes” are genes that are essential for basic cellular functions. For every bacterial species, there is a set of five to ten (usually seven) genes that are analysed in an MLST. By performing a PCR and sequencing of these set of genes for a specific isolate you get a Sequence Typing (ST) profile according to the distinct alleles. As the house-keeping genes are relatively stable within a strain, this is a method that can be used when comparing isolates from an outbreak on a larger scale (192) and information can be shared between laboratories. This makes MLST the standard method for comparing strains on a higher epidemiological level. High level of genetic similarities, but not within the same ST can be clustered into Clonal Complexes (CCs) through algorithms.
PCR of specific genes in the MLST schemes can be performed with primers targeting conserved alleles for rapid identification, as with the *pabB*-gene in *E. coli* ST131. Furthermore, sequencing of a single gene, such as the *spa*-gene in *staphylococci* is another rapid typing method.

**WGS Single Nucleotide Polymorphism Typing (WGS SNP)**

Whole genome sequencing (WGS) single nucleotide polymorphism (SNP) typing, compare the SNPs of different isolates after WGS, to establish clonality (193). The candidate SNPs vary in number between organisms, depending on size, complexity, diversity and plasticity. The utilization relies on software that can define qualitative SNPs to compare. A variant is the WGS Analysis of Nucleotide Identity (ANI), which uses software to compare the percentage of SNP differences. The percentage defined to indicate a clone varies between species.
Aims of this study

The overall aim of this thesis was to investigate underlying factors that facilitate dissemination of resistant bacteria in hospital settings.

The specific aims were:

- To explore the lethal acquisition of vancomycin resistance of the VanD-type during selective pressure \textit{in vivo}
- To investigate the zoonotic potential of MRSP as an emerging human pathogen
- To analyse the relocalisation of hospitalized patients preceding colonization by a VRE clone involved in an outbreak
- To identify the source of spread of ESBL-producing \textit{K. pneumoniae} and carbapenemase-producing \textit{P. aeruginosa}
- To explore the reason for the shift in in epidemiology among AmpC- and ESBL-carrying \textit{E. coli} in a setting

Additional aims were:

- To examine if hospitalized patients are carriers of the \textit{VanD} gene
- To find the potential source of MRSP among patients
- To evaluate WGS as a tool for epidemiological analysis
- To examine differences in bacterial survival on common materials
Materials and Methods

Isolates, patients and screening

The following isolates and patients were included in the thesis:

**Paper I:** Two isolates of *E. faecium* from blood cultures collected from a septicemic patient before and during vancomycin treatment at Uppsala University Hospital in June 2009.

Screening for *VanD* was performed by faecal samples from all patients at the haematology ward for three weeks, and 60 random patients admitted to the hospital, with symptoms of colitis.

**Paper II:** Four isolates of MRSP collected from March to July 2011 from four patients presenting with symptoms mimicking infections with *S. aureus* at different wards at Uppsala University Hospital.

Screening for MRSP was performed by cultures from nose and present wounds on all staff members who owned dogs, at the wards visited by the patients.

**Paper III:** Eighteen isolates of *VanB*-carrying *E. faecium* from 18 patients identified from the first weeks of a large outbreak of VRE, including more than 300 patients in total at Gävle Hospital in 2013.

The 18 patients included were screened for VRE with rectal swabs every month for a year, or until three negative results or deceased.

**Paper IV:** Four isolates of ESBL-producing *K. pneumoniae* from four patients admitted to the neurosurgical intensive care unit at Uppsala University Hospital between November 2009 and May 2010.

Environmental screening for ESBL-producing *K. pneumoniae* was carried out, consisting of more than 100 samples from the room and ward where the patients had stayed.
**Paper V:** Eight isolates of MBL-producing *P. aeruginosa* from eight patients admitted to either Karlskrona or Karlshamn hospital in Blekinge region between February 2006 to June 2007.

Patients admitted to the medical and surgical wards at Karlskrona and Karlshamn hospitals were screened during the outbreak episodes. Furthermore, patients and staff at the ICU at Karlshamn hospital were screened and 124 environmental samples from affected wards were collected.

**Paper VI:** Forty-six randomly chosen *E. coli* isolates with reduced susceptibility to third-generation cephalosporins collected from January 2006, through December 2008, at the Department of Clinical Microbiology, Uppsala University Hospital. Half were producers of CTX-M and the other half produced AmpC.

**Identification of species**
Bacteria were identified to the species level with conventional methods (Paper I, III-VI), PCR (194, 195) (Papers I and III), and sequencing of 16S rDNA (Paper II).

**Antibiotic susceptibility**
The disc diffusion method and Etests were used to determine the antibiotic susceptibility to relevant antibiotic drugs (Papers I-VI). In Paper II, broth dilution was added. Bacteria were classified as susceptible, intermediate or resistant according to the recommendations of SRGA or EUCAST depending on when the tests were performed.

**Resistance mechanisms**

**Phenotypic resistance**
The ESBL and AmpC phenotypes in *E. coli* and the MBL phenotype in *P. aeruginosa* were confirmed with disc diffusion synergy tests with clavulanic acid and aminophenylboronic acid (*E. coli*) or EDTA and MPA (*P. aeruginosa*) (196-199) (Paper IV-VI).

**Genotypic resistance**
PCR was used to detect:
- *van*-genes in *E. faecium* (200) (Papers I and III)
- *meca*-gene and *nuc*-gene in MRSP (201) (Paper II)
- *blaVIM* and *blalMP* in *P. aeruginosa* (202) (Paper V)
- blatem, blashvs and blactx-ms (203, 204) in E. coli (Paper VI)
- genes encoding pAmpC in E. coli (68) (Paper VI)

WGS was used to confirm resistance genes and explore their surroundings (Papers I-III, V).

**Mobile genetic elements**

PCR was used to identify:
- Plasmids by replicon typing (37) in E. coli (Paper VI)
- Integrons of class I & II (205) in E. coli (Paper VI). The gene cassettes of the integrons were, in addition, sequenced (Papers VI)
- SCCmec-type in MRSP (206) (Paper II)

WGS was used to identify or confirm the presence of transposons and integrons and their content (Papers I, III and V).

**Virulence factors**

PCR (Papers II and VI) and WGS (Papers I-III and V) were used to identify virulence genes (207-210).

**Clonality**

**PFGE**

PFGE was performed to demonstrate genetic relatedness (203) between isolates (Papers I-V).

**PCR**

*E. coli* clone O25b-ST131 was detected with PCR for the *pabB* gene (68) and genetic relatedness among AmpC was examined by rep-PCR/AP-PCR with three primers ERIC1R, ERIC2 and A-70-10 (211) (Paper VI).

**Sequencing**

*Spa*-typing was performed on the MRSP isolates (212) (Paper II).

**WGS**

WGS was used to identify MLSTs (Papers I, II, III and V) and SNP variations (Paper III).
Whole Genome Sequence software analysis and quality

WGS read quality was assessed by FastQC software (Paper V) and MIRA v3.9.9-4.9.5 was used to de-novo assembly the reads into contigs (Paper I-III and V). Assembly statistics was evaluated with Quality Assessment Tool for Genome Assemblies (QUAST) v4.4 for quality control (Paper III and V). Specific genes were searched for manually with Basic Local Alignment Search Tool (BLAST) or automatically with online software, variously depending on study (Paper I-III and V). Comparisons of whole genomes were performed with MUMmer 3.0 (Paper I) and BRIG (Paper III), SNP differences were identified using variant-calling tools included in TorrentSuite v3.6.2 (Paper III).

Survival in the environment

In Paper VI a method for examining survival in a clinical environment was developed. Inocula from an overnight incubation of bacteria were spread onto 12 different hard and soft materials commonly encountered in the hospital environment. Prior to the experiment, the materials had been disinfected or sterilized. After 1, 3, 7, 14, 21 and 28 days, surviving bacteria were collected and cultured.

Statistical analysis

Fisher’s exact test was applied when antibiotic resistance rates were compared, whereas Wilcoxon signed rank test was used when comparing number of virulence factors and survival times. The total number of days of survival on each material was calculated for every bacterial isolate (Paper VI).
Results and Discussion

Transmission of resistance genes and multiresistant bacteria

Transfer of genes encoding vancomycin-resistance from the intestinal microbiota (Paper I)

A patient at the haematology ward had, like her co-patients, repeatedly been screened for VRE of the VanA and VanB types. Suddenly she became septic, and blood cultures yielded growth of *E. faecium* isolate 8256, susceptible to vancomycin. She was treated with vancomycin but did not respond as expected. New blood cultures were therefore obtained five days later, yielding growth of *E. faecium* isolate 8294. Isolates 8256 and 8294 had identical antibiograms, with one important exception; isolate 8294 was resistant to glycopeptides. PCR of isolate 8294 was negative for the *vanA* and *vanB* genes, but positive for the *vanD* gene. The DNA-profiles of the two isolates were identical when compared with PFGE, indicating that the involved *E. faecium* strain had acquired the resistance during treatment. The PFGE finding was confirmed by WGS when the genomes of two *E. faecium* isolates were matching, except for the *vanD* gene situated in the transposon Tn1545.

The *vanD* gene has always been considered to be chromosomal like the *vanC* gene (213). In previous reports the *vanD* gene has not been associated to specific transposons and conjugation has not been proven *in vitro* (113, 115, 214). This case proved the opposite. The rapid and lethal acquisition during vancomycin treatment was probably due to the conjugative properties of Tn1545 (215). Apart from the patient infected with the *vanD*-positive isolate, a total of eight patients were faecal carriers of the *vanD* gene when screened with PCR, and half of them were staying in the same ward as the septic patient. The carriage rate was lower than previously reported (27-44% of tested patients) (216), but if the sampling was representative is not known. However, no specific species or strains could be isolated as carriers of this gene in any of these patients, as noted in earlier studies (216). Domingo *et al.* have suggested *Ruminococcus* spp. as a potential source (116) of the *vanD* gene in the intestinal microbiota. Retrospectively, VanD positive screening cultures could have been more thoroughly cultured anaerobically to possibly identify the *vanD* carrying species (116).
The main source of endogenous infections, the commensal flora, is also affected by the antibiotics we consume. As demonstrated, bacteria pick up new resistance mechanisms and our commensal flora can be a pool of resistance genes. Speculatively the species carrying the *vanD* gene was spreading on the affected ward, but as VanD nor the carrying species are screened for, the presence was never noticed in advance.

The *vanD*-carrying *E. faecium* isolate detected is unique in many aspects. Firstly, due to the limited isolates previously reported (113, 115, 117-121, 214, 217, 218), secondly because transfer of resistance genes, has not been demonstrated *in vitro* in spite of attempts (113, 115, 214) was demonstrated *in vivo*. Thirdly, because none of the previous reports have demonstrated the *vanD* gene in connection to a transferable element.

When resistance genes are transferred to a pathogenic species the outcome can be fatal. Horizontal gene transfer enables competent strains to accumulate advantageous mobile genetic elements that enhance the possibility of survival in the environment that we create. The spread of resistance genes among species in the commensal flora can therefore affect the dissemination of pathogenic bacteria. Transfer can be expected, and the clinician needs to be aware of this risk and re-evaluate the therapy regimen. Carriage of resistance genes outside pathogenic bacteria could possibly be screened for. This paper highlights the commensal flora of patients as a potential reservoir of resistance genes that can mobilize during antibiotic pressure, which has also been suggested for the *vanB* gene (219).

Transmission of multiresistant staphylococci from animals to humans (Paper II)

Between March 2011 and July 2011 four patients were treated at the Department of Infectious Diseases and the Wound centre at Uppsala University Hospital due to skin infections caused by MRSP. PFGE showed clonality between the isolates from patient 1 and 4, indicating a transmission between patients, but not with any previous known isolate, in the database from companion animals. The isolates belonged to *spa*-type *t02* and *SCCmec*-type *II-III* when analysed by PCR and WGS confirmed that the isolates belonged to the *ST71-J-t02-II–III* clone, dominating among dogs (220). Interestingly the human cases occurred the year right after the peak incident among animals (Figure 9).

The cause of the clustering was never identified. The fact that all patients had been to the same ward suggests a common source. Many of the healthcare staff treating the patients had companion animals, but screening of these animals did not result in any additional MRSP isolates. Only one of the patients had companion animals (cats) but they were all healthy. The patients had not been in contact with each other, except for patient 1 and 4 (who had PFGE-
identical MRSP) who had been visiting the Wound Care centre at similar occasions. A patient-to-patient transmission of this bacterium very likely occurred between patient 1 and 4, indicating the potential of following spread between humans. Theoretically, other patients visiting could have been colonized carriers, but lacking risk factors and therefore not developing infections requiring culturing. Moreover, as MRSP is found in the environment around colonized animals (162, 221) environmental screening may have given additional information on the source.

This clustering is remarkable since none of the patients were in direct contact with animals. MRSP is normally only found among people who are in direct contact with animals (136). However, in some previous cases the source has never been identified (159) and humans have been suggested as vectors of MRSP between animals in veterinary clinics (222). As such, human-to-human transmission should be possible as well. Furthermore, the limited number of previous cases of MRSP infections in humans (223-225) makes this clustering exceptional.

The subsequent years following the outbreak a small number of patients infected with MRSP have occurred every summer in the setting. The patients were carriers of MRSP for an extended period of time, and not just transient, indicating that the bacteria have found a new niche.

![Figure 9. Isolates of MRSP from companion animals in Sweden over 10 years. Numbers according to the National Veterinary Institute (SVA)](image)
From the environment (Paper III-VI)

**Rooms (Paper III)**

In Gävleborg county, where the VRE outbreak occurred, 2.27 disposable hospital beds/1000 inh. were registered in 2013. This was the lowest number of disposable beds in Sweden that year and in turn Sweden had the lowest number in the EU (226). Data from the year after showed that the region had the highest number of overcrowding and the fifth highest number of delocalization in the country.

The VRE outbreak was discovered in October 2013. During the four months, from July-October 2013, the 18 patients included in the study were hospitalized during 22.5 days in median (range 10-114) and had two admittance episodes (range 1-6 episodes). Length of stay is a risk factor for VRE and as the healthcare consumption was considerable, this indicates a co-selection of vulnerable patients. Throughout these admittance episodes each patent was placed in 3.5 individual rooms in median (range 1-10) and two separate wards (range 1-5). These 18 patients had during the period all together visited 11 different wards and 54 different rooms. The wards and rooms used by the 18 patients were plotted in Figure 10, where one also sees that ward 120, 111 and 110 were used by almost all patients at some stage. One may seriously question if the extensive relocalisation of patients during these admittance episodes were medically motivated or a sheer result of the organization of the healthcare and lack of beds.

The PFGE analysis revealed five subgroups of the clone during the outbreak divided into two major clusters. However, the WGS SNP analysis divided the clone into four different lineages which were not fully concordant with the PFGE analysis (Figure 10). The WGS result was better correlated to where the patients had stayed, indicating that WGS may be a superior analysis (193) when analysing outbreaks of a species with high mutation rate. The lineages were well correlated to the wards where the patients had stayed (Figure 10), and the clone likely evolved separately once established at the specific ward. Some rooms especially at ward 120 were frequently occupied (at different times) by patients later found to be colonized. This implies that the rooms and/or equipment were contaminated (176, 227) due to insufficient cleaning protocols preceding the discovery of the outbreak. No environmental sampling nor any screening of staff, were carried out, which could have given additional insight to the source of the outbreak.

Lack of isolation and hospitalization in the same units and rooms as VRE carriers is a definite risk factor (11, 228). Staying in a room where a VRE carrier has stayed within the previous 2 weeks almost three-fold the hazard ratio of acquisition of VRE in a susceptible population (228). With environmental contamination the risk of environment-to-patient transfer of Enterococci is understandable.
When designing new hospitals, single rooms have been premiered, which is good from a hygienic standpoint. However, the resulting lack of beds in proportion to the population, when not enough rooms (beds) are at disposal, may increase the relocalisation. Or even worse, that single rooms are used for multiple patients despite the spatial limits, making the situation worse (229).

As these 18 colonized patients during a short time visited 54 rooms in 11 different wards it is clear how quickly a clone can contaminate almost an entire hospital, when considering the other 300 patients and where they may have been. Enterococci are known to survive outside hosts for long periods and decontamination of hospital environments is challenging when outbreaks occur (4). The extensive relocation of patients probably facilitated the contamination of the hospital environment and the initial spread of VRE. When hospital beds are continuously decreasing, and relocation of vulnerable patients increase, the risk of dissemination of resistant bacteria must be taken into consideration. Furthermore, the methods to analyse outbreak clones is probably trending towards WGS as the price of this methods is reduced and holds several advantages in epidemiological analysis.

Figure 10. The 18 patients followed from July to October 2013 plotted every time they were admitted to a hospital room
Equipment (Paper IV-V)
Between November 2009 and April 2010 four patients admitted to the neurosurgical ICU (NICU) at Uppsala University Hospital were infected or colonized by ESBL-producing K. pneumoniae. The patients had all been treated with broad spectrum antibiotics (ciprofloxacin, piperacillin-tazobactam and cefotaxime) and all had been treated with invasive ventilation, which are known risk factors for acquiring ESBL-producing K. pneumoniae (126). Additionally, a fifth patient was later found colonized at a referring hospital indicating a subsequent transmission (Figure 11).

The bacterial isolates were resistant to all tested antibiotics except for carbapenems and PFGE confirmed that the isolates were clonal and hence an outbreak was confirmed. An outbreak investigation was initiated and when going through the medical records from the four patients it was noted that the patients had been staying in the same room, but not at the same time. There was a remarkably long time between cases 2 and 3 (Figure 11) to expect a direct transfer between patients by the healthcare workers and other reservoirs were looked upon. An incorrect use of the sink in the room was noticed, as it was used as a combined clean water source, e.g. for cleaning tracheal tubes and as a waste disposal. Samples were collected from the plughole, yielding growth of ESBL-producing K. pneumoniae, with identical DNA pattern to that of the patient isolates when examined by PFGE.

To determine whether contents from the plughole were dispersed on to surfaces outside of the drain, an identical model of the sink was used, and safranin was injected into the plughole. The water was turned on for 5 s and the sink was thereafter examined for red safranin stains. Stains were found all over the sink, including the rim telling that bacteria in the plughole of a sink will easily contaminate objects and people in close proximity.

To intervene, the sink and the plumbing in the room were replaced. Routines were changed and combined use of sinks as waste disposal and freshwater source was abolished. The plughole was cultured every three months for a year and all patients staying in this room were screened on discharge, but no more patients have acquired this ESBL-producing K. pneumoniae strain. The sink was still free from the outbreak strain and other multidrug-resistant
bacteria at the last control indicating a successful intervention. The design of sinks and other damp environments should be done to avoid environmental contamination with aerosols or splashes. The use of water sources should be clearly separated from waste disposals.

Water sources, such as sinks, are known risk factors for prolonged outbreaks and colonization of *P. aeruginosa* (230), but the damp environments as a reservoir for *K. pneumoniae* had been less looked into. Subsequent studies with recurrent outbreaks of multi-drug resistant *K. pneumoniae* have also found sinks and showers as reservoirs (231, 232), confirming sinks as a source of transmission for *K. pneumoniae*.

A similar outbreak scenario (Paper V) was observed at two hospitals in Blekinge region. Between February 2006 and June 2007, eight admitted patients were found colonized by VIM-2 producing *P. aeruginosa*. They too had underlying conditions and previous antibiotic treatments know to impose a risk for acquiring resistant *P. aeruginosa* (130), including visits to the ICU. As seen in Paper IV they had been staying in the same rooms, but at separate times (apart from patient 5 and 6), depicted in Figure 12.

The isolates collected from these patients were resistant to all antibiotics tested except for colistin and gentamicin, and PFGE confirmed that the isolates were clonal, indicating an outbreak. Screening of other patients and staff at the affected wards, yielded no additional cases. Nevertheless, environmental screening samples from the sinks in the ICU and a room at the surgery ward at Karlskrona visited by the last patient showed growth of the same clone of *P. aeruginosa*, confirmed by PFGE. The sinks were replaced and/or treated with disinfectants and no additional growth was found afterwards.

The isolation frequency of MBL-producing *P. aeruginosa* is very low in Sweden. Most isolates, have been derived from patients recently hospitalized abroad; suggesting that travel is of importance for acquiring the bacteria (233,
This is the first known nosocomial outbreak of MBL-producing *P. aeruginosa* in Sweden. Even though not as evident as paper IV, it is likely that the transmission occurred from the sinks, as the possibility of patient-to-patient transmission was limited due to the separation in time.

Reservoirs of *P. aeruginosa* in association with hospital water taps, sinks, plumbing systems and shower drains have been implicated as a source of nosocomial infections in hospital outbreaks (235-238). Environmental sources in a healthcare setting, can become contaminated during an outbreak and later become the source of a new outbreak. VIM-2 producing *P. aeruginosa* of ST 111 has been isolated in Malmö/Lund (234, 239) both before and after this outbreak. Patients are regularly transferred between these hospitals, possibly causing a circulation of this strain between hospitals in the region for more than a decade, as a result of lack of prompt intervention. Consequently, patients have died from this resistant strain.

*P. aeruginosa* contrasts with the other bacterial species covered in this thesis, as it is not a commensal but an environmental bacterium. Hence, it will not thrive unless we create a beneficial environment but given the right prerequisites *P. aeruginosa* will long last, as shown. Water is key for Gram-negative survival, and future ICU rooms maybe should not have sinks at all nor running water from common sources. Considering the high cost of ICU care, the cost of sterilized water would be negligible. Besides the dissemination inside hospitals, the sewage system from hospitals contribute to the dissemination of resistant bacteria to the environment (240, 241). The water efflux ends up in fresh water sources when treatment plants fail to remove pathogenic organisms and their genes. The sewage systems from hospitals contain increased levels of antibiotics as well, adding insult to injury as the resistance genes are sustained. An alternative solution could be hospital outlets separated from the common sewage system with water treatment that completely removed antibiotics, microorganisms, and their genes. Finally, a last problem is evident, as the sewage lining is coated with bacteria in biofilm, this creates a melting-pot for resistant bacteria to exchange resistance genes (242), selected by increased levels of dispersed antibiotics. Techniques to decrease biofilm formation in pipelining has been employed (239), but larger scale interventions are probably needed.

In summary, not solely the rooms but also the design and use of equipment in rooms can pose a source of dissemination of resistant bacteria. As seen in Paper IV and V, when contaminated sinks contributed to the transfer of ESBL-producing *K. pneumoniae* and MBL-producing *P. aeruginosa* to vulnerable patients. The potential of *P. aeruginosa* to survive in moist environments is well known, but the ability of *K. pneumoniae* to survive outside a host in a damp environment was clearly shown and longer than potentially expected. This highlights the importance of proper hygiene routines as well as functional design of hospital equipment to avoid unnecessary transfer. Furthermore, the
P. aeruginosa strain isolated, has been found several times in the region during more than a decade, exposing the need to rapidly find and eliminate the source to avoid recurrent outbreaks.

Materials (Paper VI)
The survival of ESBL- and AmpC-producing E. coli strains on different hospital-associated materials was tested and the result is listed in Table 1. Marble was the material on which the isolates survived for the shortest period. The longest survival period was provided by synthetic protection coats and polyethylene exam gloves. Apart from the interest in specific anti-bacterial coatings, the wide range of survival on different materials has not been given much attention in previous literature. Contamination of the environment surrounding a colonized patient is known (243), hence the survival time should be of importance for the possibility of transmission. The choice of materials in hospital and nursing home settings may have to take this into consideration. Furthermore, making objects and interior easy to clean rather than cosy in a hospital environment should be prioritized.

The ESBL-producing isolates had a significantly longer survival (p=0.03) on all materials compared with the AmpC-producing isolates. The median survival time of ESBL-producing isolates was 10.5 days on hard materials and at least 28 days on soft materials. The corresponding figures for AmpC-producing isolates were 1 day and 14 days, respectively. This could tell part of why ESBL-carrying E. coli is increasing. The study was based on few isolates and a more extensive study would be needed to reconfirm the results before making comprehensive conclusions. Nevertheless, the ability to survive was longer than expected.

The isolation frequency in urine at Uppsala University Hospital of E. coli and K. pneumoniae with reduced susceptibility to third generation cephalosporins during the study period, from January 2006 to December 2008, is illustrated in Figure 13. The isolation frequency of ESBL-producing K. pneumoniae decreased markedly after the initiation of the intervention program. The K. pneumoniae outbreak strain was mainly replaced by ESBL-producing E. coli in the urine samples by the end of the study period. Similar situations have been recorded at other settings described by Canton and Livermore (61, 244), indicating a global trend. The decrease of ESBL-positive K. pneumoniae could be due to the dissemination of K. pneumoniae being more sensitive to the changes of the intervention program (203). Furthermore, ESBL-carrying K. pneumoniae is more likely spread within the hospitals, while ESBL-carrying E. coli disseminate in the community too, where the interventions have limited effect.

Five (22%) of the ESBL- and one (4%) of the cAmpC-producing isolates included in Study VI belonged to the O25b-ST131 clone. The dissemination
potential of the O25b-ST131 clone is well described (67, 245) and the emergence of the O25b-ST31 clone accounts for part of the increase in ESBL-producing *E. coli* (67, 245-247) in the setting, but surely not all. Among the cAmpC-producing isolates, two small clusters were identified with rep-PCR/AP-PCR. The largest consisted of 5 isolates and the other of 2 isolates, indicating the potential for AmpC-producing isolates to spread locally if given the right prerequisites. Even though one AmpC-producing isolate belonged to the O25b-ST131 clone, this did not seem to cause any spread.

Recent studies also reveal that *E. coli*, can survive outside the intestines for longer periods than previously thought and be part of the natural environment (248), even though lineages may differ from clinical strains. Although only a few isolates were tested in Paper VI, there was a clear tendency that the ESBL-producing isolates survived longer in the environment and this can be part of their success in dissemination as contaminated objects can transfer the bacterium for extended period of time. Likewise, in the lack of prompt cleaning routines, even strains with short survival can transfer, as seen with the clonal AmpC-isolates.

**Figure 13.** Number of urine-isolates resistant to 3rd generation cephalosporins from Uppsala University Hospital
<table>
<thead>
<tr>
<th></th>
<th>AmpC-producing E. coli isolates*</th>
<th>ESBL-producing E. coli isolates*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A  B  C  D  E  F</td>
<td>G  H  I  J  K  L</td>
</tr>
<tr>
<td><strong>Hard Materials</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Marble bench</td>
<td>0  0  3  1  1  0</td>
<td>28  3  3  7  7  14</td>
</tr>
<tr>
<td>Stainless steel</td>
<td>0  3  3  3  1  1</td>
<td>28  28  21  28  21  21</td>
</tr>
<tr>
<td>Laminated bench</td>
<td>0  3  3  3  1  3</td>
<td>28  21  14  7  21  14</td>
</tr>
<tr>
<td>Glass</td>
<td>1  0  7  1  0  0</td>
<td>14  3  1  21  3  3</td>
</tr>
<tr>
<td>Wooden shelf</td>
<td>1  1  3  1  1  1</td>
<td>7  3  0  1  1  1</td>
</tr>
<tr>
<td><strong>Soft materials</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vinyl glove</td>
<td>7  3  14  14  14  7</td>
<td>28  28  28  28  21  28</td>
</tr>
<tr>
<td>Synthetic surgical scrub</td>
<td>14  7  14  3  14  7</td>
<td>28  28  14  28  28  14</td>
</tr>
<tr>
<td>Cotton-polyester coat</td>
<td>3  3  14  3  3  3</td>
<td>28  7  7  7  14  14</td>
</tr>
<tr>
<td>Polyethylene glove</td>
<td>28  28  28  21  28  28</td>
<td>28  28  28  21  28  28</td>
</tr>
<tr>
<td>Latex surgical glove</td>
<td>3  0  3  3  1  1</td>
<td>21  1  0  14  3  3</td>
</tr>
<tr>
<td>Nitrile glove</td>
<td>14  14  14  14  21  14</td>
<td>28  28  28  28  28  21</td>
</tr>
<tr>
<td>Synthetic protection coat</td>
<td>28  28  28  28  28  28</td>
<td>28  28  28  28  28  28</td>
</tr>
</tbody>
</table>

Virulence

Multi-drug resistance and increased virulence are well correlated to dissemination of pathogenic bacteria, either through co-selection or selective advantage (249-252).

**In MRSP**

The isolates of MRSP were cultured from four patients; three of them had diabetes mellitus and three of them had previous skin diseases, all had been treated with antibiotics before admittance. Diabetes seems to be overrepresented among humans infected by *S. pseudintermedius* (253, 254) as well as underlying skin disease. Three of the patients had bullous skin lesion, ulcers and general signs of infection, while the fourth patient was found colonized when screened due to general signs of infection.

The isolates were only PCR-positive for the *siet*-gene (*Staphylococcus intermedius* exfoliative toxin), when screened for four known virulence factors by PCR. As the function of the *siet*-gene in other animals is unclear (255), the PCR methods were set up for *S. aureus*, and the patients showed severe symptoms, WGS was performed. WGS identified the genes *siet* and *lukF/S-PV* of *S. intermedius*, the latter being a more likely cause of the clinical presentation as it is a known virulence factor in *S. aureus* (18, 256). Other hypothesised virulence factors in MRSP (257), were not investigated at the time of the study, and therefore never looked for.

As MRSP clone *ST71-J-t02-II–III* have emerged as a major pathogen among companion animals (220), it is likely to seen a spill-over to humans, as seen with MRSA from animal strains (158). Especially when carrying virulence factors known to cause symptoms in humans (18, 256). Previous infections not associated to bites with this clone has been reported (224) clearly indicating a zoonotic potential of this virulent clone, as the bacterium caused infections and not just colonization. Additionally, subsequent studies have confirmed additional cases in humans by this clone (254). The MRSP ST71 has shown significantly greater adherence to both canine and human corneocytes than other ST (257) and methicillin-susceptible *S. pseudintermedius*, which may explain both the dissemination of MRSP ST71 among animals and that this strain may have adapted to the human skin.

**In Vancomycin-resistant *E. faecium***

Multiple genes have been suggested as virulence factors in *E. faecium*, but only a few have experimentally shown to enhance virulence in animal models, such as the *esp* and *acm* genes (258, 259). There has been a notable increase in isolation frequency of *E. faecium* in recent years (112) and hospital associated strains seem to differ substantially from community acquired strains. The overrepresentation of certain genes for adhesion molecules (*esp, acm, ebp, hyl* and several *fms*) among the hospital associated strains point toward a selective
advantage (260). The rise of *E. faecium* as a nosocomial pathogen depicts not only the co-selection of virulence and antibiotic resistance, but also a selection of a successful molecular machinery to accumulate new genes. Hence, the antibiotic pressure within hospital environments not only speed up the evolution of antibiotic resistant isolates but also creates a genetic capitalism among strains, where a “winner takes it all” (261).

The VanB strain responsible for the VRE outbreak (Paper III) carried *esp, acm, ebpABC, hyl*. It shared almost identical virulence profile to other hospital associated strains (260). These genes encode adhesins, which enables the bacteria to colonize tissue, cause biofilms and stick to abiotic materials (262), therefore they may presumably also enhance the ability to persist in the hospital setting. Just as the vanB isolates the vanD isolate carried the *esp* gene which is believed to be involved in biofilm formation (263). The *esp* gene is commonly found in isolates causing infections and the gene from the VanB isolates were further analysed. It was part of a highly conserved region, enhancing the theory of its role as a positive selective trait, as little has changed over time.

**In ESBL-producing E. coli**

There was a significant difference (*p*=0.04) in the number of virulence factors carried by isolates producing ESBL (median 4, range 1-10) and AmpC (median 3, range 1-4). The impact of the quantity was unclear as the highest number of virulence factors was found among isolates derived from faeces. Conclusions regarding the effect of virulence factors on epidemiology is better correlated if isolates from only symptomatic patients are used. The two most frequent virulence genes were *fimH* (type 1 fimbrial adhesion) and *traT* (serum survival gene), and this was independent of bacterial groups. After these two genes, *bmaE* (M fimbrial adhesion) followed in AmpC-producers, while *iutA* and *fuyA* (both siderophores) were more common in isolates with ESBL-production, which has also been the case in other studies (246, 247). This can point toward a selective advantage of these genes. The O25b-ST131 clone has shown to carry a significant amount of virulence factors with a lesser diversity than other strains. Additionally it is linked to healthcare associated infections, indicating a co-selection of resistant and virulent *E. coli* strains in hospital settings (246, 247).

The co-selection of resistance and virulence factors creates a closed loop. Virulent strains cause disease, that we treat with antibiotics in hospitals, which selects for resistant and virulent strains in the hospital setting, causing new infections with hospital-associated strains. Speculatively, enhancement of avirulent strains could be an option, if we create the conditions for these strains to thrive. Alternatively, therapies against certain virulence factors or targeting expression of virulence rather than killing bacteria could be a preventative option (264). Blocking virulence factors, such as adhesion molecules, or the
signalling systems that change the state between planktonic and biofilm are other theoretical preventative options. However, virulence factors enhance competitiveness of bacteria, and as such the next evolutionary step would be to override such blockages. Secondly, interfering with bacteria that are part of the commensal flora, may increase the potential of other species. On the other hand, using antibiotics as we are doing today, seems to be leading to a dead end and new strategies are warranted.

Resistance mechanisms

MRSP

The cultures of MRSP yielded growth on chocolate and SAID agar and were both coagulase positive and DNase positive, phenotypically identifying the isolates as *S. aureus*. The isolates were resistant to all clinically relevant antibiotics tested except for linezolid, fusidic acid and rifampicin. However, the inhibition zones for oxacillin were in the range of 6-10 mm, whereas those for cefoxitin classified the isolates as susceptible (range 23-31 mm, mean 29.5 mm) when the breakpoints for *S. aureus* were used. Cefoxitin, which is used for human staphylococcal species, has been found unreliable in detecting *mecA* in veterinary staphylococci (265), hence the proper identification of species is important.

PCR was positive for the *mecA*-gene but negative for the *nuc*-gene. The results drew attention as it was not typical for MRSA and the 16S rDNA identified the species as *S. pseudintermedius*. *S. pseudintermedius* does produce thermonuclease (the product of the *nuc*-gene) (14), but probes are species-specific (266). The isolates harboured the combined SCCmec-type II-III, being responsible for the resistance to β-lactams.

The laboratory difficulties to identify *S. pseudintermedius* are well known (12). As the species is not expected in a clinical laboratory specialized in human medicine, it is even harder (18). Especially considering that it is phenotypically similar to *S. aureus*. MALDI-TOF did not identify the species in the study, but an updated database has solved this problem. However, phenotypic identification of species is still commonly used. When clones of virulent and resistant bacterial species are emerging in veterinary medicine, laboratories focused on humans must be aware. An increased collaboration between veterinary and human laboratories is warranted to see trends among shared species and to have techniques to identify them properly.

Companion animals, staff or relatives can be vectors of bacteria as seen in Paper II and Attachment paper VII. Resistant bacteria and resistance genes, derived from the use of antibiotics in animals, can adapt to new niches and MRSP clearly has a zoonotic potential. The wide-spread global use of antibi-
otics in agriculture, food-animals and companion animals should be questioned. If healthy food-animals need antibiotics, their living conditions need improvement. Furthermore, we must see antibiotics as a limited resource privileged to humans, and newer antibiotics should not be approved in veterinary medicine to avoid rapid resistance development.

**Vancomycin-resistant *E. faecium***

The VanD isolate (Paper I) belonged to the hospital associated ST17 and the VanD subgroup 4. In contrast to vanA and vanB, the vanD operon seems to be highly heterogenous, in spite of the few reports (114, 214, 218), indicating repeated *de novo* acquisition of vanD genes, rather than clonal spread. This pose a laboratory challenge developing universal primers targeting the vanD gene (214). An additional rare feature was that no mutation was observed in the chromosomal *Ddl* gene. The *Ddl* gene (D-ala-D-ala ligase) is the original enzyme producing the high affinity precursor and is often mutated with IS in isolates carrying vanD (119, 218, 267). When the original *Ddl* is mutated the bacterium is dependent on the *vanD* gene for peptidoglycan production, hence the *Ddl* mutations must be preceded by vanD acquisition in a step-by-step fashion. Furthermore, the *vanD* gene cluster was shown to be 99% similar to one of the few previously isolated *E. faecium* strains, 10/96A (114, 118). This is interesting since the activity of D,D-dipeptidase and D,D-carboxypeptidase of the *vanD* operon in *E. faecium* 10/96A have negligible activity (114). These enzymes hydrolyse the original high affinity peptidoglycan precursors in vanA and vanB isolates, but have shown little effect in previous vanD isolates (114, 115, 218). This has been regarded insignificant as the *Ddl* gene has been mutated anyway (114). The *E. faecium* 8294 should then theoretically be susceptible to vancomycin as the original pathway would be functioning. Either there was a deficiency in *Ddl* expression or the activity of the enzymes were sufficient after all, but this was never investigated. Finally, the *vanD* operon of the *E. faecium* 8294 was situated in the transposon Tn1545. The previous reports were not able to identify any mobile genetic element in connection to vanD. However, this was the first vanD isolate analysed by WGS and subsequent studies also employing WGS have found the vanD operon associated to large mobile elements (214). It may be that previous studies have missed this due to the older techniques used.

The VanB isolates (Paper III) belonged to hospital associated ST192 and carried the complete *vanB2* cassette in the Tn1549/Tn5382 transposon. Furthermore, they carried the macrolide efflux determinant *mrsC* and macrolide methylase *ermB*, the lincosamid resistance gene *InrB*, tetracycline efflux pumps *tetLM* and aminoglycoside resistance genes *ant*(6)-Ia and *aph*3'-III. All isolates had the same mutations in the quinolone resistance determining regions of *gyrA* and *parC*. The acquisition of the Van-gene to strains that are
already multi drug resistant, depicts the step-by-step accumulation of resistance in hospital associated strains (110). Howden et al. suggested that the vanB gene on Tn1549 is transferred from the commensal flora to E. faecium (219) of ampicillin resistant Vancomycin sensitive E. faecium of hospital associated strains. An initial dissemination of competent strains is followed by the acquisition of a resistance mechanism with large clinical implications.

The ST191 strain that was initially found in other hospitals in the neighbouring region (111), was also responsible for this outbreak. The dominance by this clone in the region depicts the difficulties in eradication once established. It is therefore important to prevent the dissemination of hospital associated clones, before they are established at wards were the most vulnerable patients are treated and vancomycin-pressure is high. Otherwise selection for vancomycin resistance, as seen in Paper I, can be expected.

**Carbapenem-resistance in P. aeruginosa**

From February 2006 to June 2007 there were 816 clinical isolates of P. aeruginosa, in Blekinge region, from 507 patients, of which 8.5% were resistant to imipenem. P. aeruginosa isolates from nine patients were resistant to both imipenem and ceftazidime and MBL was phenotypically confirmed with Double Disk test and MBL E-test. MBL was not confirmed in the last isolate and it had a different PFGE-pattern from the other isolates, indicating mutations in porins and/or efflux pumps.

The WGS of one of the clonal isolates in Paper IV revealed that the clone belonged to the high-risk multi-drug resistant CC111, known to be virulent and cause biofilms (90, 94, 238, 268). This could add to the trait that the clone was found in the sinks. It carried Integron class 1, but the blaVIM-2 gene was not within the gene cassette as previously demonstrated (87, 91, 95), indicating a subsequent rearrangement of genes. Genes for chloramphenicol resistance catB7 and fluoroquinolone resistance oqxB were identified. As well as two aminoglycoside resistance genes aph3-IIIb (O-phosphotransferase) and aacA29b (N-acetyltransferase), which are commonly associated to the VIM-2 gene, pointing towards a co-selection. The oprD-gene, responsible for an outer membrane porin, included frame-shifts, which could add to the high resistance rate toward carbapenems and quinolones. Though, efflux pumps were not studied.

*Whole Genome Sequence quality and analysis*

WGS is heavily reliant on software algorithms to interpret the sequence output. The techniques to rapidly sequence genomes have somewhat outpaced the ability to analyse the information yielded. Multiple software algorithms are available for all stages of the analysis, but few are thoroughly validated.

Assessment of the quality of the whole genome sequencing (both reads and assembly) is of increasing importance and provision of this information is now mandatory for publication in most journals. Information on total assembly
length (bp), number of larger contigs, read coverage per contig, N50 (the size of the contig at the position where 50% of the genome is covered when the contigs are arranged from largest to smallest), GC content and mapping against a reference genome are common quality indicators. Ideally the reads should be long and of good quality, the coverage high and the contigs large with small gaps in between. The GC content should match the species and the genome should largely match a reference genome.

The possibility to accurately assemble the reads into contigs is very dependent on the initial sequence quality, but also on the software algorithms ability to handle complex genetic regions (for example, repetitive sequences) and correctly scaffold the contigs in order. In Paper V the proper ST-type could not be identified due to insufficient reads or assembly of a contig covering the *acs* locus. Separate PCR sequencing of this gene could have provided the information missing. Furthermore, lack of reference genomes to establish a core genome for a species poses an uncertainty, when using software to choose qualitative SNPs to analyse epidemiological differences (Paper III) without knowing the natural variability within the genome. Additionally, the automated gene searches by online database software are depending on updated and correct data, whereof some are provided by small companies or individual researchers (269).

**ESBL**

The ESBL-producing *E. coli* isolates were more resistant to non-β-lactam antibiotics than the AmpC-producing isolates. Multiresistance (defined as resistance to three additional classes of antibiotics) was detected in 11 of the ESBL-producing isolates. The corresponding figure for AmpC-producers was 2. This can affect the dissemination when a co-selective pressure from other classes of antibiotics is applied.

The result of the screening for plasmid-mediated β-lactamases is summarized in Table 2. Two types of pAmpC were found: *CMY-2* (n=2) and *DHA-1* (n=1), in concurrence with similar studies (77). Among the 23 ESBL-producing isolates, *CTX-M-15* predominated (n=18), followed by *CTX-M-14* (n=5), which are the dominating CTX-M types (64, 246, 247). Four isolates had both *CTX-M-14* and *CTX-M-15*, which is peculiar as this should not pose a selective advantage. However, it may be that there is a co-selection of these genes due to proximity to other advantageous genes, or no disadvantage of carrying both.

Only class I integrons were detected in the two groups. Their gene cassettes contained genes encoding resistance to aminoglycosides (aminoglycoside adenyl transferase) and/or trimethoprim (dihydrofolate reductase), displaying the various mechanisms of genetic exchange carried by these isolates. Replicon types were identified in all but two isolates with plasmid-mediated β-lactamases (Table 2). The *IncF* plasmids dominated totally among both the ESBL- and pAmpC-producers. *IncF* plasmids are the most frequently detected
plasmids in Enterobacteriaceae and their role in horizontal gene transfer of CTX-M genes is well established (270, 271). The introduction of resistance genes in wide host range plasmids is thought to attribute to the global dissemination of resistant clones (64). The combination of successful plasmids with successful strains creates highly competitive clones.

**TABLE 2.** Molecular characterization of *E. coli* isolates with plasmid-mediated β-lactamases.

<table>
<thead>
<tr>
<th>Isolate (Source/Year)</th>
<th>O25b-ST131</th>
<th>β-lactamase type</th>
<th>Replicon type</th>
<th>Integron class (cassette content)</th>
<th>No. of virulence factors</th>
</tr>
</thead>
<tbody>
<tr>
<td>ESBL.0611364 -</td>
<td>CMY-2</td>
<td>II-Y-FII</td>
<td>-</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td>ESBL.0703774 -</td>
<td>DHA-1</td>
<td>FIB</td>
<td>-</td>
<td>1 (aadA1)</td>
<td>3</td>
</tr>
<tr>
<td>UM0708749 -</td>
<td>CMY-2</td>
<td>FIB-FII-FIA</td>
<td>I (no content)</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td>U0707707 -</td>
<td>CTX-M-15, TEM</td>
<td>FIB-FIA-FIC</td>
<td>-</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>UM0711746 -</td>
<td>CTX-M-15</td>
<td>FIC</td>
<td>-</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>ESBL.07020682 +</td>
<td>CTX-M-15, TEM</td>
<td>FIB-FIA-P-FIC</td>
<td>I (dfr17, aadA5)</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>S0705198 -</td>
<td>CTX-M-15</td>
<td>FIB-L/M</td>
<td>-</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>ESBL.0702314 +</td>
<td>CTX-M-15</td>
<td>FIB-FIA</td>
<td>I (dfr17, aadA5)</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>ESBL.0702644 -</td>
<td>CTX-M-15, SHV</td>
<td>A/C-FIB-L/M-Y-P</td>
<td>-</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>ESBL.0703663 -</td>
<td>CTX-M-14, TEM</td>
<td>FIB-FIA</td>
<td>-</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>ESBL.0703442 -</td>
<td>CTX-M-14, TEM</td>
<td>FIB-P-FIC</td>
<td>-</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>UM0704079 -</td>
<td>CTX-M-15</td>
<td>-</td>
<td>-</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>U0703218 -</td>
<td>CTX-M-15, TEM</td>
<td>FIB-I1-P</td>
<td>I (dfrA12, aadA2)</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>U0703411 -</td>
<td>CTX-M-15, TEM</td>
<td>FIB-FIA</td>
<td>-</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>S0700430 +</td>
<td>CTX-M-15, TEM</td>
<td>FIB</td>
<td>I (dfr17, aadA5)</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>ESBL.0611219 -</td>
<td>CTX-M-14, TEM</td>
<td>B/O-FIB-L/M-FIA-FIC</td>
<td>I (dfr17 aadA5)</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>ESBL.0610487 -</td>
<td>CTX-M-15, TEM</td>
<td>B/O-FIB-L/M</td>
<td>-</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>ESBL.0602688 -</td>
<td>CTX-M-15, TEM</td>
<td>FIB-L/M-I1</td>
<td>I (dfr17 aadA5)</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>ESBL.0603534 -</td>
<td>CTX-M-14</td>
<td>FIB-I1-FIA-P-FIC</td>
<td>-</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>U0608239 -</td>
<td>CTX-M-15, TEM</td>
<td>FIB</td>
<td>I (dfrA12, aadA2)</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>S0604097 -</td>
<td>CTX-M-15</td>
<td>FIB-FIA</td>
<td>-</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>U0712214 +</td>
<td>CTX-M-15</td>
<td>FIA-FIC</td>
<td>-</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>S0801553 -</td>
<td>CTX-M-15, TEM</td>
<td>FIB-FIA</td>
<td>-</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>U0801628 -</td>
<td>CTX-M-14, TEM</td>
<td>FIB-FIA-P</td>
<td>I (dfrA14)</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>U0803312 +</td>
<td>CTX-M-15</td>
<td>FIA</td>
<td>I (dfr17, aadA5)</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>UM0801406 -</td>
<td>CTX-M-15</td>
<td>-</td>
<td>I (dfrV, dfrA5)</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>

Source: U/UM=urine, ESBL=faeces, S=wound. Year of isolation is given with **bold** figures. Cassette content: aad = aminoglycoside adenyl transferase; dfr = dihydrofolate reductase
Summary

There is a continuous exchange of bacteria between humans, animals and inanimate objects in the environment. Hospitals are a special microbial niche, as vulnerable patients are gathered and exposed to large amounts of antibiotics, which select for resistant and virulent bacterial strains. Therefore, the exchange of bacteria should be kept at a minimum within this setting.

The transfer of resistance genes from the microbiota to pathogenic bacteria, resistant bacteria from pets to humans, and resistant bacteria from objects and materials to patients, contribute to the dissemination of resistant pathogenic bacteria in hospital settings. Furthermore, relocalisation of patients may cause dissemination of resistant bacteria both within hospitals, but also between hospitals in larger geographical areas.

Virulence factors cause disease and some possibly enhance the ability of bacteria to colonize and survive in the environment. The accumulation of resistance genes, transferred on mobile genetic elements, leads to multiresistant bacteria. As virulence and resistance genes tend to be co-selected, competitive virulent and multiresistant hospital-associated bacterial strains have emerged, with the ability to spread both locally and globally.

Clusters or outbreaks of resistant bacteria merely makes it easier to study the dissemination as it reveals the spectacular shortcomings in healthcare. By finding the factors that contributed to spread of resistant bacteria and possibly eliminating them, transmission can hopefully be decreased.
Conclusions

Dissemination of resistant bacteria in hospital settings is facilitated by:

- Selection and acquisition of resistance genes from the intestinal microbiota
- Resistant pathogens among animals that find a niche in humans
- Extensive relocation of patients between possibly contaminated rooms
- Sinks contaminated with resistant bacteria
- Extended bacterial survival on materials in the hospital environment

Additional conclusions are:

- Patients without VRE carry the VanD gene.
- Patient-to-patient transmission of MRSP may occur, the pet owning hospital staff is not a likely source within the investigated setting.
- WGS is a reliable tool for epidemiological analysis, providing both epidemiological data and information on specific genes.
- There are large variations in survival on different materials and between different strains
Populärvetenskaplig sammanfattning på svenska


Få nya antibiotika utvecklas och det är därför ytterst viktigt att förhindra spridningen av resistenta bakterier. Sjukhusmiljön är extra känslig för spridning av resistenta bakterier, då infektionskänsliga människor tångs ihop på liten yta och samtidigt utsätts för ett stort selektivt tryck via alla antibiotikabehandlingar. Risken för spridning ökar om sjukhusmiljön förörenas med resistent bakterier och vårdhygienen brister hos patienter, anhöriga och vårdpersonal.

I den här avhandlingen ingår sex studier där uppkomst, introduktion och spridningen av resistenta bakterier har studerats i sjukhusmiljöer. Vi har i olika omfattning tittat på bland annat gener som kodar för resistens och virulens (förmåga att framkalla sjukdom), bakterieisolatens släktspår vid misstänkta utbrott, bakteriers överlevnadsförmåga på olika material och i sjukhusutrustning, samt hur omflyttning av patienter kan förenkla bakteriers spridning.


Den andra studien inkluderade fyra patienter med hudinfektioner som först misstänktes bero på Staphylococcus aureus. Ytterligare analyser klargjorde dock att den hos hundar och katter förekommande bakterien Staphylococcus pseudintermedius var orsaken. En multiresistent klon av denna art (MRSP)
hade nyligen spridits snabbt bland djur i stora delar av Europa. Genom introduktion i en känslig sjukhusmiljö visade den sin zoonotiska potential, och för första gången kunde vi dokumentera att den smittade från människa till människa.

I den tredje studien inkluderades 18 av de första patienterna i vad som skulle visa sig bli ett större utbrott med vancomycinresistenta enterokocker (VRE). Cirka 300 patienter drabbades. Den orsakande klonen tillhörde en epidemiisk typ som tidigare hade hållit till i Mälardalen. Med hjälp av helge

nomsekvensering kunde förloppet av spridningen nystas upp på ett helt annat sätt än med den äldre metoden pulsfärlsgelélektrofores. Genom att följa uppgifter om var patienterna hade vårdats, avslöjades det att patienterna i omfattande utsträckning hade flyttats runt på sjukhuset månaderna innan utbrottet. Detta bidrog sannolikt till den stora spridningen av VRE.


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Björn Olsen, my co-supervisor, taught my mum about birdwatching, which she still enjoys. I do not.

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References


52. Abraham EP, Chain E. An Enzyme from Bacteria able to Destroy Penicillin Nature. 1940;146:837.


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