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Survival of infectious agents and detection of their resistance and virulence factors

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Abstract
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In the first study, three different transport systems for bacteria were evaluated. The CLSI M40-A guideline was used to monitor the maintenance of both mono- and polymicrobial samples during a simulated transportation at room temperature that lasted 0-48 h. All systems were able to maintain the viability of all organisms for 24 h, but none of them could support all tested species after 48 h. The most difficult species to recover was *Neisseria gonorrhoeae,* and in polymicrobial samples overgrowth was an observed problem. The aim of the second study was to study the presence of TSST-1 and three other important toxin genes in invasive isolates of *Staphylococcus aureus* collected during the years 2000-2012 at two tertiary hospitals. The genes encoding the staphylococcal toxins were detected by PCR, and whole-genome sequencing was used for analyzing the genetic relatedness between isolates. The results showed that the most common toxin was TSST-1, and isolates positive for this toxin exhibited a clear clonality independent of year and hospital. The typical patient was a male aged 55-74 years and with a bone or a joint infection. The third study was a clinical study of the effect of silver-based wound dressings on the bacterial flora in chronic leg ulcers. Phenotypic and genetic silver-resistance were investigated before and after topical silver treatment, by determining the silver nitrate MICs and by detecting *sil* genes with PCR. The silver-based dressings had a limited effect on primary wound pathogens, and the activity of silver nitrate on *S. aureus* was mainly bacteriostatic. A silver-resistant *Enterobacter cloacae* strain was identified after only three weeks of treatment, and cephalosporin-resistant members of the *Enterobacteriaceae* family were relatively prone to developed silver-resistance after silver exposure *in vitro*. The last study was undertaken in order to develop an easy-to-use method for simulating the laundering process of hospital textiles, and apply the method when evaluating the decontaminating efficacy of two different washing temperatures. The laundering process took place at professional laundries, and *Enterococcus faecium* was used as a bioindicator. The results showed that a lowering of the washing temperature from 70°C to 60°C did not affect the decontamination efficacy; the washing cycle alone reduced the number of bacteria with 3-5 $\log_{10}$ CFU, whereas the following tumble drying reduced the bacterial numbers with another 3-4 $\log_{10}$ CFU, yielding the same final result independent of the washing temperature. To ensure that sufficient textile hygiene is maintained, the whole laundering process needs to be monitored. The general conclusion is that all developmental work in the bacterial field requires time and a large strain collection.

Keywords: Transportation system; swab; polymicrobial samples; Neisseria gonorrhoeae; bacteremia; exotoxins; Staphylococcus aureus; TSST-1; silver; silver resistance; wound dressing; sil genes; laundry; tumble drying; bacterial decontamination

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To my family
Janne, Hanna & Pär

List of Papers

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

I   Tano, E., Melhus, Å. (2011) Evaluation of three swab transport systems for the maintenance of clinically important bacteria in simulated mono- and polymicrobial samples. *APMIS*, 119:198-203

II   Tano, E., Hyldén M.-O., Melhus, Å. Overrepresentation of the toxic shock syndrome toxin-1 gene among elderly men with bacteremic bone and joint infections caused by *Staphylococcus aureus*. *Manuscript*

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

IV   Tano, E., Melhus, Å. (2014) Level of decontamination after washing textiles at 60°C or 70°C followed by tumble drying. *Infect Ecol Epidemiol,*4:24314

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Related paper

Goscinski, G., Tano, E., Löwdin, E., Sjölin, J. (2007) Propensity to release endotoxin after two repeated doses of cefuroxime in an *in vitro* kinetic model: higher release after the second dose. *J Antimicrobiol Chemother,* 60: 328-333.

Contents

Abbreviations

AP-PCR	Arbitrarily primed polymerase chain reaction
ATCC	American Type Culture Collection
BHI	Brain heart infusion
BLAST	Basic local alignment search tool
CC	Clonal complex
CCUG	Culture Collection University of Gothenburg
CFU	Colony forming units
CLSI	Clinical and Laboratory Standards Institute
DNA	Deoxyribonucleic acid
ESBL	Extended-spectrum beta-lactamase
EUCAST	European Committee on Antimicrobial Suscepti-bility Testing
MBC	Minimal bactericidal concentration
MIC	Minimal inhibitory concentration
MLST	Multilocus sequence typing
MRSA	Methicillin-resistant *Staphylococcus aureus*
NCTC	National Collection of Type Cultures
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
PVL	Panton-Valentine leukocidin
SNP	Single nucleotide polymorphism
Spa	Staphylococcal protein A
SRGA	Swedish Reference Group for Antibiotics
SSI	Statens Serum Institut, Copenhagen
SSSS	Staphylococcal scalded skin syndrome
ST	Sequence type
SUH-M	Skåne University Hospital, Malmö
TSS	Toxic shock syndrome
UUH	Uppsala University Hospital
WGS	Whole-genome sequencing

Preface

My first contact with research was a project at the Department of Infectious Diseases at Uppsala University Hospital many years ago. The goal was to develop a method for studying the efficacy of antibiotics during treatment of intracellular bacteria. It was a demanding job and a great challenge, and it sparked my interest in research. Ever since, my main interest has been development of methods and quality assurance of these methods. In time, this interest led to this thesis.

The discovery that bacteria caused infectious diseases was made more than 100 years ago. Over the years, two fields have developed in bacteriology: medical bacteriology and clinical bacteriology. Medical microbiology has a strong research basis, whereas clinical bacteriology has a more applying role, since its primary task is to promptly assist other clinical disciplines with the information needed to diagnose and correctly treat infectious diseases. Since 1978, when I graduated as a biomedical analyst, my base has been in clinical bacteriology. The tasks today are basically the same as when I started. Culturing bacteria is still the most common method, although the molecular biological methods are increasingly used both for typing and for identifying resistance mechanisms or virulence factors.

Apart from performing diagnostics, laboratories at university hospitals, have an important role when it comes to developing, evaluating and implementing new methods. All laboratories should support and guarantee the best possible care, and new knowledge should be usable/applicable and benefit the patients at a reasonable cost.

The optimal situation is a continuous dialogue between the ward and the laboratory, when identifying the cause of the infection and giving treatment advice. This way of working is a necessity in order to improve and develop both laboratory techniques and treatment strategies.

Clinical microbiology reports phenotypic changes in bacteria. Medical microbiology, with their technical resources and specialized knowledge, identifies changes on the genetic level. In recent years, the two fields have come closer to each other due to multiresistant bacteria. The accumulated knowledge provides the foundation or forms the base for new methods and/or changes of laboratory tests which will become tools for improving the future management of patients.

Introduction

Infections

Infections are one of the most common causes of morbidity and mortality in the world (WHO). They are caused by a broad spectrum of microorga-nisms. Although infections with parasites and viruses are the most frequent, bacteri-al infections are the leading causes of hospital stays and medical treatments.

Bacterial infections can be local or disseminated. The spontaneous reco-very rate is high for local infections such as urinary tract infections. With increasing severity of the infections, the need for adequate treatment also increases (Pogue JM *et al.* 2014). To be able to treat a patient adequately, it is necessary to identify the causative agent (Uematsu H *et al.* 2014).

Several different tools can be used to identify bacteria. Most important is the bacterial culture, which makes it possible to perform a susceptibility testing and thereby verify that the chosen antibiotic treatment will be suc-cessful. With the emergence of multiresistant bacteria (Glasner C *et al.* 2013), this testing has become more difficult and more extensive (Baquero F. *et al.* 2014). To continuously improve and up-date methods have become a necessity.

The laboratory testing process

Laboratory services have a major impact on clinical decision-making. It has been estimated that 60-70% of the most important decisions on admission, medication and discharge are based on laboratory test results (Forsman RW 1996). Crucial for all testing processes at a clinical microbiology laboratory is therefore that they are of good quality and never jeopardize the safety of the patients.

Unlike many other activities in a hospital, the laboratory testing processes are well-defined and thereby easier to control. They are usually divided into three phases: the pre-analytical, analytical, and post-analytical. In practice, there is also a fourth phase after the post-analytical phase, when the micro-organisms are destroyed.

Laboratory medicine is far better than clinical disciplines in controlling the quality. In Swedish clinical microbiology laboratories, the quality is reg-

ulated by accreditation. According to the standards used in accreditation, each and every step in the testing process needs to be paid attention to.

The pre-analytical phase

The first phase in a laboratory testing process is the pre-analytical phase. It starts with the selection and order of a test and ends with the acceptance and sorting of the sample by the laboratory. Although limited attention is paid to this step, it is where the majority of laboratory errors occur. In a review by Bonini and co-workers (2002), the pre-analytical errors ranged from 32% to 75% of all errors. Kalra (2004) reported similar pre-analytical error rates but with less variation (46-68%). Most studies on this topic have been performed by clinical chemists and on blood samples, but it seems reasonable to assume that the results would not be much different if they were carried out by clinical microbiologists (Morris *et al.* 2011).

Common pre-analytical errors are inappropriate test requests, order entry errors, misidentification of patients, inappropriate containers, inadequate sample collections/transports, inadequate sample volumes, and labeling errors (Kalra 2004, Hammerling 2012). None of these processes are performed by, or under the direct control of, laboratory staff, why it has been pointed out that there is actually two phases in the pre-analytical phase and not one; a pre-pre-analytical phase and the "true" pre-analytical phase (Plebani 2006).

It has been demonstrated that many of pre-analytical errors can be avoided if laboratory staff and not the clinical staff carry out the collection, identification, labeling, handling and transport of samples (Söderberg *et al.* 2009, Kemp *et al.* 2012). It is not very likely that this will be the future solution of this problem. One way for the clinical microbiology laboratory to reduce some of the pre-analytical errors is, however, to provide the clinical staff with well-functioning transport systems and describe their limitations.

Transport systems

The ideal transport medium should be able to maintain the viability and relative proportions of bacteria in the samples. Furthermore, the sensitivity should not be affected by inappropriate temperatures during storage and transport. Unfortunately, there is no such medium.

For several decades, Stuart transport medium was used as the routine transport medium. This medium was the first for transporting all kinds of clinical swab samples, and it was invented by Dr. R. D. Stuart (Stuart 1959). It is a non-nutritional semi-solid medium, which contains a reducing agent to prevent oxidation and methylene blue as the reduction indicator. It was later replaced by Amies medium, an improved modification of Stuart's original

formula (Amies 1967). Today, there are several types of transport media, and some of them are specifically developed for certain species or types of bacteria (Rubin *et al.* 2008, Stoner *et al.* 2008, Hirvonen *et al.* 2014). See Figure 1. As the automation of clinical microbiology laboratories increases, the need for liquid transport media will also increase (Mischnik *et al.* 2012).

Tissue biopsy is considered the gold standard to diagnose wound infections, but it is hardly ever used. For the collection of non-liquid samples, swabs have been the most frequently used tool in health care settings for collection of most types of secretions. It may not always be the best approach (Rondas *et al.* 2013), but swabs are usually inexpensive and easy to use.

The ideal swab should enhance the sampling and allow for release of a sufficient representative portion of the specimen material. In late years, it has also become more important that the swab can preserve the integrity of the DNA for molecular techniques (Chernesky *et al.* 2006). The swabs have consequently been improved. Initially, they were cotton-tipped and with or without charcoal. Nowadays, the cotton is usually exchanged for calcium alginate, Dacron polyester, rayon or flocked nylon.

Figure 1. Examples of different transport media for microorganisms.

Bacteria used when testing transport systems

When evaluating transport systems, there are certain bacterial properties that need to be tested more than others. To remember every aspect is difficult, but due to the procedure described by the Clinical Laboratory Standards

Institute, M40-A, the evaluation has become standardized (CLSI 2003). This standard makes it also easier to compare the results of different studies over time and space.

Some bacteria are extremely fastidious. To the more fastidious belong the anaerobic species *Fusobacterium nucleatum* and *Peptostreptococcus anaerobicus*. Their susceptibility to oxygen makes it difficult to transport them more than 6-8 h. Even strictly aerobic bacteria, such as *Neisseria gonorrhoeae*, can be difficult to transport for longer periods than 24 h. They are therefore often included when evaluating new transport systems.

Bacteria can also differ when it comes to their ability to cause infections. *Haemophilus influenzae*, *Neisseria meningitidis*, *Streptococcus pyogenes*, *Staphylococcus aureus,* and *Escherichia coli* are all leading causative agents of septicemia. Clinical microbiology laboratories must therefore to be able to isolate them and follow any change in their virulence or antibiotic resistance.

The opportunists *Enterococcus faecalis, Enterococcus faecium, Klebsiella pneumoniae* and *Pseudomonas aeruginosa* are often involved in healthcare-related infections and have a high ability to develop multiresistance. They are common findings in polymicrobial cultures (Mattera *et al.* 2014) and survive easily in different transport systems. Especially the Gram-negative bacteria have a tendency to overgrow other species. Polymicrobial samples are not included in the current standard.

The analytical phase

Most of the time in a clinical microbiology laboratory is dedicated to the analytical phase. It starts when the sample is prepared for testing, and ends when the results are verified and interpreted. It has been stated that "quality design in a laboratory must begin with analytical quality because it is the essential quality characteristic of any laboratory test; unless analytical quality can be achieved, none of the other characteristics matter" (Plebani 2006).

In this phase, the clinical laboratories usually excel, and in clinical chemistry laboratories the overall analytical error rate can be as low as 0.1% (Sakyi A *et al.* 2015). The microbiological literature on this topic is quite scarce. Since the techniques differ too much, it is impossible even to speculate what the error rate might be in bacteriological test processes.

A major part of the processing of a bacterial sample consists of identifying the bacteria and testing their susceptibility to antibiotics. Since several exotoxins are associated with particular disease symptoms, it is not always enough to identify a bacterium to the species level. If it, in addition, causes an outbreak, it is necessary to type the causative agent epidemiologically. For several of these analyses, new techniques have been developed in recent years.

Staphylococcus aureus

S. aureus is a Gram-positive, coagulase-positive, facultative anaerobe that has its ecological niche in the anterior nares. See Figure 2. About 30-40 % of adults are carriers (Kenner *et al.* 2003, Sakwinska *et al.* 2010, Legrand *et al.* 2015), and colonized persons have a higher risk of developing staphylococcal infections. Even if the bacterium can act as a harmless commensal, it is a leading cause of a wide spectrum of infections worldwide, ranging from benign folliculitis to acute endocarditis, septicemia, necrotizing pneumonia and TSS. *S. aureus* was declared to be the most significant cause of serious infectious diseases and infectious disease deaths in the United States 2005 (Klevens *et al.* 2007).

S. aureus is one of the first bacteria for which antibiotic resistance was reported (Abraham 1940), and the penicillinase-production spread rapidly among *S. aureus* strains. During the 1940s and 1950s, *S. aureus* became multiresistant and a major nosocomial problem (Thornsberry 1988). This problem was initially solved by the use of methicillin and similar semi-synthetic penicillins. However, in the 1960s MRSA emerged (Parker and Jevons 1964). The resistance to methicillin is encoded by the *mecA* gene, which changes one of the penicillin-binding proteins (Hartman and Tomasz 1984).

Since the 1980s, *S. aureus* has been a common cause of healthcare-associated infections. Today, the isolation frequency of MRSA exceeds 25% in many countries, and most isolates are community-acquired (Chen and Huang 2014, Williamson *et al.* 2014, Sanders and Garcia 2015). Resistance to vancomycin, one of the last line drugs, has occurred more than once (Gardete and Tomasz 2014). With few treatment options, the mortality rates increase (Dolapo *et al.* 2014), but hopefully they will not reach staggering 75-83%, like they used to do during the pre-antibiotic era (MacNeal and Frisbee 1939, Mendell 1939).

S. aureus is an extremely successful pathogen. Its variability is well-documented, but, interestingly, most STs can be found in only a few clonal complexes or lineages that are distributed globally. To the dominating line-ages belong CC1, 5, 8,15, 22, 30, 45, 59, 80, 97 and 121(Nübel *et al.* 2011).

Most strains produce virulence factors associated with adherence, tissue destruction, spreading, iron uptake, lysis of host cells, and manipulations of the host's immune responses. Genes coding for these virulence factors are usually found in the core genome, but there are also mobile genetic elements (e.g. bacteriophages, plasmids pathogenicity islands, staphylococcal chromosomal cassettes, and transposons). About 15% of the genes of a *S. aureus* strain are found on these elements, and they encode antibiotic resistance and some very potent exotoxins causing SSSS, necrotizing pneumonia and TSS (Grumann *et al.* 2014). PCR or other molecular methods are used to detect these genes.

Figure 2. S. aureus cultivated on a blood-agar plate.

Exfoliative toxins A and B

The two exfoliative or epidermolytic toxins (ETs) A and B are two serine proteases, which selectively hydrolyze desmoglein-1 (Bukowski *et al.* 2010), a transmembrane protein responsible for the integrity of adhesive structures between cells. These toxins are directly responsible for bullous impetigo and SSSS, infections characterized by skin exfoliation. The typical SSSS patient is a neonate or an infant. After a short period with fever, malaise and lethargy, the child develops an erythematous rash and bullae. When the bullae break, the affected area(s) will be unprotected by epidermis like burn wounds, with risks of dehydration and secondary infections.

Less than 5% of *S. aureus* isolates carry the *eta* and/or *etb* genes (Becker *et al*. 2003), but the frequency increases if the isolates are methicillin-resistant (Koosha *et al.* 2014).

PVL

PVL is a toxin composed by two parts, LukS-PV and LukF-PV, which are encoded by two corresponding genes. The two components are secreted from the bacterial cell, before they induce lysis by assembling to a pore-forming heptamer on the membranes of monocytes, macrophages and neutrophils (Kaneko and Kamio 2004). In contrast to ETA and ETB, the role of PVL in staphylococcal infections has been controversial (Ellington *et al.* 2007, Shallcross *et al.* 2013). There is, however, a clear link between PVL and necrotizing pneumonia (Diep *et al.* 2010*,* Löffler *et al.* 2013). This toxin may also be involved in skin and soft tissue infections, including furunculosis, abscesses, carbuncles, especially if they are chronic or recurrent (Masiuk *et al.* 2010, Mine *et al.* 2011), and necrotizing fasciitis (Miller *et al.* 2005).

In a British study from 2005, less than 5% of the *S. aureus* isolates harbored PVL genes (Holmes *et al.* 2005). In recent years, the PVL genes have been identified as a stable marker of community-acquired MRSA strains. The methicillin-resistant and PVL-producing clone ST8/USA300 predominates in the USA, whereas the corresponding clone in Europe is ST80 (Holmes *et al.* 2005, Vourli *et al.* 2009).

TSST-1

The superantigens of *S. aureus* belong to the most potent T-cell mitogens known. Typical for superantigens is that they can directly activate up to 20% of the T-cells (Proft and Fraser 2003), resulting in a massive release of pro-inflammatory cytokines.

There are 23 staphylococcal superantigens described, of which TSST-1 is one of the more important ones. It has been associated with sepsis, acute endocarditis, pneumonia, and osteomyelitis. It is also one of the causative agents of TSS, one of the most well-recognized diseases caused by superantigens. In the 1980s, this syndrome became associated with tampons with a high absorbency (Davis *et al.* 1980). There are, however, cases of non-menstrual staphylococcal TSS, and they can occur in connection with almost any type of staphylococcal infection. TSS is characterized by high fever, scarlet fever-like rash, peeling of skin, vomiting, diarrhea, and hypotension. Multiorgan failure often follows and sometimes death (Spaulding *et al.* 2013).

TSST-1 is encoded by the *tst* gene. This gene can be found in nasal isolates from healthy carriers in up to 25% (Tenover *et al.* 2008), and it is harbored mainly by strains belonging to CC30/USA200 (Spaulding *et al.* 2013). In contrast to the PVL genes, the *tst* gene is not associated with MRSA.

Bacterial susceptibility to antimicrobial substances

Appropriate use of antimicrobial substances is a challenge in today's health care. Reliable and reproducible testing methods are necessary to provide the physician with the information he or she needs to make the right decisions. The susceptibility testing is considered to be one of the most important analyses performed in clinical microbiology laboratories.

Various methods can be used to test the antimicrobial susceptibility. In Swedish clinical microbiology laboratories, disc diffusion and gradient diffusion predominate. Commercially available systems such as VITEK2 (bio-Mérieux) are also used but to a lesser extent. These methods have all their advantages and drawbacks, but none of them are the gold standard. The broth dilution is, but it is usually avoided in clinical settings due to its labor intensity.

When testing the susceptibility, the isolate is classified as either susceptible or resistant on the basis of clinical breakpoints. These breakpoints are derived from a combination of data concerning MICs, pharmacokinetics, pharmacodynamics and clinical outcomes. The clinical breakpoints are recommended by mainly EUCAST and CLSI (EUCAST, CLSI 2015).

There are only clinical breakpoints for antibiotics and not biocides. Biocides are chemical agents that are capable of destroying microorganisms, and their use has increased in late years. In health care they are widely used as disinfectants and preservatives, but they can also be found in several consumer products. Their use is not without problem. In a recent report from the European Commission, it was suggested that biocides may have a role in the development of antibiotic resistance (SCENIHR 2009). One biocide which has grown in popularity is silver.

Silver

Silver is a non-essential heavy metal with toxic effects on both bacteria and humans (Drake and Hazelwood 2005). Before the 20th century it was used for several conditions, including leg ulcers, sexually transmitted infections, and warts (Landsdown 2002). With the introduction of antibiotics, its use diminished rapidly. During treatment of burn wounds in the 1960s it came back in the form of silver sulphadiazine cream (Silver 2003). Its true renaissance was, however, at the turn of this century, when silver-based dressings appeared. Soon a number of medical devices were impregnated with silver, and it did not take long before silver-products were available for the ordinary consumer. Today, silver in different forms can be found in textiles, washing machines, tooth brushes, telephones, shoes, etc., and its wide use has been questioned (Silver 2003).

The antibacterial action of silver has not been fully elucidated, but silver ions seem to interfere with the DNA replication and processes taking place in the cell wall/membrane. They can also inactivate proteins (Landsdown 2002, Percival 2005). Initially, it was suggested that bacteria had a low propensity to develop resistance to silver, but there are several reports indicating something else (Percival 2005). The genetic background to the silver resistance, the *sil* operon, has been described by Gupta and co-workers (1999). The operon consists of silver- binding proteins (SilE and presumably SilF), efflux pumps (SilABC and SilP) and regulator proteins (silS and silR). See Figure 3.

The genetic resistance is not always expressed phenotypically, but without any breakpoints it is not easy to tell if an isolate is resistant to silver or not. A possibility is to compare the silver nitrate MICs of a range of bacteria with the silver concentrations that can be reached locally in clinical situations. Another is to use an epidemiological cut-off. It corresponds to the upper MIC value of the wild–type distribution, and it separates wild-type

strains from those with acquired resistance to the antimicrobial substance that is tested (EUCAST).

In addition, a proper medium for the susceptibility testing has to be found; the silver ions may react with the proteins and the sodium chloride in the medium, resulting in inactive silver precipitates (Landsdown 2002).
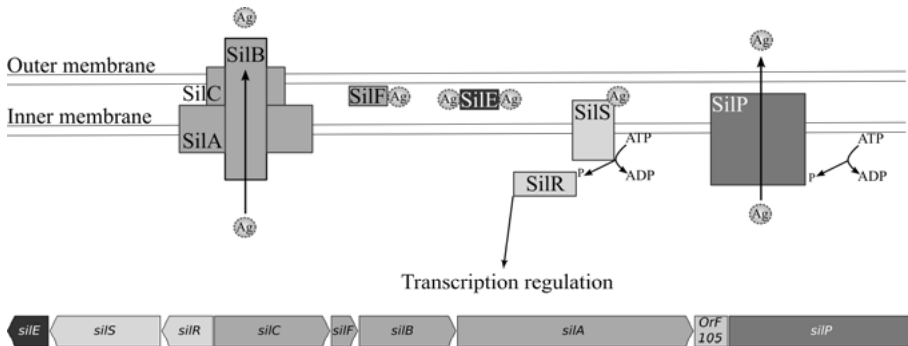

Figure 3. The *sil* operon and its proposed transcriptional products. Top: proposed function of genes from the *sil* operon (adapted from Gupta *et al.* 1999, Randall *et al.*2015). Bottom the *sil* operon of plasmid pUUH239.2.

Epidemiological typing methods

Molecular typing of bacterial isolates is necessary when following the epidemiology of highly virulent or multiresistant bacteria and when tracking outbreak strains and their sources. The choice of an appropriate molecular typing method depends significantly on the problem to solve and the epidemiological context in which the method is going to be used, as well as the geographical scale and time of its use (Sabat *et al.* 2013).

It is no longer enough to keep a local record of multiresistant strains; some of them have such a high epidemic potential that they are pandemic (Nübel *et al.* 2011, Mediavilla *et al.* 2012). For early warnings, international networks are required, and for this the produced data must be portable, i.e. easily transferable between different systems. The typing method must also be robust, standardized, and applicable for not just a few important pathogens but for a broad range in both human and veterinary medicine (Liu *et al.* 2015).

PFGE

PFGE has been considered to be the gold standard for epidemiological typing of most bacteria, and it has been in clinical use since the 1990s (Arbeit *et al.* 1990). The technique uses restriction enzymes (usually SmaI) to digest the chromosomal DNA in an agarose plug. The DNA fragments are thereafter separated by electrophoresis to produce a band pattern. The discriminatory power of PFGE is quite good, and it has a high epidemiological concordance. Over 90% of the genome is addressed, but the technique is time-consuming, demanding and labor-intensive. Its portability is limited, and the results are usually slightly subjective.

AP-PCR

Random amplification of polymorphic DNA (RAPD), or the variant AP-PCR, is much faster and easier to use than PFGE. It is also less expensive, but it has problems with the reproducibility and it is not portable (Power 1996).

Spa-typing and MLST

A more reliable method is the single locus sequence typing (SLST). In Sweden, the most widely used method in this group is spa-typing. It is based on the sequence variation in the C region of the *spa* gene (Frénay *et al.* 1996). It has a lower discriminatory ability than PFGE, but it is cost-effective. It also has a high reproducibility, a standardized international nomenclature, and a stability which makes it useful on a local, national and international level (Grundmann *et al.* 2010). Some types are, however, misclassified due to recombinations and homoplasies.

In MLST, six or more housekeeping genes are added in the analysis. After the genes have been amplified and sequenced, each allele is assigned an arbitrary number. Based on the combination of identified alleles, the ST is determined. MLST has the advantages of SLST, but it is much more expensive, takes more time, and the discrimination is sometimes insufficient. It is, however, the most widely accepted method for describing *S. aureus* epidemiology, especially if it is combined with staphylococcal cassette chromosome *mec* typing (Chua *et al.* 2014). Both spa-typing and MLST have the benefit of being able to be carried out *in silico*, using WGS data.

WGS

The next generation sequencing instruments have made it possible to explore the whole genome (or about 90% of it) and discover genome-wide variations down to the resolution of a single base pair.

The cost is still high, but the costs and time used for the analysis have been reduced quickly during the last 5 years, making WGS more interesting for clinical applications. In contrast to the other methods, WGS can provide

timely information on genetic relatedness, unique features, pathogenic potential and antimicrobial resistance of an isolate (Price *et al.* 2012). The problem is no longer to produce the sequence data but to interpret it, and it seems likely that this technique will eventually replace several, if not all, techniques mentioned above.

The post-analytical phase - and thereafter

During the post-analytical phase, the clinical microbiology laboratory verifies the results, feed them into the laboratory computer system and produce oral and written reports to the physicians. Like the pre-analytical phase, this is an error-prone phase. Of all the mistakes reported, 9-31% of them take place during this step (Bonini *et al.* 2002). Common mistakes are erroneous verification of analytical data, improper data entry and failure in reporting (Hammerling 2012). Even if the report is correct, it may come too late or never reach the decision-making physician (Kilpatrick and Holding 2001). Furthermore, mistakes can be made by the physician while interpreting and reacting to the laboratory results. None of these mistakes are under the control of the laboratory. This part of the post-analytical phase is therefore sometimes referred to as the post-post-analytical phase (Plebani 2006).

For the clinical microbiology laboratory, the testing process is now over, but for the clinician it is time for decisions and action. When bacteria are involved, multi-targeted approaches are often necessary. These may include antibiotics, vaccinations, and infection control measures.

Laundering is part of the infection control, but although a few percent of the hospital budget is used for decontaminating tons of laundry, not much attention is paid to this process. The literature is correspondingly scarce, and it is often taken for granted that it works despite reduced time, energy, water, detergents and biocides in the washing machines. Once again the clinical microbiology laboratory can assist.

Aims

The overall aim of this thesis was to develop, evaluate and implement methods, from the pre-analytical phase to the final destruction of bacteria, for diagnostics and research in clinical microbiology.

Specific aims

I   To evaluate three systems for transportation and recovery of important clinical bacteria, using simulated mono- and polymicrobial samples kept at room temperature for up to 48 h.

II   To establish methods for detecting the toxin genes *eta*, *etb*, *lucS-PV-lucF-PV* and *tst,* and to use these methods for investigating the frequencies of these toxin genes in relation to patient age, gender, diagnosis and over time among invasive isolates from two Swedish tertiary hospitals.

III To study how silver-based wound dressings affected the bacterial flora in chronic leg ulcers, and to develop a method to monitor changes in the susceptibility to silver nitrate.

IV   To develop a method to simulate the laundry process of hospital textiles, and to apply the method in evaluating the decontamination efficacy of the laundering process at two different washing temperatures followed by tumble drying.

Material and Methods

Bacterial strains (I-IV)

All used reference and control strains are presented in Table 1. In addition, clinical isolates from the Departments of Clinical Microbiology at UUH and SUH-M, Sweden, were used as follows: In **study II**, 528 clinical isolates of *S. aureus* from blood cultures were investigated, 374 were obtained from UUH and 154 from SUH-M. In **study III,** 56 isolates of different species collected from patients with chronic leg ulcers under treatment with silver-based wound dressings were included. Two culture collection strains and twelve clinical strains isolated at UUH and SUH-M were added during the *in vitro* experiments.

All bacterial isolates were stored at -70°C in a glycerol solution (10%).

Table 1. Included reference and control strains in the four studies.

Strain	Study	Comments
E. coli ATCC25922	I, III	
E. faecalis CCUG9997	I	
E. faecium NCTC7171	IV	
F. nucleatum ATCC25586	I	
H. influenzae ATCC10211	I	
K. pneumoniae CCUG54718	III, IV	Multiresistant, ESBL+
N. gonorrhoeae ATCC43069	I	
N. gonorrhoeae CCUG41810	I	
N. meningitidis ATCC13090	I	
P. aeruginosa CCUG17619	I	
P. anaerobius ATCC27337	I	
S. aureus ATCC29213	I	
S. aureus CCUG35601	I, II	MRSA
S. aureus CCUG47167	II	PVL+
S. aureus NCTC8325	II	PFGE control
S. aureus S913	II	TSST-1+
S. aureus SSS10937	II	ETA+/ETB+
S. pyogenes ATCC19615	I	

Preparation of inocula (I, IV)

Study I: A 0.5 McFarland standard inoculum (approximately 1.5 x 10^8 CFU/ml) of each organism was prepared. The inocula were diluted to yield a working concentration of about 1.5 x 10^7 CFU/ml. An aliquot of 100 µl of each bacterial suspension was used to inoculate the swabs, which were thereafter directly placed into their transport containers. Polymicrobial inocula were prepared as described above, with the exception that two pure bacterial suspensions were mixed 1:1. The following strains were mixed: *E. faecalis* (CCUG 9997) and *E. coli* (ATCC 25922); *P. aeruginosa* (CCUG 17619) and MRSA (CCUG 35601); *S. aureus* (ATCC 29213) and *S. pyogenes* (ATCC 19615)*.

Study IV: A single colony from an overnight culture of either *K. pneumoniae* CCUG54718 or *E. faecium* CCUG33573 was inoculated in BHI and incubated 5 h at 35°C to obtain a bacterial concentration of 10^8-10^9 CFU/ml. One ml of each bacterial suspension was used in the heat tolerance experiments. For the laundry process, all test samples (10 x 10 cm pieces of cloth, Figure 4) were inoculated with 1 ml of the enterococcal suspension.

Figure 4. Inoculation of test samples with an enterococcal suspension.

Preparation and amplification of DNA (II, III)

In paper II DNA was extracted from the bacteria using Amplicore Respiratory Preparation Kit (Roche), and in paper III by heating a bacterial suspension for 10 min at 95°C. PCRs were thereafter run. Amplified products were separated by electrophoresis in agarose gels. See Figure 5.

Figure 5. Amplified PCR-products of the *tst* gene separated in an agarose gel. Line 3, 5 and 8: products from different *S. aureus* isolates; line 12: molecular size marker.

Determination of the viability of bacterial cells (I, III, IV)

The bacterial suspensions were serially diluted in PBS. At least three samples (10 or 100 µl) from the original bacterial suspension and/or dilutions of it were subsequently spread on agar plates and incubated at 35-37°C in the adequate environment. After incubating the bacteria for 18-48 h, the CFUs on the plates were counted.

Antibiotic susceptibility testing (II, III)

The antibiotic susceptibility was tested by disc diffusion (Oxoid) as recommended by SRGA or EUCAST (after 2011). See Figure 6.
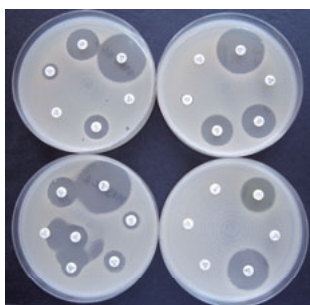

Figure 6. Antibiotic susceptibility testing of an ESBL-producing *E. coli* strain.

Determination of silver nitrate MICs and MBCs (III)

Silver nitrate MICs were determined by broth macro-dilution (Figure 7) according to the SRGA guidelines. Duplicates were used in all experiments. A silver nitrate MIC >512 mg/l classified the bacterium as silver-resistant.

For determination of MBCs, viable cell counts were performed on bacterial suspensions in test tubes with concentrations equal and above the MIC (no visible growth). The MBC corresponded to the lowest concentration that killed 99.9% of a bacterial inoculum.
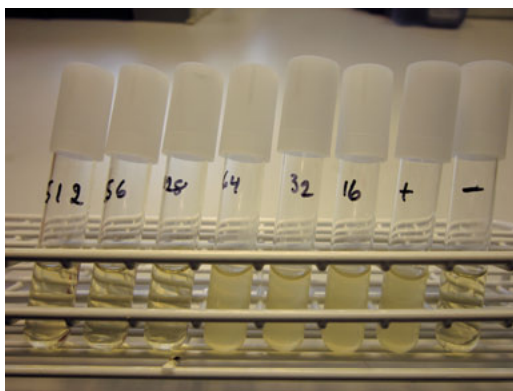

Figure 7. Determination of silver nitrate MIC.

Exposure of bacteria to silver *in vitro* (III)

To induce silver resistance, 1 CFU of an isolate was inoculated into IsoSensitest broth. After an overnight incubation at 37°C, 10 µl of the bacterial suspension was transferred into a series of tubes with increasing concentrations of silver nitrate (8-512 mg/l). The tubes were incubated overnight, and a new inoculum was taken from the tube with the highest silver nitrate concentration with visible bacterial growth. The experiment was repeated until MIC was >512 mg/l or 10 passages had been performed.

Stability control of the silver resistance was carried out by subcultivating the bacteria on plates without silver and controlling that they were still able to grow in silver nitrate concentrations >512 mg/l after each passage.

Epidemiological typing

AP-PCR (III)

AP-PCR was used to compare bacterial isolates with the same origin and phenotypic characteristics over time. The technique is similar to that used when running a conventional PCR, but there are some important exceptions: the primers are unspecific and of short length, a single primer is used in each reaction mix, and the annealing temperature is low (in this case 36°C). The PCR-products were separated by gel electrophoresis.

PFGE (II)

This method was used for comparison of *S. aureus* isolates PCR-positive for the *tst* gene. Agarose plugs with genomic DNA were prepared. The DNA was digested with *Sma*I. Restriction fragment length polymorphism was detected with the Gene Path Electrophoresis System (BioRad, USA). Band patterns were analyzed with BioNumericsSoftware.

WGS (II)

The genomes of eight randomly chosen *tst*-positive *S. aureus* isolates were sequenced on IonTorrent™ with a read length of 400 bp. The reads were assembled into a draft genome using the AssemblerSPAdes plugin in TorrentSuite 4.2. MLST and spa-typing was carried out using BLAST and online databases.

Heat tolerance (IV)

To control the heat tolerance, inocula of *K. pneumoniae* CCUG54718 and *E. faecium* CCUG33573 were exposed to 65°C and 85°C in a thermo-block. Viable counts were performed at different time intervals. The enterococcal strain was used to further explore its survival in temperatures between 60-90°C in up to 30 min.

Processing of test samples during laundering (IV)

Fourteen test samples were used in each experiment. Four were used as controls, while the remaining test samples passed through the washing process. In the first 10 experiments, the test samples were washed at 70°C, tumble

dried and transported back to the laboratory. In experiments 11-13, the test samples were excluded from the drying process.

Within 20 h after completion of the laundering, the test samples were examined. Stomacher plastic bags with the test samples were filled with peptone water and processed in the Stomacher® 400 (see Figure 8). The samples were removed, and the suspensions were vortexed and cultured directly or filtered and then cultured.

After the first 13 experiments, ten more followed. In these latter ten experiments, three changes were made: the laundering process took place at another professional laundry, the temperature was lowered to 60°C and the tumble drying process was excluded for 5 test samples in all experiments.

Figure 8. Stomacher® 400 used for processing the test samples in Study IV.

Results and discussion

Study I

The ability to identify the causative agent of an infectious disease relies to a large extent on good sampling technique, good transport conditions and good laboratory practices. During the 1950s Stuart developed a semi-solid medium, which was later modified by Amies. In recent years, a liquid variant of this latter medium has been developed. All these changes are adaptions to a centralization, and recently also an automation, of the clinical microbiology laboratories (Bourbeau *et al.* 2013)

The initial plan for this study was to evaluate a new variant of Amies medium (M40) without charcoal, using Stuart medium with at charcoaled swab as a comparator. Just before the study was initiated, the prototype for the ESwab was presented by Copan, and it was included in the study. This transport system consists of Amies medium in the liquid form and a nylon-flocked swab. Both lack charcoal.

Swab systems are usually used in areas where contaminations and polymicrobial findings are common. Pure cultures are therefore not the rule. In the M40-A protocol, only monomicrobial samples are used. To explore the maintaining ability of the transport systems under more realistic conditions, polymicrobial samples, simulating common findings in infected wounds, were added. The experiment was pushed even further by keeping the samples at room temperature and not refrigerated as recommended.

After 24 h of simulated transportation, all added bacteria were recovered from all transport systems but at different rates. M40 and Amies broth showed similar results and outperformed the SSI transport medium (Stuart medium).

Both Copan systems promoted growth of *H. influenzae* and *S. pyogenes*. Furthermore, Amies broth was able to act as a growth promotor for *P. anaerobius*. The SSI transport medium was the best maintainer of *N. gonorrhoeae* ATCC 43069. Similar results were obtained after 48 h, except for the fact that none of the Copan transport systems could maintain the viability of *N. gonorrhoeae*. This was the reason why a second strain of *N. gonorrhoeae* was included in the study. The experiment was repeated with two gonococcal strains at three different inoculum concentrations. The results showed that the recovery was strain and concentration dependent.

Noteworthy is that the oldest transport system, SSI's, showed the best results where *N. gonorrhoeae* was concerned. The results are maybe not entirely unexpected, since the bacterium was widely used during the development of this transport medium (Stuart 1959). The new Amies broth exhibited, however, such poor results for *N. gonorrhoeae* that it should not be used for samples in which gonococci can be a part, before more extended studies have shown that it is OK to use.

When less fastidious bacteria were mixed, the bacterial numbers increased up to 220 times after 24 h, and the replication continued for another 24 h for the Gram-negative bacteria. This can be a problem, especially when fast growing bacteria are present together with fastidious bacteria. The fastidious bacteria may be overgrown and thereby difficult to find. Uncompensated multiresistant bacteria can be slow growers (Foucault *et al.* 2009, Linkevicius *et al.* 2013), and it has been suggested that their impaired growth can make them harder to find.

Due to how swab systems are used, it is essential that they work for polymicrobial samples. The most problematic species in polymicrobial samples was *P. aeruginosa.* Overgrowth of this species has been reported with several types of swab transport systems (Rishmawi *et al.* 2007, Morosini *et al.* 2006). Transport and storage at +4°C can reduce the overgrowth, (Van Horn *et al.* 2008).

To summarize, none of the tested systems was able to maintain both the viability and the relative proportions of bacteria present in the samples after 24 h. Prolonged periods of transportation should therefore be avoided. When selecting a swab transport system, consideration must be given to the sample type, the conditions that prevail locally, and the performance in a clinical setting.

There are lots of things that could be improved in the pre-analytical phase. It is possible that the bacterial culture only reflects a minor part of the microbiome present. It would therefore be of interest to study this further, and to study how different bacteria require different transport conditions.

During the last year the development in clinical laboratories has been rapid. M40 was designed for situations when Gram-staining directly from the swab was desirable. Already, it is outdated, and the focus has moved on to liquid media that are compatible with robots for specimen inoculation (Bourbeau *et al.* 2013) and with different types of instruments for molecular applications (Silbert *et al.* 2014).

When evaluating these new media, it is important that not only monomicrobial samples are used. The test situation should resemble the reality, and polymicrobial samples are thereby a necessity. Furthermore, many reference strains are adapted to the life in a laboratory. Their ability to survive differ-

ent conditions might differ from that found in newly isolated clinical strains. Clinical strains should therefore also be added in evaluations.

There are signals that the frequencies of gonococcal and pneumococcal infections are decreasing with the new liquid media. These signals are only coming from the laboratories and not the clinicians, which is a warning sign. CLSI released a new edition of M-40-A in July 2014, M-40-A2. This update includes guidelines for evaluating liquid transport media with accompanying swabs (CLSI 2014).

Study II

Two important discoveries, both rewarded with the Nobel Prize, made this study possible: the knowledge that DNA codes for all proteins an organism produces, and the development of the PCR technique (Hongbao 2005). It was later followed by different sequencing techniques that allow(ed) the DNA codes to be identified (França *et al.* 2002, Quai *et al.* 2012, Grada *et al.* 2013)

S. aureus is a leading agent of several infectious diseases, and its ability to cause disease is a result of the virulence factors it produces (Lowy 1998). Some of the genes encoding the more potent virulence factors are carried by mobile elements (Grumann *et al.* 2014). They are therefore not found in every strain, but it could be advantageous if these genes were identified early in the infectious process in order to improve the outcome. A physician would not hesitate to prescribe both penicillin and clindamycin to a patient with TSS caused by *S. pyogenes*. For a patient with a staphylococcal TSS it is not quite as natural for unclear reasons. The positive effects of the combined treatment would, however, probably be the same. Detection of the toxin gene in question would support the clinician when making treatment decisions.

Many published papers describe methods that can be used for identifying staphylococcal toxins and their respective frequencies. Few studies go further and explore the clinical significance of these toxins in severe infections. Apart from implementing the method at the clinical microbiology laboratory at UUH, the purpose of this study was to examine how common the presence of ETA/ETB, PVL and TSST-1 was in blood isolates during the years 2000-2012 in relation to patient data.

A preliminary study was conducted on all *S. aureus* isolates collected at SUH-M during the years 2000-2003. The results indicated that the *tst* gene was most prevalent, and that it was present in isolates from patients with osteomyelitis or septic arthritis. The study continued thereafter at UUH, where a total of 1,640 blood cultures with *S. aureus* isolates were identified during the time period 2000-2012. For cost reasons, only a selection of these isolates was analyzed (see Figure 9).
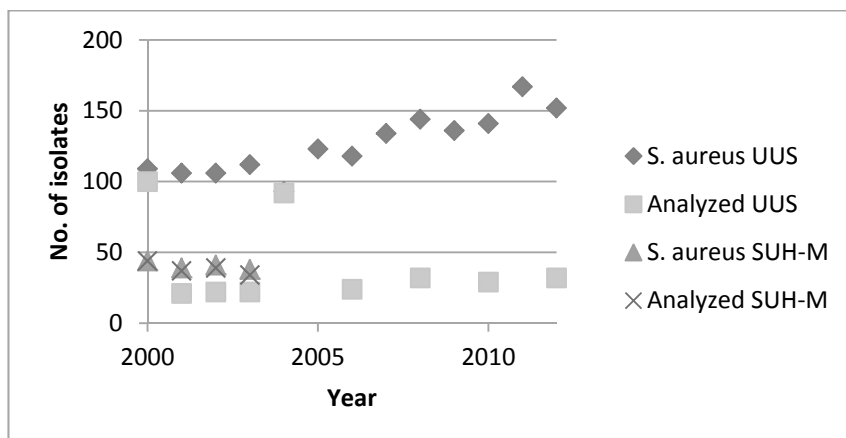
Figure 9. The total number of S. *aureus* in blood cultures and the proportion of these included in the study.

While working with the isolates, it was noticed that some of them lacked the *nuc* gene. This gene was used to control that the bacterium was correctly identified and that the DNA extraction had worked. *Nuc*-negative isolates were re-analyzed after new DNA preparations, but a few isolates remained negative and had to be excluded. *Nuc*-negative S. *aureus* isolates have in late years become a problem, especially if they are methicillin-resistant (van Leeuwen *et al.* 2008).

Even in the UUH material, the *tst* gene predominated. Resistance profiles were studied and all isolates except one, a methicillin-resistant PVL-producer, were fully susceptible to the tested antibiotics. These findings were expected and in accordance with other reports.

The genetic relatedness of the *tst*-positive isolates from both hospitals was examined. A great similarity between the band patterns of the isolates from the two hospitals was observed, although no connection in time or space could be shown. Was it due to the method used?

PFGE is best suited for comparison of strains in local outbreak situations, but it has problems with the resolution for species with a high degree of clonality. Furthermore, for long-term studies over larger geographic areas, MLST is usually preferred (Peacock *et al.* 2002). Recently the ability to analyze bacteria by WGS became possible. With this technique a large number of genes can be studied simultaneously without any specifications, and several epidemiological typing methods based on sequencing can be applied and compared (Leopold *et al.* 2014, Bartels *et al*, 2014, Stephen *et al*. 2015).

In this study, the genomes of a selection of isolates were sequenced. The sequence data yielded both the MLSTs and spa-types of the isolates. One isolate was, however, impossible to spa-type due to a recombination. The WGS also offered an opportunity to analyze the SNPs to get an even higher

resolution, but these analyses are not yet finished. The results show that most *tst*-positive isolates in Uppsala and Malmö belong to CC30.

The most problematic part with WGS is not the sequencing but the processing of the sequence data. Without a good dialogue with a bioinformatician familiar with the bacterial genome, it is difficult to extract all the information the sequences can yield.

Finally the patient population was investigated, and the typical *tst*-positive patient was a male aged 55-74 years with a bone or joint infection. The relatively low frequency of toxin-positive *S. aureus* isolates makes it difficult to achieve cost efficiency with a general screening approach. For selected cases with typical symptoms, the detection of staphylococcal toxins can be valuable for optimal treatment and epidemiological reasons.

Study III

Since the discovery of antibiotics in the 20th century, they have been used for treatment of infectious diseases. With the emergence of multiresistant bacteria, the use of biocides such as silver has increased. Even if silver is used in a lot of consumer products and medical devices, the knowledge about the silver effects on bacteria over time and in clinical settings is limited. A study on chronic leg ulcers treated with silver-based dressings was therefore performed.

A major problem was that there were no breakpoints or methods established for testing the susceptibility to silver. In addition, the silver nitrate precipitated in Mueller-Hinton broth and several other types of broths. The only broth that worked was the IsoSensitest broth. Several Gram-positive and Gram-negative strains were thereafter tested in this broth, and all of them had a silver nitrate MIC < 32 mg/l. This became the breakpoint for the wild type bacteria. The breakpoint for the resistant population was set at >512 mg/l. This concentration could not be reached locally by the silver-based dressings, other research groups had used similar levels, and it was so high that there could be no doubt that the bacterium was resistant (Kremer *et al*. 2012, Randall *et al*. 2015).

The effects of silver after three weeks of treatment seemed to be limited, since the flora in the ulci was almost the same after the treatment as before. To control that the same strains were still present, an AP-PCR was carried out. The AP-PCR confirmed the suspicion: the initial bacteria were not eradicated.

The antimicrobial silver effects seemed to be especially poor for primary wound pathogens, i.e. the Gram-positive *S. aureus* and beta-hemolytic streptococci. This finding was investigated further by determining the MBCs for all *S. aureus* isolates and for three Gram-negative strains. For the latter

strains, the silver nitrate MICs were almost the same as the silver nitrate MBCs. However, silver nitrate was not bactericidal for *S. aureus.*

A silver-resistant *E. cloacae,* which also was resistant to antibiotics, was found in one culture after the silver treatment. This finding was the most interesting in the study. Except for a *S. aureus* with resistance to fusidic acid, no other bacteria had a reduced susceptibility to antibiotics. The question was if there could be an association between silver resistance and resistance to antibiotics.

Fourteen strains with different antibiotic resistance profiles were therefore exposed to silver *in vitro*. Five of them developed resistance to silver, and four of them were resistant to cefotaxime and ceftazidime before the silver exposure. One strain exhibited a reduced susceptibility to carbapenems after the exposure.

The stability of the silver resistances was tested by sub-cultivations in silver-free medium, and one isolate lost the silver resistance. This isolate was the only one that had not shown any resistance to antibiotics before the silver exposure. The underlying genetic mechanism for the silver resistance was the *sil* genes in all cases but one. The silver resistance in this isolate was not stable.

The results of this study show that silver-based wound dressings had limited effects on bacteria *in vivo*, and that they can in fact promote development of silver resistance in bacteria. The methods applied are frequently used in different studies on antibiotic drugs. This demonstrates that they can also be useful when studying biocides.

Study IV

Clinical microbiology is usually associated with the identification of microorganisms and detection of their properties, but it also plays an important role in infection control. Contact tracing and decontamination controls would not be possible if the laboratories had not developed methods for these purposes.

Study IV was initiated during a major outbreak with an ESBL-producing *K. pneumoniae* strain (Lytsy *et al.* 2008). At the same time, there was a wish from the laundry industry to achieve an environmentally as gentle process as possible without reducing the hygienic quality. An important factor in this context was the energy costs. They started a discussion about lowering the laundering temperature from 70°C to 60°C. Industry representatives contacted the laboratory at UHH to discuss the impact and possible consequences of a lower washing temperature, as textiles from medical facilities are heavily contaminated with often multiresistant bacteria with high virulence.

The study was performed at two professional laundries, but the first challenge was to select a suitable bacterium that was heat tolerant and easy to identify even when the test samples became contaminated with bacteria from the surroundings. The outbreak strain was too unpredictable, and its disseminating capacity could pose a problem in the laundries if anything went wrong. *E. faecium*, a low-virulent bacterium often used for this type of experiments (Wilcox *et al*. 1995, Orr *et al*. 2002), was chosen instead. Its heat tolerance was equivalent to that of the outbreak strain.

The first part of the study served as a test of how the experimental design worked. The contaminated test samples were handled as much as possible as the ordinary laundry. The results showed a significant reduction of the bacterial load when the samples had passed the entire laundering process, which also entailed tumble drying (Figure 10). This observation led to a modification of the experimental protocol, and half of the test samples were excluded from the drying process. When the washing temperature was lowered to 60°C, the first laundry could not manage it any longer. The second laundry was further away, and as a consequence a courier had to be used.

Figure 10. This washing machine and tumble dryer are placed at the Dept. of Clinical Microbiology at Uppsala University Hospital. They were not used for the study.

The results showed that the decontamination of the test samples was comparable at 70°C and 60°C. The study was, however, small and no other microorganisms were included. To draw any major conclusions is therefore not possible. The most remarkable finding was the impact of the drying process on the final result. Regardless if a textile is tumble dried or ironed, the high temperature used will kill microorganisms (Rutala *et al*. 1997, Patel *et al*. 2006, Lakdawala *et al*. 2011). The results of the study have been used when discussing how and where health care workers' clothes should be laundered, and if an ordinary washing machine is inadequate for washing clothes in old people's homes.

Conclusions

I   None of the tested transport systems were able to maintain both the viability and the relative proportions of bacteria present in the bacterial samples after 24 h. **(Study I)**

II   No transport system works for all situations. **(Study I)**

III   The next standard (M-40-A3) for evaluation and validation of bacterial transport systems needs to include polymicrobial samples that reflect common combinations in clinical samples. **(Study I)**

IV   More attention needs to be paid to the pre-pre-analytical phase in the testing process to improve the quality and the patient safety in the most error prone phase. **(Study I)**

V   Of the studied staphylococcal toxins in blood isolates, TSST-1 was the most frequent, and this was independent of time and space. The typical patient was a male aged 55-74 years with a bone or a joint infection. **(Study II)**

VI   To screen for staphylococcal toxins in blood isolates is only cost-effective if the patient exhibits typical clinical symptoms, there are signs of therapeutic failure, or there is a need for additional epidemiological data. **(Study II)**

VII A well-organized long-term culture collection is a prerequisite for the development, evaluation and improvement of clinical diagnostics in the bacterial field. **(Study II)**

VIII While the PCR technology makes it easy to detect specified bacterial genes, whole-genome sequencing can detect most genes in the bacterial genome and at the same time type the strain epidemiologically. **(Study II)**

IX   The activity of silver-based wound dressings was limited, and important wound pathogens were not eradicated. **(Study III)**

X   Silver may contribute to the selection of antibiotic-resistant bacteria. **(Study III)**

XI   Determination of silver nitrate MIC is a usable method for monitoring silver-resistance, but it is necessary to establish an international test standard with accepted breakpoints. **(Study III)**

XII   A method for simulating a complete laundering process in a professional laundry was developed, and it showed that sufficient textile hygiene was maintained for bacteria when the washing temperature was lowered to 60°C. **(Study IV)**

XIII   For a good result, the washing cycle has to be followed by tumble drying, and the whole laundering process has to be monitored. **(Study IV)**

XIV   Only bacterial aspects were studied in this laundering model. No conclusions can therefore be drawn about how the lowering of the washing temperature affected viruses and fungi. Further studies are needed. **(Study IV)**

Sammanfattning på svenska

Infektioner utgör en betydande orsak till sjukdom och död i världen. Parasiter och virus är de mikroorganismer som orsakar flest infektioner, medan infektioner orsakade av bakterier i större utsträckning kräver sjukhusvård och medicinsk behandling.

Ca 60-70% av all infektionsdiagnostik bygger på laboratorieanalyser. Med en allt mer problematisk resistensutveckling har behovet av en snabb och korrekt bakteriediagnostik ökat. Allt förbättringsarbete inom detta fält är därför viktigt. Syftet med denna avhandling var att utvärdera och införa metoder för diagnostik och forskning inom ämnet klinisk bakteriologi.

I delarbete I studerades hur bakterier i ren- och blandkulturer klarade transport i tre olika transportsystem avsedda för s.k. pinnprover. Provtagningspinnarna inokulerades med en standardiserad mängd bakterier. De bakteriearter som ingick hade speciella egenskaper (krävande, ej syretålande, höggradigt sjukdomsframkallande) och/eller speglade hur vanligt förekommande de är i patientprover. Varje bakterieart/artkombination inokulerades i transportrör och utsattes för en simulerad transport i rumstemperatur som varade upp till 48 timmar. Antalet levande bakterier räknades före och efter 24 och 48 timmars transport. Efter 24 timmar identifierades samtliga tillsatta bakteriearter i alla tre transportsystemen. I två av transportsystemen visade några bakteriearter t.o.m. en tillväxt som vissa fall var 100-faldig. I försöken med blandade kulturer ökade vissa bakterier i antal så mycket att de växte över andra, mindre snabbväxande arter, som därmed blev svåra att hitta. Gonorré-bakterien *Neisseria gonorrhoeae* var den bakterie som hade svårast att överleva transporter som varade längre än 24 timmar. Studien visade att det inte finns ett enda transportsystem som fungerar för alla bakterier i alla situationer, utan man måste anpassa sig efter den kliniska frågeställningen och de förhållanden som gäller lokalt.

Syftet med delstudie II var att sätta upp en PCR-metod för kliniskt bruk för att möjliggöra identifiering av mer aggressiva varianter av det gula varets bakterie, *Staphylococcus aureus*. Denna bakterieart kan bära på flera olika toxiner (gifter) som gör att patientens infektion får ett allvarligare förlopp. Toxiner finns dock inte hos alla isolat, varför syftet var att även kartlägga hur vanligt förekommande olika toxiner är vid bakteriemier (bakterier i blodet). I studien ingick stafylokocker som hade isolerats från blod hos patienter på universitetssjukhusen i Malmö och Uppsala under åren 2000-2003 re-

spektive 2000-2012. Resultaten visade att genen för toxinet TSST-1 var den vanligast förekommande. Detta toxin associerades med den s.k. tampongsjukan under 1980-talet, men det kan förekomma i andra sammanhang. I studien detekterades det framför allt hos isolat från män i åldersgruppen 55-74 år med infektionsfokus i benvävnader och leder. Att undersöka toxingener hos alla *S. aureus*-isolat som laboratoriet hittar i blododlingar är inte möjligt av kostnadsskäl. Om patienten däremot tillhör ovannämnda patientgrupp, kan det vara motiverat att undersöka om toxin-genen finns eftersom det kan påverka valet av antibiotika.

Delarbete III är en studie av hur bakterier i kroniska sår påverkas av silvret som finns tillsatt i vissa typer av sårförband. För detta behövdes metoder sättas upp för att kunna följa en eventuell utveckling av resistens mot silver. Fjorton patienter med kroniska sår behandlades i minst 3 veckor med silverförband. Sårodlingar togs före och efter behandlingen. Antibiotika- och silverresistensen undersöktes både med traditionella resistensbestämningsmetoder och en nyligen publicerad PCR-metod. Bakterieodlingarna visade att silverförbanden hade en marginell inverkan på bakteriefloran. Dessutom framväxte en tarmbakterie (*Enterobacter cloacae*) som hade utvecklat silverresistens efter bara tre veckors behandling. Kompletterande laboratorieförsök visade att silverjoner huvudsakligen hämmade *S. aureus* tillväxt men avdödade gramnegativa tarmbakterier. Dessutom kunde bakterier utveckla silverresistens när de utsattes för ökande koncentrationer av silver, och lättast att utveckla denna resistens hade de bakterier som redan var resistenta mot antibiotika. Slutsatsen drogs att silverförband troligen inte är så effektiva mot bakterier och bör användas med försiktighet inom sjukvården.

I det sista arbetet, delarbete IV, sattes en modell upp för att studera hur bakteriologiskt ren sjukhustvätt blir efter tvättning. Frågeställningen var om tvätten blir lika ren om man sänker tvättemperaturen från 70ºC till 60ºC för att spara energi. Provlappar av tyg förorenades med en känd mängd bakterier. Därefter skickades provlapparna till ett professionellt tvätteri, där tvättning och torktumling skedde. Tvättningen skedde vid de två olika temperaturerna och med eller utan torktumling. Efter tvätt skickades textilierna i retur till laboratoriet för analys av antalet överlevande bakterier. Modellen fungerade bra, och resultaten visade att det blev ingen skillnad i bakteriologisk renhet om tvättemperaturen sänktes från 70ºC till 60ºC. Dock framgick det klart att torktumlingen hade en stor betydelse för att göra provlapparna bakteriellt rena. Hela tvättprocessen, inklusive torktumlingen, behöver därför monitoreras för att man ska få den renhet som önskas hos sjukhustextilier.

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Propensity to release endotoxin after two repeated doses of cefuroxime in an *in vitro* kinetic model: higher release after the second dose

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Objectives: To study endotoxin release from two strains of *Escherichia coli* after exposure to two repeated doses of cefuroxime in an *in vitro* kinetic model.

Methods: Cefuroxime in concentrations simulating human pharmacokinetics was added to the bacterial solution with a repeated dose after 12 h. In another experiment, tobramycin was given concomitantly with the second dose of cefuroxime. Samples for viable counts and endotoxin analyses were drawn before the addition of antibiotics and at 2 and 4 h after each dose.

Results: The propensity to release endotoxin, expressed as $\log_{10}$ endotoxin release (EU)/$\log_{10}$ killed bacteria, was higher after the second than after the first dose, 0.80 ± 0.04 and 0.65 ± 0.01, respectively, in the ATCC strain and 0.80 ± 0.04 and 0.65 ± 0.02, respectively, in the clinical strain ($P < 0.001$). Endotoxin was released earlier after the second dose ($P < 0.001$). Addition of tobramycin at the second dose reduced the endotoxin release in comparison with that of cefuroxime alone ($P < 0.001$).

Conclusions: The propensity to liberate endotoxin is higher after the second dose of cefuroxime than after the first, resulting in a higher release of endotoxin than expected from bacterial count. The release after the second dose can be reduced by the addition of tobramycin.

Keywords: aminoglycosides, *Escherichia coli*, morphology

Introduction

Numerous *in vitro* and animal experiments as well as some clinical studies have shown that endotoxin concentrations increase after antibiotic treatment of Gram-negative infections.[1–4] Antibiotics differ in their capacity to cause endotoxin release depending on their mode of action. β-Lactam antibiotics, acting on the cell wall, lead to a higher endotoxin release than aminoglycosides and other groups of antibiotics affecting bacterial protein synthesis.[4,5] Among the β-lactam antibiotics, there are also differences in capacity to liberate endotoxin depending on their affinities for the various penicillin binding proteins (PBPs) that are located in the cell wall.[1] Furthermore, affinities for PBPs have been shown to be dose dependent.[6,7] Ceftazidime has been demonstrated to bind to PBP 3 at low doses, leading to the formation of long filamentous structures with an increased endotoxin production before lysis. With increasing doses, ceftazidime also has a high affinity for PBP 1, leading to rapid lysis without elongation and with less endotoxin release.[7] Similar mechanisms have been suggested for other cephalosporins, such as cefuroxime, for which higher doses have been shown to free less endotoxin per killed bacterium *in vitro*.[8] It has also been demonstrated that the relation of the affinities affects the length of the rods and subsequent endotoxin liberation.[9] In addition, combination of antibiotics may affect the endotoxin release since tobramycin has been shown to significantly reduce the cefuroxime-induced PBP 3 release.[8]

An important factor for the magnitude of endotoxin release is the number of killed bacteria, as demonstrated in *in vitro* experiments exposing the bacteria to constant concentrations of antibiotics.[8,10] This would imply that clinically the largest amount is liberated after the first dose when the bacterial count is at its highest. However, in a patient reported by Dofferhoff *et al.*,[1] in whom the endotoxin concentrations were repeatedly measured after administration of two doses of a PBP 3-binding antibiotic, it was shown that the free plasma levels increased more following the second dose. From this it may be hypothesized that the increase of endotoxin per killed bacterium might be higher at the second dose. The main purpose of the present investigation was therefore to further explore this and to study the endotoxin

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release from two *Escherichia coli* strains after exposure to two repeated doses of cefuroxime using an *in vitro* kinetic model simulating the human concentration–time profile. In addition, a secondary aim was to study whether the combination with tobramycin at the second dose resulted in a reduction similar to that previously shown when combined with the first dose.

Materials and methods

Cultures and media

Two *E. coli* strains were used in the experiments, a clinical strain isolated from a patient with septicaemia (B049-3036) and a reference strain (ATCC 25922).

Brain heart infusion (BHI) made with pyrogen-free water was used as medium. Prior to each experiment, the test strains were inoculated in a pyrogen-free glass tube containing BHI and incubated for 4.5 h at 35°C, resulting in a logarithmic-phase culture of around 10^8 cfu/mL.

Antibiotics

The antibiotics were obtained as reference powders with known potencies. Cefuroxime was purchased from Glaxo Wellcome AB (Gothenburg, Sweden) and tobramycin from Eli Lilly Sweden (Stockholm, Sweden).

Minimum inhibitory concentrations

MICs were determined in duplicate by 2-fold macrodilution in broth with an inoculum of $\sim 10^5$ cfu of the test strain per mL.

In vitro kinetic model

The *in vitro* kinetic model has been described in detail elsewhere.[11] It consists of a spinner flask with a total volume of 110 mL with a filter membrane (0.45 μm) fitted in between the upper and bottom part, impeding elimination of bacteria. A magnetic stirrer ensures homogeneous mixing of the culture and prevents membrane pore blockage. In one of the side arms of the culture vessel, a silicon membrane is inserted to enable repeated sampling. A thin-plastic tube from a vessel containing fresh medium is connected to the other arm. The medium is drawn from the flask at a constant rate by a pump (type P-500, Pharmacia Biotech. Norden, Sollentuna, Sweden), while fresh, sterile medium is sucked into the flask at the same rate by the negative pressure built up inside the culture vessel. The flow-rate of the pump was adjusted to obtain the desired half-life of the drug. The apparatus was placed in a thermostatic room at 37°C during the experiments.

Experimental procedure

The bacteria were added to the culture vessel at time zero, resulting in a starting inoculum of $\sim 5 \times 10^6$ cfu/mL. Cefuroxime was diluted in PBS to a concentration of 10 000 mg/L, whereupon 0.66 mL of the solution was added to the spinner flask BHI medium. The initial cefuroxime concentration in the vessel was 60 mg/L, corresponding to the free fraction seen after an intravenous dose of 1.5 g which is an often recommended dose in adults with severe sepsis.[12] The half-life of 1.5 h for cefuroxime was chosen in order to simulate human pharmacokinetics.[13] The same dose was added to the vessel through the thin-plastic tube after 12 h when the inoculum had reached a

level approximating that of the starting inoculum. Six experiments were performed with each strain.

In additional experiments, the bacteria were exposed to the same concentrations of cefuroxime but at the time of the second dose it was combined with tobramycin. Tobramycin was diluted in PBS to a concentration of 40 000 mg/L and 0.055 mL was added to the culture compartment resulting in a final concentration of 20 mg/L. The tobramycin concentration was chosen to correspond to the peak value seen after a single dose of 6 mg/kg often given in human sepsis.[14] Two experiments with each strain were performed.

Samples for viable counts were drawn through the silicone membrane before addition of antibiotics and at 2, 4, 14 and 16 h. After dilution in PBS, the samples were seeded onto Columbia agar plates and incubated at 35°C. The samples were serially diluted 10-fold in PBS. At least three samples (10 or 100 μL) from the original bacterial suspension and/or dilutions were subsequently spread on agar plates, incubated at 35°C and counted after 48 h. To avoid antibacterial carryover, all samples were placed on the same spot on the agar plates and the samples allowed to diffuse into the agar for 3–5 min before spreading. If there was, after growth, a clear zone where the sample had been placed, the agar plate was divided into sections and the clear section was excluded from counting. The limit of detection of the viable counts was 1×10^1 cfu/mL. Growth controls were performed assuring normal growth during the experimental conditions up to 4 h when the high number of bacteria interfered with the filter.

Concomitantly with the samples for viable counts, and immediately after addition of the second antibiotic dose, samples were drawn for analyses of endotoxin. Since it has been shown that most of the endotoxin is released within 4 h after antibiotic administration, samples for analyses were not drawn after this time point.[8] In two of the control experiments, samples for endotoxin analysis were obtained at 0, 2 and 4 h.

Determination of endotoxin

Endotoxin-free glass tubes were used for all endotoxin assays, the culture vessel was pre-heated at 180°C and the stirrer was washed in pyrogen-free water. Sterile, pyrogen-free plastic syringes and pipettes were used, and non-pyrogenic filters of 0.45 μm were employed to analyse the free endotoxin. The samples were immediately filtered and kept frozen at −70°C pending analysis. Analyses of endotoxin were performed in duplicate with the limulus amebocyte lysate assay (Endochrome-KTM; Charles River Endosafe, Charleston, SC 29407, USA).

Morphological studies

The morphology of *E. coli* was examined using scanning electron microscopy. For this purpose, a separate experiment was performed with the ATCC strain given the same antibiotic concentrations as those used in the previous experiments and with samples drawn before the antibiotic doses and at 1 and 2 h after each dose. Samples of 10 mL of broth culture were removed and centrifuged at 1400 **g** for 10 min whereupon the bacteria were resuspended in glutaraldehyde 2.5% in 0.2 M sodium cacodylate buffer. The samples were then dehydrated for 10 min periods in increasing concentrations of acetone. Finally, the bacteria were critical point dried and gold-coated in a sputter coater at 10 mA and 1200 V for 2 min. A LEO Gemini 1530 scanning electron microscope at an accelerating voltage of 2 kV was used for examinations.

Calculation and statistics

Reduction in cfu at various time points after the first dose was calculated by subtraction of the remaining number of cfu at 2 and 4 h from that at time zero. Similarly, reduction in cfu after the second dose was calculated by subtraction of the remaining number of cfu at 14 and 16 h from that at 12 h. In the further calculations, the reduction in cfu was considered to be equivalent to the number of killed bacteria.

The endotoxin release was similarly calculated by subtracting the endotoxin values at time zero and at 12 h from those obtained 2 and 4 h later.

The amount of endotoxin that was eliminated by the pump was calculated using the formula of first order kinetics and assuming a linear increase in concentration during the first two hours and between 2 and 4 h after each dose. It has previously been shown that the logarithmic release of endotoxin is proportional to the logarithmic number of killed bacteria.[10] In order to reduce the effect of the variation in viable counts and to relate the endotoxin release to the bacterial killing, the propensity to release endotoxin, expressed as $\log_{10}$ endotoxin release per $\log_{10}$ killed bacteria, was calculated. Values obtained after the first dose were compared in the primary analysis with those obtained after the second dose. This ratio, as well as the logarithmic values of the endotoxin release and the number of killed bacteria, approximated normal distribution. Therefore, in the primary analysis a repeated measures ANOVA was performed. This statistical method was also used in the comparison between the release of endotoxin after the first and second doses and to test the effect of the addition of tobramycin at the second dose. A *P* value of <0.05 was considered significant. All values are expressed as mean $\pm$ SE, except the endotoxin concentrations which were the result of bacterial release, pre-exposure values caused by the preceding growth and elimination by the pump. Endotoxin concentration values are expressed as median and range. The software STATISTICA (StatSoft, Inc., Tulsa, OK, USA) was used in the statistical calculations.

Results

The MIC values of cefuroxime were 2.0 mg/L for *E. coli* B049-3036 and 4.0 mg/L for *E. coli* ATCC 25922, whereas the MICs of tobramycin were 1.0 and 2.0 mg/L, respectively. In the control experiments, the mean $\log_{10}$ bacterial count after 4 h had increased from 6.9 at time zero to 7.9 for the B049 strain and from 6.2 to 7.5 for the ATCC strain. Corresponding endotoxin values were 3600 and 54 000 endotoxin units (EU)/mL, and 330 and 15 000 EU/mL, respectively.

The bacterial killing rates of *E. coli* B049 and *E. coli* ATCC 25922 after exposure to cefuroxime are shown in Figure 1, and the $\log_{10}$ number of killed bacteria at 2 and 4 h after the first and the second dose of the ATCC and B049 strains is demonstrated in Table 1. Irrespective of the bacterial killing rate, more bacteria were killed after the first than after the second dose, due to the somewhat higher number of bacteria at time zero than after 12 h. As expected, most bacteria were killed during the first two hours after administration of the cefuroxime doses. There was a larger variation in viable counts at 12 h than at time zero, and consequently there was also a larger variation in the number of killed bacteria after the second dose.

The endotoxin concentration at various time points is demonstrated in Table 2. There was a large variation, mainly due to the variation in the number of killed bacteria. The reduction in
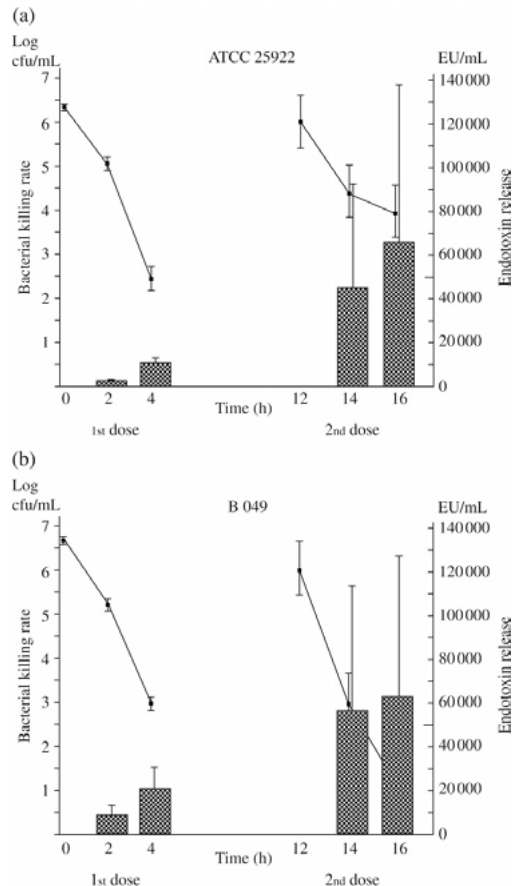

Figure 1. Bacterial killing rate (mean $\pm$ SE) and endotoxin release (geometric mean $\pm$ antilog of the SE of the logarithmic values) after the first and second dose of cefuroxime in the ATCC strain (a) and the B049 strain (b). The lines represent bacterial counts and the bars endotoxin release. Note that considerably fewer bacteria were killed after the second dose due to the higher number of bacteria at time zero than at 12 h.

endotoxin concentration that occurred between 2 and 4 h after the second dose was due to a larger elimination than release during this period.

Table 1. $\log_{10}$ number of killed bacteria after dose 1 and dose 2 in the ATCC and B049 strains

	ATCC	B049
2 h after dose 1	6.27 ± 0.04	6.65 ± 0.04
4 h after dose 1	6.30 ± 0.04	6.66 ± 0.04
2 h after dose 2	6.06 ± 0.62	6.07 ± 0.54
4 h after dose 2	6.07 ± 0.62	6.07 ± 0.54

Table 2. Median endotoxin concentration (range) at various time points in the ATCC and B049 strain solutions

	ATCC (EU/mL)	B049 (EU/mL)
Before dose 1	159 (57–318)	3580 (1530–5320)
2 h after dose 1	1490 (875–2830)	8300 (5140–30 300)
4 h after dose 1	9850 (4890–16 000)	13 700 (6470–40 900)
Before dose 2	2520 (959–6790)	6100 (1240–11 200)
2 h after dose 2	87 400 (4220–219 000)	111 000 (3530–306 000)
4 h after dose 2	51 200 (3240–207 000)	30 094 (1830–142 180)

Table 3. Propensity to release endotoxin expressed as the ratio between the $\log_{10}$ endotoxin release and the $\log_{10}$ number of killed bacteria after dose 1 and dose 2 in the ATCC and B049 strains

	ATCC log EU/log bacteria	B049 log EU/log bacteria
2 h after dose 1	0.52 ± 0.01	0.60 ± 0.02
4 h after dose 1	0.65 ± 0.01	0.65 ± 0.02
2 h after dose 2	0.78 ± 0.03	0.80 ± 0.04
4 h after dose 2	0.80 ± 0.04	0.80 ± 0.04

Geometric mean of the endotoxin release after correction for elimination via the outflow of the pump was at 2 and 4 h after the first dose 1700 and 12 000 EU/mL, respectively, for the ATCC strain and 9600 and 22 000 EU/mL, respectively, for the B049 strain (Figure 1). Corresponding values after the second dose were 45 000 and 67 000 EU/mL for the ATCC strain ($P <$ 0.01) and 58 000 and 63 000 EU/mL (NS) for the B049 strain, respectively.

In Table 3, the propensity to liberate endotoxin is demonstrated. When the endotoxin release was related to the number of killed bacteria, there was a marked reduction in the variation and in both strains. The increase in the tendency to release endotoxin after the second dose in comparison with that after the first was highly significant ($P < 0.001$ for both strains). The time course was also significantly different, with an earlier release after the second dose ($P < 0.001$ for both strains).

After addition of tobramycin to the second dose of cefuroxime, the propensity to release endotoxin was significantly reduced in comparison with that after cefuroxime alone for both the ATCC and the B049 strain ($P < 0.001$ for both strains) (Table 4).

Morphology

Morphological changes are demonstrated in Figure 2. After addition of cefuroxime into the bacterial suspension, an extensive elongation of the bacteria into filamentous forms occurred after 2 h, whereas only minor changes were seen at 1 h. At 12 h, before the second dose, elongated forms were noted among bacteria of normal size that were not seen before the first dose. After the second dose, filamentous forms together with

Table 4. Propensity to release endotoxin expressed as the ratio between the $\log_{10}$ endotoxin release and the $\log_{10}$ number of killed bacteria after addition of tobramycin to the second dose of cefuroxime

	ATCC log EU/log bacteria	B049 log EU/log bacteria
2 h after dose 2	0.56 ± 0.01	0.48 ± 0.02
4 h after dose 2	0.58 ± 0.01	0.47 ± 0.01

spheroplasts were observed already at 1 h. When tobramycin was added, the filamentation was less pronounced.

Discussion

In this *in vitro* kinetic model, in which the concentration–time profile of human serum levels of cefuroxime was simulated, it was demonstrated that the propensity to release endotoxin was higher after the second than after the first dose of cefuroxime for the two *E. coli* strains studied. The difference between the doses ranged from 0.15 to 0.26 $\log_{10}$ EU/$\log_{10}$ number of killed bacteria. This implies that the difference between the doses will be greater at higher numbers of killed bacteria and in effective treatment this will covariate with the bacterial concentration at the time of the dose. With this magnitude of the difference, and provided that 10^6 bacteria are killed, the $\log_{10}$ reduction of viable count has to surpass $\sim$1.1–2.0 $\log_{10}$ cfu/mL if the endotoxin release caused by the second dose should not exceed that of the first one. Alternatively expressed, the endotoxin release after the second dose would theoretically be 8–36-fold higher than that after the first dose provided that 10^6 bacteria are killed on both occasions.

The starting inocula of 10^6–10^7 cfu/mL in our experiments were within the range of the variations found at sites of clinical infections, namely 10^4–10^9 cfu/mL in pus and peritonitis[15] and 10^3–10^9 cfu/mL in meningitis,[16] respectively. Thus, increased propensity to release endotoxin might explain the increased plasma endotoxin concentration observed after the second dose in the report by Dofferhoff *et al.*[1]

A possible mechanism behind the higher endotoxin release after the second dose of cefuroxime might be a continuing release from remaining filaments caused by the first dose. This hypothesis is supported by Jackson and Kropp[6] who found an increased endotoxin release at sub-MIC concentrations of several β-lactam antibiotics that offers an explanation of a sustained release. Our electron microscopy findings just before the second dose may also be in agreement with this. However, at that time, the endotoxin concentration was relatively low, indicating that even if there may be some sustained release, the contribution of this to the total release after the second must be limited. A change in PBP affinity with a higher binding to PBP 3 than to PBP 1 represents another possibility, but the presence of spheroplasts after the second dose does not favour this hypothesis. Thus, the mechanism is not clear, but nevertheless it might be speculated that there is an enduring antibiotic effect on cell wall synthesis after the first dose, resulting in a quicker and
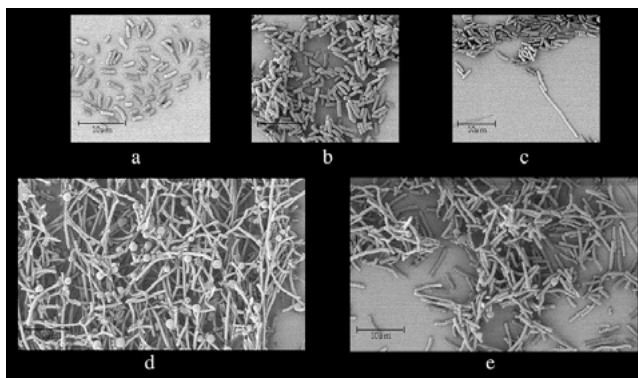
Figure 2. Scanning electron microscope pictures of the *E. coli* ATCC 25922 strain: before exposure to cefuroxime (a), at 1 h after the first dose of cefuroxime (b), at 12 h after the first dose, i.e. just before the second dose (c), at 1 h after the second dose of cefuroxime (d) and at 1 h after the second dose of cefuroxime given in combination with tobramycin (e).

more extensive bacterial elongation after the second dose. Since most of the elongated forms at 2 h after the first dose were eliminated at 4 h, the remaining filamentous structures seen 10 h later may be a sign of disturbed bacterial growth, but this needs further investigation.

In several experimental *in vitro* studies when the bacteria have been exposed to one dose resulting in constant antibiotic concentrations, it has been shown that addition of aminoglycosides lowers the endotoxin release induced by β-lactam antibiotics if given concomitantly.[8,10,17] However, if administration of the aminoglycoside is delayed 2 h this effect is no longer seen.[8] In the present study, however, it was shown that addition of tobramycin reduced the cefuroxime-induced release also at the second dose even if this release was increased and other mechanisms of action may be present. The effect of a repeated combined aminoglycoside β-lactam antibiotic treatment could not be studied because the clinical concentrations used resulted in all bacteria being killed already after the first dose at the inoculum sizes possible in our *in vitro* kinetic model.

The clinical relevance of antibiotic-induced endotoxin release is still under debate.[18] Endotoxin that is released has been shown to be biologically active,[17] but because of the variable levels of endotoxin binding serum proteins, the host response can be markedly altered.[19] Thus, even if it has not been shown to affect mortality in prospective controlled clinical studies, antibiotic-induced endotoxin release may be of importance in the most seriously ill patients with septic shock, in whom further deterioration due to an increased endotoxin load may affect mortality. In order to rapidly reduce the initial bacterial load and reduce endotoxin release, addition of a single large dose of an aminoglycoside has been proposed.[10] The present data suggest that if there is a clinical deterioration after the first β-lactam dose, tobramycin may exert its effects despite preceding high β-lactam concentrations and subsequent sub-MIC concentrations. However, the theoretical advantages of an initial aminoglycoside combination must be weighed against possible side effects and this should be further evaluated in experimental and clinical studies.

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