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The faecal flora: a source of healthcare-associated infections and antibiotic resistance.

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a source of healthcare-associated
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Synen är noll

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På nära håll

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Tage Danielsson: Samlade tankar från roten. 1985-86

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Abstract

Healthcare-associated infections (HAI) are important causes of mortality and morbidity, and antibiotic treatment is often necessary. Development and availability of new antibiotics are closely followed by development of resistance among microorganisms. During antibiotic therapy, a fraction of the antibiotic given is found in the gut. The human gut is an important reservoir of bacteria. Microorganisms residing or passing the gut is referred to as the gut flora or microbiota. The results of this thesis showed spread of *Enterococcus* spp between patients on a general intensive care unit, causing septicaemia. After improved hygiene, reorganisation of rooms and thorough cleaning of the unit, together with revision of antibiotic strategy, the incidence of septicaemia with *Enterococcus* spp fell. Investigation of patients treated for acute intra-abdominal infections showed a shift in the aerobic faecal flora from antibiotic-susceptible *Enterobacteriaceae* spp towards *Enterococcus faecium*, yeasts and species of *Enterobacteriaceae* more resistant to antibiotics, after antibiotic treatment and hospital care. Investigation of recurrent outbreaks of *Serratia marcescens* sepsis in patients admitted to a neonatal intensive care unit showed different clones with each outbreak. Multiple hygiene interventions and revision of antibiotic strategy subsequently obviated recurrent outbreaks of sepsis, but spread of *S. marcescens* was not reduced until compliance with basic hygiene guidelines remained stable above 80%. We also found that low gestational age at birth, ventilator treatment and central venous or umbilical catheters are independent risk factors for late onset sepsis. Investigation of the faecal microbiota in patients with acute appendicitis or diverticulitis revealed that disturbance of the faecal microbiota already existed on admission, with higher numbers of *Enterobacteriaceae* and less *Bacteroides*, *Faecalibacterium*, *Ruminococcus* and *Prevotella* prior to antibiotic treatment and hospitalisation, than the control population. After treatment and hospitalisation diversity increased significantly in the diverticulitis group, approaching the healthy controls in composition.

Sammanfattning på svenska

Vårdrelaterade infektioner är en viktig orsak till sjuklighet och ibland dödlighet inom sjukvården, ca 10 % av ineliggande patienter i Sverige, drabbas av en vårdrelaterad infektion. Ofta är antibiotika behandling nödvändig. Utveckling av nya antibiotika följs ofta av resistensutveckling bland mikroorganismer. Under antibiotika behandling passerar en del av antibiotikan tarmen där det finns och passerar ett stort antal bakterier – tarmfloran även kallad tarmens mikrobiota. I denna avhandling verifierades ett utbrott på en allmän intensivvårds avdelning av Enterokocker. Spridningen skedde mellan patienter, och gav i några fall upphov till blodförgiftning. Efter förbättringar av hygien rutiner, omorganisation av rum och en noggrann storstädning av enheten upphörde blodförgiftningarna med enterokocker. Vid undersökning av patienter som antibiotika behandlats och sjukhusvårdats för akut infektion i buken, visade det att den aeroba tarmfloran förlorade antibiotika känsliga *Enterobacteriaceae* arter och att *Enterobacteriaceae* arter resistent mot fler antibiotika samt *Enterococcus faecium* och jästsvampar tillkom. Undersökning av återkommande utbrott av blodförgiftning med *Serratia marcescens*, på en neonatal intensivvårdsavdelning, visade att spridning förekom inom varje utbrott men att olika bakteriestammar låg bakom de olika utbrotten. Ett stort antal vårdhygieniska åtgärder och ändring av antibiotika strategier förhindrade, allteftersom återkommande blodförgiftnings fall med *S marcescens*. Men spridningen av *S marcescens* på avdelningen minskade inte förrän följsamheten till basala hygienrutiner stadigt låg över 80 %. Kort graviditetslängd, respirator behandling, central venkateter och katetrar i navlekärl är oberoende riskfaktorer för sen blodförgiftning. Vid undersökning av patienter med akut blindtarmsinflammation eller akut inflammerade tjocktarmsfickor framkom att tarmfloran (mikrobiotan) redan var rubbad med ökad förekomst av *Enterobacteriaceae* och minskad förekomst av *Bacteroides*, *Faecalibacterium*, *Ruminococceae* och *Prevotella* arter redan vid ankomst till akutmottagningen, jämfört med friska kontroller. I gruppen med inflammerade tjocktarmsfickor ökade diversiteten och närmade sig kontrollgruppen efter avslutad behandling.

List of Papers

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

Paper I

Samuelsson A, Isaksson B, Jonasson J, Monstein H-J, Berg S. Clustering of enterococcal infections in a general intensive care unit. *Journal of Hospital infection*. 2003;**54**:188-95.

Paper II

Samuelsson A, Isaksson B, Jonasson J, Nilsson L.E, Eriksson O, Chabok A, Hanberger H. Changes in the aerobic faecal flora of patients treated with antibiotics for acute intra-abdominal infection. *Scandinavian journal of infectious diseases*. 2012;**44**:820-7.

Paper III

Samuelsson A, Isaksson B, Jonasson J, Hanberger H, Olhager E. Late onset neonatal sepsis, risk factors and interventions: an analysis of recurrent outbreaks of *Serratia marcescens* 2006-2011. *Submitted*

Paper IV

Samuelsson A*, Wefer H*, Fahlén A, Agréus L, Nixon Andreasson A, Chabok A, Lundin D, Hanberger H, Engstrand L. Disturbed intestinal microbiota (dysbiosis) and micro dynamics in patients treated for appendicitis and diverticulitis. *Manuscript*

*These author contributed equally

Abbreviations

CPAP	Continuous Positive Airway Pressure
APACHE III	Acute Physiology And Chronic Health Evaluation
CRP	C-reactive protein
CT	Computer Tomography
DDD	Defined Daily Doses
EBC	Enterobacteriaceae
ESBL	Extended-Spectrum Beta Lactamses
GICU	General Intensive Care Unit
HAI	Healthcare Associated Infections
ICU	Intensive Care Unit
LOS	Late Onset Sepsis
LPK/WCC	Leukocyte Particle Concentration / White Cell Count
NF	Non-Fermentative gramnegative bacilli
NICU	Neonatal Intensive Care Unit
PBP	Penicillin Binding Protein
PPK	Platelet Particle Concentration
PROM	Premature Rupture of the Membranes
SMI	Swedish Institute for Communicable Disease Control
spp	Species
TISS	Therapeutic Intervention Score System
UHL	University Hospital Linköping

Introduction

Healthcare-associated infections

A healthcare-associated infection is an infection occurring in a patient during the process of care in a hospital or other healthcare facility. The infection was not present or incubating at the time of admission or at the time of visit to a healthcare facility. They may appear in any setting, and may also appear after discharge(1). The source of HAI is either exogenous or endogenous. Endogenous infections may be prevented, for example, by preoperative skin disinfection of the patient, preoperative antibiotic prophylaxis, good clinical praxis and rigid hygiene routines at insertion of urinary catheters and central venous access devices (2). Prevention of endogenous infections is by preventing the patient from being infected by his/her own microbial flora. Exogenous infections are caused by infective agents from the surroundings, including medical devices and medical personnel, where the hands are the most important way of transmission (2). Healthcare-associated infections, including bloodstream infections, are an important cause of mortality and morbidity on the NICU(3-5). According to Borghesi et al. "Immunological immaturity, frequent use of invasive procedures and prolonged hospitalisation account for the high incidence of infections among pre-term infants" (6). Sepsis in new-born infants is often classified as either early onset sepsis (EOS), appearing up to 48 h after delivery and mainly due to bacteria acquired before or during delivery, or late onset sepsis (LOS) appearing 48-72 h postpartum and often caused by healthcare-acquired bacteria(7).

In 1847 Ignaz Semmelweis, at the time responsible for the First Division of Maternity Service at Allgemeine Krakenhaus in Vienna, observed the effect of hand-washing on mortality on the ward.

He observed that women delivered by physicians or medical students had a postpartum mortality rate, due to puerperal or childbed fever, of 13-18%. Women delivered by midwives, however, had a corresponding rate of 2%. The intervention of hand-washing with disinfectant of the hands of physicians and medical students reduced the postpartum mortality to 2 % (8). It was subsequently discovered that the physicians and medical students often came directly from a postmortem having not cleaned their hands in any way. This connection between a hygiene precaution and infection was

established before the theory of “germs” causing infectious diseases by Pasteur in 1870(9), and the experimental proof of ability of bacteria to cause infection by Robert Koch in 1890(10). After the discovery of bacteria and their infective properties, models of transmission could be established.

Transmission

Modes of transmission of microorganisms from person to person are divided into contact transmission, droplet transmission and airborne transmission. Transmission from sources other than infected individuals is associated with common environmental sources or vehicles such as contaminated food or water. The most common way of bacterial transmission in the healthcare setting is contact transmission divided into direct and indirect contact transmission(11). The body surfaces of the healthcare provider that comes into contact with the patient in the care situation are usually the hands and forearms(12). Hand-mediated cross-transmission is a major factor in the current infection threat to patients in hospital (2). Transmission of microorganisms from hands, in the healthcare setting is dependent on five sequential steps:

1. organisms are present on health-care receiver’s skin or on objects or surfaces in the near surroundings;
2. organisms must be transferred to health-care provider’s hands;
3. organisms must be able to survive a sufficient length of time on the healthcare provider’s hands or in the environment, to be able to come into contact with a recipient;
4. inappropriate or lack of cleansing of healthcare provider’s hands between contamination and contact with the next patient or devices;
5. the contaminated hands must come in contact with a healthcare receiver, or surfaces or objects in the near surroundings (13).

1. Infected wounds and eczemas are populated by pathogenic bacteria but even intact human skin is frequently colonised with pathogens such as *Staphylococcus aureus*, *Proteus mirabilis*, *Klebsiella* spp and *Acinetobacter* spp. The most heavily colonised body sites are the inguinal and perineal regions, but the axillae, trunk and upper extremities are also often colonised by pathogens.(13) . Humans shed about 10 000 epithelial particles every minute and around 10% of these particles hold viable microorganisms (14) that consequently contaminate the immediate surroundings. The intestinal tract and its vast content of microorganisms is also an important source of contamination of the environment, where faecal incontinence and diarrhoea facilitate dissemination of pathogens into the healthcare environment .(15).

Hand-touch sites are often contaminated by hospital pathogens such as MRSA, VRE, *Acinetobacter* and *Clostridium difficile* spores (16-19). An increasing number of studies show that cleaning or disinfection of the hospital environment can reduce transmission of healthcare-associated pathogens (20).

2. Healthcare providers contaminate their hands while performing “clean activities” such as taking pulse or blood pressure or lifting patients.(21) Touching the groin of a patient two times for 15 seconds, followed by hand-washing with soap and 5 minutes thereafter manipulating a sterile foley catheter led to *Enterobacteriaceae* being transmitted in 11 of 12 tests (22). Waters et al. investigated the carriage of *K. pneumoniae*, *E. coli*, *P. aeruginosa*, *S. marcescens* and *E. cloacae* on the hands of the nursing staff at a NICU, and infants infected by these agents over a two-year period. In 9% of bloodstream infections, conjunctivitis, skin and soft tissue infections, respiratory tract infections or infections in the central nervous system caused by these agents, PFGE identical clones were found on the nurses’ hands. In this study *K. pneumoniae* and *S. marcescens* were more likely to be shared between nurses and infants (23). Bacterial contamination of hands increases with time in contact with the patient (24, 25).

3. In an experimental model with healthy volunteers Noskin et al. showed that viable enterococcal strains survive up to 60 minutes on gloved or un-gloved fingertips (26). Enterococci were also found on inoculated environmental surfaces up to 1 week, and there was no reduction in colony count during the first 24 h. Fryklund et al. showed that 50% of clinical *E. coli* and *K. pneumoniae* isolates survived on fingertips 6 respectively 2 minutes(27) . Zachary et al. investigated healthcare workers' gowns (umbilical region and cuffs) for VRE after structured physical examination of 49 VRE carriers. Thirty-seven per cent of healthcare workers had VRE on their gowns after the examination (28). This emphasises the importance of bare forearms accessible for disinfection, and the importance of aprons to protect the gown.

4. With inappropriate hand disinfection, hands remain contaminated by potential pathogens and the risk for transmission remains after disinfection. The wearing of rings and artificial fingernails impair the effect of hand disinfection with even more pathogens remaining (29-33). The mode of handwashing and disinfection and the choice of soap and disinfectant also play a part in the reduction of pathogens on healthcare provider's hands (34-36). Nurses uniforms are shown to be contaminated while on duty with *S. aureus*, *Clostridium difficile* and VRE (37) to a large extent. Doctors' long-sleeved coats have been shown to often carry *S. aureus* on cuffs and in pockets after clinical work (38). Staphylococci have been isolated from a large fraction of neck ties worn by physicians on a British ICU (39). In an experimental setting viable *S. aureus* was found on polyester/cotton coats 24 h after contamination. Contamination with *S. marcescens* and *P. aeruginosa* showed viable bacteria when measured at 8 and 2 h respectively (40). Survival of and transmission of pathogens outside the natural environment is essential for spread of healthcare-acquired infections. Long- sleeved uniforms easily get wet during hand disinfection and this provides a moistened environment perfect for the survival of bacteria, but also an interface with correctly disinfected hands.

5. de Vries et al. described long-term carriage of *S. marcescens* on the hands of a healthcare worker with secondary cases of infection or colonisation with the same clone, among patients on a neurosurgical unit. The healthcare worker went on a 3 month leave and *S. marcescens* colonisation disappeared, but on the workers return *S. marcescens* colonisation among patients (41) re-occurred.

Factors that influence transfer of microorganisms from surface to surface are: the type of microorganism; the type of source and destination surfaces; and the moisture level and size of inoculum. Factors that influence the ability of a patient to become colonised by a microorganism are: the presence of medical devices; the disruption of normal mechanical and other host defence mechanisms; patient morbidity; and exposure to medications such as broad spectrum antibiotics(13). Even so, new colonisation with a potential pathogen isn't always followed by a clinical infection. The spread of pathogens such as MRSA in patients appears as colonisation (42) or as life-threatening infections (43). It is impossible to predict outcome of transmission of pathogenic bacteria in the individual case which is why transmission in general must be prevented. Studies have shown that 13-35 % of outbreaks of HAI are due to cross-transmission in the healthcare setting (44).

Preventing healthcare-associated infections

The main goal of infection control is to reduce HAI. Standard infection control measures include the meticulous practice of hospital environment hygiene, hand hygiene, use of personal equipment and safe use and disposal of sharps (2). An inventory of healthcare-related harm and death in Sweden 2008 pointed out risk areas for harm and death related to healthcare, where HAI was of great importance. The following areas of improvement were identified: prevention of urinary tract infections; prevention of central line infections; and prevention of surgical site infections(45).

Outbreak

An outbreak is identified as a temporal increase in infection or colonisation of a specific bacterial species in a distinct population, caused by enhanced transmission. It could be caused by a single epidemic strain or a combination of strains (46). In a healthcare setting an attentive physician or

nurse, or a laboratory worker in the microbiological laboratory is often the first to notice an unusual increase in a usual pathogen, or sporadic cases of an unusual disease. Recognition of an outbreak is the first step in harm limitation. The most important reason to start an investigation of an outbreak is to identify and eliminate any source in order to prevent further cases (47). Since not every transmission of pathogenic bacteria results in an infection, there could be widespread distribution of a microbial agent before it results in a symptomatic infection. This results in non-identifiable potential sources for a new outbreak, for example the discovery of asymptomatic carriers found in MRSA outbreaks or in outbreaks of *Enterobacteriaceae* with ESBL (48-51). In outbreaks of ESBL-EBC and MRSA, the infectious agent is a common pathogen with a striking feature (in these cases the antibiotic resistance pattern) that catches attention, and results in complementary epidemiological typing. Spread of common pathogens with no striking feature rarely leads to epidemiological typing. Consequently small outbreaks of common pathogens with no striking features probably pass by without recognition.

Outbreak interventions

The main principle of coping with outbreaks in the healthcare setting is to identify the causative agent and its way of transmission and, if necessary, revise antibiotic routines. The aim of hygiene measures is to prevent transmission, without knowing whether a patient is harbouring pathogens or not, by assessing each individual for risk-factors that facilitate transmission (e.g. wounds, diarrhoea, exposure to chicken pox etc.)(52).

Basic hygiene guidelines

In Sweden, efforts to curb healthcare-associated infections are based on the concept of basic mandatory hygiene guidelines stipulated by the National Board of Health and Welfare.

Basic hygiene guidelines are designed to be used in all healthcare provider- patient contact situations such as patient examination, patient nursing, and treatment. The guidelines comprise the following points.

- 1) Clothing should be short-sleeved.
- 2) Working clothes should be changed at least every day, more often if dirty.
- 3) Hands and forearms should be free from jewellery such as rings, bracelets and watches.
- 4) Regular disinfection of hands with alcohol-based disinfectants or agent with corresponding effect.
- 5) Disinfection of hands before and after direct contact with each patient.
- 6) If hands are visibly contaminated, hand-wash with water and liquid soap is recommended before disinfection.
- 7) After contact with a patient suffering from gastroenteritis, hand-wash with water and liquid-soap is recommended before disinfection.
- 8) Hands should be dry before hand-disinfection.
- 9) When there is a risk for contamination of working clothes with patient blood, body-fluids, secretion or excretions, disposal plastic aprons or gowns must be worn.
- 10) Disposable gloves must be worn when procedures carry the risk of exposure to blood, body-fluids, secretion or excretions.
- 11) Disposable gloves must be removed directly after each risk moment, and must be changed between procedures.

This list complies with the evidence-based guidelines for preventing HAI in hospitals in England (2).

Human pathogens and commensals

In this thesis I have focused on certain human pathogens because they are frequent causes of infection, or are commensals as natural inhabitants in the human colon or faeces not causing infection. *Serratia marcescens* and enterococci were the focus of my studies as they were the cause of outbreaks.

Enterobacteriaceae

Members of the *Enterobacteriaceae* family are important human pathogens and may cause urinary tract infections, gastrointestinal infections, abscesses, pneumonia, and meningitis. They are glucose fermentative gram-negative rod shaped, and are well represented in the human gastrointestinal tract. *Enterobacteriaceae* account for nearly 50% of septicemia cases, more than 70% of urinary tract infections, and a significant percentage of gastrointestinal infections(53). *Escherichia coli* may cause infection in both normal and compromised hosts and is the most common cause of urinary tract infections and septicemia in the healthcare setting, and is a common agent of intra-abdominal infections (54). Other clinically important members of *Enterobacteriaceae* group, causing extra-intestinal infections are *Klebsiella*, *Enterobacter*, *Serratia*, *Proteus*, *Providentia*, *Morganella* and *Hafnia*(54). The three first-mentioned are often involved in outbreaks of HAI(55, 56).*Serratia marcescens* has constitutional resistance genes and thereby the ability to develop resistance to cephalosporins and penicillins during treatment. *S. marcescens* is a natural inhabitant of soil and water and sometimes found in the human gastrointestinal tract (57, 58)

Pseudomonas, Stenotrophomonas and Acinetobacter

Important human pathogens in this group are *P. aeruginosa*, *S. maltophilia* and *A. baumannii*. These bacteria are opportunistic bacteria that cause infections in the compromised host, often seen in patients at burn units, NICU and ICU. They have intrinsic antibiotic resistance that makes infections difficult to treat.(53) *P. aeruginosa* and *A. baumannii* can survive long time in a moist environment and several outbreaks of HAI with are described.(59-63)

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infections difficult to treat (53). *P. aeruginosa* and *A. baumannii* can survive long periods of time in a moist environment and several outbreaks of HAI have been described (59-63).

Enterococcus, Staphylococcus and Streptococcus

Enterococci are gram-positive facultative anaerobic bacteria that are natural inhabitants of the gastrointestinal and genito-urinary tracts in humans. They can grow and survive for a long time in harsh environments. *Enterococcus faecalis* and *Enterococcus faecium* are frequently found as opportunistic pathogens. Severe infections such as endocarditis and bacteraemia with high mortality rates are not unusual. Enterococci are intrinsically resistant to the inhibitory and bactericidal activities of many commonly used antimicrobial agents, including some beta-lactam antibiotics (53). Resistance to vancomycin and high levels of aminoglycosides is an increasing problem and multi-drug resistant enterococci have emerged as an important cause of healthcare-associated infection (64). Healthcare-associated infection with enterococci is either endogenous or the effect of indirect contact between patients via staff or through medical devices. A contaminated environment may facilitate the spread. A number of investigators have sampled environmental surfaces in units where infected or colonised patients are located (20, 65). Inanimate objects as diverse as electronic rectal thermometers (66) and air-fluidised microsphere beds (67, 68) have been implicated in the transmission of *E. faecium*.

S aureus is an important pathogen causing skin diseases such as folliculitis and furunculosis, surgical site infections, abscesses, osteomyelitis, pneumonia and sepsis etc. (54). Approximately 20% of a population are nasal carriers of *S aureus* and numerous other sites are colonised such as the axillae, groin and gastrointestinal tract. This provides a reservoir from which endogenous as well as exogenous spread may occur (69).

Anaerobic bacteria

In clinically significant anaerobic infections, there is often a mixture of species involved and the relevance of each bacterium is difficult to evaluate. In this thesis they are regarded as members of the human faecal microbiota and are presented in the section on faecal flora. Anaerobic bacteria are often found in abscesses and other intra-abdominal infections (54).

Antimicrobial agents and antibiotic resistance

Since Alexander Fleming discovered the antibacterial properties of penicillin in 1927(70, 71), antimicrobial agents have been used in treating infections and as infection prophylaxis in surgery. The importance of antimicrobial agents in many advanced medical interventions such as organ transplantation, cancer treatment and prosthetic surgery is considerable (72). Development and commercial availability of new antibiotics are closely followed by development of resistance among microorganisms. Early examples of this were sulphonamide-resistant *Streptococcus pyogenes* in military hospitals in the 1930s and penicillin-resistant *Staphylococcus* strains in 1948 (73). Holmberg et al estimated that infection caused by an antibiotic-resistant bacteria doubles the duration of hospital stay, double the mortality, and probably the morbidity, compared with infection with corresponding antibiotic-susceptible infections (74). Bacterial resistance occurs through intrinsic or acquired mechanisms. Intrinsic mechanisms are those naturally occurring in the bacterial chromosome where some beta-lactamases and antibiotic efflux systems are examples. Acquired mechanisms occur as mutations in genes targeted by the antibiotics, or exchange of transferable mobile genetic elements (75). In the following paragraphs antibiotics used in the studies in this thesis are briefly presented with mode of action, antibacterial spectrum and elimination.

Beta-lactams

One of the largest groups of antibiotics is the beta-lactams comprising penicillins, cephalosporins, carbapenems and monobactams. Beta-lactam antibiotics are characterised by beta-lactam ring structure, which is essential for their antibacterial effect. Their action is to inhibit cell wall synthesis by binding to penicillin-binding-proteins (PBP) and block cell wall assembly. Resistance to beta-lactam

antibiotics is gained via four mechanisms; hydrolysis of the beta-lactam ring by beta-lactamase enzymes; reduction in penetration of the antibiotic through the lipopolysaccharide membrane to the PBP; enhanced efflux of the drug from the periplasmic space; and alteration in PBP structure target resulting in reduced binding affinity.(54)

One important emerging group of beta-lactamases are the extended spectrum beta-lactamases (ESBL), where the gene for beta-lactamase production is sited on mobile genetic elements. These mobile genetic elements can cross bacterial species boundaries. ESBL production leads to degradation of cephalosporins beta-lactam (76) and recently carbapenem beta-lactam (77).

Penicillins: the penicillin group includes natural penicillins (e.g. penicillin G), penicillin V, penicillinase-stable penicillin (e.g. isoxasolyl –penicillin), aminopenicillins (e.g. ampicillin) and ureidopenicillins (e.g. piperacillin) (54). Common clinical uses of penicillin include treatment of infections with *Streptococcus pyogenes*, and susceptible strains of *Streptococcus pneumoniae*. Penicillinase-stable penicillins (e.g. isoxasolyl –penicillin) are used to treat methicillin-sensitive *Staphylococcus* infections. Aminopenicillins (e.g. ampicillin) and ureidopenicillins (e.g. tazobactam) have the same spectrum and in addition are active against *Neisseria*, *Haemophilus influenzae*, some *Enterobacteriaceae* spp, and to some extent *E. faecalis*. The combination of ampicillin or piperacillin with the beta-lactamase inhibitor clavulanic-acid and tazobactam respectively (inhibitor of class A beta-lactamases), extends the antibacterial spectrum of these penicillins. The piperacillin-tazobactam combination is used in treating infections caused by *P. aeruginosa* and also has effect against some ESBL-producing strains of *E. coli* and *Klebsiella* spp as well as an effect on anaerobes. Penicillins are mainly eliminated through the kidneys, but a small fraction is secreted in the bile (78). In the case of piperacillin-tazobactam the fraction excreted in the bile, is probably less than 5 % (79).

Cephalosporins: The cephalosporins form a large group with various antibacterial spectra. Due to low toxicity and broad spectrum they are widely used to treat infectious diseases in almost all areas (54). Cefuroxime is active against streptococci and staphylococci (e.g. penicillin-sensitive *S. pneumoniae*, *S.*

agalactiae and methicillin-sensitive *S. aureus*) and are effective against gram-negative bacteria (e.g. *E. coli*, *K. pneumoniae*, *P. mirabilis*, *H. influenzae*)(80). Cefotaxime is active against the same bacteria as Cefuroxime, but has better activity against *Enterobacteriaceae*, including *Salmonellae spp* and *Shigellae spp*(80). Ceftazidime has good activity against *Enterobacteriaceae*, *P. aeruginosa*, *H. influenzae* and *Neisseria spp*, its activity against *Staphylococci* and *Streptococci* is poor(80). *Enterobacter spp*, *Citrobacter spp*, *Serratia spp*, *Morganella spp* and *Providentia spp* are able to develop resistance against cephalosporins during treatment. The mechanism of this “resistance development during treatment” is either up-regulation of existing beta-lactamase production or spontaneous mutation (81, 82). Cefuroxime, cefotaxime and ceftazidime are mainly and rapidly excreted by the kidneys with only a very small fraction secreted in the bile where the concentration is even lower than the plasma concentration (83-85). An exception to this is Ceftriaxone that is slowly excreted by the kidneys with as much as 40-50% of a dose is recovered in the faeces (2, 84)

Carbapenems and monobactams: Carbapenems (e.g. imipenem, meropenem, ertapenem) bind to the PBP of gram-positive and gram negative organisms with high affinity. In gram-negative organisms they are transported via a specific outer membrane protein (OrpD) and have excellent beta-lactamase stability. Their antibacterial activity against gram-positive bacteria as well as gram-negative bacteria is broad and they even have a well-documented anaerobe effect. Imipenem and meropenem are often used in serious and life-threatening infections. Carbapenems often have effect against ESBL or AmpC-carrying *Enterobacteriaceae* with the exception of the emerging problem with ESBL CARBA.(77) Resistance against Imipenem in *Pseudomonas spp* and *Acinetobacter spp* is due to loss of or reduction of outer membrane protein (OrpD) or beta-lactamase production. In *P. aeruginosa* efflux pumps also cause imipenem resistance (78).

Aztreonam is a monobactam, only used in this thesis as a diagnostic disk in cultures, since it is only active against gram-negative aerobic bacteria (54).

Carbapenems are predominantly excreted by the kidneys, but a small fraction is secreted in the bile in a microbiologically active form and passes the gut. About 1-2% of the dose of meropenem administered is found in the faeces(86) but 10% of ertapenem is found in the faeces (87)

Fluoroquinolones

The action of quinolones (e.g. ciprofloxacin) is by blocking DNA gyrase or topoisomerase and thereby transcription of DNA inhibiting bacterial growth. Ciprofloxacin has activity against *Enterobacteriaceae* spp, *P. aeruginosa*, *Acinetobacter* spp, *Campylobacter* spp, *Aeromonas* spp, *Vibrio cholerae*, *H. influenzae*, *Moraxella catarrhalis*, *Legionella pneumophila*, *Neisseria* spp and *Pasteurella multocida*(80). Ciprofloxacin also has activity against gram-negative anaerobic bacteria (78). Clinically important resistance to fluoroquinolones is due to mutations in the bacterial DNA gyrase and/or topoisomerase IV(88). Ciprofloxacin is eliminated via the liver and kidneys (78). After intravenous administration about 15% of the Ciprofloxacin dose is found in the faeces and 60% in the urine (80, 89), after oral administration about 11-30% of the dose is found in the faeces (90).

Aminoglycosides

The aminoglycosides inhibit protein synthesis by blocking the large subunit, 30s, of the bacterial ribosome(78) . Aminoglycosides(e.g. gentamicin, tobramycin) have good antibacterial activity against *Staphylococci* spp, *Enterobacteriaceae* spp , *Pseudomonas* spp and *Acinetobacter* spp (91). Resistance to aminoglycosides is due to a large number of aminoglycoside-modifying enzymes. The genes of such enzymes are often found on integrons and other mobile genetic elements. Acetyltransferases, for example are capable of modifying tobramycin, gentamicin, netilmicin and amikacin. Nucleotidyltransferase alters the activity of tobramycin, and phosphotransferase affect amikacin susceptibility (75). Gentamicin is mainly excreted by the kidney, but a small fraction is secreted in the bile and the mean biliary concentration is usually 30-40% of the serum concentration (92).

Sulphonamides and Trimethoprim

Sulphonamides and trimethoprim act on dihydropteroate synthetase and dihydrofolate reductase respectively, in prokaryotic cells thereby disrupting the purine synthesis. Purine is the building block of RNA and DNA. Trimethoprim-sulphamethoxazole is a combination with activity against *Staphylococcus* spp, *Streptococcus* spp including *S. pneumoniae*, *E. coli*, *Klebsiella* spp, *Enterobacter* spp, *Proteus* spp, *Salmonellae* spp, *Shigellae* spp and *Stenotrophomonas maltophilia* among others(80). Resistance is due to mutations in the enzyme gene reducing the substance's affinity for the enzymes and by a reduction in purine synthesis inhibition (75). Trimethoprim-sulphamethoxazole is also mainly excreted by the kidney and the concentration of trimethoprim in bile is slightly higher than in serum (93).

Nitroimidazoles

Metronidazole has activity against a variety of anaerobic bacteria, micro-aerophilic bacteria and protozoa e.g. *Bacteroides* spp, *Prevotella* spp, *Fusobacterium* spp, *Porphyromona* spp and *Peptostreptococcus* spp (78). The antibacterial and antiprotozoal effect is due to the cytotoxic activity of free radicals (54). Metronidazole is largely excreted by the kidneys, only 13.9% is found in the faeces (94). When given parenterally metronidazole penetrates well into inflamed intra-abdominal organs (95) and when given as prophylaxis preoperatively a high concentration in colonic wall tissue is achieved that is maintained through-out surgery(96).

The faecal flora

The human gut flora is a large dynamic bacterial community, an adult bacterial flora is considered to harbour about 10^{14} microbes, that is ten times the number of cells in the adult human body(97).The community of microorganisms residing or passing the gut could also be referred to as the gut microbiota (98). This thesis focuses on the bacterial content of the microbiota, and the concept microbiota is used to describe the results from 16sRNA gene sequencing. If one considers the different bacterial species in the gut flora as different cell lineages with the capacity to communicate

with one another, the capacity to consume, store and redistribute energy, and the capacity to maintain and repair itself through self-replication, the gut flora may well be regarded as being a microbial organ (99). The majority of gut microbes reside in the large intestine and rectum, where the environment is suitable for a large variety of microorganisms. The gut flora contributes to the body's metabolism by fermentation of non-digestible dietary residues and endogenous mucus. It also salvages energy from fatty acids and produces vitamin K and facilitates the absorption of ions (100). One fatty acid produced in the colon is butyrate, an important energy source for the colonic epithelium (101). The faecal flora also has a protective effect against pathogens, by taking space and producing antibacterial substances and play an important role in the maturation of the human immune system (100).

Colonisation of the human gut with microbes starts immediately after birth. The number of species and the diversity increases during the first year of life. The composition of the flora is influenced by the maternal colonisation, diet, environmental exposure and any antimicrobial therapy (102). It is presumed that this initial colonisation is important in shaping the composition of the gut flora in the adult individual. After the first year of life the microbiota stabilises and resembles that of a young adult. Comparison of faecal samples from monozygotic twins living apart, their marital partners and unrelated individuals implicate that host genotype is of greater impact than the diet in determining the microbial composition of the gut microbiota (103).

Collecting samples from the large intestine is complicated. When collecting samples during gut surgery, the patient is anaesthetised, often has an intestinal disease that has led to the procedure, and has received antibiotics as preoperative prophylaxis. A preferable way of collecting samples is through an endoscope, but this often requires laxative treatment before the procedure, and isn't always suitable in the acutely ill patient, and probably affects the microbiota. Another problem is to maintain an adequate anaerobic sample milieu pending culture, since the vast majority of the

microbiota is anaerobic. Most studies on the colonic bacterial content is performed on faecal samples, studies have shown that the bacterial composition of the large intestine and of faeces is similar (104).

Bacterial genera (groups) in cultured human faeces samples are dominated by the obligate anaerobic genera *Bacteroides spp*, *Eubacterium spp* (and former members of the genus) and *Bifidobacterium spp*. The facultative aerobics are dominated by *Enterobacteriaceae spp*, *Enterococcus spp* and *Streptococcus spp*. Other genera found are *Prevotella spp*, *Clostridium spp*, *Fusobacterium spp*, and gram-positive anaerobic cocci, *Ruminiococcus spp*, *Lactobacillus spp*, *Actinomyces spp* and *Propionibacterium (104)*.

In 16S rRNA gene studies so far, Firmicutes and Bacteroidetes dominate the colon microbiota. Less than 5 % consist of Proteobacteria, Actinobacteria and other phyla together (105).

Bacterial classification and nomenclature

To compare the results between the different methods in my thesis, some bacterial classification and nomenclature aspects should be taken into account.

Early classification systems used morphological, physiological and biochemical criteria to delineate the species of bacteria, these being the typing techniques available at that time. This shows the phenotypic features with no regard of evolutionary relationships. The basic unit in this system was the species. During the 1980's molecular biology developed rapidly, DNA-DNA hybridisation of whole bacterial genomes and sequencing of the 16s or 23s RNA-gene led to a new dimension in taxonomy. Vandamme et al. proposed the term polyphasic taxonomy to approach bacterial classification (106). Polyphasic taxonomy is a synthesis of chemotaxonomic and phenotypic features with phylogeny derived from 16S RNA gene analysis, in consensus. One classification work often referred to is Bergey's Manual of Systematic Bacteriology (107), where this concept is used.

There is no official classification of bacteria but an official nomenclature. Since 1980 priority of bacterial name is based on the approved list of bacterial names (12) and new names are only accepted if they are published in the International Journal of Systematic and Evolutionary Microbiology (IJSEM), published by the Society for General Microbiology (SGM). This is the official journal of bacterial names of the International Committee on Systematics of Prokaryotes (ICSP) of the International Union of Microbiological Societies (IUMS). Prior to 1980, because of the great diversity of bacterial species, duplicity of new species occurred when they were published in different journals using different names at different times. There are also bacteria that cannot be cultured and are thus characterised by microscopic morphology or by differences in molecular sequence.

All living cells are divided between the three domains; Bacteria, Archaea and Eucarya. The domain Bacteria is divided into 23 phyla which are subdivided into 28 classes. Three important phyla containing a great number of clinically relevant bacterial species in humans are Proteobacteria, Firmicutes and Actinobacteria. Other phyla of importance in the human are Bacteroidetes, Chlamydiae and Spirochaetes.

Proteobacteria includes the majority of gram-negative bacteria such as the families: Brucella, Ehrlichia and Rickettsia (Alphaproteobacteria); Burkholderia, Bordetella and Neisseria (Betaproteobacteria); Aeromonas, Legionella, Vibrio and Enterobacteriaceae (Gammaproteobacteria); Campylobacter and Helicobacter (Epsilonproteobacteria).

Firmicutes are gram-positive organisms including Bacillus, Clostridium, Staphylococcus, Mycoplasma, Enterococcus, Streptococcus and Lactobacillus.

Actinobacteria are gram-positive organisms including Bifidobacterium, Mycobacterium and Corynebacterium. The phyla Bacteroidetes include Bacteroides, Flavobacterium and Sphingobacteria (53).

Identification of bacteria at a routine microbiology laboratory is often limited to biochemical tests and standard molecular typing procedures such as 16s rRNA gene PCR with comparison of characteristics to reference types.

Culture methods

During cultivation, bacteria in bacterial samples multiply under different circumstances. Typing is based on phenotype properties such as the ability to grow on various culture media (using different substrate for energy harvesting), antibiotic -susceptibility, growth in various oxygen concentrations or with carbon dioxide in the micro-atmosphere, and the result of gram-staining amongst others (53). With these culture methods the phylogenetic information obtained is minimal and epidemiological information limited.

Molecular typing methods

Molecular typing of bacteria is usually based on 3 consecutive procedures:

- 1) Amplification of a selected region of the bacterial chromosome using PCR
- 2) Sequencing of the amplified segment
- 3) Searching for a similar sequence in a reference nucleotide sequence database(53)

PCR

The polymerase chain reaction (PCR) is a well-established method for multiplying selected DNA sequences. It is based on DNA synthesis driven by a thermo-stable DNA-polymerase as primer for the extension reaction. Apart from DNA-polymerase and a double-stranded template DNA, a mixture of nucleoside triphosphates and two synthetic oligonucleotides with known sequences (the primers) are required. One primer must be complementary to the 3'-end of the + (plus) strand of the intended amplification segment of the DNA template. The other primer must, correspondingly, be complementary to the 3'-end of the – (minus) strand of the template. In the first step of the PCR reaction the temperature is raised above the denaturation temperature of double-stranded DNA (~95°C). The template then becomes single-stranded. In the next step the temperature is lowered to

(~60°C) so that the primers can anneal with the corresponding complementary sequences on + and – strands of the template, respectively. The DNA-polymerase then starts synthesis of a new strand of DNA that is complementary to the template sequence by extending the 3'-end of the primer. Only nucleotides that are complementary to the template DNA strand will be incorporated.

Pyrophosphate is released in the reaction. The high temperature – low temperature cycle is repeated many (25 – 50) times, and for each cycle the number of DNA copies (PCR products or amplicons) doubles (108). Twenty cycles will produce a million copies from each template DNA molecule. The PCR reaction is thus capable of producing sufficient amounts of DNA for sequence analysis even if there were only a few DNA molecules in the sample to start with. The full-length DNA of a bacterium could be used for typing and phylogenetic studies but it is a large molecule and is too time and resource consuming at present. Selected regions of the DNA coding for the small subunit of the bacterial ribosome (16S rRNA) has proved useful in both phylogenetic studies as well as classification at family or even species level (109). The 16S rRNA gene is about 1550 bp long and has a variety of well-conserved regions and regions of hypervariability. The gene polymorphism and the size of the 16S rRNA gene provide a distinguishing and statistically valid measure for bacterial identification (109).

By targeting highly conserved regions with -primers, multiplying and sequencing the highly variable regions of the 16S rRNA gene, and comparing the results with a reference gene database, identification of family or species is possible.

Pyro-sequencing of the PCR product

Pyrosequencing is a widely used technique for determining the order of nucleotides in a DNA strand.

It is based on sequencing a single strand of DNA by the synthesis of a complementary strand as primer extension. In this reaction, the four nucleoside triphosphates (dATP, dCTP, dGTP, and dTTP) are dispensed one at a time in a predetermined order to the reaction vessel, while one monitors the release of pyrophosphate indicating incorporation of the added base. If the dispensed nucleotide is not complementary to the template it will not be incorporated into the newly synthesised DNA

strand and no pyrophosphate will be released. The excess of dispensed nucleoside triphosphate is degraded by the enzyme apyrase before the next base is added for synthesis.

Pyrosequencing may briefly be described in 6 steps.

1. One strand of the PCR product to be sequenced is used as template and hybridised to an oligonucleotide primer. The choice of primer determines the starting point for DNA synthesis. If the whole PCR product is to be sequenced the corresponding PCR primer would be appropriate.
2. The process begins with dispensation of the enzymes: DNA-polymerase, needed for synthesis; apyrase, a nucleoside triphosphate degrading enzyme; and two enzymes used for the detection of pyrophosphate release by giving a chemiluminescent signal. In the next dispensation the substrates adenosine 5' phosphosulphate (APS) and luciferin are dispensed to the reaction vessel, which then contains all the requirements for DNA synthesis apart from deoxynucleoside triphosphates. Thereafter, dATP, dCTP, dGTP, and dTTP are dispensed one at a time in a predetermined order (e.g. cyclical A, C, T, G, A, C, G, T ...). Actually, dATP α S, which is not a luciferase substrate, is added instead of dATP.
3. If the nucleotide added is incorporated into DNA pyrophosphate is released.
4. The released pyrophosphate is converted to ATP in the presence of APS by the enzyme ATP-sulphurylase, providing energy for Step 5.
5. Light is emitted when oxidation of Luciferin is catalysed by Luciferase. The reaction is driven by ATP as the energy source.
6. The light signal is monitored in real-time and correlated with the nucleotide introduced. The signal amplitude is proportional to the amount of nucleotide incorporated. Thus a homopolymer e.g. AA in the nucleotide sequence will appear as a double strength peak

signal. However, the proportionality is not absolute. This makes it virtually impossible to determine the exact length of longer homopolymer sequences e.g. AAAAAA in DNA, which is a weakness of the pyrosequencing method (110) .

DNA sequence databases

Frequently used DNA sequence databases from known bacterial isolates and well-characterised reference strains, as well as bacteria that cannot be cultured are GeneBank (111) , Greengene (112) and RDP-II(Ribosomal Data Project II)(113), where the last two focus on rDNA genes. BLAST (Basic Local Alignment Search Tool, National Library of medicine, US) (114, 115) is a tool for matching the DNA sequence to a known species or family.

UniFrac

UniFrac is a method used for computing differences between microbial communities based on DNA-sequence data (116). UniFrac is a beta-diversity measure that uses phylogenetic information to compare environmental samples. When used with standard multivariate statistical techniques including principal coordinate's analysis (PCoA), it identifies factors that explain differences between microbial communities (117). Such methods are valuable when searching for gradients that affect microbial distribution; gradients could be human disease states (118).

Pulsed-field gel electrophoresis

In pulsed-field gel electrophoresis (PFGE), the whole DNA content of a bacterial isolate, is exposed to a defined restriction endonuclease with six or more base pair recognition sites. The cleavage usually results in fewer than 30 fragments in the size range 20 to 600 kbp. These fragments are separated on an agarose gel by periodically altering the angle of an electric field. The patterns of the DNA fragments are digitalised and computer analysed. PFGE provides isolate-specific bacterial fingerprints

(46). PFGE is resource- and time- consuming; the process takes 2-4 days. Two isolates with no observed difference in PFGE patterns are considered to be indistinguishable, and probably from the same origin within a relevant time span (clone). Isolates differing by one to four bands are considered to be subtypes (46) or, if there is a relevant space and time span, closely related and probably part of the same outbreak (119). When interpreting epidemiological typing results it is important to regard the result in it's time-frame and current epidemiological context. It could also be of interest when comparing current outbreak isolates with previous ones. Epidemiological typing can be helpful when tracing the source or way of transmission of bacteria. PFGE has a high discriminatory power and reproducibility, and is suitable for almost all bacterial species (46).

Aims

The aims of the thesis were:

- To perform an epidemiological investigation of enterococcus septicaemia clustering (Paper I) and *Serratia marcescens* sepsis clustering (Paper III).
- To evaluate the impact of outbreak interventions (Papers I and III).
- To investigate changes in the rectal flora and the emergence of antibiotic resistance in *Enterobacteriaceae* and non-fermentative gram-negative bacteria, in patients with intra-abdominal infection receiving antibiotics (Paper II).
- To study risk factors for *Serratia marcescens* septicaemia (Paper III).
- To investigate the microbial diversity of the microbiota of patients suffering from appendicitis or diverticulitis, and the microbial dynamics after antibiotic therapy and hospitalisation. (Paper IV)

Material and Methods

Study designs

Paper I was an open retrospective descriptive study.

Paper II was an open prospective observational study.

Paper III has two parts, one was an open retrospective observational study and the other was a retrospective case-control study.

Paper IV was an open prospective observational study.

Settings

Papers I and III were conducted on a general intensive care unit (GICU) and a neonatal intensive care unit (NICU), respectively, at the University Hospital in Linköping (UHL). UHL is a tertiary care university-affiliated 780-bed teaching hospital and serves a region in the southeast of Sweden with around 1 million inhabitants. The hospital's intensive care is divided into the GICU, the NICU and several subspecialised ICUs; burn ICU, coronary care unit, neurosurgical ICU, and thoracic surgery ICU, where patients often have critical single organ conditions.

The GICU serves all medical and surgical departments in the hospital and gives care to patients with a range of diseases or multiple critical conditions, including neurosurgical and cardiopulmonary diseases, and children, other than neonates. At the time of the study, 800–950 patients were admitted per year. Approximately 15% were trauma patients and 25% were other emergency admissions. Less than 10% of patients were admitted for planned postoperative intensive care after extensive surgery. The GICU mortality was 4.7–5.5%.

The NICU provides care to approximately 450 infants per year. The unit consists of six to seven cots for full intensive care with ventilation and CPAP treatment, and eight cots for treatment of preterm infants born between 28–32 gestational weeks for treatment of jaundice, feeding problems etc. Most

pregnant women within the region, at risk of delivery of infants born before 29-30 gestational weeks, are referred to Linköping University Hospital. Infants admitted to the NICU are between 23 weeks of gestation to three months of age. About 60 patients need ventilation care and 120 patients need continuous positive airway pressure (CPAP) per year. Between 2006 and 2011; 20 (± 3) infants prior to gestational week 27 (extremely preterm) were born. In the Express study from Swedish NICUs, survival to discharges is 72 % and the incidence of sepsis is around 40 % (5).

In **Papers II and IV**, surgical emergency wards and surgical units from eight hospitals in the mid and south-east regions of Sweden participated; hospital in Norrköping, Västerås, Gothenburg, Örebro, Helsingborg, Uppsala, Linköping and Karlstad.

Identification of outbreak

In **Paper I**, the outbreak was first noticed by workers in the microbiology laboratory in January 1996. They observed enterococci in blood cultures from 5 patients within ten days, all from the same ICU, when there used to be only one case per year. The hygiene department and the ward were alerted.

In **Paper III**, the initial outbreak on the NICU at UHL was first noticed in January 2007 by laboratory workers when they isolated *S. marcescens* from a number of patients over less than a week. Cultures were from more than four patients, including one blood culture. Until 2006 *S. marcescens* was rarely seen on the NICU. No isolates were identified from 2003 until August 2006.

Inclusion criteria cases, control and registered data

In **Paper I** all patients admitted to the ICU from 1 March 1995 to 29 February 1996 were included as study population and all patients admitted to the ICU from 1 March 1996 to 29 February 1996 were included as control population. The characteristics of the two groups were compared according to individual patient disease severity assessed by the APACHE II score(120); the extent of therapeutic interventions assessed by TISS (121, 122); growth on microbiological cultures from the clinical

microbiology laboratory's computer system; antibiotic consumption assessed by delivery data from the hospital pharmacy; ICU occupancy and length of stay on the ICU.

In **Paper II** adult patients aged eighteen and over, admitted to a surgical ward with a suspected diagnosis of complicated intra-abdominal infections requiring surgical intervention were included.

Surgical diagnoses:

- 1) Cholecystitis (including gangrenous) and either rupture, perforation or progression of the infection beyond the gallbladder wall. Surgical intervention or CT/Ultrasound confirmed diagnosis.
- 2) Diverticular perforation/abscess requiring surgical intervention or CT/ultrasound confirmed abscess without surgical intervention.
- 3) Perforation of appendix with local purulent fluid on surgical intervention or abscess confirmed by CT/Ultrasound without surgical intervention.
- 4) Acute gastric or duodenal perforation, only if operated on >24 hours after perforation occurred.
- 5) Traumatic perforation of the intestines, only if operated on >12 hours after perforation occurred.
- 6) Peritonitis due to visceral or other focus of infection (but not spontaneous bacterial peritonitis associated with cirrhosis and chronic ascites).
- 7) Intra-abdominal abscess including liver and spleen abscesses.

Data registered for the study included age, gender, inclusion diagnosis, surgical interventions, discharge diagnosis, co-morbidity, previous antibiotics and travel outside Sweden during the previous 6 months. Antibiotic therapy was chosen by the attending physician according to local guidelines and traditions.

In **Paper III** all patients admitted to the NICU between 2006-01-01 and 2011-10-31 with *S. marcescens* cultures, were included in the outbreak analysis. In the risk-factor study, patients with a discharge diagnosis of sepsis during the same period were included.

The sepsis diagnosis was based on the following criteria:

- a. Growth in blood culture and at least 2 of criteria 1-4
- b. No growth in blood culture and at least 3 of criteria 1-4

Sepsis criteria:

- (1) Leukocyte particle concentration (LPK) <5mg/mL or > 20mg/mL
- (2) Platelet particle concentration (PPK) < 100 mg/mL
- (3) C-reactive protein (CRP) >15 mg/L
- (4) Impaired respiratory function with respiratory rate >70 breath per minute, grunting/gasping or increased ventilatory support in ventilated infants that could not be explained by other factors (123-125).

LPK, PPK and CRP were taken within 24 hours after onset of clinical symptoms. When there were several values, the highest was chosen. Risk factors recorded were: age of gestation at birth and gestational age at onset of sepsis; birth weight; on-going ventilator treatment including nasal intermittent pressure ventilation; CPAP treatment indwelling umbilical vein and/or umbilical artery catheter and/or central venous catheter; premature rupture of membrane (PROM) and maternal fever during delivery (126).

The control case was defined as the first child admitted to the NICU for at least 48 hours intensive care, directly after each LOS-case had been admitted. When several LOS were admitted, an equal number of consecutive children admitted to the NICU for at least 48 h were taken as controls.

In **Paper IV** a sub-population from patients in **Paper II**, patients with a discharge diagnosis of appendicitis or diverticulitis were investigated. Chemical laboratory parameters registered on admission were C-reactive protein (CRP) and white cell count (WCC). Current antibiotic treatment, previous antibiotics, travel outside Sweden during the previous 6 months, age and gender were recorded. Faecal samples from healthy controls were gathered randomly from individuals within the population-based randomised colonoscopy study (Popcol) (127). This cohort has previously been described in detail (128, 129). Briefly, the control population consisted of 33 individuals between 18 and 70 years of age, living within two adjacent parishes in Stockholm and born in Sweden (individuals born abroad were excluded, since gut microbiota is already established during infancy(130)), and who had not been treated with antibiotics within the previous three months.

Bacterial and yeast cultures

Clinical bacterial samples cultured in **Papers I and III**, were taken when clinically relevant, from various sources (wounds, urine, blood, medical devices, tracheobronchial, secretions etc.). In **Paper III** screening samples for *S. marcescens*, were taken from the nose or throat and perineum or rectum on admission, once a week and when a patient was discharged between routine screening days. Rayon swabs with transport medium (Copan, Brescia, Italy) were used.

Rectal swabs from all patients in **Paper II and IV** were obtained on admission (Sample 1) and 2-14 days after the end of antibiotic treatment (Sample 2). Rayon swabs with transport medium (Copan, Brescia, Italy) were used. At the laboratory the rayon swab tips were immediately cut off and immersed in 1.0 ml of freeze broth (Nutrient broth, No 2, Lab m Bury, United Kingdom, containing glycerol 15%) in a sterile glass tube and shaken thoroughly. The freeze broth was transferred to a plastic storage tube and stored at -70°C pending analysis. At analysis the samples were thawed and once again shaken. The rayon swab was streaked over a chromogenic UTI-agar plate (Oxoid, Basingstoke, United Kingdom) and streaked over a second UTI-agar plate using a dish rotator (131). To facilitate identification of different species, antibiotic discs (ampicillin aztreonam, ceftazidime,

cefotaxime and linezolid) were placed on one of the plates. After overnight incubation at 35°C, different bacterial colonies were identified visually. Bacterial isolates considered to be different species or subspecies were identified at species or family level using standard biochemical methods (53). 25 µL of the freeze medium were cultured in Yeast-nitrogen-base- broth (BD, Franklin Lakes, New Jersey, USA) with gentamicin 50mg/L and chloramphenicol 50 mg/L (YNB-broth) and incubated at 35°C until growth or for a maximum of five days. The YNB-broth was streaked over a Sabouraud-agar plate with chloramphenicol 50 mg/L and gentamicin 50 mg/L and cultured two days at 35°C. Colonies growing on the plate were visually inspected and classified as Yeasts after gram staining and microscopy.

Environmental cultures in **Papers I** and **II** were taken, using pre-moistened rayon swabs or convex 6 cm trypticase soy imprint-agar-plates, from medical devices and surroundings wherever persistent *S. marcescens* or enterococci were suspected. In **Paper III** over 200 environmental cultures were taken from stethoscopes, laryngoscopes, thermometers, clean pacifiers, liquid soap, baby oil, ultra-sonar gel, water for incubator humidification, clean articles from the washer-disinfector, resuscitation table, computer keyboard, portable phones, clean laundry, breast pumps, incubators, hands of nursing staff and neonatologists, faecal samples of nursing staff and neonatologist, surfaces in patients room and milk kitchen and surfaces in the delivery room at the operating theatre. The environmental samples and rectal/perineum samples from the staff were analysed using standard biochemical tests (53).

Antibiotic susceptibility testing

Antibiotic susceptibility testing was performed by disc diffusion with or without MIC (Etest AB Biodisk, Solna, Sweden) using methods, breakpoints and sets of antibiotics according to guidelines of the Swedish Reference Group for Antibiotics (SRGA). The isolates were classified as sensitive (S), intermediate (I) or resistant (R).

In **Paper II** isolates defined as I or R showed decreased susceptibility. All aerobic gram-negative rods were tested with imipenem, ceftazidime, ciprofloxacin, gentamicin and piperacillin-tazobactam.

Enterobacteriaceae were further tested with cefuroxime, cefotaxime and trimethoprim-sulphamethoxazole. The dominant bacterial population and also subpopulations of *Enterobacteriaceae* and non-fermentative gram-negative rods were tested for antibiotic susceptibility. Each species was counted once only per patient and sample when measuring distribution of species.

In order to detect emergence of resistance, several isolates per species from each patient were analysed. The occurrence of decreased susceptibility in all of the isolates for each species and patient was measured. Isolates of *Enterobacteriaceae* with decreased susceptibility to cefotaxime or/and ceftazidime were suspected as being producers of ESBL. Phenotypic double-strip Etest (AB Biodisk, Solna, Sweden) with cefotaxime with and without clavulanic acid, ceftazidime with and without clavulanic acid or cefepime with and without clavulanic acid were used to confirm classical ESBL-enzymes such as CTX-M, TEM and SHV designated ESBL_A. Etest with cefotetan with or without cloxacillin were used to confirm plasmid AmpC, designated ESBL_M. When the minimum inhibitory concentration (MIC) ratio of cephalosporin/cephalosporin with clavulanic acid ≥ 8 or a phantom/deformation zone occurred, an ESBL-phenotype was considered. When the minimum inhibitory concentration (MIC) ratio of cefotetan/cefotetan with cloxacillin ≥ 8 or a phantom/deformation zone occurred, an AmpC-phenotype was considered. *Klebsiella pneumoniae* isolates with phenotypic AmpC were considered to have a plasmid AmpC genotype. Isolates with ESBL_A-phenotype were genetically confirmed using PCR as described previously (132) to detect enzymes of CTX-M, and SHV and TEM genotype. *E. coli* isolates with AmpC phenotype were genetically confirmed by multiplex PCR according to Perez and Hanson (133) to verify plasmid mediated AmpC.

Pulsed field gel electrophoresis

Isolates differing by more than three bands were considered to represent different strains in PFGE (119).

In **Paper I** eight bloodstream isolates from six patients, six isolates from other sources (from patients with bloodstream infections or other patients in the same room) and nine surveillance cultures positive for enterococci were chosen for the epidemiological study. Sma I was used as restriction endonuclease and the ethidium-bromide stained separated DNA fragments were compared visually.

In **Paper III** epidemiological typing was performed on *S. marcescens* isolates, 11 isolates from blood and 36 isolates from other sources. The isolates of *S. marcescens* were sent to the Swedish Institute for Communicable Disease Control (SMI) where epidemiologic typing was performed with PFGE using restriction endonuclease Xba 1. The results were compared with all other *S. marcescens* isolates analysed at the SMI.

DNA extraction, PCR and pyrosequencing

In **Paper IV**, DNA from faecal and rectal samples were extracted with Ultraclean faecal DNA isolation (MoBio, Naxo ltd) from 100 mg of each faecal sample or 100 µl freezing media for rectal samples according to the manufacturer's instructions. Final elution was made with 2x50 µl elution buffer (solution S5). For each sample, three 50 µl PCR mixes were prepared containing 1X PCR buffer, 200 µM dNTP PurePeak DNA polymerase (Pierce Nucleic Acid Technologies, Milwaukee, USA), 50 mM of each primer, 0.5 U Phusion F-530L enzyme (Finnzyme, Massachusetts, USA) and 1 µL template DNA. Primer pairs used to amplify the hypervariable 16 rRNA regions V3-V4 were 341f (5'CCTACGGGNGGCWGCAG) with adaptor B and 805r, (5' GACTACHVGGGTATCTA ATCC) with adaptor A, and a sample specific sequence tag of 7 nucleotides as described previously (134). A PCR-negative template control was also made for each primer pair. The PCR conditions used were 95°C for 5

minutes, followed by 25 cycles of 95°C for 40 sec, 58°C for 40 sec, and 72°C for 1 min, followed by a final extension of 72°C over 7 min the faecal samples.

The samples and the negative template control were then run on an agarose gel (1% w/v in TBE buffer) for quality control. The three PCR reactions were pooled, and for the faecal samples 45 µl of each pooled PCR reaction was purified using Agencourt AMPure beads (Beckman Coulter, California, USA) according to manufacturer's instructions with final elution in 1X TE buffer. The purified PCR products were diluted to a concentration of approximately 3 ng/µl before pooling them together for the multiplexed pyrosequencing. All concentrations were determined using the Qubit system (Invitrogen, California, USA) The DNA pools were finally sequenced on the 454-FLX GS-100 using a standard titanium kit (Roche 454 Life Sciences, Branford, CT, USA)(135). Due to the large number of samples, these were split into two titanium runs.

Statistical and data analyses

In **Paper I** the results are presented as mean and standard deviation. Statistical analysis was performed using Student's *t*-test and Fisher's exact test, a *p*-value <0.05 was considered statistically significant.

In **Papers II** and **IV** the aerobic cultured flora was summarised as indicator variables. The indicator variables were the presence or absence of each particular species; quantity was not taken into consideration. The gain or loss of a bacterial species was compared between Samples 1 and 2 using McNemars test and *p*-value < 0.05 was considered significant.

In **Paper II**, the material was divided into a group with Enterobacteriaceae (EBC) and non-fermentative gram negative bacteria (NF). The EBC group included all *Enterobacteriaceae* spp and the NF group included *P. aeruginosa*, *Pseudomonas* spp (*Pseudomonas* other than *P. aeruginosa*), and *Acinetobacter* spp. Each combination of antibiotic and group was analysed separately. The fraction of EBC with decreased susceptibility in Samples 1 and 2 from each patient was calculated. The fraction

of NF with decreased susceptibility in Samples 1 and 2 was calculated for each patient. The fraction of decreased susceptibility was defined as the number of species with decreased susceptibility divided by the number of species found. Finally the mean fractions of species with decreased susceptibility in the study population were compared between Samples 1 and 2 with two-way analysis of variance with time as fixed factor and patient as random factor. P values <0.05 were considered significant.

In **Paper III** continuous variables are presented as median and range (minimum-maximum), and categorical variables are presented as fractions. Differences between groups were compared with simple binary logistic regression and expressed as odds-ratio, with 95% confidence limits, when significant. Significance level was $p < 0.05$. Stepwise logistic regression was performed.

In **Paper IV**, sequences produced by 454 sequencing were processed with the QIIME pipeline (Quantitative Insight Into Microbial Ecology)(129). The sequences were filtered according to quality using the default parameters in QIIME. Sequences with an average quality score <25 or with a length <200 nt were discarded. The remaining sequences were trimmed to remove barcodes and primer sequences. Using the UCLUST(136) algorithm built into the QIIME pipeline, sequences were clustered at 97% identity producing 10238 OTUs (Operational Taxonomic Unit). A representative sequence was chosen by taking the most abundant sequence from each OTU and was assigned a taxonomy using the RDP classifier (Ribosomal Database Project), also built into QIIME, with a minimum confidence of assigning a taxonomy of 80%. The representative sequences were subsequently aligned against the Greengenes 16S gene database using the PyNAST software (137).

Three metrics for alpha diversity were calculated; number of observed species; Chao1 for richness estimation and Shannon index for diversity. These metrics were calculated on a subset of 1210 randomly chosen sequences per sample in order to reduce diversity bias. Five samples containing fewer than 1210 sequences were thus removed from the alpha diversity comparison. Beta diversity measures were calculated on the same sub-sampling level. Using the QIIME pipeline, UniFrac

distances were produced and used to make PCoA (Principal Coordinates Analysis) plots. To analyse diversity differences between the groups, for each metric a p-value was calculated using Wilcoxon's rank sum test in order to investigate the level of significance.

Continuous demographic variables are presented as median and range (minimum-maximum), differences between two groups were analysed using the Mann-Whitney U-test and between three groups using the Kruskal-Wallis test. Categorical demographic variables are presented as fractions and differences between groups were analysed with Chi2-test. $p \leq 0.01$ was considered significant.

Outbreak interventions

In **Paper I** interventions were employed:

- (1) A temporary closure of the unit with thorough cleaning and reorganisation of rooms
- (2) Isolation of infected patients as far as possible;
- (3) More restrictive use of antibiotics;
- (4) Further education of the staff in order to ensure increased awareness of the problems of healthcare-associated infections. Moreover, improved hygienic measures were implemented in close co-operation with the hospital Department of Hygiene.

Reorganisation of rooms consisted of closure of the four-bed room and a new four-bed room with double the floor space was brought into use. This room was organised with all patients along one wall, thereby reducing the risk for healthcare-associated bacterial spread by increasing the distance between beds. The room was used exclusively for patients with no known infections. Four new single-bedded isolation rooms with a separate disinfection room and an ante-chamber were prepared. Furthermore, two additional rooms were made available, both with a separate disinfection room and an ante-chamber and with the possibility for nursing two patients in each. Antibiotic treatments were continually discussed with a consultant from the Department of Infectious Diseases. The overall aim was to reduce usage of third-generation cephalosporins and quinolones together

with increased diversity in choice of antibiotic treatment. The length of each treatment period was decided when it commenced. The use of metronidazole instead of oral vancomycin for the treatment of colitis due to healthcare-associated *Clostridium difficile* was emphasised. The staff were further educated about nosocomial spread of enterococci, particularly modes of transmission. The importance of disinfection of hands with alcoholic disinfectant before and after each patient contact, the use of gloves and gowns and environmental cleaning were strongly emphasized.

In **Paper III** the interventions employed were, concerning; hand hygiene and use of personal protection, hospital environmental hygiene including cleaning and storage of devices and equipment; handling of indwelling venous catheters; and the distribution of patients in rooms:

1. January-March 2007

- 1.1. Isolation of infected/colonised infants.
- 1.2. *S. marcescens* screening of all patients on admission and once a week while on the unit
- 1.3. Disposal of tube feeding syringe after every meal and no re-use, instead of manual washing twice a day in-between use.
- 1.4. Education on basic hygiene guidelines was given to all staff on the unit.
- 1.5. Improved routines for cleaning of incubators, including a checklist for the procedure. All incubators were cleaned with soap and water and, after drying, cleaned with alcohol-based surface disinfectant.
- 1.6. Improved routines for the handling of central venous or umbilical catheters.
- 1.7. Infant pacifiers disposed each day. These were previously disinfected and reused.

2. October-December 2007

- 2.1. Compliance to basic hygiene rules were monitored each month starting in October 2007. (see below).
- 2.2. The staff members were not allowed to use any form of long-sleeved coat during patient care.

- 2.3. New cleaning routines for the water reservoir in the incubators humidifiers, with daily cleaning and disinfection with alcohol-based surface disinfectant. These were previously cleaned twice a week.
- 2.4. New cleaning routines for breast pumps, including alcohol-based surface disinfectant after each use.
3. January-March 2008
 - 3.1. Infant clothing and bed sets were sent to the county's central laundry where cleaning with a quality-assured laundry process is used, instead of being washed at the unit's disinfection room.
 - 3.2. Documentation of the infant's cot placement on the unit in order to facilitate identification of infants at risk when a new case of *S. marcescens* occurred.
 - 3.3. Pasteurisation of donated breast milk.
 - 3.4. Separate milk kitchen staff, not taking part in patient care, working in shifts with no more than one or two persons per shift.
 - 3.5. Updated cleaning routines around each cot including alcohol- based surface disinfection not only when a surface was contaminated but also routinely three times a day between shifts.
 - 3.6. Minimising the use of common material between cots, including separate tables, trash bags, pencils, chairs etc.
 - 3.7. More frequent cleaning of the parents' day-room and kitchenette, a dishwasher was installed.
 - 3.8. Routine use of disposable plastic gowns when in contact with the incubator or bed during patient care.
 - 3.9. Infants younger than 25 weeks gestation with an indwelling umbilical catheter were not allowed to participate in Kangaroo care (holding the infant skin-to-skin beneath clothing of the mother or father).

- 3.10. One person each day was responsible for incubator cleaning.
- 3.11. Incubators cleaned with alcohol-based surface disinfectant after diaper change or other dirty work.
- 4. April-June 2008
 - 4.1. Checklists for cleaning routines for all technical equipment with date and signature for each cleaning procedure.
 - 4.2. First-line antibiotic treatment was adjusted according to MIC results of the *S. marcescens* isolate. See antibiotic strategy for sepsis below.
 - 4.3. One ICU room dedicated to care of infants 27 weeks of gestation and younger, with separate staff. When the infant became 27 weeks, they were moved to another ICU room.
- 5. July-September 2008
 - 5.1. New incubators, with a separate closed humidification system brought into use.

Antibiotic strategy for sepsis prior to April 2008

Benzympenicillin in combination with tobramycin were given to all new-born infants requiring treatment for infection, and as prophylaxis to all children, regardless of gestational age, who received an indwelling umbilical vein and/or umbilical artery catheter and/or central venous catheter. It was also given as first-line therapy in sepsis. Second-line sepsis therapy consisted of cefotaxime with or without vancomycin depending on suspicion of catheter-related sepsis or not. As third-line therapy meropenem was used.

Antibiotic strategy for sepsis after April 2008

Benzympenicillin in combination with gentamicin was given as first-line treatment and prophylaxis. When symptoms of emerging sepsis occurred, and gram-negative sepsis was suspected in the extreme preterm infant, meropenem was chosen as first-line treatment.

Monitoring compliance with basic hygiene guidelines

Seventeen to twenty members of staff were observed each month as regards compliance in the care situation; the personnel observed were unaware of being observed. The use of hand-disinfectants, use of gloves, use of plastic apron, absence of wrist watch, ring or bracelet on hand and forearm, presence of short fingernails without nail polish were all noted. If all conditions were fulfilled, then compliance with basic hygiene guidelines was considered complete. Compliance to dress code was observed and registered in the same way. Short-sleeved work dress provided by the unit, and short hair or hair prevented from falling onto the patient, were considered correct. The percentages of staff with correct compliance with basic hygiene guidelines and compliance to dress code were calculated separately. Feedback on the percentage compliance was given to the staff each month.

In figure 1 the ICU stay and results of PFGE on 7 blood-isolates and one catheter isolate are shown. Patients 1 and 5 had the same clone (Bm) of *E. faecium*, as two patients had in bile and urine samples, respectively. Patients 3, 5 and 6 had the same clone (As) of *E. faecalis* in blood samples and this clone was also found in three surveillance samples (from a warming-ceiling, a cleaned mattress and hands of staff). One patient had the same clone (Cm) of *E. faecium* in a wound, as was found in one surveillance sample from the handle of a digital thermometer (taken approximately one month after the patient died). The remaining surveillance isolates were not found to be identical to any patient sample, but eight surveillance samples with *E. faecium* were of the same clone (Am).

Paper II

Samples from 140 patients (70 women and 70 men) were investigated in the final analyses. Surgery was performed in 83 patients (59.2%) the most common procedure being appendectomy (44; 31.4%) followed by colectomy and cholecystectomy (13; 9.3% respectively). Fifty-seven (40.7 %) patients were not operated upon. Eighty-two (59%) patients had a concomitant diagnosis. Co-morbidity was dominated by hypertension 34 patients (24%), diabetes 11 patients (8%), malignancy 6 patients (4%), asthma 6 patients (4%), chronic arthritic disease 5 patients (4%) and chronic obstructive pulmonary disease 4 patients (3%). Twenty-seven patients (19%) had received antibiotic treatment within six months preceding inclusion, and 34 patients (24%) had travelled outside Sweden. Data was missing for 8 patients regarding previous travel and antibiotic treatment.

Antibiotic treatments in the carbapenem group included imipenem, meropenem and ertapenem. Correspondingly, cefotaxime, cefuroxime, ceftibuten and cefadroxil were used in the cephalosporin group. The quinolone group included levofloxacin, moxifloxacin and ciprofloxacin.

Ninety-five of 140 patients (67.9 %) received a combination of antibiotics with frequent changes. Uniform treatment was defined as receiving antibiotics from one antibiotic class only or one combination of antibiotics the whole treatment episode. The median length of antibiotic treatment was 10 days with a range of 1 to 95 days. Of all patients, 8 (5.7%) received a carbapenem, 8 (5.7%) piperacillin-tazobactam, 23 (16.4%) received a combination of cephalosporin and metronidazole, 4 (2.9%) received fluoroquinolone treatment as a single treatment or in combination with metronidazole, 1 patient (0.7%) each received a cephalosporin or a combination of cephalosporin and aminoglycoside as only treatment.

Culture results are shown in Table 1. The three most common aerobic bacteria isolated in Sample 1 were *E. coli* (90.0% of all patients) followed by *E. faecalis* (57.9%) and yeast (31.7%). In Sample 2 yeast was the most common isolate (70.7% of the patients), followed by *E. faecalis* (64.3%) and *E. coli* (57.9 %.)

Other differences observed between Samples 1 and 2 were a decrease in *P. aeruginosa* (16.4% in Sample 1 and 5.7% in Sample 2), an increase in *E. faecium* (21.4 and 50.0%, respectively) and *Enterobacteriaceae* not further typed (3.6 and 10.7%, respectively).

There was a significant loss of *E. coli* and *P. aeruginosa* and a significant gain in *E. faecium* and yeasts between Samples 1 and 2. The group of *Enterobacteriaceae*, which were not identified further with routine laboratory tests, also gained in numbers.

Table 1 Number and proportion of the 140 patients colonised with different species at the two sampling times.

Species	Sample 1		Sample 2	
	Number of patients colonised	Proportion of patients colonised (%)	Number of patients colonised	Proportion of patients colonised (%)
<i>Escherichia coli</i>	126	(90.0)	81	(57.9)
<i>Enterococcus faecalis</i>	81	(57.9)	90	(64.3)
Yeast	44	(31.7)	99	(70.7)
<i>Klebsiella pneumoniae</i>	36	(25.7)	47	(33.6)
<i>Enterococcus faecium</i>	30	(21.4)	70	(50.0)
<i>Citrobacter species</i>	23	(16.4)	38	(27.1)
<i>Klebsiella oxytoca</i>	23	(16.4)	29	(20.7)
<i>Enterobacter cloacae</i>	23	(16.4)	21	(15.0)
<i>Pseudomonas aeruginosa</i>	23	(16.4)	8	(5.7)
<i>Pseudomonas species</i>	6	(4.3)	6	(4.3)
<i>Enterobacteriaceae species^a</i>	5	(3.6)	15	(10.7)
<i>Morganella morganii</i>	5	(3.6)	1	(0.7)
<i>Acinetobacter species</i>	4	(2.9)	7	(5.0)
<i>Stenotrophomonas maltophilia</i>	4	(2.9)	4	(2.9)
<i>Proteus vulgaris</i>	3	(2.1)	6	(4.3)
<i>Enterococcus species non faecium, non faecalis</i>	3	(2.1)	1	(0.7)
<i>Enterobacter agglomerans</i>	2	(1.4)	4	(2.9)
<i>Proteus mirabilis</i>	2	(1.4)	3	(2.1)
<i>Providentia species</i>	2	(1.4)	3	(2.1)
<i>Serratia species</i>	1	(0.7)	4	(2.9)
<i>Gram-negative bacteria^b</i>	1	(0.7)	2	(1.4)
<i>Kluyvera species</i>	1	(0.7)	1	(0.7)
<i>Vibrio vulnificus</i>	1	(0.7)	0	(0.0)
<i>Hafnia alvei</i>	0	(0.0)	2	(1.4)
<i>Enterobacteriaceae species</i>	136	(97.1)	108	(77.1)
<i>Pseudomonas species /Acinetobacter species</i>	30	(21.4)	21	(15.0)

^a *Enterobacteriaceae* species not further typed

^b Gram-negative bacteria not *Enterobacteriaceae*.

The gain or loss of a bacterial species was compared between Samples 1 and 2 using McNemars test.

The total number of species present was tested with the pair-wise student's t-test.

P < 0.01 was considered significant and marked as bold numbers.

Development of antibiotic resistance is shown in Table 2. The mean fraction of *Enterobacteriaceae* with decreased susceptibility to cefotaxime per patient, increased from 0.357 before antibiotic treatment to 0.507 after antibiotic treatment. The corresponding figures for cefuroxime and piperacillin-tazobactam were an increase from 0.600 to 0.800 and from 0.307 to 0.450 respectively. There were no differences in the mean fraction of *Enterobacteriaceae* with decreased susceptibility to ciprofloxacin, gentamicin, imipenem and trimethoprim-sulphamethoxazole, per patient, before and after antibiotic treatment.

In the group of non-fermentative gram-negative rods the mean fraction of decreased susceptibility to antibiotic, per patient, decreased for imipenem from 0.043 to 0.007. In this group there were no significant differences in antibiotic susceptibility to ceftazidime and ciprofloxacin per patient, before and after antibiotic treatment.

Table 2 The difference in mean fraction of *Enterobacteriaceae* (EBC) with decreased susceptibility and *Pseudomonas spp/ Acinetobacter spp* (NF), between the two samples.

Antibiotic	EBC			NF		
	Sample 1 Mean ^a	Sample 2 Mean ^a	p	Sample 1 Mean ^b	Sample 2 Mean ^b	p
Cefotaxime	0.357	0.507	0.045			
Ceftazidime	0.286	0.379	0.158	0.043	0.043	1.000
	0.600	0.800	0.032			
Cefuroxime						
Ciprofloxacin	0.414	0.400	0.841	0.021	0.021	1.000
Gentamicin	0.050	0.036	0.549			
Imipenem	0.0	0.014	0.157	0.043	0.007	0.049
Piperacillin-Tazobactam	0.307	0.450	0.041	0.014	0.021	0.640
Trimethoprim-sulphamethoxazole	0.343	0.293	0.425			

p<0, 05 considered significant

^aMean fraction of *Enterobacteriaceae spp*(EBC) with decreased susceptibility to named antibiotic per patient.

^bMean fraction of *Pseudomonas spp/ Acinteobact spp*(NF) with decreased susceptibility to named antibiotic per patient.

The mean fractions of species with decreased susceptibility in the study population were compared between Samples 1 and 2 using two-way analysis of variance with time as fixed factor and patient as random factor.

Two (1.4%) patients had ESBL-producing *E. coli* in Samples 1 and 2. One (0.7%) patient had ESBL-producing *E. coli* in Sample 1 only and one (0.7%) patient in Sample 2 only. ESBL-enzymes found were CTX-M 15/28, CTX-M 14 analogous and CTX-M 3/22. Other enzymes found were TEM-1, TEM-2 and SHV-1 alike. Four (2.8%) patients had *Klebsiella pneumoniae* isolates with plasmid Amp-C before

antibiotic treatment and two (1.4%) still had these after antibiotic treatment. No other ESBL-producing *Enterobacteriaceae* were found.

Paper III

Forty-seven children were colonised with *S. marcescens* during the study period. In March 2007, *S. marcescens* was found on used pacifiers after cleaning in wash-disinfector and stored in a clean box. All other environmental cultures were negative for *S. marcescens* including all 46 faecal samples from the staff. Results of the outbreak analysis are shown in Figure 2. Epidemiologic typing showed that clone A caused the two cases in 2006. In the first quarter of 2007, clone B was the cause of sepsis. In the third quarter of 2007 two children were colonised with clone B, the other 5 infants were colonised with clone C, including the three cases of sepsis. The spread of clone C continued in the two first quarters of 2008 with 13 infants colonised of whom 4 developed sepsis. One child was colonised with a unique clone during the third quarter of 2008. In the first quarter of 2009 there were 13 cases with clone F, one of which developed *S. marcescens* sepsis. In the first quarter of 2011 there were two cases of *S. marcescens* sepsis among infants on another NICU after primary care on the Linköping unit. These sepsis cases occurred one and four days after discharge and had the same clone as the two colonised children at the same time on the NICU in Linköping. When these two children were admitted to the other unit they were no longer subjected to the interventions typed. The colonisation with *S. marcescens*, clone U, from the second quarter 2011 was unique. The last case in the third quarter of 2011 was colonised on admission and was not epidemiologically typed. After the major cluster of *S. marcescens* colonisation in the first quarter of 2009 we identified a breakpoint of colonisation even though there was a minor spread in the first quarter of 2011. The last intervention was adopted during the second quarter of 2008.

Compliance with basic hygiene guidelines was initially low with 65% compliance the fourth quarter of 2007. This increased gradually to above 80% in the first quarter of 2009 since when it has remained the same. Observations from May 2008, and May, September and November 2009 are missing and not included in those quarters.

Figure 2: *S. marcescens* colonisation and sepsis.

In the case-control study in **Paper III**, medical records of 190 patients with sepsis as discharge diagnosis were studied. Seventy-six patients fulfilled the sepsis criteria, 36 patients had EOS and 40 patients had LOS. In the LOS group there were 47 separate episodes of sepsis in 40 patients. Eleven patients had 12 separate episodes of LOS-ser. The records of one infant with *S. marcescens*

septicaemia in August 2006 were not complete and this case was not included in the risk factor study, but is included in Figure 2. There were no *S. marcescens* sepsis cases in the EOS group. Forty-seven control cases were included.

In the comparison of the LOS-group with controls, the risk for LOS decreased significantly with every day of gestation age at birth and kg of birth weight with odds ratios 0.98 and 0.17 respectively. The risk for LOS also increased for each day with a central vein or umbilical catheter, and each day of ventilator treatment, with odds ratios 2.6 and 1.6 respectively.

In the comparison of LOS-ser with LOS-non-ser, there were no significant differences. In analysis with stepwise logistic regression, birth-weight was excluded because it was dependent on gestational age at birth. No other risk factors were found in this analysis.

Paper IV

In the study group with appendicitis the median age was 47 (19-82) years, in the diverticulitis group 62 (39-84) years and in the control group 48 (23-70), the median age in the diverticulitis group being significant higher. The difference in gender distribution was not significant. The patients in the appendicitis and diverticulitis groups had raised CRP (136(7-350) and 121(6-337)) and WCC (16.6(5.5-25.8) and (15.6(9.9-68.0)) respectively, previous antibiotic treatment (6 patients (15.0%) and 3(16.7)) , days in hospital (5(2-10) and 6(2-19)), days of antibiotic treatment (12(2-23) and 14(4-21)), perforation of the appendix and large intestine respectively(7 patients (16.7%) and 4(21.1)) with no significant differences. A significant difference was found in the rate of surgery 38 patients (90.5 %) in the appendicitis group and 4 patients (21.1%) in the diverticulitis group.

All patients received antibiotic therapy with broad in-vitro effect on aerobic and anaerobic bacteria. Twenty-six patients in the appendicitis group and 10 patients in the diverticulitis group received intravenous carbapenem (meropenem, imipenem or ertapenem). Cefalosporin (cefotaxime or

cefuroxime) in combination with metronidazole was chosen as antibiotic therapy in 16 appendicitis patients and nine diverticulitis patients.

Looking into the sequencing data of the faecal microbiota at genus level (figure 3); there were distinctive differences when comparing healthy controls to patients diagnosed with appendicitis. Healthy controls have a microbiota dominated by *Bacteroides*, *Faecalibacterium*, *Ruminococceae* and *Prevotella* while appendicitis patients show an intestinal flora with a higher abundance of *Escherichia/Shigella* and unclassified *Enterobacteriaceae*. The same pattern, however not quite as distinct could be seen for the diverticulitis patients.

Figure 3: Taxonomic composition by genus

Before: Sample taken just before antibiotic treatment start, in a patient with appendicitis or diverticulitis.

After: Sample taken 2-14 days after end of antibiotic treatment

Looking at data on phyla level (figure 4) there was a distinct difference between the study populations and the control population, in which *Proteobacteria* and *Bacteroidetes* occurring to a

greater extent in the study population, whereas *Firmicutes* and *Actinobacteria* are more abundant in the control population.

Figure 4: Taxonomic composition on phyla level

Before: Sample taken just before antibiotic treatment start, in a patient with appendicitis or diverticulitis.

After: Sample taken 2-14 days after end of antibiotic treatment

The diversity (figure 5) was significantly lower in the two study groups compared with healthy controls in samples before treatment. In the diverticulitis group the diversity increased significantly after antibiotic treatment and hospitalisation.

Figure 5: Diversity (Shannon Index)

Before: sample taken just before start of antibiotic treatment start, in a patient with appendicitis or diverticulitis.

After: sample taken 2-14 days after end of antibiotic treatment.

Differences between groups are calculated using Wilcoxon’s unpaired rank test. p -value < 0.01 is considered

In cultures from the microbiota there was a significant loss of *E coli* and a significant gain of *Citrobacter species* in the appendicitis group. In the appendicitis group as well as in the diverticulitis group there were a significant gain of *E faecium* and Yeasts.

The loss of *E. coli* and gain of *Citrobacter species* and enterococci between the standard cultivation methods was consistent with 16S rRNA sequence analyses in the appendicitis group.

Discussion

In **Paper I** a multi-clonal outbreak of enterococcal sepsis on a general ICU was identified. The environment was soiled with enterococci and transmission between patients was identified in seven cases, and between patients and environment in two cases. The most severe manifestation of the infection was sepsis. After implementation of the intervention programme no further outbreak of enterococcal sepsis occurred. We were not able to determine if any particular part of the intervention programme was more important or whether this was a result of the intervention programme at all. Enterococci are able to survive in the environment for up to three months (138) and studies have shown the importance of appropriate cleaning to eradicate enterococci from a ward (139, 140). It is reasonable to presume that thorough cleaning was important. The use of cephalosporins and antibiotics with anaerobic effect are known risk-factors for infection or colonisation with enterococci (141). Furthermore over-all mortality and severity of illness was unchanged. The reduction over all was probably due to decrease in occupancy since DDD per ICU patient was not changed. Reduction in antibiotic usage is generally beneficial for the bacterial ecology of the hospital and for ICU cost containment. Extraction of figures from hospital pharmacy-delivery data is not optional for assessing antibiotic consumption and appropriate usage. With the advent of computerised patient records, where notes on indication for therapy at the time of prescription are mandatory, details on indication for antibiotic treatment will become much more dependable. Increase in bed occupancy often leads to understaffing which could enhance MRSA transmission(142) due to a decrease in compliance to hygiene guidelines and a reduction in the capacity isolate patients or perform cohort-care. In this study, increased bed occupancy and suboptimal use of the rooms were probably predisposing factors for the outbreak. Adherence to basic hygiene guidelines is an important measure in the prevention of healthcare-associated infections (143). The present data also illustrate how difficult infection control can be when endemic infection rates are unknown because of poorly kept of infection-records. The importance of active surveillance through continuous registration infections seems obvious. The consequence of this is that it is difficult to draw definitive conclusions

from our results. Basic hygiene routines, optimal antibiotic therapy, (with choice based on resistance pattern and given as soon as possible instead of the overuse of broad-spectrum antibiotics) and ability to isolate of infected patients should be standard in all ICUs.

In **Paper II** we found a shift in faecal floral pattern from *E. coli* to other *Enterobacteriaceae*, *E. faecium* and yeasts in samples from patients after treatment with antibiotics for acute intra-abdominal infection in the clinical setting. Most studies on the effect of antibiotic treatment on the gut flora have been performed in healthy human volunteers (144). The fraction of decreased susceptibility to cephalosporins and piperacillin-tazobactam in *Enterobacteriaceae* increased after antibiotic treatment. The loss of *E. coli* and gain of other *Enterobacteriaceae*, and the shift to *E. faecium* was mainly a shift to species with greater intrinsic antibiotic resistance. This indicates that the emergence of antibiotic resistance is not due to mutations but selection of more resistant species. In multicentre studies similar to ours, a substantial increase in bowel colonisation with resistant *Enterobacteriaceae* in patients treated with piperacillin-tazobactam or ceftriaxone in combination with metronidazole was seen, which is in agreement with our findings (145).

Most of the antibiotics observed in this study are mainly eliminated via the urinary tract, the amount found in faeces is often low (approximately 1-5%) (79, 83, 86, 92, 93, 146), but nevertheless the aerobic composition of the faecal flora was affected. We could not ascertain what effects the antibiotic regimens had on the faecal flora because this study did not have the power for that.

Some studies indicate that harbouring of antibiotic-resistant *Enterobacteriaceae* in the faeces increases the risk for being infected by that subtype after hospital admission (147, 148). Thus the shift towards more antibiotic-resistant species must be taken into consideration when designing treatment regimens for secondary intra-abdominal infections.

In **Paper III**, we identified different clones with each outbreak, except for overlapping of clone B and C during fourth quarter of 2007, where an infant from a referring hospital already was colonised with clone B at admission and caused a spread. A decrease in LOS-ser, and no further major outbreak of

S. marcescens colonisation after early 2009 was observed after implementing the five -stage intervention programme. We were not able to identify any specific effects of individual intervention measures. The spread of *S. marcescens* decreased, however, when compliance with hygiene rules remained at a steady high level after the 4th quarter of 2008, with one exception in early 2010. This may reflect a delay in the adoption of new routines, and we believe that our intervention programme had a positive effect on the occurrence of *S. marcescens*. Observation of compliance with basic hygiene guidelines helps maintain awareness of the importance of all hygiene and cleaning routines(149). The decrease in the *S. marcescens* sepsis rate after change of antibiotic policy illustrates the importance of modifying existing antibiotic regimens according to local culture findings. It is also important to discuss measures with the microbiological laboratory when there are recurrent breakthroughs of sepsis, during empiric antibiotic treatment. In our case we were obliged to MIC-tests from disc-diffusion testing in order to reveal the most suitable aminoglycoside, taking variations in *in vivo* concentrations into consideration. The transmission of *S. marcescens* via a pacifier illustrates the importance of adequate cleaning and storing routines, and the risk of sharing devices. We were not able to identify the source of *S. marcescens* despite a large number of cultures from the environment. Even though *S. marcescens* was not found on the hands of the nursing staff, handling is an important way of transmission and hand hygiene is crucial in preventing contact transmission(13). The importance of revising hygiene and cleaning routines, as well as antibiotic strategies has been described by Voelz (150). During an outbreak situation it is important to ensure that hygiene guidelines are properly followed, adequate and updated if necessary. The spread of bacteria between patients should be considered a failure in hygiene routines and must lead to adequate control measures even if the source of the outbreak has not been identified.

We also found that central venous or umbilical catheter, ventilator care and low gestational age at birth are independent risk factors for LOS, which is in accordance with other studies. (151, 152). We

did not see any significant differences between non-*Serratia* LOS and LOS with *S. marcescens*. This could be due to the small numbers in each group. Looking back over the six year observation period, we believe that allocation of infants born prior to 28 weeks of gestation, to a special management cohort facilitates the awareness of their fragility and underlines the importance of compliance to hygiene routines. We believe that prophylactic cohort-care also protected these children from being colonised and infected with *S. marcescens*.

In **Paper IV**, the patients arriving at the emergency department with acute appendicitis or diverticulitis already had significantly disturbed faecal microbiota prior to antibiotic treatment and hospitalisation. At genus level, the healthy controls had a flora dominated by *Bacteroides*, *Faecalibacterium*, *Ruminococceae* and *Prevotella*, while appendicitis patients showed an intestinal flora with a higher *Escherichia/Shigella* and unclassified *Enterobacteriaceae* content. The absence of *F. prausnitzii* is associated with several inflammatory diseases of the bowel (153). Chassaing et al. suggested that the ratio of *F. prausnitzii*/*E. coli* can be used to evaluate the microbiota disturbance level (dysbiosis) in patients with inflammatory bowel disease, and identify those at high risk for recurrent relapses of Crohn's disease(154). This could indicate that a change in the gut microbiota is part of the pathogenesis of both appendicitis and diverticulitis. Microbial diversity increases after treatment with broad spectrum antibiotics and hospitalisation. Conventional aerobic cultivations show a shift towards *E. faecium* and Yeasts indicating disturbed microbiota. The shift from *E. coli* towards other *Enterobacteriaceae* in the appendicitis group could be an expression of displacement of one species by another with more intrinsic antibiotic resistance as shown in Paper II. Freezing of the samples could have influenced faecal composition with a reduction in the proportion of *Bacteroidetes* (155). This should not have affected the dynamics between the two sampling occasions in the study population.

Conclusion

- A multi-clonal outbreak of enterococcal sepsis on a general ICU was identified. The environment was soiled with enterococci and transmission between patients and between patients and environment had occurred. The most severe manifestation of the infection was sepsis.
- After improved hygiene routines including reorganisation of rooms and thorough cleaning of the GICU together with revision of antibiotic regimens reduced the incidence of septicaemia with *Enterococcus* spp and prevented clustering of enterococcal infections, was observed.
- Antibiotic treatment and hospital care, of patients treated for acute intra-abdominal infections, leads to a shift in the aerobic faecal flora from antibiotic-susceptible *Enterobacteriaceae* towards *E. faecium*, yeasts and more antibiotic-resistant species of *Enterobacteriaceae*.
- Epidemiological typing showed that recurrent outbreaks of *Serratia marcescens* was caused by different clones each outbreak, but within one outbreak the major part was caused by a single clone, demonstrating spread between neonates at a NICU.
- Multiple hygiene interventions and revised antibiotic regimens eventually obviate recurrent outbreaks of *Serratia marcescens* on the NICU. Spread of *Serratia marcescens* was not reduced until the observed compliance with basic hygiene guidelines remained stable above 80%.
- Low gestational age at birth, weight at sepsis onset, ventilator treatment and central venous or umbilical catheters are independent risk factors for late onset-sepsis, among patients on the NICU.
- In patients with acute appendicitis or diverticulitis the faecal microbiota is already disturbed with a higher content of *Enterobacteriaceae* spp and less *Bacteroides*, *Faecalibacterium*, *Ruminococcus* and *Prevotella* than the control population. In the group with acute

appendicitis there was no significant difference in the diversity before and after antibiotic treatment and hospitalisation. In the diverticulitis group the faecal diversity increased significantly and approached the healthy controls after antibiotic-treatment and hospitalisation.

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