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COLONIZATION, INFECTION AND DISSEMINATION IN INTENSIVE CARE PATIENTS

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Stockholm 2007
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"Innan vi lever
är livet inte något,
men det tillkommer oss att ge det en mening
och värdet är inget annat
äns den mening vi väljer"
Sartre
ABSTRACT

Nosocomial infections are a substantial problem in hospitals all over the world and the incidence is among the highest in the intensive care unit, affecting mortality and morbidity for the individual patient and cost for the society. In order to prevent these infections it is important to gain knowledge about colonization and infection pathways as well as about bacterial dissemination between patients.

The overall aim of the present study was to investigate bacterial and fungal colonization pattern, dissemination pattern within and between patients and the incidence of some ICU-acquired infections in intensive care patients, treated in a multidisciplinary Swedish university hospital ICU. Colonization and dissemination patterns of microorganisms were studied by microbiological analyses and antimicrobial susceptibility was monitored over time. Subtyping was performed by using phenotyping as well as genotyping methods, such as the Phene-Plate system and PFGE (pulse-field gel-electrophoresis). Fungal colonization index and other risk factors for acquiring invasive candida infection, were studied in patients with a length of stay of at least seven days. In addition, the status of the immunosystem was monitored with HLA-DR expression once a week in these patients.

The main results and conclusions of this thesis can be summarized as:

- Intubated intensive care patients are often heavily colonized in the lower airways with potentially pathogenic microorganisms, aerobic and anaerobic bacteria as well as yeasts.
- Different colonization routes were demonstrated for different species; primary colonization of the oropharynx or concomitantly in the lower airways, was shown for *Staphylococcus*, *Enterococcus*, Enterobacteriaceae and *Candida* spp., while *Pseudomonas* and other non-fermenting gram-negative rods and several anaerobic species often showed primary colonization of the trachea.
- The dissemination rate of CoNS between ICU patients was high, 70% of patients treated for more than three days were involved in at least one transmission event.
- Prolonged ICU stay was correlated to an increased rate of cross-transmission between patients as well as a significantly higher risk of being colonized with multi-resistant strains.
- The diversity of colonizing CoNS was significantly decreased in ICU patients with a length of stay of at least five days.
- The endogenous spreading of resistant clones within patient’s skin and mucosal areas increased with time.
- The incidence of invasive candida infections was high in the ICU patient population studied, despite a frequent use of antifungal agents. This was
probably due to that the majority of the patients were burdened by several risk factors.

- High colonization index (≥ 0.8) and recent extensive abdominal surgery was identified as significant risk factors for acquiring invasive candida infection in ICU patients with a length of stay of at least seven days.

In conclusion, the results of the present study emphasize the importance of compliance to barrier treatment, implementation and continuously follow-up of infection control programmes. Furthermore, the results underline the importance of a prudent use of antimicrobial agents for therapy and prophylaxis, based on daily reconsideration of the treatment according to microbiological and laboratory results and the patient’s condition, especially in this vulnerable patient population.
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<th>Description</th>
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<tr>
<td>BAL</td>
<td>Broncho-alveolar lavage</td>
</tr>
<tr>
<td>CASS</td>
<td>Continuous subglottic suctioning</td>
</tr>
<tr>
<td>CI</td>
<td>Colonization index</td>
</tr>
<tr>
<td>CARS</td>
<td>Compensatory anti-inflammatory response syndrome</td>
</tr>
<tr>
<td>CoNS</td>
<td>Coagulase-negative staphylococci</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming units</td>
</tr>
<tr>
<td>CPIS</td>
<td>Clinical pulmonary infection score</td>
</tr>
<tr>
<td>CRBSI</td>
<td>Catheter-related bloodstream infection</td>
</tr>
<tr>
<td>CRI</td>
<td>Catheter-related infection</td>
</tr>
<tr>
<td>CRRT</td>
<td>Continuous renal replacement therapy</td>
</tr>
<tr>
<td>CVC</td>
<td>Central venous catheter</td>
</tr>
<tr>
<td>CVP</td>
<td>Central venous pressure</td>
</tr>
<tr>
<td>ESBL</td>
<td>Extended spectrum β-lactamases</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>Histocompatibility leukocyte antigen-D-related</td>
</tr>
<tr>
<td>ICI</td>
<td>Invasive candida infection</td>
</tr>
<tr>
<td>IVD</td>
<td>Intravascular device</td>
</tr>
<tr>
<td>ICU</td>
<td>Intensive care unit</td>
</tr>
<tr>
<td>LOS</td>
<td>Length of stay</td>
</tr>
<tr>
<td>MOF</td>
<td>Multiorgan failure</td>
</tr>
<tr>
<td>MRSA</td>
<td>Methicillin resistant Staphylococcus aureus</td>
</tr>
<tr>
<td>MRSE</td>
<td>Methicillin resistant Staphylococcus epidermidis</td>
</tr>
<tr>
<td>NCCLS</td>
<td>National Committee for Clinical Laboratory Standards</td>
</tr>
<tr>
<td>PFGE</td>
<td>Pulsed-field gel electrophoresis</td>
</tr>
<tr>
<td>PSB</td>
<td>Protected specimen brush</td>
</tr>
<tr>
<td>SIRS</td>
<td>Systemic inflammatory response syndrome</td>
</tr>
<tr>
<td>VAP</td>
<td>Ventilator-associated pneumonia</td>
</tr>
<tr>
<td>VRE</td>
<td>Vancomycin-resistant enterococci</td>
</tr>
<tr>
<td>QEA</td>
<td>Quantitative endotracheal aspirate</td>
</tr>
<tr>
<td>WCC</td>
<td>White Cell Count</td>
</tr>
</tbody>
</table>
1 INTRODUCTION

At the beginning of the third millennium it is almost impossible for anyone in the
developed countries to be unaware of the importance of nosocomial infections.
Unfortunately, it has become apparent that earlier hopes of controlling or eradicating
infectious diseases are, for the most part, unjustifiable optimism. Infectious disease
problems concerning for example HIV/AIDS, different kinds of hepatitis and
meningitis, and the ravages of multi-resistant bacteria are regularly reported in
newspapers and television. The costs in human misery are considerable, as are the
additional economic and societal costs of dealing with infectious diseases in the
society.

Except for a few successes with viral diseases and vaccination programs, our
effort to control infectious diseases, particularly those caused by bacteria, have been
quite insufficient. We have failed to make such diseases a thing of the past and with
the development of an increased frequency of multi-resistant bacteria, it appears as
“the bugs are fighting back”. Bacterial infections and infection control measures are
therefore of continued and increasing importance to the general public, to health
professionals and in particular to those who practice critical care medicine. Despite
this, infection and infection control issues are often given relatively low prominence
in academic intensive care. However, there are signs of an increasing interest among
both hospital staff and the general public during the last decade. Still, these issues are
often paid with lip service in everyday practice. An everyday example is the simple
rule to disinfecting the hands with alcohol-based solution before and after
examining/touching the patient or the patients’ bed, which is often ignored.

During the last years there has been an increasing focus worldwide in these issues
and the World Health Organization (WHO) has recently (2005), started a campaign
addressing this problem “Clean Care is Safer Care: the first Global Challenge of the
WHO World Alliance for Patient Safety” led by amongst others Prof. D. Pittet and
MD L. Donaldson from Geneva [1, 2].
Figure 1 Pathways of possible bacterial transmission.

Cleanse hands:
- immediately before having direct contact with patient
- after direct contact with patient
- after contact with inanimate object(s) in the immediate vicinity of the patient

Cleanse hands if moving from a contaminated body site to a clean body site during patient care.

Appropriate technique for hand cleansing is critical. Except when hands are visibly soiled, alcohol-based handrubbing is recommended rather than handwashing with soap and water.

Published with permission from Prof. Pittet D, HUG Geneva
2 NOSOCOMIAL INFECTIONS AT THE ICU

Nosocomial infections are a substantial problem in hospitals all over the world. Despite a lot of effort and improvement in technical and medical fields, the problem with nosocomial infections due to antibiotic resistance and transmission of multi-resistant bacteria seems to increase over time. Focus on cost-effectiveness often forces hospital administrators to set limit to the newest and most expensive types of medical care, as well as numbers of staff, resulting in under staffed wards which contributes to nosocomial spread of infections.

Critically ill patients are treated in the intensive care unit (ICU). These patients are very susceptible to infections due to acquired defects in host defense mechanisms from the immuno-suppressive effect of for example the underlying disease, recent surgery, trauma and concurrent drug therapy. This is a population of patients which often have multiorgan failure (MOF) and as a consequence of this, are exposed to invasive procedures like ventilator-treatment and continuous renal replacement therapy (CRRT). These patients often have several invasive devices. They are often exposed to broad-spectrum antibiotics, which may alter the normal bacterial flora and predispose to infections with more resistant bacteria than in other wards. In context, these patients have many risk factors and have a higher frequency of nosocomial infections than patients in regular hospital wards. Infection occurs in 15-40% of all ICU admissions and the crude mortality rate is between 10-60%, but this includes mortality from the underlying condition [3, 4]. In the recently published SOAP study which investigated the incidence of sepsis in 198 European ICUs comprising 3.147 patients, during a fortnight period, the most common site of infection was the lung (68 %) and the abdomen (22 %). Although, in the patients who had an ICU-acquired sepsis the dominating infection was pneumonia (79.6 %), followed by urinary tract infections (17.9 %) and catheter-related infections (13.6 %), where abdominal infections were less common (10.8 %) [4]. The EPIC study, which was a one-day point-prevalence study of 1,417 ICUs and 10,038 patients conducted in 1995, a similar prevalence of ICU-acquired infections was demonstrated and seven risk factors for infection were identified: length of ICU stay, mechanical ventilation, trauma, central venous-, pulmonary artery- and urinary catheterization and stress ulcer prophylaxis [3].
2.1 VENTILATOR-ASSOCIATED PNEUMONIA

The most common ICU-acquired infection is ventilator-associated pneumonia (VAP), which is associated with high morbidity and a mortality ranging from 10 to 50% [5, 6].

2.1.1 Definition and diagnosis

The diagnosis of ventilator-associated pneumonia is often difficult without an obvious “golden standard” procedure. Different and sometimes diverging methods involving several criteria are being used to establish diagnostic tools. Clinical pulmonary infection score (CPIS) can be used in the clinical setting. It consists of six parameters (clinical, laboratory and radiological investigation) and where a score > 6 supports the diagnosis of pneumonia, see table 1 [7]. Furthermore, although the criteria for ventilator-assisted pneumonia (VAP) differs around the world, commonly it is based on the clinical suspicion of pneumonia in patients having received invasive ventilator-treatment for more than > 48 hours in combination with X-ray investigation, laboratory investigations and microbiological findings as listed below:

- New, persisting or unchanged consolidation on the chest X-ray.
- At least two of the following criteria: fever < 35°C or > 38.5°C, WCC < 4 or > 12 x 10⁹ and/or purulent tracheal secretions.
- Positive cultures: according to the following thresholds:
  - PSB 10³ CFU/ml
  - BAL 10⁴ CFU/ml
  - QEA 10⁵-⁶ CFU/ml

There is an ongoing discussion in the literature about sampling techniques from the lower airways; there are invasive as well as non-invasive techniques, both of which have advantages and disadvantages, as well as blind or eye-directed protected specimen brush (PSB) sampling [8-14]. To date there is no study that has showed any significant impact on mortality of using either invasive or non-invasive diagnostic methods. However, a significant decrease in antibiotic consumption has been demonstrated in favor of the invasive methods [15]. It has been realized that administration of antibiotics can alter the results of bronchoscopic cultures, and even more importantly, that the mortality increases for every hour that the onset of antibiotic therapy is being delayed [16]. Therefore, there is a powerful argument for the early use of quantitative endotrachial aspirate (QEA) which is available in every institution in contrast to more invasive methods.
<table>
<thead>
<tr>
<th>Criterion</th>
<th>Range</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature, °C</td>
<td>≥36.5 and ≤38.4</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>38.5 and ≤38.9</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>≥39 and ≤36</td>
<td>2</td>
</tr>
<tr>
<td>Blood leukocytes, mm$^3$</td>
<td>≥24,000 and ≤11,000</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>&lt;4,000 or &gt;11,000</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>+ band forms ≥500</td>
<td>2</td>
</tr>
<tr>
<td>Oxygenation, PaO$_2$/FiO$_2$ mmHg</td>
<td>&gt;240 or ARDS</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>≤240 and no evidence of ARDS</td>
<td>2</td>
</tr>
<tr>
<td>Pulmonary radiography</td>
<td>No infiltrate</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Diffused (or patchy) infiltrate</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Localized infiltrate</td>
<td>2</td>
</tr>
<tr>
<td>Tracheal secretions</td>
<td>≤14+µl of tracheal secretions</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>≥14+µl of tracheal secretions</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>+ purulent secretion</td>
<td>2</td>
</tr>
<tr>
<td>Culture of tracheal aspirate (semi-quantitative: 0–1–2 or 3+)</td>
<td>Pathogenic bacteria cultured ≤1+ or no growth</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Pathogenic bacteria cultured &gt;1+</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>+ same pathogenic bacteria seen on the Gram stain &gt;1+</td>
<td>2</td>
</tr>
</tbody>
</table>
2.1.2 Incidence and etiology

The incidence of VAP varies between 10-53 cases/1000 days of ventilator treatment [17] or 5-10 cases/1000 hospital admissions [6], in different parts of the world, with different categories of patients, where the neurosurgical ICU wards often report the highest incidence. The incidence in Scandinavia appears to be lower but the registration and documentation of the frequency is scattered and information is sparse [18-21]. The most common pathogens are *Haemophilus influenzae* and *Streptococcus pneumoniae* in early onset VAP (< 5 days after intubation), while methicillin-resistant *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia* and *Acinetobacter* spp. dominates as causative agents in late-onset VAP (> 5 days after intubation) [6, 15, 22].

2.1.3 Prevention

- Basal infection control measures with sufficient compliance to hand disinfection before and after contact with the patients is the most important measure to prevent VAP [23, 24].
- Elevated head with an angle of at least 45° has significantly lowered the incidence of VAP in one study [25], although there was no effect on mortality. The semirecumbent position is a low cost and simple procedure to prevent VAP. Still, the practical difficulties of maintaining an elevated bed angle of 45° have recently been shown but there are conflicting results concerning this issue [26, 27].
- Sucralfate rather than ranitidine is preferred to prevent VAP (it preserves the acidity in the ventricle which prevent bacterial growth), but an increased risk of stomach bleeding complications should be considered [28].
- Continuous aspiration of subglottic secretion (CASS) has in some studies [29-32] been shown to significantly decrease the incidence of VAP, especially early-onset VAP, which is in line with the theory that microaspiration from the upper airways and ventricle below the cuff to trachea is the main mechanism for early-onset VAP. There are risks associated with a continuous negative pressure in a sensitive area like the trachea which is not possible to examine daily. In a recent study on 14 sheep, all the animals exposed to CASS had tracheal lesions at post-mortem examination [33]. An option can be to aspirate carefully through the subglottic channel on an hourly basis or to rinse with sodium chloride solution three times per day. However, this procedure is costly and there is a need for special equipment and highly motivated staff. At present, there is no consensus on this issue. A meta-analysis favors CASS in reducing the incidence of VAP by nearly 50 % by decreasing both time on mechanical ventilation and length of stay in ICU.
for patients expected to be ventilated > 72 hours, while other authors exclude this procedure [34, 35].

- Early tracheostomy makes it easier to clean the mouth and the teeth thoroughly, which decrease the bacterial load and prevents microaspiration below the cuff of bacterial content. Tracheostomy facilitates for having the patient fully awake and co-operative. However, there are controversies in the literature on this subject, whether this increases the incidence of VAP or not. More recent studies indicate a favorable effect on days of ventilation and mortality [36-41].

- Noninvasive ventilator treatment (NIV) should always be considered as an alternative to conventional mechanical ventilation in selected patients.

Another important measure in order to prevent VAP is probably to limit the time of ventilator treatment by means of an active approach. Also, having the patient sedated as little as possible, and not giving any muscle-relaxant drugs as well as aiming for spontaneous breathing modes on the ventilator leading to an efficient weaning as soon as possible. Last, but not least, is an antibiotic policy with de-escalation therapy and short-term courses of antimicrobial treatment combined with daily reconsideration of treatment [42, 43], leading to decreases in the antibiotic load on the ward and thus decreased risk of development of virulent resistant strains that can cause VAP in this vulnerable patient population.

### 2.2 CATHETER-RELATED INFECTIONS

#### 2.2.1 Definition and diagnosis

The terminology used when discussing this subject can be confusing, catheter-related infections (CRI) are commonly only concerning the intravascular catheters and it should be taken into account which type of catheters that are used and studied. In the majority of studies only short-term and long-term central venous catheters are included, but in some studies arterial catheters and pulmonary catheters are studied as well. In recent years the term intravascular device-related infection (IVD-related infection) or intravascular device-related bloodstream infection (IVD-related bloodstream infection) have been more commonly used. Catheter-related infections include colonization of the catheter device, skin exit-site infections and device-related bloodstream infections. There are two diagnostic definitions that are important to distinguish:

- **CRI or IVD-related infection.** These infections are in Sweden most commonly diagnosed by a semi-quantitative culture performed according to the method of Maki, i.e. the catheter tip is rolled on an agar plate which then is cultured and $\geq 15$ CFU/plate is regarded as a positive culture [44].
Nosocomial infections at the ICU

- **CRBSI (catheter-related blood stream infection) or IVD-related bloodstream infection.** In Sweden the diagnosis is commonly obtained either by paired quantitative blood cultures, or by using differential time to positivity, see Table 2.

Internationally, there are several different methods used for establishing these diagnoses, for an overview of methods and performance of analyses, see Table 2. In a large meta-analysis by Safdar et al., which compared different diagnostic methods, the recommendation was to use paired quantitative blood cultures as the method of choice and not to culture the catheter tip routinely in the absence of clinical symptoms or suspicion of infection [45]. In a recent study which compared three methods that left the IVD in situ during the analysis, (paired quantitative blood culture, differential time to positivity and intra-luminal brushes were analyzed), the authors concluded that all three methods had an acceptably high specificity and sensitivity and recommended differential time to positivity as a first line method and intra-luminal brushes when problems to aspirate blood from all lumens of the CVC occurred [46].
## Table 3. Overview of different diagnostic methods for CRI and CREBSI
Adapted from ref nr 45.

<table>
<thead>
<tr>
<th>Diagnostic Method</th>
<th>Description</th>
<th>Criteria for Positivity</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Methods requiring device removal</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Qualitative catheter segment culture</td>
<td>A segment from the removed catheter is immersed in broth media and incubated for 24–72 h</td>
<td>Any growth</td>
</tr>
<tr>
<td>Semi-quantitative catheter segment culture</td>
<td>A 5-cm segment of the catheter is rolled 4 times across a blood agar plate and incubated</td>
<td>( \geq 15 ) CFU</td>
</tr>
<tr>
<td>Quantitative catheter segment culture</td>
<td>A segment from the removed catheter is flushed with broth (95) or sonicated in broth (68), followed by serial dilutions, surface plating on blood agar, and incubation</td>
<td>( \geq 1000 ) CFU</td>
</tr>
<tr>
<td><strong>Methods not requiring device removal</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Qualitative blood culture through the device</td>
<td>One or more conventional blood cultures are drawn through the device</td>
<td>Any growth</td>
</tr>
<tr>
<td>Quantitative blood culture through the device</td>
<td>A blood culture drawn through the device and processed by pour-plating methods or a lysis-centrifugation technique (isolator, Wampole Laboratories, Cranbury, New Jersey)</td>
<td>( \geq 100 ) CFU/mL</td>
</tr>
<tr>
<td>Paired quantitative blood cultures</td>
<td>Concomitant quantitative blood cultures are drawn through the device and percutaneously</td>
<td>Cultures are positive from both sites and the concentration of microorganisms in the culture from the device is 3- to 5-fold greater than in the peripherally drawn culture</td>
</tr>
<tr>
<td>Differential time to positivity</td>
<td>Concomitant conventional blood cultures are drawn through the device and percutaneously and are monitored continuously</td>
<td>Both blood cultures are positive and the catheter-drawn blood culture turns positive 2 h earlier than the peripherally drawn culture</td>
</tr>
<tr>
<td>Acidine orange leukocyte cytopsin</td>
<td>Approximately 1 mL of blood is aspirated from the catheter, the cells are lysed with sterile water, and the specimen is centrifuged, stained with acidine orange, and examined microscopically</td>
<td>Visualization of any microorganisms</td>
</tr>
</tbody>
</table>
2.2.2 Incidence and etiology

IVD-related infections are common nosocomial infections and the incidence varies considerably depending on type of intravascular device (cuffed, tunneled, impregnated, long-term or neither of the previous, single-, double- or triple-lumen). Other factors are patient-related, for example compromised immunity. An overall incidence is reported to range between 0 to 14 episodes per 1000 catheter days, with the lower range after interventional programs to reduce infection rate. There are differences depending on site for vascular access, where the femoral access seems to have the highest rate of infection and the subclavian site the lowest. Arterial catheters also have a lower incidence than venous catheters. Long-term catheters (≥ 10 days) have higher incidence than short-term catheters (< 10 days) [47-50]. In the recently published SOAP study, the overall incidence of bloodstream infections was 20%, and of the ICU-acquired episodes of sepsis (23.7%), the proportion of catheter-related infections was 13.6 % [4]. Information concerning Scandinavia is lacking, but in a recent study from Sweden the incidence was lower, only 1.55/1000 days, and the investigators concluded that this was due to a good compliance to maximal barrier sterile precautions, but notably the majority of CVC were single-lumen (42%) using short-term catheters (median duration 7.5 days) and there was a low incidence of multi-resistant bacteria in this study. The only risk factor found for IVD-related infection in this study was duration of catheterization [51]. The most common pathogens that cause IVD-related bloodstream infection are CoNS, *S.aureus*, *Candida* spp. and enterococci [52]. The pattern in Sweden shows similar results [51]. *Candida* spp. is now the third most common pathogen in the ICU [53], and the incidence for candida bloodstream infection has increased during the last decade with a shift towards non-albicans species such as *Candida glabrata* and *Candida tropicalis*, which are commonly more resistant to fluconazole [54-56].

2.2.3 Prevention

There are four different pathways acquiring an IVD-related infection, where the external and intra-luminal pathways are the two most important ones (see Fig 2). Several mechanisms contribute to the development of infection such as host defense ability, bacterial adhesiveness to the device (especially CoNS), skin colonization at insertion site and manipulations with the infusion hubs. Additional risk factors such as the catheter material, its localization or the type of care are viewed as specific targets for preventive measures and will be discussed further. The third pathway, haematogenous seeding of the catheter during blood stream infection of any origin does occur, but is uncommon. Finally, contaminated fluids or drugs administered
intravenously constitute another process for IVD-related infections, which sometimes results in outbreaks

**Figure 1** Different pathways of colonization and infection for intravascular device-related bloodstream infections, where the internal and external route are the two most common.

![Diagram](image)

### 2.2.3.1 Specific measures for preventing IVD-related infections

- Compliance to hand hygiene [1, 24]
- Education programs and interventions [24, 57, 58].
- Maximal barrier precautions during insertion of devices, which includes large sterile drapes, thoroughly and proper disinfection of the skin with alcohol-based chlorhexidine gluconate 0.5% or chlorhexidine 2%, sterile gown, gloves, cap and mask during the procedure. In a study from Berenholtz and coworkers, the infection rate declined from 11.3 to zero IVD-related infections per 1000 catheterization days, by using maximal barrier precautions combined with a checklist and empowering the ICU nurse to stop the procedure if the checklist was not fulfilled [49].
- If possible, (no contraindications or insertion of dialysis catheter), chose the subclavian vein for insertion. The jugular internal vein as well as the femoral vein show higher incidences of infections in many studies and this is also reported for arterial catheters (femoral versus radial) [48, 50].
- Select as few lumens in the CVC as possible for sufficient treatment of the particular patient and use injection membranes except for the lumen where CVP is measured. Ensure careful fixation of the catheter to prevent dislocation of the CVC or dressings [48, 52].
• Use semi-permeable dressings that allow inspection of insertion site. Change dressing and disinfect skin every 48-72 hours, if change is not clinically indicated more frequently [48, 52].
• There are many types of coated catheters which in different studies have lowered the incidence of infection. However, they are expensive and a meta-analysis revealed that the effect of the coating seemed to be lost after 7-10 days [59].

2.3 ABDOMINAL AND URINARY TRACT INFECTIONS

In two large European surveillance studies the EPIC and the SOAP study, abdominal and urinary tract infections (UTI) were common in the ICU setting [3, 4]. Still, these infections are frequently hospital-acquired and also a reason for ICU admission rather than ICU-acquired, in contrast to ventilator-associated pneumonia and catheter-related infections.

Since the majority of antibiotic agents are excreted through the kidneys and urine, UTI is uncommon as an ICU-acquired infection due to the frequent use of these drugs.

Abdominal infections, caused for example by perforation of the bowel and secondarily as a complication of surgery, are common reasons for ICU admission.

This group of patients has an increased risk of acquiring fungal infections due to many risk factors, such as colonization with Candida spp, recent onset of haemodialysis, use of invasive medical devices, parenteral nutrition, exposure to broad-spectrum antibiotic and abdominal surgery. It is important to consider prophylactic treatment in these patients and develop different strategies to predict and calculate the individual risk in each patient as reported in the literature. Specific rules to map risk factors, Candida colonization index and Candida score have been suggested to identify patients that would especially benefit from early antifungal treatment [60-63]. Several studies have also shown that an ICU stay of > 7 – 10 days is an independent risk factor for invasive fungal infection [64, 65]. To prevent these infections and still maintain a balance against overuse of antifungal agents is a laborious task, but extremely important, as ICU-acquired fungal infections have high morbidity and mortality and pose an increased cost for the society and considerable suffering for the individual and their relatives [66-68].
3 GENERAL INFECTION CONTROL MEASURES

Well-trained and competent staff, adequate equipment and good design of patient treatment rooms are especially important in the intensive care setting where most of the patients are very susceptible to infections. Whilst spacious and appropriate physical facilities are clearly important, these are no substitute for educated and highly motivated staff, since inadequate number of staff is shown to contribute to the acquisition and spread of nosocomial infections [69]. Concerning the physical facilities there must be an adequate space around each patient bed of minimum 20 m² to prevent dissemination of bacteria between patients. A minimum of one isolation cubicle per six patients, with facilities for both negative and positive air pressure is desirable. There should also be facilities for washing and/or disinfecting hands, e.g. one hand basin and one dispenser of alcohol-based hand disinfection for every other patient [70]. Gloves and gowns should be placed nearby each patient bed for easy access, as well as dedicated stethoscopes to each patient. Compliance to barrier treatment and alcohol-based hand disinfection before and after examining each patient are cornerstones in preventing nosocomial infections. It is therefore important with continuous education and motivation of staff and physicians as well as of consultants and other staff that come to the ICU. Hand disinfection removes transient flora including important opportunistic ICU pathogens, (e.g. \textit{S. aureus} and enterococci) as well as fastidious organisms (e.g. viruses). Contaminating microorganisms survive long enough on hands, from 30 minutes to several hours and can thereby be transmitted from patient to patient by the ICU staff, see Fig 1.

Whilst compliance to hand hygiene regimens is often insufficient amongst health care workers, especially among physicians, it is well documented that improvement in hand disinfection results in a decreased rate of nosocomial infections. In a study from Pittet and colleagues, the incidence of nosocomial infections decreased from 20 to 10 % during the study period of five years in response to a hospital-wide infection control program. The program included several interventions, such as education and observation of all staff, continuous feedback of alcohol-based hand disinfection fluids consumption, as well as the infection rates in the hospital throughout the whole study [24].
Picture 1  Nurse investigating an ICU patient properly dressed in gown when having physical contact with the patient.
4 IMPORTANT ICU PATHOGENS

4.1 STAPHYLOCOCCUS SPECIES

The genus of staphylococci consists of nonmotile, nonsporeforming, spherical and Gram positive cocci. These microorganisms are 0.5-1.5 \( \mu \text{m} \) in diameter, occurring singly, in pairs and in grape-like clusters. They are facultative anaerobes which tolerate high concentration of salt. They are commensals in the normal flora of the skin and mucous membranes. Phylogenetically, this genus belongs to the Micrococaceae family. Traditionally, staphylococci can be divided into two groups according to their ability to clot blood plasma: coagulase positive staphylococci, mainly \textit{Staphylococcus aureus} and coagulase negative staphylococci (CoNS). \textit{S. aureus}, \textit{S.saprophyticus} and \textit{S. epidermidis} are the three main pathogenic species with \textit{S. aureus} being the most virulent one.

4.1.1 Staphylococcus aureus

\textit{S. aureus} can cause a wide spectrum of diseases such as wound infections, abscesses, sepsis, pneumonia and toxic shock syndrome etc. The bacteria has maintained its position as the leading pathogenic organism in the ICU and caused 30 % of the ICU-acquired infections in the recent SOAP study as well as in the EPIC study conducted in 1995 [3, 4]. \textit{S. aureus} is one of the commonest pathogens in late-onset VAP and there is an increasing trend all over the world of methicillinresistant clinical isolates i.e. MRSA. Several studies have shown an increased cost and excess length of stay in VAP caused by MRSA [71-73]. To date the treatment options are limited with vancomycin being the most widely used agent although rifampicin and more recently developed agents such as linezolid, daptomycin and tigecyclin are possible alternatives.

4.1.2 Coagulase negative staphylococci

\textit{Staphylococcus epidermidis}, the most important pathogen among the CoNS are reported to be the third most common causative agent of nosocomial infections and the most frequent cause of nosocomial bloodstream infections [74, 75]. CoNS is a part of the normal skin microflora but can also colonize the nasal mucosa, the lower airways and invasive devices [20, 76, 77]. Usually the colonization with \textit{S. epidermidis} on invasive devices and implants occurs in two steps, first a primary attachment to the foreign body, followed by a cell proliferation denoted “run for the surface”. Biofilm formation is important for cell accumulation and thus, contributes
Important ICU pathogens

to the virulence of the bacteria. The biofilm of *S. epidermidis* consists of clusters of multilayered cells embedded in extracellular slime substance, on average 160 µm thick and exceeding 50 cells. The function of such biofilm is to work as a diffusion or penetration barrier to both host defense and to antibiotics. These species have the ability to survive in the ICU surroundings on medical devices and equipment for weeks up to months [78]. Due to these specific properties, *S. epidermidis*, are specifically prone to cause catheter-related infections (CRI).

CoNS infections pose a serious problem especially among immunocompromised patients and are often difficult to treat since CoNS strains are commonly multiresistant [79-83]. In contrast, other members of the CoNS that frequently colonize human skin and mucous membranes such as *Staphylococcus haemolyticus*, *Staphylococcus capitis*, *Staphylococcus lugdunensis* and *Staphylococcus xylosus* are uncommon pathogens in human diseases.

### 4.2 ENTEROCOCCI

The excessive and widespread use of cephalosporins during the last few decades has increased the importance of the enterococci in the clinical setting, especially in nosocomial infections. Cephalosporins are inactive against this group of bacteria and thereby promote selection and spread of resistant strains. The group includes *Enterococcus faecalis*, *Enterococcus faecium*, *Enterococcus durans* and *Enterococcus avium*, where the former two are of greatest clinical importance in the ICU setting. *E. faecalis* is by far the most frequently isolated species, but the prevalence of the more resistant *E. faecium* has increased and could be a result of the augmented use of carbapenems. Outbreaks of vancomycin-resistant enterococci (VRE) are occasionally seen in Swedish hospitals, while these very reluctant strains are a consistent and increasing threat in many countries such as USA and the southern Europe. Thus, enterococci is a group of bacteria that with their inherent tolerance against unfavorable conditions, renders them prone to survive for long periods in the hospital setting even on dry surfaces and accordingly, further facilitate transmission between patients. There are only few antimicrobial agents available to treat these bacteria and the main focus is currently on good infection control practice and restricted antibiotic use to prevent the emergence and spread of these pathogens.

### 4.3 ENTEROBACTERIACAE

These opportunistic pathogens include amongst others *Escherichia coli*, *Klebsiella* spp and *Enterobacter cloacae* which are all part of the normal flora in the large bowel. They may cause infections, mainly urinary tract infection (UTI) and pneumonia, following antibiotic treatment, as they are often resistant to first-line
treatment such as amoxicillin. In recent years, outbreaks caused by multi-resistant strains due to generation of extended spectrum of β-lactamase (ESBL) have been an increasing clinical problem world wide including Sweden. These infections can often be successfully treated with either a combination therapy or by a carbapenem.

4.4 PSEUDOMONAS AERUGINOSA

This water-borne bacterium is one of the commonest pathogens causing ventilator-associated pneumonia (VAP), especially late-onset VAP. This non-fermentative gram-negative rod is often multi-resistant and can survive for long periods on equipment in the patient’s surroundings. Cross-infection does occur but endogenous infection is probably more common through the use of broad-spectrum antibiotics that select for resistant strains. The treatment is often difficult due to the ability of the bacteria to create a protective slim-layer, which makes it problematic for the antibiotic to penetrate into the cell wall. As *Pseudomonas aeruginosa* develops resistance quickly, the general recommendation is to treat with a combination therapy and not single line antibiotic.

4.5 STENOTROPHOMONAS MALTOPHILIA

This non-fermentative bacterium is a pathogen of increasing importance in the ICU setting. It is widely found in the ICU environment and has been isolated on equipment (dialysis machines, ventilation circuits and nebulisers) as well as in disinfectant solutions [84, 85]. *Stenotrophomonas maltophilia* mainly causes lower respiratory tract infection and bacteraemia but can also cause peritonitis as a complication to gastro-abdominal surgery. These infections are mainly related to previous antibiotic exposure, especially carbapenems.

4.6 ACINETOBACTER SPP.

These gram-negative coccobacilli can survive for long periods in a dry environment and are often intrinsically resistant to many antibacterial agents. They often colonize the respiratory tract and wounds and are a special threat in burn units. Outbreaks can be difficult to control and there are case reports where entire ICUs have been forced to close due to such outbreaks [86]. Risk factors for acquiring these strains are emergency procedures and previous antibiotic use, especially fluoroquinolones.

4.7 CANDIDA SPECIES

Fungal infections are an increasing problem in hospitals during the last decade, especially in the ICU setting which has emerged as epicenters for infections such as
candidaemia and invasive candida infection (ICI). The escalating problem in ICUs is probably due to an increasing population of immuno-compromised patients, or otherwise vulnerable patients. The risk for fungal infection is increased by several risk factors that are frequently occurring among ICU patients, such as colonization with *Candida* spp., new onset haemodialysis, use of invasive medical devices, parenteral nutrition and recent extensive abdominal surgery. *Candida* spp. is the fourth most common isolates amongst nosocomial bloodstream infections and the third amongst ICU-acquired IVD-related bloodstream infections. Furthermore, the incidence of candidaemia has been shown to be highest in the ICU and general surgery units.

### 4.7.1 Candida albicans

*Candida albicans* is still the most frequently isolated yeast pathogen in the clinical setting, but a shift towards non-albicans *Candida* spp. have been reported over the last years [54-56]. *C. albicans* can cause several different infections, such as UTI, abdominal abscesses, endocarditis, endophthalmitis, pneumonitis and septicaemia. The mechanism is thought to be as follows. First, there is an endogenous colonization of the gastrointestinal channel, then a candidaemia in the blood through translocation, which in turn causes a spread to different target organs such as liver, lung, and heart, i.e. disseminated candidiasis. Instances of fluconazole resistance have developed, due to the increased use of these agents in prophylaxis and general treatment, but are still not a big issue in the clinical setting.

### 4.7.2 Non-albicans species

*Candida glabrata, Candida lusitaniae, Candida tropicalis, Candida krusei, C. dubliniensis, Candida pelliculosa and Candida norvegensis* are some of the species in this group. *C. glabrata* is reported in many studies to be the most common non-albicans pathogen followed by *C. tropicalis*, both are prone to cause infection in elderly and immuno-compromised patients and are often resistant to fluconazole. During recent years, an increasing rate of non-albicans systemic fungal infections has occurred, which is mainly ascribed to the extended use of fluconazole for which theses species have none or borderline susceptibility.
5 ANTIMICROBIAL AND ANTIFUNGAL DRUGS COMMONLY USED IN THE ICU

Despite the importance of infection control measures we will never be able to prevent all infections and proper antibiotic use will always be one of the cornerstones when saving lives in patients whom have acquired an infection. De-escalation therapy, i.e. to start with a broad-spectrum antibiotic when the infecting organism is unknown, and administrate the drug after that cultures have been obtained and then narrow the spectrum as soon as the results of the microbial cultures are known, have been widely used in Sweden for a long time. Recently a new study demonstrated the importance of an early administration of adequate antibiotic (within an hour) and the impact on mortality with every hour of delayed administration, in patients with septic shock and hypotension (see Fig 3) [16]. Thus, a knowledge concerning antibiotic agents and their antimicrobial spectrum is essential to every physician that works in the ICU. Basically, the majority of drugs administered in the ICU are given intravenously since the enteral route is often not available and absorption of drugs from the gastrointestinal channel is unreliable. A strict antibiotic policy is very important and narrow-spectrum agents are desirable, but patients have often been exposed to several antibiotics before admission to the ICU [20]. So, in reality there is a large use of broad-spectrum antibiotic in the ICU, yet, an active policy with a daily reconsideration and evaluation of the antibiotic treatment is crucial. An overview of agents commonly used in Swedish ICU is given below.
Cumulative effective antimicrobial initiation following onset of septic shock-associated hypotension and associated survival. The x-axis represents time (hrs) following first documentation of septic shock-associated hypotension. Black bars represent the fraction of patients surviving to hospital discharge for effective therapy initiated within the given time interval. The gray bars represent the cumulative fraction of patients having received effective antimicrobials at any given time point.

Figure 3 Adapted from ref 16
5.1.1 Beta-lactam agents

Penicillins, cephalosporins, carbapenems and monobactams are all composed of the beta-lactam ring and are thus classified as beta-lactam agents. These bactericidal drugs act by inhibiting the cell wall synthesis by binding to the penicillin-binding proteins. Resistance can develop through bacterial synthesis of beta-lactamases which are enzymes degrading the beta-lactam molecule. The encoding genes can be both chromosomally and plasmid generated.

5.1.2 Penicillins

Penicillins (such as benzyl-penicillin, phenoximehtyl-penicillin, ampicillin and isoxasolyl penicillins) have been used for more than 60 years and still exert an excellent effect on streptococci and most pneumococci. Thus, these agents are the first-line treatment of community-acquired pneumonia, otitis and tonsillitis. They also have good activity against *Meningococcus* spp., *Treponema pallidum*, *Borrelia* spp. and most anaerobic gram-positive bacteria, but variable activity against staphylococci, enterococci and gram-negatives. Penicillins are easily degraded by most beta-lactamases. Treatment with combinations of penicillin and a beta-lactamase inhibitor overcomes the resistance mediated by most beta-lactamases. The combination of piperacillin and tazobactam is an alternative for the treatment of serious infections such as severe pneumonia, intra-abdominal infections and sepsis with primary focus in the lung or abdomen as well as in neutropenic patients. Tazobactam is a beta-lactam that lacks antibacterial activity but that acts as an enzyme inhibitor by binding irreversible to beta-lactamases. As all other penicillins, piperacillin and tazobactam is eliminated by renal excretion.

5.1.3 Cephalosporins

Cefuroxime, ceftazidime and cefotaxime are the agents most commonly used in this group. Cefuroxime and cefotaxime have a broad-spectrum activity and are recommended choices in both community-acquired and nosocomial pneumonia, UTI, meningitis, abdominal, skin- and soft tissue infections. In abdominal infections it is important to add cover for anaerobic bacteria and for enterococci, if suspected. Ceftazidime is highly effective against gram-negative bacteria including *P. aeruginosa* but is not considered sufficient when gram-positive infection is suspected. All cephalosporins are known to increase the risk for antibiotic-associated *Clostridium difficile* infection. Coagulase-negative staphylococci are commonly resistant (30-40%), due to methicillin resistance. All these agents are mainly eliminated by renal excretion, thus dose-adjustment is required if the kidney function is impaired.
5.1.4 Carbapenems

Meropenem and imipenem are both frequently used carbapenems in the ICU setting. They have a very broad spectrum against both gram-positive and gram-negative bacteria including anaerobic bacteria. The carbapenems are stable against most β-lactamases including the extended-spectrum beta-lactamases (ESBL). *Enterococcus* spp. and *Stenotrophomonas maltophilia* are often intrinsically resistant while *P. aeruginosa* often develop resistance during treatment. The indications for these agents are severe infections mainly the in lung, abdomen, CNS (meropenem) and for initial treatment of pyrexia of unknown origin in neutropenic patients. Carbapenems are excreted renally and the dose must be reduced in case of impaired renal function.

5.2 QUINOLONES

Quinolones exert a bactericidal effect by acting on the bacterial DNA-gyrase complex and topoisomerase IV. Resistance against quinolones is mainly chromosomally mediated by mutations in target genes, or by increased efflux of the drug. Resistance rates are increasing and in many countries these antibiotics are not recommended for single-line treatment of severe infections. The quinolones are largely eliminated by the kidneys (75-85%) but also via the liver and through transintestinal excretion. Ciprofloxacin and levofloxacin are the most commonly used quinolone agents in intensive care. Ciprofloxacin is highly active against gram-negative bacteria including *P. aeruginosa* and is therefore an alternative when treating UTI and exacerbations of bronchitis and other infections caused by gram-negative bacteria. Levofloxacin is active against most gram-positive and gram-negative aerobic bacteria but MRSA is resistant. It is active against *Mycoplasma pneumoniae*, *Chlamydia* spp. and *Legionella pneumophilia* and levofloxacin is thus often used in the treatment of pneumonia when there is suspicion of an atypical infection.

5.3 GLYCOPEPTIDES

Glycopeptides inhibit the bacterial cell wall synthesis of gram-positive bacteria at an earlier stage than the beta-lactam agents. Acquired glycopeptides-resistance through the genes *vanA* and *vanB* is an increasing clinical problem, mainly seen in *E. faecalis* and *E. faecium* (< 10 % of clinical isolates). Resistance to glycopeptides or decreased susceptibility in *Staphylococcus* spp. has been reported but is still very uncommon (< 1 %). The drug is eliminated via excretion by the kidneys and it is crucial to reduce the dose in case of renal insufficiency and to carefully monitor serum concentrations of the drug due to the risk of nephro- and ototoxicity. Teicoplanin and vancomycin are the currently clinically available agents within this group of drugs and the use of vancomycin is dominating in the ICU. Treatment is indicated for serious infections
with multi-resistant gram-positive bacteria such as MRSA, MSSE and *Enterococcus* spp. (VRE). Vancomycin given orally is not absorbed at all but may be used to treat antibiotic-associated *Clostridium difficile* infections.

### 5.4 OXAZOLIDINONES

Linezolide, the first agent in this new class, exerts a unique antibiotic mechanism by binding to 50S-ribosome-subunits and thereby inhibiting the initiation of protein synthesis. Linezolide has a bactericidal effect against most *Streptococcus* spp., (including *S. pneumoniae*) and a bacteriostatic effect against *Staphylococcus* spp. and *Enterococcus* spp. Resistance is still uncommon but is reported for *Enterococcus* spp. and *Staphylococcus* spp. Linezolide may be used as an alternative to vancomycin in the treatment of pneumonia and soft-tissue infections caused by multi-resistant gram-positive organisms especially in patients with impaired renal function. Despite a predominant renal elimination (80-85%), the half-life of the drug is not increased in kidney failure.

### 5.5 MACROLIDES

The macrolides (e.g. erythromycin, clarithromycin, roxithromycin and azithromycin) inhibit the bacterial protein synthesis by binding to the ribosome and most of them are administered per orally. There are two main mechanisms of resistance: the first is mediated by methylating enzymes encoded by acquired *erm* genes, leading to decreased binding to the 23S ribosome; the second is encoded by the *mef*A gene, which leads to efflux of active substance from the bacterial cell. These agents have a bacteriostatic effect mainly on gram-positive bacteria except for *Enterococcus* spp. but are also active against *Mycoplasma pneumoniae*, *Chlamydia* spp. and *Legionella pneumophila*. Erythromycin can be an alternative for treatment of pneumonia where there is a suspicion of atypical infection, penicillin allergy or as an adjuvant drug to penicillin in pneumococcal pneumonia. Main limitations to its use are due to extensive hepatic metabolism and the interactions with many other drugs commonly used in the ICU, for example: ciclosporine, digoxine, fenytoine, carbamazepine, methylprednisolone, midazolam, nitrazepam, tacrolimus, teophyllin and zopiclone. The drug can also induce a prolonged QT interval with an increased risk for Torsades de Pointes.

### 5.6 TRIAZOLE DERIVATES

These agents are fungistatic and affect the fungi by interfering with the cytochrome P 450 system, acting in several different enzymes which in turn inhibit the ergosterol synthesis. Fluconazole is the most commonly used drug and has similar
characteristics given either intravenously or orally, with a bioavailability of > 90%. It is excreted in the urine and the elimination is dependent on the creatinine clearance. The half-life of the drug in patients with a normal renal function is about 30 hours and with a doubled loading dose on the first day, 90 % of steady state is reached already on day two. The drug is active against several fungi and is most frequently used in the treatment of *Candida* (mainly *C. albicans*) and *Cryptococcus* infections. However, *C. glabrata* are less susceptible and *C. krusei* as well as *Aspergillus* spp. are resistant. Voriconazole is a newer broad-spectrum triazole derivate that is effective against all *Candida* spp. and *Aspergillus* spp., thus being an alternative in the treatment of fluconazole-resistant non-albicans isolates. In *Aspergillus* spp. infections voriconazole is the drug of choice and has in one study been shown to have better effect and decreased mortality on invasive aspergillosis in immuno-compromised patients than liposomal amphotericin [87].

### 5.7 AMPHOTERICIN B

In order to reduce the toxic effects of amphotericin B, this substance is mainly given as a lipid formulation where amphotericin B is dissolved in liposomes resulting in an improved pharmacokinetic profile. The drug is fungicidal or fungistatic depending on the drug concentration achieved in the target organs. Liposomal amphotericin B binds to ergosterol in the fungal cell wall, leading to an increased permeability and leakage of intracellular components. Liposomal amphotericin B is active against both *Candida* spp. and *Aspergillus* spp. and is indicated in severe and deep fungal infections as well as empirical therapy in suspected fungal infections in neutropenic patients with fever. The drug is also active against visceral Leishmaniasis.

### 5.8 ECHINOCANIDINES

Caspofungin is a semi-synthetic lipopeptide substance (echinocandin) inhibiting the synthesis of β (1,3)-D-glucane, which is an essential component in the cell wall of many filamentous fungi and yeasts. The drug has fungicidal effect against *Candida* spp. and is fungistatic against *Aspergillus* spp. Caspofungin is an option in invasive candidiasis in adults and an alternative in invasive aspergillosis where liposomal amphotericin B or voriconazol is not possible to use due to intolerance or treatment failure. Caspofungin is excreted in urine and faeces. The elimination is very slow and dependent on distribution-mechanisms and plasma-protein binding.
Aims of the study

6 AIMS OF THE STUDY

The overall aim of the present study is to explore colonization pattern, dissemination in, and between patients, of bacteria and fungi. In addition, to investigate the incidence of some ICU-acquired infections (VAP and ICI), in intensive care patients at a multidisciplinary ICU, in a Swedish university hospital.

The specific aims were:

- To assess the impact of oropharyngeal and gastric colonization on the emergence of tracheal colonization over time in mechanically ventilated patients. Special emphasis was made on elucidating the role of anaerobic bacteria in the lower respiratory tract. Furthermore, to investigate the frequency of VAP in the study group. (Paper I)

- To investigate gastric and respiratory tract colonization and the rate of transmission of CoNS within and between patients treated with mechanical ventilation. (Paper II)

- To study the impact of the patient’s length of stay on the diversity and dissemination within and between patients, and resistance patterns of CoNS colonizing the patients. (Paper III)

- To investigate Candida colonization pattern and colonization index (CI), in combination with other risk factors and their relation to invasive candida infection (ICI) and clinical outcome in patients with a length of stay of at least 7 days and, if possible, to identify specific patient groups that may benefit from antifungal prophylaxis. (Paper IV)

- To assess the impact of patients’ immunological status on the risk of acquiring ICI, by repeated measurements of the expression of histocompatibility leukocyte antigen-DR (HLA-DR). (Paper IV)
7 MATERIAL AND METHODS

7.1 PATIENTS

Subjects included in all studies were ICU patients with a variable length of stay (< 2 hours – 77 days). The majority of the patients were intubated and treated with mechanical ventilation for some time of their ICU stay. The ICU where all the studies were performed is a highly specialized unit at a university hospital, thus, a considerable proportion of the patients was either transplanted, immuno-suppressed and/or neutropenic, in addition, exposed to extensive abdominal surgery and/or dialysis treatment (CRRT). This rendered the population extremely susceptible to nosocomial infections, both due to common pathogens as well as opportunistic pathogens such as CoNS and Candida spp.

7.1.1 Paper I and II

Forty-eight consecutive patients, expected to need invasive ventilator treatment for at least three days, during two periods (April-August 1998 and September-December 1999) were included in the study. The only formal exclusion criterion was patient age less than 18 years. In addition, seven patients were excluded due to lack of informed consent (n = 1), extubation before day 3 (n = 3), death before day 3 (n = 2), and transfer to another hospital before day 3 (n = 1). Finally 41 patients were included in the study. The majority of the patients were extubated in the ICU (n = 35), but one withdrew consent and five were transferred to other hospitals and sampling was therefore discontinued in these patients. Two of the patients were ventilated for more than 33 days when sampling was discontinued according to the protocol. Fifteen of the patients were females and the median age of the 41 patients was 59 years (range 20-82). The median APACHE-II score during the 24 h after admission was 19 (range 0-44), and the ICU mortality was 10/41. Ten of the patients (24%) had undergone transplantation of liver (n = 5), bone marrow (n = 2), kidney (n = 2) or liver and bone marrow (n = 1). The patients were mechanically ventilated for 3->33 days (median 6 days). Twelve of the patients were ventilated for 12 days or more. One patient had a permanent tracheostomy at admission and 16 of the patients were exposed to at least one reintubation or tracheostomy operation. Twelve of the patients had clinical pneumonia at time of admission. Nine of the patients had not received any antibiotic treatment at the time of admission. During the ICU stay, 39 of the 41 patients were treated with at least two antibacterial agents often as combination therapy and 13 received five to seven different antimicrobial agents, including antiviral and antifungal therapy. The most frequently used antibiotics were cefuroxime (n = 17),
Material and methods

imipenem (n = 15), metronidazole (n = 14), ceftazidime (n = 13) and cloxacillin (n = 9). Only two patients were treated with vancomycin while one patient was not given any antimicrobial treatment. Of note, 15 patients were treated with corticosteroids, 10 had other immunosuppressive therapy, while none was given any muscle-relaxant drug during their stay at the ICU.

The samples from the first twenty consecutive patients included in the main study (paper I) were further analysed according to bacterial transmission and multi-resistance (paper II). Of these, fifteen patients were male and five females. The median age was 59 years (range 34-82), the median Apache score was 24 (range 9-40), the median ICU stay 9 days (range 3- >33) and the median time on mechanical ventilation was 6 days (range 3- >33). The ICU ward consisted of four single-bed rooms and three rooms with three beds each at the time of this investigation. On average, one nurse was shared by two patients and one to two nurse assistants.

Relatives’ informed consent was obtained before patients entered the trial and the study was approved by the Local Ethics Committee at Huddinge University Hospital, Karolinska Institutet, Stockholm, Sweden.

7.1.2 Paper III

Thirty-five consecutive patients were included in the study performed in the multidisciplinary ICU April-July 2001. Informed consents were obtained from relatives before patients entered the trial. The study was approved by the Local Ethics Committee at Karolinska University Hospital Huddinge, Sweden. Two categories of patients were included. The first group, referred to as shortstayers (SS), consisted of 20 consecutive patients, 13 men and 7 women, mean age 62 years (range 3-86), in whom sampling was performed within 2 hours of admittance to the ICU. Eight of these patients had not received any antimicrobial treatment during at least seven days before sampling, while eight patients had received a single dose of cefuroxime and metronidazole as pre-surgical prophylaxis, and four were treated with at least two antibiotic agents, including a carbapenem. The second group, referred to as longstayers (LS), included patients who had stayed in the ICU for at least 5 days (median 14 days, range 5-102 days). Sampling of LS was performed consecutively at four occasions with 2 weeks intervals and included all patients who fulfilled the inclusion criteria on the sampling day. Due to repeated sampling of all current LS every second week, one individual could be included more than once. This approach was chosen since a particular patient could at each sampling period have a significant impact on the colonization pattern on the other patients in the ward. The aim was to include at least 20 sampling occasions in this group and at the fourth sampling period, six weeks after the start of the study, a total of 23 LS subjects, 10 men and 13 women, mean age 58 years (range 26-78), were included. Due to repeated sampling every
second week, five LS patients were included twice or more meaning that 15 individuals were included as LS (8 men and 7 women), mean age 57 years (range 26-78), although each individual was statistically regarded as a “new” subject when analyzing a particular sampling occasion. All patients in the LS group were treated with at least two antimicrobials, of which one was a carbapenem, during their ICU stay. None of the sampled SS patients were included in the LS group.

7.1.3 Paper IV

Fifty-nine consecutive patients treated at the ICU, with a length of stay (LOS) of at least 7 days within the period Mars 2004 to July 2005, were included for sampling. Informed consent was obtained from patients or relatives and the study was approved by the Ethics Committee, Karolinska University Hospital, Stockholm, Sweden. Of the 59 patients, there were 38 men and 21 women with a mean age 59 years (range 19-81), the mean LOS was 19.8 days (range 7-77 days). The dominating reasons for ICU admission were abdominal surgery with complications, respiratory failure, severe septic shock, and trauma. The patients were treated with mechanical ventilation for an average of 16 days (range 0-74), the mean Apache Score was 12.8 (range 3-22) and the SOFA score day 7 for all included patients was on average 8.2 (range 2-21). All patients had at least one invasive medical device and the majority had two or more, (central venous line, arterial line, central dialysis catheter and/or endotracheal tube). Most of the 59 included patients were treated with at least one broad-spectrum antibiotic, 43 received carbapenems and 20 were treated with vancomycin. A majority (66%) of the studied patients were treated with immunosuppressive drugs (17/59) and/or low-dose cortisone (≤ 200 mg glucocorticosteroides/24 h) (39/59), and 34 patients were exposed to at least one antimycotic drug during their ICU stay; 22 received fluconazole, 17 received liposomal amphotericin B, three patients were treated with voriconazole and three with caspofungin.

7.2 COLLECTION OF SAMPLES

7.2.1 Oropharyngeal samples (Paper I, II and III)

The oropharyngeal samples were taken from both the tonsils and the pharynx wall avoiding contamination from the tongue with a depressor. A sterile swab and a charcoal transport medium were used.
7.2.2 Gastric channel samples (Paper I and II)

From the stomach, secretions were aspirated directly into a sterile tube after disinfection of the outer surface and equal volumes of sterile transport medium were added.

The gastric samples were taken in the mornings 3 hours after turning off the enteral nutrition. In the transplanted patients there were often difficulties obtaining gastric samples due to concomitant immunosuppressive therapy, which was partly administered through the gastric tube.

7.2.3 Subglottic samples (Paper I, II, and IV)

Samples from the subglottic space were aspirated with a sterile syringe from a channel of the tube (EVAC Hi Lo; Mallinckrodt, Athlone, Ireland) after disinfection of the outer surface of the channel with 70% isopropanol, and put into a sterile tube with equal volumes of transport medium.

7.2.4 Tracheal samples (Paper I, II, and IV)

From the trachea, secretions were aspirated directly into a sterile tube with a sterile suction device, (in Paper I and II equal volumes of sterile transport medium were added).

7.2.5 Cutaneous samples (Paper III)

Sampling sites were vestibulum nasi, the axilla, the perineum and the skin area on the right side of the neck (corresponding to the insertion site for a central venous catheter in the internal jugular vein). The samples were collected with a sterile cotton swab which was rolled and pressed to an area of 1-2 cm$^2$, depending on sampling site, during 5-10 seconds. The cotton swab was then placed in a transport medium and delivered within 30 minutes to the laboratory where it was stored at -70°C until assayed.

7.2.6 The oral cavity and rectal samples (Paper IV)

The oral cavity and the rectum were sampled with sterile cotton swabs which were gently rolled and pressed to the area for 5-10 seconds and then placed in a collection tube with a transport medium.
7.2.7 Drainage samples (Paper IV)

Samples from drainage were collected aseptically by aspiration with a sterile syringe, and put into a sterile collection tube, after disinfection of the outer surface of the drain with 70% isopropanol.

7.2.8 Urine samples (Paper IV)

The urine samples were collected by aspiration after disinfection of the membrane of the catheter and put directly into sterile collection tube.

7.2.9 Blood samples (Paper IV)

The blood was drawn directly into bottles specially designed for culturing fungi.

7.3 LABORATORY ANALYSES

All microbiological procedures, pheno- and genotyping of particular isolates, as well as immunological analyses were performed at Karolinska University Hospital Huddinge, Stockholm, Sweden.

7.3.1 Microbiological cultures (Paper I-IV)

7.3.1.1 Cultures and methods for identification of bacterial species (Paper I-III)

All samples (paper I-III) were diluted in tenfold serial dilution to $10^{-6}$ in pre-reduced media. If needed, subglottic and tracheal secretions were lysed with an equal amount of sputolysin. The suspensions were inoculated on selective and non-selective agar media as previously described [88]. Aerobic agar plates were incubated for 24 hours at 37°C, while agar plates for anaerobic microorganisms were incubated for 48 hours at 37°C in anaerobic jars (GasPak; BBL, Cockeysville, MD, USA). After incubation, different colony types were counted and isolated in pure cultures for identification to species or genus level by morphological, biochemical and serological tests, and for anaerobic microorganisms also by gas liquid chromatography. The lower limits of detection were 100 CFU/ml of oropharyngal secretion, 20 CFU/ml gastric juice, and 20-40 CFU/ml subglottic and tracheal secretions, respectively. In paper II-III the CoNS were further analyzed by biochemical and molecular methods.
7.3.1.2 Cultures and methods for identification of Candida species (Paper IV)

Blood cultures were incubated at 35°C for 10-14 days using the BACTEC 9040 system (aerobic, anaerobic and mycology media) (Becton Dickinson, Franklin Lakes, NJ, USA). For the other specimens, culture was performed on Sabouraud’s agar, with or without antibiotics (chloramphenicol and/or gentamicin) and on chromogenic medium (CHROMagar®; Meridian, Paris, France), incubated at 35°C for 48 h, followed by 30°C for 72 h. For the identification of Candida species, assimilation pattern was determined by the API 32C® (bioMérieux, Lyon, France). Green yeast colonies on CHROMagar® were also identified down to species level by the pyrosequencing technique to discriminate between Candida albicans and Candida dubliniensis. Filamentous fungi cultures were further incubated at 43°C and identified according to their macroscopic and microscopic characteristics.

7.3.2 Determination of antibiotic susceptibility. (Paper I-III)

The minimal inhibitory concentration (MIC) of penicillin, erythromycin (AstraZeneca, Södertälje, Sweden), oxacillin, (Sigma-Aldrich, Stockholm, Sweden), gentamicin (Biochrom, Berlin, Germany), clindamycin, ciprofloxacin and vancomycin (Eli Lilly, Stockholm, Sweden) against the CoNS isolates were determined using the agar dilution method performed as recommended by the National Committee for Clinical Laboratory Standards (NCCLS) [89]. Staphylococcus aureus ATCC 29213 and Escherichia coli ATCC 25922 were used as reference strains. The cut-off for resistance was set according to the NCCLS (penicillin R \( \geq \) 0.25 µg/ml; oxacillin R \( \geq \) 0.5 µg/ml; erythromycin R \( \geq \) 8.0 µg/ml; gentamicin R \( \geq \) 16 µg/ml; clindamycin R \( \geq \) 4.0 µg/ml; ciprofloxacin R \( \geq \) 4.0 µg/ml; and vancomycin R \( \geq \) 32 µg/ml).

7.3.3 Phene-plate (Paper III)

Phenotyping with PhenePlate™ system: CoNS isolates were sub-typed to the clonal level according to their phenotype with the PhenePlate™ (PhP) system using PhP-CS plates (PhPlate Microplate Techniques AB, Stockholm, Sweden) designed for typing CoNS [90, 91]. The PhP system is based on repeated measurements of 23 biochemical tests which generate as diverse results as possible within a group of closely related species. The results of different isolates were compared pairwise, and the similarities were calculated as correlation coefficients. Dendrograms were created by clustering, using the unweighted pair group method of arithmetic mean (UPGMA). By running duplicate identical reference strains in each round of analysis and subsequently clustering reference strains from different assays, an identity-level (ID-level) of 99% was chosen. In context, isolates with identical or very similar
biochemical profiles i.e. with a correlation coefficient $\geq 0.99$ were regarded as belonging to the same PhP type. All data processing was performed with the PhP software (PhPlate AB, Stockholm, Sweden). Phenotypes originating from individuals who had been included twice or more were also clustered separately in order to study stability over time within these patients.

### 7.3.4 Pulsed-field gel electrophoresis (Paper II-III)

**Genotyping with PFGE.** All collected CoNS isolates (n=199) in paper II and some of the samples (61 CoNS isolates, 22 phenotypes) in paper III were further subtyped with pulsed-field gel electrophoresis (PFGE), using a modified protocol based on a previous description by de Lencastre *et al* [92]. Chromosomal DNA from the CoNS isolates was prepared and the DNA containing disks were restricted overnight with *Sma*I (Promega Corporation, Madison, Wi., USA) at 37°C and loaded in a gel run for 20 hours at 11.3°C in a contour-clamped homogenous electric field (CHEF) apparatus (Bio-Rad GenePath™ system, Bio-Rad Laboratories, Hercules, Calif., USA). Digestion patterns were visualized by staining with ethidium bromide. The similarity coefficients were calculated according to Dice. The control strain *S. epidermidis* ATCC 12 228 was included at least twice on each gel. Inclusion of the control strain served as a control of running conditions and subsequent software aided normalization of the banding patterns. Calculation of similarity matrix and creation of dendrograms was done by using Molecular Analyst® Software program (Bio-Rad Laboratories) using unweighted pair group method using arithmetic averages (UPGMA). According to Tenover *et al*, isolates showing up to three bands difference are considered probably genetically related, and isolates with 4-6 bands difference possibly related [93]. However, in the present studies, sampling was performed in a single ICU during a short time span. Therefore were isolates with identical band pattern considered as one genotype, while isolates with 1-3 bands difference were classified as a PFGE group. Blind duplicate samples, other than the control strain, were run in the software analysis to check the appropriateness (Dice coefficient 93-100%) of the dendrogram created. The presence of two isolates of the same genotype in two patients were considered as one transmission event, while specific PFGE groups isolated form more than one patient were referred to as probable transmission events.

### 7.3.5 HLA-DR analyses (Paper IV)

Fresh blood samples collected from each patient at day 7 and then every 7th day were analysed to monitor the immunological status by measuring the expression of histocompatibility leukocyte antigen-D-related (HLA-DR). The analyzes were
performed by three colour flow cytometric analysis (FCM), using a FACS Calibur equipped with a 15mW argon laser and Cellquest Pro software (Becton Dickinson, Franklin Lakes, NJ, USA). Daily controls of optics and fluorescence intensity were performed using standardized beads (Calibrite; Becton Dickinson). The blood samples were collected in EDTA anticoagulant tubes and immediately transported to the laboratory. Staining was performed on whole blood within an hour using PC5 labelled CD45 (clone 2D1), fluorescein isothiocyanate (FITC)-labelled CD14 (clone M P9) and phycoerythrin-labelled HLA-DR (clone L243) all from Becton Dickinson. Red blood cells were lysed, using FACS lysing solution (Becton Dickinson). Monocytes were identified as CD45+CD14+ and the results of HLA-DR expression on monocytes were recorded both as the fraction (%) of HLA-DR positive monocytes of the total monocyte population for each sample and as mean fluorescence intensity (MFI) of the entire monocyte population. A level of HLA-DR expression of > 85% was considered as a normal value. In accordance with previous studies, two cut off values (< 70% and < 30%) were chosen as limits for decreased HLA-DR expression [12-14]. The MFI of the entire monocyte population of each sample was analyzed in individual patients and compared between different subgroups of patients.

### 7.4 STATISTICAL ANALYSES

The results from different subgroups of patients were compared by using odds ratio test and the CHI square test.
8 RESULTS

8.1 COLONIZATION PATTERN IN INTENSIVE CARE PATIENTS INTUBATED ≥ THREE DAYS (PAPER I)

The patients were often heavily colonized with microorganisms that was not correlated to a healthy normal oropharyngeal or gastric flora already on admission to the ICU. A majority of them harboured enterococci, coagulase-negative staphylococci (CoNS) and Candida spp. in the subglottis and/or trachea and nine of the patients were colonized by anaerobic bacteria in their lower airways during the first 24h of intubation. The majority of the patients were colonized with rather constant levels of dominating microorganisms over time, while in 4/41 a more than 100-fold increased concentration and in 9/41 a markedly decreased concentration were found over time. With time there was an increasing load of enterococci, Enterobacteriaceae, non-fermenting gram-negative rods and gram-negative anaerobes. Decreasing levels occurred mainly with Candida spp. A progression of colonization of a specific species from the oropharynx or the stomach, the subglottis and the trachea over time was seen in 15 of 41 patients. The majority of strains isolated from the subglottic and the tracheal regions were concomitantly isolated from the upper respiratory tract. Of note, a high proportion of Pseudomonas spp., other non-fermentative gram-negative rods and some anaerobic species were primary colonizers of the lower airways before, or without, appearing in other sites. A primary tracheal colonization with these species may be explained by a preferential tropism to tracheal epithelial cells.

8.1.1 Colonization with aerobic microorganisms.

Staphylococci were isolated from 38 of the patients (93%) and always from the trachea in patients intubated for at least 6 days. All of these 38 patients harboured CoNS. From eight patients Staphylococcus aureus was isolated, in 6/8 from the lower airways in levels of $10^4$-$10^7$ CFU/ml. Thirty-four of the patients (83%) were colonized with Enterococcus spp. during the study period, of which 29 harboured Enterococcus faecalis and 19 Enterococcus faecium. Non-fermentative gram-negative rods were often isolated in high numbers from the lower airways (n = 16 patients), mainly Pseudomonas aeruginosa and Stenotrophomonas maltophilia.

Eighty percent of the patients were colonized by Candida spp. (Candida albicans, Candida glabrata and/or Candida krusei) in any of the sites and 73% in the lower airways. All the transplanted patients were colonized by Candida spp. in the subglottic and/or tracheal secretions while 20 of the 31 non-transplanted patients harboured Candida in those sites. High concentrations, $>10^4$ CFU/ml, of Candida spp.
were found in six of the 10 transplanted patients and in 13 of the 31 non-transplanted patients.

### 8.1.2 Colonization with anaerobic bacteria.

In contrast to other investigators, anaerobic bacteria were isolated from the subglottical and/or tracheal secretions in the majority (59%) of the patients, always in mixed cultures with either aerobic bacteria or yeasts. From day 12 to day 23, a range of between 40-100% of all samples from the lower airways yielded anaerobes. The most frequently isolated anaerobic bacteria were *Peptostreptococcus* (n = 16), *Prevotella* (n = 12), *Veillonella* (n = 7), *Fusobacterium* (n = 4), *Actinomyces* (n = 9), *Bifidobacterium* (n = 4), *Clostridium* (n = 3) and other anaerobic gram-positive rods, mainly *Eubacterium* spp. (n = 12). Anaerobic species were often present in all four loci at the same time.

### 8.2 COLONIZATION AND DISSEMINATION WITH CONS IN PATIENTS TREATED WITH MECHANICAL VENTILATION ≥ THREE DAYS (PAPER II)

Seventeen of the 20 patients were colonized with CoNS on at least one occasion during the study period. One to 25 isolates per patient were analyzed further by microbiological and molecular methods. Sixteen patients were colonized by CoNS in the lower airways, i.e. the subglottic area and/or the trachea during the study period, of whom ten patients already at the onset of intubation.

#### 8.2.1 Colonization pattern within patients.

Among the 199 isolates originating from 17 intubated ICU patients, 74 clusters referred to as PFGE groups, each including isolates with 0-3 bands differences were revealed by PFGE. One to 12 PFGE groups were isolated from each patient. The first site within a patient where a specific PFGE group of CoNS was detected was in the oropharynx (n = 23), the stomach (n = 9), the subglottic space (n = 9), and the trachea (n = 21), respectively. Simultaneous colonization at two or more sites occurred for 12 PFGE groups. For seven PFGE groups we were able to follow a colonization route from oropharynx and/or the stomach to the subglottic space and/or trachea.

#### 8.2.2 Transmission of CoNS between patients.

One isolate of each unique genotype per patient was further analyzed regarding its relation to other genotypes. Five unique genotypes were each detected in more than
one individual patient, strongly indicating transmission between patients. However, a total of 8 PFGE groups were found, each of which was isolated from at least two patients. Each PFGE group consisted of either one single genotype or of several probably related genotypes displaying 1-3 bands difference. Eleven to 14 patients were involved in at least one transmission event based on genotype, or PFGE group classification, respectively. The length of ICU stay these patients overlapped for all but two clusters. According to the genotypic analyzes, both patient 6 and patient 20 had isolates belonging to the same PFGE type although patient 6 was discharged approximately 3 weeks prior to admission of patient 20. In addition, patient 17 was discharged one week prior to the admittance of patient 23. Thirteen patients were each involved in 1-6 probable transmission events, while one patient (no 8) was involved in eight transmission events, that is, shared genotypes with eight other patients, during her ICU stay of nearly 3 months.

8.2.3 Antimicrobial susceptibility assay.

A total of 182 isolates were analyzed regarding the minimum inhibitory concentrations (MIC) of penicillin, oxacillin, erythromycin, clindamycin, gentamicin, ciprofloxacin and vancomycin, respectively. The rate of resistance according to the NCCLS against penicillin was 95%; 86% for oxacillin, 48% for erythromycin, 42% for clindamycin, 54% for gentamicin, 66% for ciprofloxacin, and 0% for vancomycin, respectively. Multi-resistance was commonly seen, 21% of the isolates were resistant to six, 34% to at least five, and 59% were resistant to at least four of the seven tested antibiotics. Resistance patterns were mainly, but not always, uniform within PFGE-groups. Discrepancies were found regarding ciprofloxacin and gentamicin susceptibilities. Genotype B, which was isolated from four patients, consisted only of isolates with phenotypic resistance to all of the tested antimicrobials, except vancomycin.

8.3 RESISTANCE AND DISSEMINATION OF CONS IN PATIENTS WITH LONG- RESPECTIVELY SHORT ICU STAY (PAPER III)

8.3.1 Antibiotic susceptibility of CoNS isolates

A total of 868 CoNS isolates deriving from patients with a length of stay of at least five days, longstayers (LS) and 403 isolates from recently admitted (≤ 2 hours) patients, shortstayers (SS) were analyzed for antimicrobial susceptibility. None of the tested CoNS isolates were resistant to vancomycin or linezolid. The highest resistance rates were seen for oxacillin and ciprofloxacin, being 92% and 83% respectively. LS subjects were at significantly higher risk of being colonized with CoNS isolates
resistant to oxacillin, clindamycin, ciprofloxacin and gentamicin, while resistance to fusidic acid was more common among SS patients. The majority of the isolates (69%) was considered multiresistant, i.e. expressed resistance to at least four of the tested antimicrobial agents. The risk for being colonized by a multiresistant isolate was significantly higher for the LS group.

8.3.2 Diversity and dissemination of CoNS within patients

The phenotypic analysis revealed a high diversity of phenotype groups within the patients with a range from one to 14 phenotypes per patient according to the PhenePlate system. The number of phenotypes from the different sampling sites ranged from zero to eight, with highest diversity in perineum, followed by the skin, axilla, nostril and oropharynx. Prolonged stay in the ICU setting increased the risk of oropharyngeal colonization of CoNS, OR 5.8 (95% CI 1.4; 23.4). The SS group harboured a significantly greater diversity of PhP types (median 9.5 range 5-14) per patient, while the numbers of phenotypes ranged between 1-9 (median 8) among the LS subjects (p < 0.05). The frequency of subjects in each group who were colonized by specific phenotypes in at least 3, 4 or 5 sites were 45%, 30% and 5% among SS; and 100%, 83% and 61% among LS, respectively (p < 0.001). Colonization patterns within the five individuals who were included twice or more varied markedly over time. Each patient harboured 1-6 phenotypes (median 2) that remained stable over time, and 5-17 (median 11.5) transient phenotypes. Both persistent and transient clones from all these five individuals were involved in dissemination events.

8.3.3 Dissemination of CoNS between patients

A total of 22 separate phenotypes were isolated from at least two individuals, indicating a spread of CoNS between patients. Sixty-one isolates, presumably involved in transmission events, were subjected to genotyping by PFGE in order to confirm or reject clonal relations within these clusters. Genotyping revealed a total of 32 clones of which 16 colonized more than one individual. Twelve of the 15 individuals in the LS group, of whom four at repeated occasions, and six SS patients, were involved in at least one transmission event. One of the clones was isolated from 10 individuals, including two SS patients, indicating an epidemic strain. There was generally a very good agreement between the two typing methods. Six of the 22 phenotypes did not generate identical or closely related PFGE patterns. Isolates within four of these were genetically related, i.e. exposed >3 but ≤ 6 bands difference. The concordance between the two methods was 82%.
8.4 FUNGAL COLONIZATION AND INCIDENCE OF INFECTION IN ICU PATIENTS WITH A LOS OF ≥ SEVEN DAYS (PAPER IV)

8.4.1 Colonization pattern and colonization index

*Candida albicans* was isolated from 35 patients, *Candida glabrata* from 11, while 14 patients where colonized by other non-albicans species, often in combination with *C. albicans*. The other non-albicans species were distributed as follows, *Candida lusitaniae* (*n = 3*), *Candida tropicalis* (*n = 3*), *Candida krusei* (*n = 2*), *C. dubliniensis* (*n = 3*), *Candida pelliculosa* (*n = 1*) and *Candida norvegensis* (*n = 1*). In addition, four patients were also colonized with non *Candida* fungi, *Saccharomyces cerevisiae* (*n = 3*) and *Aspergillus fumigatus* (*n = 1*). Eleven of the patients were colonized by at least two species, of whom two patients by three species. A single patient was colonized by four species (*C. albicans, C. glabrata, C. tropicalis* and *A. fumigatus*). Of the 59 patients included, 14 were not colonized by yeasts at any time (CI = 0). At the first sampling occasion at day 7, 42 % of the patients (25/59) had a CI ≥ 0.5, eight patients were colonized at each sampled site, i.e. had a CI = 1.0. At day 14, 32% of the patients (10/31), had a colonization index of CI ≥ 0.5, of whom two patients had a CI = 1.0. Only 10 patients or fewer were sampled at days 21, 28, 35, 42 and 49, when the mean CIs were 0.4, 0.4, 0.7, 0.35 and 0.5, respectively.

8.4.2 Clinical outcome and patient characteristics

Nine cases of proven ICI and one case of probable ICI occurred during day 1-12 after ICU admission. Thus, the overall observed rate of ICI was 17% (proven-probable), with a mortality of 50%, in contrast to the overall ICU mortality of 30.5%. Both a high colonization index (CI ≥0.5: OR=2.9 [95% CI 0.7-12.4]; CI ≥ 0.8: OR=23 [95% CI 4.1-129]) and recent extensive gastro-abdominal surgery (OR = 16 [95% CI 2.9-85]) were identified as risk factors for subsequent invasive fungal infection. Neither of these was significantly correlated to ICU mortality. Among the 59 patients enrolled in the study, a pattern of four diverse groups, each including patients with similar characteristics according to outcome, CI and antifungal therapy were identified. These groups are described separately in Paper IV.

8.5 HLA-DR ANALYZES AND RELATION TO ICI (PAPER IV)

The expression of HLA-DR was not significantly decreased in the studied population. The mean value of the median fluorescence intensity (MFI) was 65.6 for the whole population studied (range 9.8-434). At day 7, 28 of the 59 patients had a HLA-DR expression of ≤ 85%, mean MFI among these was 33.9, while only 10 patients had a
Results

HLA-DR expression of ≤ 70 % with a mean MFI for these 10 patients of 17.2. None of the patients had a HLA-DR expression of ≤ 30% at any time during the study period. Only three of the 18 patients who died in the ICU had a HLA-DR expression of ≤ 70%, while two of the 10 patients with proven or probable ICI (no 20 and 50) had a HLA-DR expression of ≤ 70%, MFI of these two were 24.4 for patients no 20 and 9.8 for patient no 50, respectively.
Discussion

9 DISCUSSION

Nosocomial infections are well-documented and pose an increasing threat in ICU patients. They cause major morbidity and mortality as well as being an economic burden to society. In order to minimize the incidence and the impact of these healthcare associated infections, it is of importance to gain knowledge about colonization as well as transmission routes. Nosocomial infections often proceed in two steps; first the patient becomes colonized with a nosocomial strain on the skin or mucosal surfaces, originating either from another patient, from staff or from medical equipment. Later, the strain may become invasive, often due to impaired immune response, and cause an infection. Thus, by only measuring infections, the dynamics of transmission can be greatly underestimated and important improvements in infection control measures may be overlooked. Unrecognized high colonization frequencies may therefore lead to increasing risk of nosocomial infections and antimicrobial resistance. In the present studies we have investigated bacterial colonization in upper and lower airways including the stomach of intubated patients, colonization patterns on mucosa and skin of both Candida spp and CoNS, prevalence of multi-resistant strains related to length of stay, as well as dissemination of CoNS within and between intensive care patients. The main reasons why CoNS have become important nosocomial pathogens are their ability to form biofilms, which may adhere to plastic materials used in hospitals, including catheters. Their hardy nature and ability to become multiresistant to antimicrobial agents will add to the pathogenic nature. Yeasts often have low virulence, but the incidence of severe systemic candida infections has increased markedly during the last decade, as a consequence of the increasing population of patients with immuno-deficiencies caused by severe diseases or medical interventions, in combination with a high antibiotic pressure.

Colonization with both pathogenic and potentially pathogenic multiresistant microorganisms is common among patients in the ICU due to the high antibiotic pressure at these wards (paper I) and [94]. The antibiotic consumption in individual patients may select for pre-resistant strains that are enriched and disseminates within the patients, i.e. endogenous colonization, in contrast to exogenous colonization where patients acquire bacterial strains via cross-transmission due to inadequate infection control measures. Furthermore, with an increased proportion of colonized patients in the ward (either due to admission of more colonized patients or through increased endogenous colonization of resistant strains), the risk for cross-transmissions will increase as well. This phenomenon has been named colonization pressure, and is calculated as the ratio of all colonized patients to all non-colonized patients in a ward at a specific sampling occasion. The colonization pressure has an impact on both risk for cross-transmission between patients and the risk of patients acquiring a nosocomial infection. Furthermore, it has been shown that a high
colonization pressure (>50%) is an independent risk factor for acquiring infection with VRE and MRSA [95-97].

The aim of paper I was to assess the impact of oropharyngeal and gastric colonization on the emergence of tracheal colonization over time, and its relation to the incidence of VAP in mechanically ventilated patients. The majority of patients were colonized at rather constant levels by the most predominant microorganisms during the period of intubation and mechanical ventilator treatment. Over time there was an increasing load of enterococci, Enterobacteriaceae, non-fermenting gram-negative rods and gram-negative anaerobes. Decreasing levels occurred mainly with *Candida* spp. A progression of colonization of a specific species from the oropharynx or the stomach to the subglottis and the trachea over time, which is believed to be the pathway of early onset VAP, was seen in only 15 of 41 patients. The results of the study indicate heavy colonization with levels between $10^4$-$10^8$ CFU/ml, of the lower airways in mechanically ventilated ICU patients, including potentially pathogenic aerobic and anaerobic bacteria. Different colonization patterns for different groups of microorganisms were found, indicating primary or concomitant colonization of the oropharynx for staphylococci, enterococci, enterobacteria and *Candida*, while *Pseudomonas* spp. and other non-fermenting gram-negative rods and several anaerobic species often showed a primary colonization of the trachea, indicating exogenous routes of colonization. Furthermore, an aim of the study was to investigate the incidence of VAP, which was surprisingly low, of 41 patients not a single one developed VAP. Although the study is small, the finding of no VAP in a consecutive series of 41 ventilated patients is different from a 20% incidence which is often reported in the literature. The low rate of VAP may be due to routines aimed at preventing infections, such as early tracheotomy which allows for better cleaning of the upper airways, fully awake-patients who can cooperate during respiratory physician sessions performed twice a day in most patients. The ward has also discussions with infectious disease specialists, close and daily contact with the microbiological laboratory to evaluate each patient and in cases of suspected infection, early cultures and de-escalation therapy of antibiotic use is performed. This may result in early onset of treatment, perhaps even before the development of radiological signs on chest x-ray, which otherwise has been seen as an obligate criteria for VAP.

The diversity of CoNS strains (paper III) was shown to be significantly reduced with patients’ length of ICU stay. The results of the present studies supports the theory that resistant strains that are able to be retained in the environment for long periods, become enriched and spread to different sites of the body. In paper III, at the common site for catheter insertion on the neck, at least one strain were in 78-96% of the patients with a length of stay of at least five days (LS), also isolated from other sampling sites. Corresponding rates among the recently admitted patients were 20-
50%. These results show that the probability of a unique strain disseminating from one ecologic niche to another within a patient increases with prolonged ICU stay. The clinical impact of these results is supported by a study where methicillin-resistant CoNS colonizing CVC were analyzed. The investigators of that study showed that nasal carriage of the same strain occurred in 20 of the 54 patients [76].

Concerning multi-resistance, the majority of the isolates (59 % paper II, 69% paper III), expressed resistance to at least four of the tested antimicrobial agents. The risk for being colonized by a multi-resistant isolate was significantly higher for the LS group, (for odds ratio, see table II in paper III).

In papers II and III, we studied the CoNS as a model organism to investigate the dissemination pattern both between and within patients, as well as the enrichment of multi-resistant strains in intensive care patients. In these studies we showed both a very high frequency of dissemination between patients which clearly indicates room for improvement of hygienic measures, as well as endogenous dissemination where the more virulent and multi-resistant strains became enriched and spread to different areas within the same patient. Several other studies have also shown that outbreaks of multi-resistant bacteria have been correlated to residual strains in staff in the ICU, which have been transmitted to patients and caused nosocomial infections [98, 99]. It has also been reported that the risk of acquiring antibiotic resistant bacteria (MRSA and VRE) increases if the patient is treated in a room where the most recent occupants were infected with these pathogens, strongly indicating the ability of these bacteria to survive for long periods in the surroundings [100]. Furthermore, cross-transmission of potential pathogens in severely ill patients was shown to be associated with nosocomial infections. Of all infections registered during the study period in this investigation, at least 37.5 % were due to cross-transmission of bacterial strains between patients [101]. In paper II the dissemination rate of CoNS was unexpectedly high, 14 of 20 patients were involved in at least one and up to eight transmission events. Thirteen patients were involved in 1-6 probable transmission events, while one patient, who stayed in the ICU for three months, was involved in eight transmission events, which indicates that colonization as well as the frequency of transmission events with more resistant strains increase over time. In paper III, the same high rate of transmission events of approximately 70% in patients treated for at least five days (LS) was revealed. Thus, twelve of the 15 individuals in the LS group, of whom four at repeated occasions, and six recently admitted patients (SS), were involved in at least one transmission event. The presence of an epidemic strain was detected by the finding that one clone was isolated from 10 individuals, including two recently admitted patients. We also demonstrated a high concordance (82%) between the phenotype-based PhP-system and the genotype-based PFGE method which is often considered the “golden standard”. Thus, when analyzing large number of strains, the approach to use PhP as a screening method, combined with a confirming
Discussion

genotyping method for studies on transmission of CoNS, is feasible. Similar results concerning epidemic strains of methicillin-resistant CoNS has been reported from Widerström et al., where the majority of epidemic strains were isolated from patients who had been treated in an ICU for some time during their hospital stay. In this study the investigators concluded that a possible transmission from referral hospital to county hospitals had occurred [102]. The high rate of bacterial dissemination between intensive care patients is also supported by a study from Häggren et al., in which the transmission rates of enterococci detected by rectal sampling in patients with a LOS $\geq$ five days, in either of two ICUs was investigated and in which 12 of 14 patients were involved in transmission events [103]. In another study, from our group at Karolinska University hospital, on enterococcal colonization of the upper and lower airways and transmission between intensive care patients, similar transmission rates, (13/17) patients, as those shown for CoNS were demonstrated [104]. When the preliminary results were known to us, an infection control program was implemented at the ICU and since that time a new intensive care unit has been built in which particular considerations to infection control measures has been taken into account when planning for the physical facilities. A follow-up study of bacterial dissemination rates has been performed in the new ICU, but the results are not yet analyzed.

Picture 2 The physical facilities from the new ICU, with a space of 25 m$^2$ for each patient, dispenser for alcohol–based hand disinfection at each side of the bed, as well as a hand basin for each place.
In paper IV, fungal colonization patterns and possible relationships to the incidence of fungal infections were studied. Altogether, of the 59 patients included, 14 were not colonized by fungi or yeast at any time. In the population that was colonized, the majority was colonized with *Candida* spp., and approximately 1/3 of them also with non-albicans *Candida* spp., but other non *Candida* fungi were also isolated. A majority of the patients (76%) were colonized with fungi and a considerable proportion (>40%) had a high colonization index (≥0.5). The consumption of antymycotic drugs was high and the majority of patients with a CI ≥ 0.5 were prescribed at least one antymycotic agent which could be the reason for a slight decrease in CI over time. Still, other risk factors for invasive candida infection (ICI) were considered and in the group of patient with a CI ≥ 0.5 who were not treated with antifungal prophylaxis or treatment, none developed ICI. Despite the frequent use of antifungal therapy and prophylaxis, the incidence of ICI was high (17%). A high colonization index as well as recent extensive gastro-abdominal surgery was identified as significant risk factors for subsequent invasive fungal infection. Neither of these factors was significantly correlated with ICU mortality. Of the patients included, a considerable proportion was transplanted (13/59). In concordance with this, the majority of the patients were treated with immunosuppressive medication and/or low-dose cortisone as well as broad-spectrum antibiotics. Thus, the majority of the patients had several risk factors for developing ICI, including length of stay (>7 days at the ICU). These findings of a high *Candida* colonization rate was also demonstrated in paper I, where all the transplanted patients were colonized with *Candida* spp. in the lower airways, the majority (6/10) at high concentrations (≥10^4 CFU/ml), in contrast to the non-transplanted patients amongst whom 20 of 31 patients were colonized with *Candida* spp at any time. As a consequence, in most patients with later confirmation of ICI, antifungal therapy was initiated before the CI or the results of blood cultures or cultures from other normally sterile body sites were known to the clinician. These clinical characteristics of the included patients are probably the explanation for the relatively high rates of ICI (17%) and ICU mortality (31%), compared to previous studies in unselected ICU populations [64, 105, 106]. However, the results in the present study are in line with a previous report conducted in a surgical ICU, where 29 patients with high risk for ICI developed invasive disease in 38% of cases (11/29 patients) and in whom the mortality was 6/11. The authors demonstrated in this study a high positive predictive value of the colonization index and as well as the corrected colonization index [61].

**HLA-DR expression:** we were not able to show any benefit of using HLA-DR expression as a prognostic marker of immuno-competence in our study population. A plausible explanation to this might be that the majority of our patients did not have systemic inflammatory response syndrome (SIRS) and/or septic shock at the time of
sampling. In addition, we did only monitor HLA-DR expression once a week, in contrast to many other studies which monitored on a daily basis. Thus, we may have failed in detecting the decrease in HLA-DR expression in the CARS-phase.

### 9.1 CONCLUDING REMARKS

The major findings of this thesis are:

- Intubated intensive care patients are often heavily colonized in the lower airways with potentially pathogenic microorganisms, aerobic and anaerobic bacteria as well as yeasts.

- Different colonization routes were demonstrated for different species; primary colonization of the oropharynx or concomitantly in the lower airways, was shown for *Staphylococcus*, *Enterococcus*, Enterobacteriaceae and *Candida* spp., while *Pseudomonas* and other non-fermenting gram-negative rods and several anaerobic species often showed primary colonization of the trachea.

- The dissemination rate of CoNS between ICU patients was high, 70% of patients treated for more than three days were involved in at least one transmission event.

- Prolonged ICU stay was correlated to an increased rate of cross-transmission between patients as well as a significantly higher risk of being colonized with multi-resistant strains.

- The diversity of colonizing CoNS was significantly decreased in ICU patients with a length of stay of at least five days.

- The endogenous spreading of resistant clones within patient’s skin and mucosal areas increased with time.

- The incidence of invasive candida infections was high in the ICU patient population studied, despite a frequent use of antifungal agents. This was probably due to that the majority of the patients were burdened by several risk factors.

- High colonization index (≥ 0.8) and recent extensive abdominal surgery was identified as significant risk factors for acquiring invasive candida infection in ICU patients with a length of stay of at least seven days.

*In conclusion*, the results of the present study emphasize the importance of compliance to barrier treatment, implementation and continuously follow-up of infection control programmes. Furthermore, the results underline the importance of a prudent use of antimicrobial agents for therapy and prophylaxis, based on daily
reconsideration of the treatment according to microbiological and laboratory results and the patient’s condition, especially in this vulnerable patient population.
10 ACKNOWLEDGEMENTS

Many are those, who in different ways, have contributed to my thesis. In particular, I wish to express my gratitude to:

**Professor Charlotta Edlund**, my supervisor, for teaching me patiently, the very fundamentals about scientific work and how to write. For your professionalism, scientific excellence, knowledge and availability whenever I needed you. Encouraging my ideas, though being realistic and slowing me down when I rush away. For your friendship, it is a privilege to have such a supervisor!

**Docent Hans Hjelmqvist**, my co-supervisor, for always being enthusiastic, energetic and encouraging, supporting me and constantly standing by my side during different circumstances over the years.

**Professor Carl Eric Nord**, head of the Department of Laboratory Medicine, for creating a fruitful scientific atmosphere at your department, for generously sharing your international contacts and knowledge with me.

**MD Bengt Eriksson**, head of the Department of Anaesthesiology and Intensive Care, for your dynamic and courageous leadership which has developed our department to a place where high ambitions in both clinical and scientific work is possible to realize. For interesting discussions, your never ending support and understanding through different periods of life.

**MD PhD Leif Tokics**, head of the Intensive Care Unit, you have one of the sharpest intellects and are certainly one of the most skilled clinicians I have ever met. You read me like an open book and have been supporting me in every possible way during the years. I really appreciate to work with you!

**DDS PhD Bodil Lund**, one of my co-authors, for teaching me basic and more advanced laboratory methodology, generously sharing your excellence and skills with me, for all the fun and the friendship we have together. I get happy every time I see you!

**Professor Jan Wernerman** co-author, **Professor Alf Sollevi**, **Professor Claes Frostell** and **Docent Sigga Kalman** for in different ways introducing, encouraging, guiding and advising me during my scientific studies.

**Professor Pontus Stierna**, for giving me the best advice I ever had concerning how to plan my scientific career.

**Professor Sten Walther** my tutor, **Kalle Sundkvist** and **Micle Fjälllid** my coaches, for listening, supporting, guiding and comfort me through good times and dark moments in life.
Acknowledgements

Professor Kristina Broliden and Fredrik Karpe, Reader in Metabolic Medicine, Hon. Consultant Physician, Senior Clinical Research Fellow, both dear and close friends, for your scientific brilliance, your wisdom and for sharing it with me whenever I needed your advice.

Ann-Cathrin Palmgren, for teaching me essentials about microbiology, for excellent laboratory work and just because you are such a nice and generous person!

My other co-authors, Doc. Johan Struwe, MD PhD Lena Klingspor and Doc. Göran Hedin, for stimulating discussions and new knowledge while working together.

Docent Bengt Gårdlund, for sharing your clinical experience and great knowledge with me, for interesting ideas and input in paper IV.

Lena Andersson, Anna Somell, Håkan Kalzen and all my other colleagues at the Department of Anaesthesiology and Intensive Care, all staff at the ICU and research nurses, for all the challenges, fun, sorrow and the comfort we share in every day work together.

The cheese academy: Katarina, Marie, Suzanne and Tina, my network for almost 24 years, you are a very important part of my life! For all the fun, stimulating conversation and all the crazy things we have done together. I trust you in every matter from how to choose the right cheese for every occasion to strategies when life turns up-side down.

All other friends and relatives, in Sweden, as well as France and United Kingdom, for contributing to this thesis in different ways, just by being the persons you are. I feel rich because I know you all!

Gunnar Sidenbladh, my mother, for being a role model, a solid and independent woman, for your constant and invaluable support concerning our family life, for keeping your curiosity of life alive despite your age.

Janne, for being the best possible husband and father, my soul mate and companion through life. For your never ending support in everything from computer problems to pep-talk when my self-confidence or energy is lacking. For all that has been and all that will come.

Maria and Peter, our children, for all laughs and crazy jokes that release my tensions after a hard day at work, for your empathic attitude and honesty towards all people around you, and for constantly reminding me of what is important in life.
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