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ÜLLE PARM

Early mucosal colonisation and its role in prediction of invasive infection in neonates at risk of early onset sepsis

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ABBREVIATIONS

AFLP	multienzyme multiplex PCR amplified fragment length polymorphism typing
ALV	artificial lung ventilation;
AR	ampicillin-resistant;
ARISIA	automated ribosomal intergenetic spacer analysis;
BSI	bloodstream infection;
BW	birth weight;
CD	colonisation density;
CDC	Centers for Disease Control and Prevention;
CoNS	coagulase negative staphylococci;
CLSI	Clinical and Laboratory Standards Institute;
CvC	central venous catheter;
DC	dendritic cells;
DGGE	denaturing gradient gel electrophoresis;
EOS	early onset sepsis;
ESBL	extended spectrum β-lactamases;
EUCAST	The European Committee on Antimicrobial Susceptibility Testing;
FISH	fluorescent in situ hybridization;
FN	false negative;
FP	false positive;
GA	gestation age;
GBS	group B streptococcus;
GIT	gastrointestinal tract;
HAI	hospital acquired infection;
HEPA	high-efficiency particulate air;
IAP	intrapartum antibiotic prophylaxis;
ICU	intensive care unit;
IQR	interquartile range;
LOS	late onset sepsis;
LPS	lipopolysaccharide;
MDRGN	multidrug resistant Gram-negatives;
MIC	minimum inhibitory concentration;
MRSA	methicillin-resistant *Staphylococcus aureus*;
NEC	necrotizing enterocolitis;
NICU	neonatal intensive care unit;
NP	nasopharyngeal;
NPV	negative predictive value;
OR	odds ratio;
PCR	polymerase chain reaction;
PFGE	pulsed field gel electrophoresis;
PICU	paediatric intensive care unit;
PNA	postnatal age;

PPV	positive predictive value;
PROM	premature rupture of membranes;
RFLP	restriction fragment length polymorphism method;
TN	true negative;
TP	true positive;
TPN	total parenteral nutrition;
VLBW	very low birth weight;
WBC	white blood cells.

1. INTRODUCTION

Normal microbiota is regularly found in specific areas of the body. This specificity is far from arbitrary and depends on local factors, such as pH, oxygen concentration, amount of moisture present, and types of secretions associated with each anatomical site (Cappuccino *et al.*, 2000). The gastrointestinal tract (GIT) serves as an important reservoir of normal microbiota with about 500 different microbial species have been described (Rautava *et al.*, 2002; Noverr *et al.*, 2004; Caicedo *et al.*, 2005; Mshvildadze *et al.*, 2010a; Ogra, 2010;). More than 99% of cultivable faecal microbiota is represented by 30–40 bacterial species (Fanaro *et al.*, 2003; Noverr *et al.*, 2004). In the lower intestine and colon, 96% to 99% is composed of anaerobes such as the members of the genera *Bacteroides*, *Bifidobacterium*, *Lactobacillus*, *Clostridium*, and *Streptococcus*, whereas 1% to 4% is composed of aerobes, including coliforms, enterococci, and a small number of *Proteus*, *Pseudomonas* and *Candida* spp (Cappuccino *et al.*, 2000; Noverr *et al.*, 2004)

The mucosal surfaces especially GIT are dynamic ecosystems influenced by host, intrinsic, and environmental factors. Although the importance of the gut microbiota for human health has been increasingly recognized, early bacterial colonisation in the neonatal gut and other mucosal sites is not yet completely understood (Wall *et al.*, 2009; Vaishampayan *et al.*, 2010). The relative importance of factors influencing mucosal colonisation is interacted and difficult to organize into a hierarchy. A better knowledge of the microbiota and the impact of antibiotics and other risk factors will provide an essential step towards understanding the development of mucosal bacterial community (De La Cochetiere *et al.*, 2007), contribute to improved infection control-strategies and eventually improved outcomes for critically ill neonates (Donskey, 2004; Miranda *et al.*, 2009).

Antibiotic therapy has only emerged over the last 60+ years as a practical proposition and has become one of the pillars of modern medicine. By these years significant changes in patterns of microbial colonisation in human infants have been revealed, attributable in part also to the higher numbers of Caesarean and premature deliveries, introduction of formulas, essentially sterile food, and generally cleaner living environments (Kelly *et al.*, 2007). Furthermore, advances in perinatal care have decreased neonatal and especially preterm mortality. For better outcome empiric use of antibiotics in neonatal intensive care units (NICU) has become a common though not problem-free practice.

A combination of gentamicin with a beta-lactam antibiotic such as penicillin G or ampicillin is the most recommended treatment for early onset sepsis (EOS) (Schrag *et al.*, 2002; Huang *et al.*, 2004; Mehta, 2005). Ampicillin has greater activity against some Gram-negative bacteria than penicillin G and also has activity against enterococci, *Listeria monocytogenes, Escherichia coli*, and group A and B streptococci (Ambroise, 2009). Previous studies have suggested that broad spectrum antibiotics including ampicillin when used for the empiric

treatment of EOS leads to increased rates of potentially pathogenic *Enterobacteriaceae*, to overgrowth of *Candida* spp. and selects for antibiotic resistant strains (Bonnemaison *et al.*, 2003; Donskey, 2004; Manzoni *et al.*, 2008; Auriti *et al.*, 2009). Narrow spectrum penicillins like penicillin G on the contrary, have the least potential of interfering with normal colonisation (Bennet *et al.*, 2002). Still the number of comparative studies in the field is limited.

Infants admitted to NICU are at great risk of sepsis risk factors which include preterm labour, premature rupture of membranes (PROM), indwelling catheters, feeding with total parenteral nutrition (TPN), prolonged artificial lung ventilation (ALV), and colonisation with potential pathogens (Bizzarro *et al.*, 2005; Srivastava *et al.*, 2007; Samanta *et al.*, 2011). Immaturity may predispose to bacterial translocation from GIT and other mucosa to systemic organs and tissues (Cartelle *et al.*, 2004; Donskey, 2004; Graham *et al.*, 2007; Miranda *et al.*, 2009). In an effort to anticipate septic events and guide antimicrobial therapy, some studies attempt to identify potential pathogens before infection occurs by routinely culturing a variety of surfaces from different body sites (Evans, 1988; Choi *et al.*, 2008; Smith *et al.*, 2010). So far the data are still limited and do not permit a great success.

In the Department of Microbiology of the Tartu University microbial ecology of the GIT has been investigated for a number of years, including comparisons of the faecal microbiota in healthy and sick persons (Mikelsaar, 1992; Naaber *et al.*, 1997; Bjorksten *et al.*, 1999; Mikelsaar *et al.*, 2004; Stšepetova *et al.*, 2007; Mikelsaar *et al.*, 2009) and characterization of its formation in different countries of the Baltic-Scandinavian region (Sepp *et al.*, 1997; Sepp *et al.*, 2000; Voor *et al.*, 2005). Also, an association between maternal and neonatal microbiota (Mändar *et al.*, 1996; Mändar *et al.*, 2001) and antimicrobial resistance of invasive pathogens (Lõivukene *et al.*, 2006; Sepp *et al.*, 2009) has been evaluated. This thesis focuses upon the formation of the rectal and NP opportunistic microbiota in neonates at risk of EOS admitted to NICU, identifies independent perinatal, neonatal, and environmental factors influencing the colonisation process; and defines the value of surveillance cultures in predicting late onset sepsis (LOS). Comparison of the clinical efficacy of ampicillin plus gentamicin *vs* penicillin and gentamicin in the empiric treatment of EOS is presented elsewhere (Metsvaht, 2010).

2. REVIEW OF LITERATURE

2.1. The developing microbiota and importance of early neonatal colonisation

At birth the neonatal intestine and other mucosa are sterile while no invasive procedures have been carried out on the mother and there is no PROM (Srivastava *et al.*, 2007; Mshvildadze *et al.*, 2008). During and after birth, infants are exposed to microbes that originate from the surrounding environment and/or from the birth canal (Bettelheim *et al.*, 1974b; Fryklund *et al.*, 1992; Thompson-Chagoyan *et al.*, 2007; Morelli, 2008; Mshvildadze *et al.*, 2010a; Mshvildadze *et al.*, 2010b; Ogra, 2010;). The newborn's colonisation begins at the skin and mucous membranes and enters the intestinal tract (Haenel *et al.*, 1975; Garcia-Rodriguez *et al.*, 2002; Levy, 2007; Reid *et al.*, 2011).

2.1.1. Early gut colonisation

The formation of GI microbiota is a gradual process in which several stages can be distinguished. Bacteria usually start to appear in neonate's faeces within a few hours from birth (Thompson-Chagoyan *et al.*, 2007). In general, the first phase, described as initial acquisition phase, lasts for the first two weeks of postnatal age (PNA); the second phase is the remaining period of solely breast-feeding; the third phase is the time between the beginning of food supplementation and the cessation of breast-feeding; and the fourth phase is the period of conversion to adult biota patterns beginning after the completion of weaning (Sepp, 1998; Mackie *et al.*, 1999).

During the early postnatal period aerobic and facultative anaerobic opportunistic bacteria such as *Enterobacteriaceae,* especially *E. coli* and Gram-positive cocci may reach high population levels in the intestine (Almuneef *et al.*, 2001; Rautava *et al.*, 2002; Hallstrom *et al.*, 2004; Morelli, 2008; Enck *et al.*, 2009; Ogra, 2010). Early colonisation by Gram-negative bacteria is explained by the fact, that fetal intestinal villi and crypt epithelial cells express Toll-like receptors 4 and adaptor molecules MD2, the key components of the lipopolysaccharide receptor (Levy, 2007). However, the proportion of predominant faecal colonisers by the end of the first week of life differs in different studies. For example, rates of neonates colonised by enterococci range from 12% to 100%, enterobacteria from 50% to 83%, staphylococci from 35% to 100% and streptococci from 1.2% to 45% (Rotimi *et al.*, 1981; Blakey *et al.*, 1982; Sakata *et al.*, 1985; Fryklund *et al.*, 1992; Hallstrom *et al.*, 2004; Adlerberth *et al.*, 2006). By using more modern techniques like 16S rDNA *E. coli* was found to be the largest taxonomic group on Day 6 of life (Park *et al.*, 2005).

Although these bacteria belong to potentially pathogenic species, it has been proposed, that the metabolism and oxygen consumption of these aerobic bacteria might be a positive factor in preparing the path to beneficial anaerobic

genera such as *Bacteroides, Bifidobacteria* and *Lactobacilli* (Srivastava *et al.*, 2007; Thompson-Chagoyan *et al.*, 2007; Morelli, 2008). However, previous data are mostly based on infants who have been subjected to factors, which can have a profound disruptive effect on the natural colonisation process such as Caesarean delivery (Penders *et al.*, 2006), use of antibiotics (Bennet *et al.*, 1986), or treatment in NICU (Hallstrom *et al.*, 2004). The results of the only study in infants without any major medical or dietary intervention showed that bifidobacteria are one of the first gut colonisers (Eggesbo *et al.*, 2010).

Anyway, more recently some changes in the composition of the first colonisers in industrialized countries have been reported with "classical" faecal bacteria like *E. coli* appearing late and staphylococci becoming more abundant than *Enterobacteriaceae* (Adlerberth *et al.*, 2006; Kelly *et al.*, 2007; Morelli, 2008). In a study of Adleberth *et al* (2006) by Day 3 almost 99% of infants were colonised with CoNS, and it took 2 months until enterobacteria, traditionally the first colonisers, appeared in the gut in more than 90% of infants. This phenomenon has been explained by widespread antibiotic use, dietary changes, more stringent hygienic conditions during the delivery and short hospital stay (Noverr *et al.*, 2004; Adlerberth *et al.*, 2006; Kelly *et al.*, 2007; Morelli, 2008).

2.1.2. Early nasopharyngeal colonisation

Studies, mostly published 30 to 40 years ago, have shown that immediately after birth the nasopharyngeal (Haanpera *et al.* 2008) area of 62% babies contains bacteria consistent with those found in their mothers' vaginas or faeces immediately before delivery (Bettelheim *et al.*, 1974a; Goldmann, 1981). Similar to gut colonisation the predominance of Gram-positive cocci has been demonstrated in the NP (Saiman, 2002). By the example of very low birth weight (VLBW) infants a rise of coagulase negative staphylococci (Silvestri *et al.*1999) colonisation from 12% on NICU admission to 75% by week 2 followed by a decline to 30% by week 6 has been shown (Hall *et al.*, 1990). These data are consistent with the results of Blakey *et al* (Blakey *et al.*, 1982), showing the predominance of *Staphylococcus epidermidis* (17%) colonisation in preterm neonates on day 1–4, with further rise to 43% by day 9–10 and decline to 25% by day >20.

Baltimore *et al* (1989) monitored the dynamics of Gram-negative bacterial carriage in normal infants from birth to 6 months of age and compared with colonisation in age-matched hospitalized infants (Baltimore *et al.*, 1989). The prevalence of Gram-negatives in healthy infants in the first 72 hours of life was 8% increasing to 29% during the first month. As the data were similar in hospitalized infants (overall prevalence 26%), they concluded the age of the infant to be the primary factor associated with Gram-negative microbial colonisation. In both groups the most frequent colonisers were *Klebsiella pneumoniae, Enterobacter cloacae* and *E. coli,* but also *Klebsiella oxytoca, Serratia marcescens, Pseudomonas aeruginosa* and *Acinetobacter* spp. were present. In

a more recent study conducted in Sarajevo (Ljubovic *et al.*, 2007) 37.3% of neonates in the NICU had rectal or NP colonisation by antibiotic resistant Gram-negatives in the first week of hospitalization. The most frequent NP and rectal colonisers were *K. pneumoniae, Pseudomonas* spp., *Acinetobacter* spp., whereas *Serratia* spp. was isolated only in NP samples.

The common bacterial respiratory pathogenic colonisers are *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Moraxella catarrhalis* and probably all humans are colonized with these microbes at least once early in life (Aniansson *et al.*, 1992; Garcia-Rodriguez *et al.*, 2002; Cardozo *et al.*, 2006; Mackenzie *et al.*, 2010). In the first two months of life colonisation rate of 5%, 12% and 20% for *H. influenzae*, *S. pneumoniae*, and *M. catarrhalis* have been shown, respectively (Aniansson *et al.*, 1992). Anaerobes are rarely colonising NP but may transiently occur during infection (Kononen, 2005).

2.1.3. Importance of normal microbiota

It is believed that indigenous microbiota plays an important role in the health and well-being of the host although scientific evidence of these interactions is still limited. Indigenous bacteria stimulate the development of host immunity in the intestinal tract and regulate the immune response outside the gut being the major stimulators for postnatal maturation of T cells, production of different cytokines and regulation of differentiation of dendritic cells (DC) in the intestinal mucosa (Kelly *et al.*, 2000; Rautava *et al.*, 2002; Noverr *et al.*, 2004; Pietzak, 2004; Tlaskalova-Hogenova *et al.*, 2004; Caicedo *et al.*, 2005; Levy, 2007; Wynn *et al.*, 2009; Mshvildadze *et al.*, 2010a).

The lack or change of early microbial stimulation may result in aberrant immune response later in life (Kalliomäki *et al.*, 2001a; Kalliomäki *et al.*, 2001b; Rautava *et al.*, 2002; Noverr *et al.*, 2004; Kelly *et al.*, 2007). Consistent with the "hygienic hypothesis" of allergy, an inverse epidemiological relationships between the rates of infection and autoimmunity have been described – while the rates of common infection have dropped in wealthy industrialized countries, the rates of allergy and autoimmune disease have risen (Levy, 2007, Kelly 2007, Maldonado 2007). For example, in healthy children greater faecal microbial diversity than in children with eczemas at ages 1 (mean Shannon index of diversity for healthy children = 0.75 *vs* 0.53 for exema patients; $p = 0.01$) and 4 months (0.92 *vs* 0.59; $p = 0.02$) have been shown (Forno *et al.*, 2008). As Gram-positive and Gram-negative bacteria induce partly different mediators, the optimal balance between TH1 and TH2-like immunity may be changed (Bjorksten *et al.*, 1999; Hessle *et al.*, 2000; Bjorksten, 2001; Roilides *et al.*, 2004; Bjorksten, 2006; Biasucci *et al.*, 2010).

In addition, normal microbiota plays an important role by providing a barrier for colonisation of pathogens, competing with them for nutrients, degrading their toxins, interfering with the adherence and growth, and secreting antimicrobial substances (Sprunt *et al.*, 1978; Lievin *et al.*, 2000; Noverr *et al.*,

2004; Pietzak, 2004; Caicedo *et al.*, 2005; Manzoni *et al.*, 2008; Wall *et al.*, 2009; Stecher *et al.*, 2011). However, the specific members of the indigenous bacteria that are able to inhibit colonisation by pathogens are not known (Donskey, 2004). Infants with no detectable *Bifidobacteria* in bowel, have high numbers of clostridia and *E. coli* (Mackie *et al.*, 1999; Kelly *et al.*, 2007). Also, members of the normal microbiota, such as lactic acid fermenting bacteria, producing large quantities of biologically active short-chain fatty acids as by-products of anaerobic fermentation, possess an anti-inflammatory function and thus may inhibit *C. albicans* colonisation on the epithelium of the GIT (Noverr *et al.*, 2004).

In NP resident viridans streptococci can antagonize colonisation by other streptococci, especially group A β-haemolytic streptococci (Garcia-Rodriguez *et al.*, 2002). In a study conducted more than three decades ago infants colonised by Gram-negative enteric bacilli, *S. aureus* and *S. epidermidis* were more likely to develop clinical infections (18 of 115) compared to those with normal pharyngeal flora (predominated by alpha-streptrococci; none of 108) (Sprunt *et al.*, 1978).

Furthermore, due to immaturity of various barrier mechanisms bacteria may translocate from GIT or from other mucosal surfaces into systemic organs and tissues (Cartelle *et al.*, 2004; Donskey, 2004; Graham *et al.*, 2007; Miranda *et al.*, 2009). See also chapter 2.4. Also, opportunistic bacteria may cooperate and protect each other. For example mixed-species biofilms of *S. epidermidis* and *C. albicans* may be particularly pathogenic to preterms as slime production by *S. epidermidis* may inhibit the penetration of fluconazole into mixed biofilm and *C. albicans* may protect staphylococci from the action of vancomycin (Adam *et al.*, 2002).

2.2. Factors associated with neonatal colonisation

2.2.1. Geographic region and season

The reported rates of bowel and NP bacterial acquisition and carriage vary extensively between different studies and geographical regions due to genetic background and socio-economic conditions including housing, access to health care, hygiene habits, family size, day-care contact, etc. (Mims *et al.*, 2004; Noverr *et al.*, 2004; Roilides *et al.*, 2004). In industrialized countries routine hygienic procedures, aimed at reducing the spread of bacteria in maternity and neonatal wards, have strongly influenced the colonisation pattern of the newborn infant (Noverr 2004; Morelli 2008). The 10- to 1000-fold higher counts of CoNS, enterococci, enterobacteria and lactobacilli in the intestinal microbiota of Estonian compared with Swedish neonates during the first week and also first month of life have been demonstrated in late 1990ies (Sepp *et al.*, 2000). Significantly earlier colonisation especially by *E. coli* and streptococci in Guatemalan and Pakistani children, compared with Swedish hospital-born

children has also been described (Adlerberth *et al.*, 1991; Orrhage *et al.*, 1999; Fanaro *et al.*, 2003). These differences are likely triggered by exposure to heavy bacterial loads already at birth in developing countries. Still, variations between different wards and hospitals of similar setting, likely triggered by the microbial load of the immediate environment, occur (Fanaro *et al.*, 2003; De La Cochetiere *et al.*, 2007).

An environmental risk factor, often overlooked, is the seasonal variation in the incidence of neonatal hospital acquired infection (HAI). For example, higher rates of colonisation by enterococci and multidrug resistant strains during winter/spring as compared with summer/autumn months have been shown (Hufnagel *et al.*, 2007). In Italy slightly higher rates of NP carriage of respiratory pathogens (*S. pneumoniae, H. influenzae, Moraxella catarrhalis*) in spring compared with autumn have been demonstrated (23.7% *vs* 19.5%, respectively), especially for *H. influenzae* (18.2% *vs* 13%; p>0.0001) (Marchisio *et al.*, 2001). Factors such as warm climate have been associated with a rise in colonisation rates with *Enterobacter* spp. Increased humidity or increased environmental dew point during the use of nursery air conditioners propagates airborne dissemination of *Acinetobacter* spp; the latter associated with *Acinetobacter*-related bloodstream infections (BSI) (Srivastava *et al.*, 2007).

2.2.2. Neonatal intensive care unit environment and cross-colonisation

In 1970s newborns were mostly colonised by strains spread by the hospital staff. Today we hope that better hospital hygiene interventions, such as proper hand and surface cleaning, better nutrition, adequate patient/nurse ratio, better ventilator management, use of coated urinary and central venous catheters and high-efficiency particulate air (HEPA) filters, have reduced such exposure. However, the last 15 years publications show, that NICU environment is still not problem-free as it can serve as a potential source of high pathogen loads (Curtis, 2008; Mshvildadze *et al.*, 2010a).

Microbes live on the hands of health-care workers, in air, water or environmental surfaces (De Man *et al.*, 2001; Toltzis *et al.*, 2001; Lidsky *et al.*, 2002; Hira *et al.*, 2007; Mammina *et al.*, 2007; Huang *et al.*, 1998b). Medical devices such as central venous catheters (CvC), urinary catheters and endotracheal tubes commonly used in the NICU become frequently colonised with opportunistic pathogens like *Enterobacteriaceae*, staphylococci and yeasts (Mahieu *et al.*, 2001; O'grady *et al.*, 2002; Srivastava *et al.*, 2007). For example, association between intubation for more than 72 hours and frequent NP colonisation by *Enterobacteriaceae* and/or *C. albicans* and high rates of BSI in patients with CvC are well recognised (Harris *et al.*, 1976; O'grady *et al.*, 2002).

In the NICU environment cross-colonisation followed by an outbreak of infection caused by opportunistic organisms has been described in numerous studies. The outbreaks have mainly been caused by *K. pneumoniae*, *S. marce-*

scens, E. cloacae, Acinetobacter baumannii, and *P. aeruginosa* (Verweij *et al.*, 1995; Shi *et al.*, 1996; Van Der Zwet *et al.*, 1999; Pillay *et al.*, 1999; Cartelle *et al.*, 2004; Crivaro *et al.*, 2007; Crivaro *et al.*, 2007; Zarrilli *et al.*, 2007; Mammina *et al.*, 2007; Dalben *et al.*, 2008; Cassettari *et al.*, 2009; Sanchez-Carrillo *et al.*, 2009). The origin of these outbreaks has been intensively investigated and association with NICU environment confirmed in most cases. In Italy during an outbreak of *P. aeruginosa* over 24 months the predominant type, identified by pulsed field gel electrophoresis (PFGE), was responsible for 36% of infections and at least 35% of colonisation. The same PFGE profile strain was also isolated from one sink (Crivaro *et al.*, 2009). In Scotland, during an outbreak of *Serratia marcescens* in two NICUs the outbreak strain was isolated from a laryngoscope blade and a sample of expressed breast milk (Jones *et al.*, 2000). In Sweden clonal similarity between *E. cloacae, E. coli* and *S. liquefaciens* isolates, collected from the neonates with BSI and the NICU environment has also been demonstrated (Amaya *et al.*, 2010).

Longer hospitalization is associated with colonisation by opportunistic microorganisms including *Candida* spp. (Duman *et al.*, 2005; Mammina *et al.*, 2007; Manzoni *et al.*, 2008; Westerbeek *et al.*, 2006). In a recent study every 10-day increment of NICU stay increased the odds of being colonised and infected with methicillin-resistant *Staphylococcus aureus* (MRSA) by approximately 1.3 fold (Maraqa *et al.*, 2011). By the end of the second week of life more than 95% of NICU patients have been shown to be colonised by Gram-negative bacteria, primarily *E. coli, Klebsiella* spp., *Enterobacter* spp. and *Citrobacter* spp. (Almuneef *et al.*, 2001). In addition, over the same period acquisition of resistant strains may also occur (Millar *et al.*, 2008).

Microbes acquired via horizontal transmission are frequently antibiotic resistant and are endemic in many hospitals (Donowitz *et al.*, 1981; Ayan *et al.*, 2003; Gras-Le Guen *et al.*, 2003; Bagattini *et al.*, 2006; Huang *et al.*, 2006a; Curtis, 2008; Millar *et al.*, 2008; Mears *et al.*, 2009; Cassettari *et al.*, 2009; Simmonds *et al.*, 2009). In an Italian study 55.2% of NICU patients were colonised by multidrug-resistant Gram-negative bacilli (MDRGN), with colonisation rate being associated with the length of NICU stay; in 72.4% a cross-colonisation had occurred (Mammina *et al.*, 2007). Also in Washington by molecular epidemiologic analyses clustering for 36 (78%) *P. aeruginosa*, 22 (45%) of the *E. cloacae*, and 13 (59%) *K. pneumoniae* isolates – all of them were MDRGN – has been revealed (Anderson *et al.*, 2008).

In the Netherlands during an outbreak period multiresistant *E. cloacae* was transmitted via an electronic digital thermometer (Van Den Berg *et al.*, 2000). In Turkey two outbreaks due to *K. pneumoniae* and one due to *K. oxytoca* with a mortality rate of 76.7% were caused by the same genotype strain obtained from an incubator (Ayan *et al.*, 2003). Toltzis *et al* (Toltzis *et al.*, 2001) investigated 10 antibiotic-resistant Gram-negative species most frequently isolated from NP and rectum by PFGE in a non-outbreak period. They found, that cross-colonisation occurred in 12% of all analysed isolates. In India and

Italy, extended spectrum β-lactamases (ESBL) producing *K. pneumoniae* with similar PFGE type and antibiogram were isolated from the clinical samples of neonates, and also from room surfaces, health-care workers´ hands, incubators, work surfaces, suction apparatuses, medicine trolley and sinks (Bagattini *et al.*, 2006; Tallur *et al.*, 2000). In the USA and Korea transmission of these microbes via artificial nails and designated stethoscope has been described (Gupta *et al.*, 2004; Lee *et al.*, 2004).

Colonisation with nosocomial microbes may persist for more than a year, even long after the index case has left the hospital (Goldmann, 1981; Millar *et al.*, 2008). In Australia over a 4 week period 86 samples from 36 toys of 19 infants were studied. Almost all toys (98%) were contaminated by CoNS, 39% by streptococci; and 47% by *S. aureus,* while more than three quarters were carrying MRSA. Eight (42%) of the infants had positive blood culture results and 5/8 of the isolates were genotypically identical to those colonising their corresponding toy (Davies *et al.*, 2000).

Admitted neonates may also serve as a reservoir for cross-contamination within the ward. Frebourg *et al* (1999) have demonstrated the presence of a single strain of MRSA (identified by PFGE) isolated from 33 CvC – nasal cultures pairs. Anyway, over the last several years clonal transmission of MRSA has become an increasing problem in NICUs around the world (Shiojima *et al.*, 2003; Regev-Yochay *et al.*, 2005; Mcdonald *et al.*, 2007; Anderson *et al.*, 2008; Gregory *et al.*, 2009; Heinrich *et al.*, 2011), mostly imported into the unit by colonised parents or healthcare workers but also by contaminated breast milk and medical equipment (Boyce *et al.*, 1993; Morel *et al.*, 2002; Eckhardt *et al.*, 2003; Behari *et al.*, 2004; Fujimura *et al.*, 2004).

2.2.3. Maternal factors (maternal microbiota, premature rupture of membranes, mode of delivery)

Maternal vaginal and intestinal flora is a well-recognized source of bacteria for newborns´ microbiota (Bettelheim *et al.*, 1974a; Bettelheim *et al.*, 1974b; Bettelheim *et al.*, 1974c; Goldmann, 1981; Tannock *et al.*, 1990; Penders *et al.*, 2006; Ogra, 2010). Recently Dominguez-Bello *et al* (2010) compared maternal skin, oral and vaginal microbiota sampled 1 h before delivery with neonatal skin and NP aspirate sampled <5 min, and meconium <24 h after delivery by using multiplex 16S rRNA gene pyrosequencing. They showed that the microflora in vaginally delivered infants resembled maternal vaginal flora while infants born via Caesarean section harboured bacterial communities similar to those found on maternal skin. The similarity rates of the maternal-neonatal microbial pairs vary from 85% (Bettelheim *et al.*, 1976; Mändar *et al.*, 1996) to 8% (Fryklund *et al.*, 1992) from study to study. Tapiainen *et al* (Tapiainen *et al.*, 2006) documented, that the short- and long-term (6 months) effects of the maternal faecal flora on gut colonisation in hospital-born infants were less marked than

had been expected and the faecal flora of infants resembled both the faecal flora of the mother as well as the first nurse.

Many studies have described the type of delivery as a crucial aspect in selecting the first colonisers in neonates (Goldmann, 1981; Hallstrom *et al.*, 2004; Pietzak, 2004; Morelli, 2008; Biasucci *et al.*, 2010) although some controversies exist. Vertical transmission of the same phenotype of *Enterobacteriaceae* in 12% of vaginally delivered *vs* 0% of Caesarean section delivered neonates has been shown (Fryklund *et al.*, 1992). In naturally delivered neonates within 24–72h from birth *E. coli,* streptococci, staphylococci, and enterococci can be detected in stool (Morelli, 2008). Vaginally born infants *vs* those delivered via Caesarean section harbour significantly more *E. coli* at the age of 3 (39% *vs* 9%) (Biasucci *et al.*, 2010) and 14 days (14.7% *vs* 2.8%, p=0.012) (Hallstrom *et al.*, 2004). However, no differences in bacterial counts seem to occur. Penders *et al* (2006) did not find any effect of the route of delivery on the overall intestinal microbial composition; *E. coli* counts were even somewhat higher in Caesarean section *vs* vaginally born infants; 9.59 *vs* 9.09 log10 CFU/g, respectively. The analogous results have shown by Mshvildadze *et al* (2010b) who evaluated the diversity of intestinal microbiota shortly after delivery and during hospitalization by using 16S rRNA pyrosequencing and found no significant differences in babies of mothers who had a Caesarean delivery *vs* vaginal route (diversity index 8.99 *vs* 8.13, respectively).

Neonates born via Caesarean section also become colonised by *E. coli* but also with other members of *Enterobacteriaceae*, such as *Klebsiella* spp (Kelly *et al.*, 2007). Compared to naturally delivered infants these neonates seem to have reduced numbers of anaerobes (*Bacteroides* spp., *Bifidobacterium* spp.), that may promote overgrowth of *Clostridium difficile* (Fanaro *et al.*, 2003; Westerbeek *et al.*, 2006; Kelly *et al.*, 2007; Biasucci *et al.*, 2008; Huurre *et al.*, 2008; Morelli, 2008; Adlerberth *et al.*, 2009). For example, Caesarean-delivered infants are less often colonised with lactobacilli and bifidobacteria on day 4 (4% *vs*. 59% and 0% *vs.* 23%, respectively) and with bifidobacteria on day 30 (0% *vs.* 35%, p = 0.042) compared to vaginally delivered ones (Mitsou *et al.*, 2008). Also, based on the results of denaturing gradient gel electrophoresis (DGGE) analyses *Bifidobacterium* spp was detected in 13 of 23 (56.5%) samples derived from vaginally delivered newborns but in none of the samples obtained from those born via Caesarean section (Biasucci *et al.*, 2010). On the other hand Mshvildadze *et al* (2010b) evaluated the diversity of intestinal mictobiota shortly after delivery and during hospitalization by using 16S rRNA pyrosequencing and found no significant differences between babies born via Caesarean *vs* vaginal delivery.

More virulent microbes such as MRSA may also be acquired from the mother. In Taiwan (Huang *et al.*, 2006a) MRSA colonisation was detected in 41% of infants admitted to the NICU. In nearly 90% of colonised infants, MRSA was detected in the first 2 (weekly taken) samples. As most of them (60%) were admitted in the first 24 hours of life, and the acquisition of MRSA

occurred very soon after hospitalization or even before the infants were admitted to the NICU, the authors suggest, that the infants might have acquired MRSA from the mother. Howerver, this conclusion might not be conclusive, as in NICU healthcare workers nasal carriage rate was 4.8%. Anyway, in USA the prevalence of MRSA anovaginal colonisation in pregnant women remained stable and low over the years 2005 to 2009 (0.5% to 0.6%), but the rise of the clone USA3000 (0 of 14 isolates in 2005 vs 12 of 18 isolates in 2009) lead to an increase in postpartum MRSA infections in both, women and their newborns (Top *et al.,* 2010). Maternal vaginal colonisation has been shown to be a risk factor of neonatal colonisation and infection by *Candida* spp. One third of mothers delivering preterm were colonised with *Candida* and 15 of 25 strains (60%) isolated from the mothers and their neonates were similar (Xu, 1996; Mendiratta *et al.*, 2006).

Additionally, the composition of newborn microbiota and also infections in preterm infants may originate from uterus. The presence of microbes in the amniotic fluid without rupture of membranes in the second-trimester placental parenchyma has been demonstrated (Mändar *et al.*, 2001; Onderdonk *et al.*, 2008; Mshvildadze *et al.*, 2010a). In particular, intraamniotic bacterial infections in relation to PROM may cause preterm birth followed by a serious threat of immediate postnatal infections (Asindi *et al.*, 2002; Kenyon *et al.*, 2003; Simhan *et al.*, 2005; Kirchner *et al.*, 2007; Levy, 2007; Lafeber *et al.*, 2008; Veleminsky *et al.*, 2008). For example, in the USA PROM affects over 120 000 pregnancies annually, and is associated with significant neonatal morbidity and mortality (Simhan *et al.*, 2005). In Saudi Arabia (Asindi *et al.*, 2002) association between microbes isolated from the endocervical swabs of mothers with PROM and their infants' microbiota were found, and infection risk of colonised neonates was 14%. CoNS (24%) and *K. pneumoniae* (13%) were the predominant isolates in the mothers and the same microbes were frequent in infants.

2.2.4. Maturity

Comparing term and preterm neonates delayed bacterial colonisation with limited number of bacterial species has been demonstrated in the latter. For example, Mshvildadze *et al* (2010b) did not detect any microbial DNA in the first stool sample in 2 of the 23 very premature infants and lower GA (<30 weeks compared with ≥30 weeks) was associated with lower diversity index (Simpson diversity index 6.20 *vs* 9.38; p=0.03). Another study found that the lower the birth weight (BW) the later the colonisation by Gram-negative opportunistic bacteria ($r=-0.449$; $p < 0.01$) (Almuneef *et al.*, 2001). Increased similarity of the bacterial communities in hospitalized preterm infants in contrast to breast-fed full-term infants has been shown also by Schwiertz *et al* (Schwiertz *et al.*, 2003). The microbes most commonly found in all preterm infants in this study were *E. coli*, *K. pneumoniae* and *Enterococcus* spp.

Westerbeek *et al* (2006) reviewed the results of six studies determining the intestinal microbiota in preterms, carried out 20–30 years ago. They concluded that gut colonisation with beneficial bacteria is delayed in preterm neonates, while the counts of potentially pathogenic bacteria are high. Unfortunately, no comparable data from term newborns were presented. Similar results were described by Gewolb *et al* (1999) in ELBW infants. By day 30, the predominant species were *Enterococcus faecalis*, *E. coli, E. cloacae, K. pneumoniae, S. epidermidis* and, *S. haemolyticus* whereas *Lactobacillus* and *Bifidobacterium* spp were identified in only one infant. Significant association between LBW and colonisation with multidrug-resistant Gram-negative microbes has been demonstrated (Duman *et al.*, 2005; Mammina *et al.*, 2007). Also, the colonisation rate by *Candida* spp is significantly higher in preterm compared to healthy term infants (Mendiratta *et al.*, 2006), especially GA <32 weeks is associated with either *C. albicans* (OR=1.08; 95% CI 1.30–2.49) or *C. parapsilosis* (OR=2.87; 95% CI 1.79–4.58) colonisation (Saiman *et al.*, 2001).

2.2.5. Nutritional habits

The neonate is also likely to obtain bacteria originating from the nipple and surrounding skin as well as the milk ducts in the breast (Heikkila *et al.*, 2003; Morelli, 2008). Studies have shown that microbes may translocate into the breast milk from mothers' GIT and bloodstream (Perez *et al.*, 2007). So, expressed breast milk contains up to 10^9 microbes/L in healthy mothers (Mackie *et al.*, 1999; Morelli, 2008).

Whether and how feeding habits influence intestinal colonisation is still controversial. Some studies have found higher abundance of aerobic bacteria in the GI tract of formula-fed compared to breast-fed neonates (Noverr *et al.*, 2004; Pietzak, 2004; Ogra, 2010), others describe the opposite findings (Gewolb *et al.*, 1999) or no difference between breast milk and formula at all (Penders *et al.*, 2006).

Breast milk colonised by microbes, including staphylococci, streptococci, and lactic acid bacteria, may play a positive role and act as a natural "probiotic" (Neu, 2007; Perez *et al.*, 2007). Martín *et al* (2007) suggested that also Gram-negatives, enterococci, and *S. aureus* are normal inhabitants of human milk. Anyway, *Bifidobacteria* and *Lactobacillus* spp. are among the dominant microorganisms in breast-fed infants, outnumbering *Enterobacteriaceae* by 1000-fold (Pietzak, 2004), whereas formula-fed infants are colonised more quickly and by more diverse microbiota, including *Bifidobacteria*, *Bacteroides*, clostridia, enterococci, staphylococci, streptococci and especially *Enterobacteriaceae* (*E. coli, Klebsiella* spp) (Gewolb *et al.*, 1999; Mims *et al.*, 2004; Noverr *et al.*, 2004; De La Cochetiere *et al.*, 2007; Ogra, 2010). This may be in part due to the iron in the infant formulas, which fosters the growth of more complex flora (Pietzak, 2004).

Although formula is expected to be sterile, possible contamination of milk bank or equipment used for enteral feeding should be considered. Outbreaks, caused by milk contamination by *Pseudomonas aeruginosa* (Gras-Le Guen *et al.*, 2003; Sanchez-Carrillo *et al.*, 2009) or *Klebsiella* spp. (Donowitz *et al.*, 1981; Berthelot *et al.*, 2001) have been described. Furthermore, Hurrell *et al* (2009) isolated opportunistic organisms like *E. coli, K. pneumoniae, Serratia* spp., and also *Pseudomonas* spp. from 76% of nasogastric enteral feeding tubes. Also, an association between exclusive formula feeding and cross transmission of MDRGN rods (RR=1.8; 95% CI 1.1–3.5) (Mammina *et al.*, 2007) and more frequent colonisation with *E. coli* (OR=2.90; 95% CI 1.22–6.89) has been described (Penders *et al.*, 2006). Based on the expression of surface K1 antigens (virulence marker) and serum killing, *E. coli* strains differ in breast fed and formula-fed infants (Mackie *et al.*, 1999).

There are almost no studies describing the direct impact of TPN on colonisation process. Recently Smith *et al* (2010) demonstrated an association between delayed enteral feeding and late rectal colonisation by Gram-negative bacilli. On the other hand, in neonates receiving TPN normal GIT structure and function is lost, villi become shorter, mucosal DNA is lost, protein content and enzymatic activity are reduced (Ben, 2008). As a result they have an increased risk of nosocomial infection by Gram-negative organisms and fungi (Clark *et al.*, 2004; De La Cochetiere *et al.*, 2007).

Only a few studies have described the influence of feeding habits on NP colonisation. Baltimore *et al* (1989) reported, that breastfed infants had significantly lower prevalence of Gram-negative rods in NP than non-breast-fed infants at the age >3 weeks to 2.5 months (40% *vs* 64%, respectively). They speculated that immunoglobulin A and lymphocytes found in breast milk may be important in determining the different rates of colonisation. On the other hand, breast feeding *vs* formula feeding has been shown to protect against otitis media (Garcia-Rodriguez *et al.*, 2002) although did not have any substantial influence on NP colonisation with respiratory pathogens such as *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Haemophilus influenzae*, and *Moraxella catarrhalis* (Kaleida *et al.*, 1993; Garcia-Rodriguez *et al.*, 2002).

2.2. Antibiotic treatment

Antibiotic treatment may influence colonisation either when given to the mother during the delivery (for intrapartum prophylaxis or treatment of perinatal infection) or to the neonate early after birth. However, due to methodological variations as well as the multifactorial nature of gut colonisation the data in literature contains some controversy. Changes in the composition of microbiota as a consequence of antibiotic treatment can result in the dysregulation of host immune homeostasis and an increased susceptibility to disease (Willing *et al.*, 2011).

2.3.1. Intrapartum antibiotic treatment of mothers

Intrapartum antibiotic prophylaxis (IAP) to prevent EOS caused by group B streptococci (GBS) has been in use for more than a decade and as shown in several studies has decreased infection rate significantly. For example, over the 17 years' experience (1986–2002) in Ohio antepartum antibiotic prophylaxis decreased the number of GBS BSI cases from 41 (1986–1991) to 4 (1998–2002) (Cordero *et al.*, 2004) and over the 9 years' experience (1993–2001) in Australia from a peak of 1.43/1000 live births to 0.25/1000 (Daley *et al.*, 2004). Additionally, during the IAP era the decrease of GBS sepsis has led to a reduction in the overall incidence of EOS; in a Boston study from 3.70/1000 live births in 1990–1992 to 1.59/1000 live births in 1997–2007 (Puopolo *et al.*, 2010).

However, some worrying trends have been noted concomitantly. Administration of antimicrobial agents may disturb the ecological balance between the host and the microorganisms and thus interfere with initial gut colonisation in neonates (Sullivan *et al.*, 2001; De La Cochetiere *et al.*, 2007; Tanaka *et al.*, 2009). As a result of IAP increased rates of neonatal mucosal colonisation by ampicillin resistant *Enterobacteriaceae* and increased risk of EOS caused by ampicillin-resistant *E. coli* in many (Mcduffie *et al.*, 1993; Joseph *et al.*, 1998; Schuchat *et al.*, 2000; Almuneef *et al.*, 2001; Stoll *et al.*, 2002a; Laugel *et al.*, 2003; Cordero *et al.*, 2004; Bizzarro *et al.*, 2008), although not in all studies (Edwards *et al.*, 2002; Jaureguy *et al.*, 2004; Schrag *et al.*, 2006a; Puopolo *et al.*, 2010). Stoll *et al* (2002a) and Puopolo *et al* (2010) have shown, that the mothers of infants with ampicillin-resistant (AR) *E. coli* had significantly more likely received IAP with ampicillin than those with ampicillin sensitive strains (26 of 28 *vs* 1 of 5; p=0.01; and 36.9% *vs* 16.8%, respectively). On the other hand, Jaurgery *et al* (Jaureguy *et al.*, 2004) compared the patterns of GIT colonisation in infants born with IAP (amoxicillin) and those without early antibiotic exposure and found no differences in colonisation by amoxicillin-resistant enterobacteria (75% and 77%, respectively).

The trends in invasive disease have been even more controversial. Namely, Bizzarro *et al* (Bizzarro *et al.*, 2008) reported a significant increase in the proportion of ampicillin-resistant *E. coli* EOS in the VLBW population over the study period of 1976 to 2006. No cases of AR *E. coli* were observed from 1979 to 1992, compared with 1 out of 4 during the risk factor based IAP period from 1993 to 1996 and 12 out of 17 cases during the screening based IAP period from 1997 to 2006. Intrapartum ampicillin exposure was identified as a significant risk-factor for AR (OR=17.91; 95% CI 1.59–202.37). In the Ohio study, IAP (penicillin G or ampicillin) increased *E. coli* blood isolates' resistance to ampicillin/sulbactam from 45% to 73% over the period from 1996–1996 to 1997–2002 (Cordero *et al.*, 2004). However, Puopolo *et al* (2010) found no change in the incidence of infection with AR organisms overall or among VLBW infants. In Australia, using preferably penicillin for IAP, the decrease in EOS due to GBS has been accompanied by concomitant decrease in *E. coli*

sepsis (Daley *et al.*, 2004; Tarnow-Mordi *et al.*2010), suggesting the role of the different choice of antibiotics applied in IAP.

Unfortunately, direct comparisons of different antibiotic regimens, especially involving sufficiently long time periods to allow out-selection of resistance, are scarce. Edwards *et al* (2002) assessed whether ampicillin as a broader spectrum antibiotic *vs* narrow spectrum penicillin G for IAP affects the subsequent exposure of the neonate to AR Gram-negatives and found, that either antibiotic may promote neonatal exposure to AR *E. coli* and other *Enterobacteriaceae*. The rates of AR strains in maternal genital tract cultures obtained at study entry and 8–36 h postpartum were similar, with an increase in postpartum culture rates of AR *E. coli* and other *Enterobacteriaceae* seen in both groups.

Alternately, case control and/or cohort approach applying multivariate analysis has been used. Schrag *et al* (2006a) found an association between IAP exposure to ≥2 doses of penicillin or ampicillin and invasive *E. coli* infection. However, comparing *E. coli* patients with uninfected controls while controlling for other risk factors, this association disappeared. This study highlights (1) the importance of adequate study design and statistical analysis to elucidate the true relationship between antibiotic exposure and resistant infections; and (2) the dangers of overinterpreting associations based on case patients' data alone or in analyses not controlling for other major risk factors.

2.3.2. Antibacterial treatment and colonisation by resistant microorganisms

A number of studies have looked the effect of general antibacterial therapy on mucosal colonisation in terms of development of antibacterial resistance but only a limited number of studies have monitored the impact of short-term empiric antibacterial therapy of EOS (Table 1). In some studies the purpose of antibacterial therapy has not been specified.

Table 1. The effect of antibacterial therapy on mucosal colonisation

Study	Purpose of treatment	Antibiotics	Findings
Bennet *et al.*, 1982	General	Gentamicin + ampicillin *vs* untreated	*E. coli* dominated in untreated, and *K. pneumoniae* in treated neonates
Burman *et al.*, 1993	General	ampicillin *vs* cefuroxime *vs* untreated	In ampicillin group colonisation with resistant strains to all broad spectrum penicillins and cephalosporins occurred
Duman *et al.*, 2005	General	Maternal: ampicillin or amoxicillin, neonatal: AB were not specified	Infant and maternal AB use were risk factors of colonisation with ESBLs
Gewolb *et al.*, 1999	General	Week I: ampicillin + gentamicin or cefotaxime; later: vancomycin + cefotaxime	Inverse correlation between AB days and the number of bacterial species and the total number of microorganisms.
Jain *et al.*, 2003	General	Unspecified	Indiscriminate use of III-generation cephalosporins may be responsible for the selection of ESBLs.
Mammina *et al.*, 2007	General	AB during NICU stay (unspecified)	AB treatment was not associated with colonisation by multidrug resistant Gram-negative microorganisms
Millar *et al.*, 2008	General	EOS: penicillin + gentamicin; LOS: different Abs	Exposure of specific AB is not risk factor for the carriage of a strain resistant to that AB
Penders *et al.*, 2006	General	AB during the first month	AB use results in decreased amount of bifidobacteria and bacterioides
Sprunt, 1985	General	AB during NICU stay (unspecified)	NP: AB – bacterial overgrowth of abnormal microbiota
Almuneef *et al.*, 2001	NS	ampicillin + gentamicin, III generation cephalosporins	Amount of AB use was associated with increase colonisation by resistant strains and late colonisation
Tanaka *et al.*, 2009	NS	AB free *vs* cefalexin in first 4 days *vs* maternal cefotiam hydrochloride	AB in the beginning of life conduces to overgrowth of *Enterobacteriaceae* and *Enterococcus* spp. and crush down bifidobacteria. Occurrence of resistance strains was not observed.

Study	Purpose of treatment	Antibiotics	Findings
Toltzis *et al.*, 2002	NS	Rotation of ABs: gentamicin → piperacillin-tazobactam, → ceftazidime	Rotation did not decrease resistant strains
Auriti *et al.*, 2009	EE	Two *vs* a single dose of ampicillin + netilmicin in 72 hours	The single and double dose was equally effective
Bonnemaison *et al.*, 2003	EE	Untreated *vs* amoxicillin + netilmicin *vs* amoxicillin + netilmicin + cefotaxime	In three AB group the biodiversity of intestinal flora was low with overgrowth of staphylococci and *Candida* spp.
Cotten *et al.*, 2009	EE	Different regimen, frequently ampicillin + gentamicin	Prolonged duration of the initial empirical therapy may be associated with the risk of death or NEC
De Man *et al.*, 2000	EE	amoxicillin + cefotaxime *vs* penicillin + tobramycin	Broad spectrum of AB regimen leads to colonisation by resistant strains

EE – early empirical antibacterial therapy; AB – antibiotic; NS – not specified; NEC – necrotizing enterocolitis

The results of studies focusing on risk factors of ESBL colonisation are somewhat controversial. While the majority of studies highlight prior use of third-generation cephalosporins as an important risk-factor of the emergence of ESBL producing strains (Rice *et al.*, 1990; Arlet *et al.*, 1994; Wong-Beringer *et al.*, 2002; Jain *et al.*, 2003; Glynn *et al.*, 2005), the results of a study conducted in Taiwan (Chiu *et al.*, 2005) have not confirmed this effect, though 34 of 76 of *Enterobacteriaceae* isolates from III level NICU patients were ESBLs. Duman *et al* (Duman *et al.*, 2005) determined the rate of β-lactam resistant bacteria in the commensal faecal flora of newborns and the risk factors of colonisation in Turkey. Colonisation with AR-resistant and ESBL producing microorganisms was found in 75.2% and 33.7% of 367 stool samples, respectively. Besides very low birth-weight, vaginal delivery and male sex, infant and/or maternal antibiotic use was identified as a risk factor related to colonisation with ESBL-producing microorganism (infant antibiotics – OR=14.5; 95% CI 1.19 – 164.62 and maternal antibiotics – OR=3.81; 95% CI 1.07 – 13.50). Mothers were treated with ampicillin or amoxicillin in this study but infant's antibiotic regimens were not specified. In another study the use of penicillin and amikacin but not third-generation cephalosporins was identified as a risk factor for the acquisition of ESBL-producing *K. pneumoniae* (OR=12.3; 95% CI 3.66 – 41.2) (Cassettari *et al.*, 2009).

Rotation of antibiotics has also been suggested as a measure to reduce selection pressure. Toltzis *et al* (Toltzis *et al.*, 2002) in USA tested the potential of antibiotic rotation in reduction of colonisation with resistant Gram-negative bacilli in NICU. In one team physicians chose antibiotics according to their individual practice, while in the other antibiotics were changed monthly as follows: gentamicin was followed by piperacillin-tazobactam followed by ceftazidime. For Gram-positive coverage, vancomycin or ampicillin were accepted at the discretion of the attending physician. This strategy deemed to be ineffective – a total of 10.7% infants in the rotation *vs* 7.7% in the control group were colonised by resistant Gram-negative bacilli.

Apart from resistance selection attributed to antibiotics, the delay in normal intestinal and NP colonisation may facilitate introduction of environmental pathogens or overgrowth of potentially opportunistic organisms normally present in small numbers (Bennet *et al.*, 1982; Sprunt, 1985; Gewolb *et al.*, 1999; Tanaka *et al.*, 2009), especially promote intestinal colonisation with *Candida* spp. (Bendel, 2003; Bonnemaison *et al.*, 2003; Donskey, 2004). Gewolb *et al* (1999) have shown an inverse correlation between the number of days on antibiotic treatment in the first month of life and the number of bacterial species ($r=0.491$; $p=0.007$) and the total number of organisms ($r=0.482$; $p=0.008$) in stool samples of ELBW infants on day 30. The association between the amount of antibiotics used and later colonisation has been confirmed in Yale by Almuneef *et al* (2001). They have also demonstrated the interplay of antibiotic use (ampicillin and gentamicin), development of gentamicin-resistance, and the spread of nosocomial Gram-negatives in the NICU setting.

Tanaka *et al* (2009) have monitored the influence of antibiotics administrated to neonates (first-generation cephalosporins) or their mothers (second-generation cephalosporins) for 4 days after delivery, on the development of intestinal microbiota. They showed a significant overgrowth of enterococci on day 3 and 5 and one month and arrested growth of *Bifidobacterium* on Day 3 in the antibiotic treated group as compared with untreated group. Also, after the first month, the population of *Enterobacteriaceae* was markedly overrepresented in the antibiotic therapy group as compared to the untreated population. Furthermore, infants whose mothers received antibiotics for Caesarean section, sustained similar, although relatively weaker, alteration in the development of microbiota.

2.3.3. Empiric antibacterial treatment of early onset sepsis

A combination of gentamicin with a beta-lactam antibiotic such as penicillin G or ampicillin is recommended as the treatment of choice for EOS by most handbooks and guidelines (Joung, 2008; Metha, 2005; Schrag *et al.*, 2002) and are used widely (Table 1). However, some experts advocate antibiotics of wider spectrum of activity like third generation cephalosporins (Hall *et al.*, 2011). Although, their use has remained limited because of increasing resistance (Yurdakok, 1998; De Man *et al.*, 2000; De Hoog *et al.*, 2005; Hakalehto, 2006; Ambroise, 2009), there may be a place for broader spectrum primary EOS therapy in special circumstances. Thrombocytopenia <94.5 X 10^9/L with concomitant need for vasoactive treatment; or white blood cells (WBC) below 3.5 X 10^9/L or above 38.8 X 10^9/L; or blood glucose below 1.65 mmol/L within 72 h of PNA all carry a more than 30% risk of a penicillin and gentamicin treatment failure in neonates at risk of EOS as highlighted recently by Metsvaht *et al*. (2009).

Penicillin and ampicillin are in general considered as narrow spectrum antibiotics but ampicillin has higher activity against some Gram-negative bacteria (e.g. *E. coli*) than penicillin G and is also effective against entrococci, *Listeria monocytogenes,* and group A and B streptococci (Yurdakok, 1998; De Man *et al.*, 2000; De Hoog *et al.*, 2005; Hakalehto, 2006; Schrag *et al.*, 2006b; Ambroise, 2009). Aminoglycosides are bactericidal antibiotics used for broad spectrum coverage of most Gram-negative organisms. They work by inhibiting protein synthesis on 30S ribosomes (De Hoog *et al.*, 2005; Ambroise, 2009).

A limited number of studies have looked at the effect of short-term empiric antibacterial therapy of EOS on mucosal colonisation in the era of resistance and no final conclusions can be drawn. De Man *et al* (2000) compared the effect of amoxicillin plus cefotaxime with penicillin plus tobramycin on the emergence of resistant *Enterobacteriaceae* in two NICUs in the Netherlands over a period of six months. They found an 18.8% colonisation rate with bacteria resistant to initial antibiotics with the first regimen compared to only 1.3% with the second regimen. Still, the study did not clarify whether the differences

between the two antibiotic regimens were triggered by amoxicillin or cefotaxime or both.

In a French study Bonnemaison *et al* (2003) compared the impact of two antibiotic combinations (amoxicillin + netilmicin and amoxicillin, cefotaxime and netilmicin) on the faecal flora during the first 10 days of life with untreated controls. In the untreated control group the overall colonisation began within the first 3 days of life and was of normal biodiversity, while in the group receiving the combination of three drugs the overall colonisation was delayed, a species level biodiversity was low, and rapid growth of staphylococci (mainly *S. epidermidis*) and *Candida* spp. occurred.

To avoid resistance Auriti *et al* (2009) shortened the course of antibiotic prophylaxis (ampicillin and netilmicin) and showed, that a single dose on admission was as effective as a three day course with no significant differences between the two groups in overall mortality and incidence of vertically acquired infections (23.9% *vs* 22.2%).

2.4. Association between mucosal colonisation and invasive diseases

2.4.1. Mucosal surfaces as a source of invasive disease

In NICU by the end of the first week of life more than 40% and by Day 30 almost all high risk neonates have mucosal colonisation with potentially pathogenic Gram-negative microorganisms which due to disturbed integrity of the barriers in sick neonates may result in invasive disease (Lin *et al.*, 2008; Ambroise, 2009; Ogra, 2010; Smith *et al.*, 2010). Genetical concordance of colonising and invasive strains has been demonstrated previously (Cartelle *et al.*, 2004; Graham *et al.*, 2007; Miranda *et al.*, 2009). For example in 15 of 35 (43%) cases of candidemia GIT colonisation preceeded and in 14 patients' clonal relatedness of colonising and bloodstream isolates was confirmed (Saiman *et al.*, 2000). Also, in 117 episodes of ceftazidime-resistant Gram-negative bacteraemia in 74.5% colonisation (oral swabs, urine sampling, tracheal aspirates, wounds) preceded invasive disease (Blot *et al.*, 2005).

The large intestine is a reservoir of *Enterobacteriaceae*, other Gram-negative bacilli (Foca *et al.*, 2000; Donskey, 2004; Gupta *et al.*, 2004; Duman *et al.*, 2005; Tapiainen *et al.*, 2006; De La Cochetiere *et al.*, 2007; Graham *et al.*, 2007; Srivastava *et al.*, 2007), *Candida* spp. (Saiman *et al.*, 2001; Donskey, 2004; Hallstrom *et al.*, 2004; Feja *et al.*, 2005; Mendiratta *et al.*, 2006; Manzoni *et al.*, 2008; Miranda *et al.*, 2009;) and of enterococci (Askin *et al.*, 2005; Hufnagel *et al.*, 2007). Mucosa may also serve as a source of opportunistic staphylococci and streptococci. For example NP constitutes an important ecological reservoir of microorganisms such as *Streptococcus pneumoniae* and *S. aureus* (Lima *et al.*, 2010). In Taiwan it has been demonstrated that a neonate in a NICU may harbour MRSA strains in multiple sites for a long period

(>3 months), and colonisation serves as a significant risk factor for subsequent infection (OR: 19.86; 95% CI: 9.11–45.07). By the results of PFGE analyses colonised and clinical isolates were indistinguishable in 63 out of 68 episodes, highly related in 2, and distinct in 3 episodes (Huang *et al.*, 2006a).

As CoNS infections have been associated with skin colonisation, strategies to reduce infection rates have focused primarily on the insertion and maintenance of CvCs (Ibrahim *et al.*, 2000; Mahieu *et al.*, 2001; O'grady *et al.*, 2002; Bizzarro *et al.*, 2005; Brady, 2005; Inglis *et al.*, 2007; Babazono *et al.*, 2008). The biofilm forming capacity of CoNS is thought to be the main virulence factor (Von Eiff *et al.*, 2002). However, mucosal colonisation by CoNS as an important source of bacteraemia is also suspected, suggesting that strategies to decrease mucosal colonisation are needed for effective infection control (D'angio *et al.*, 1989; Costa *et al.*, 2004). For example, genotypically similar CoNS strains have been isolated from different body sites in 12 of 13 paired BSI episodes (8 caused by *S. epidermidis*, 3 by *S. hominis* and 1 by *S. intermedius*) (Huang *et al.*, 2006b). Also, molecular relatedness between *S. epidermidis* BSI and tracheal isolates from four mechanically ventilated infants (Betremieux *et al.*, 1995) and BSI and nasal mucosal isolates in four patients (Valvano *et al.*, 1988) have been documented. The assumption that mucosal sites can be a source of BSI has been further enabled by the fact, that in 3 of 7 bacteraemic patients plasmid profiles of the catheter entry site and blood isolates of *S. epidermidis* were different (Valvano *et al.*, 1988).

2.4.2. Factors that predispose translocation

Neonates, especially those with extreme prematurity are immunocompromised with host response shifted towards immune tolerance; they have decreased T-cell function compared to adults. Lower cytokine production, diminished natural killer cell cytotoxicity, neutrophil migration, and complement pathway activity have all been described (Ambroise, 2009). Membrane protective IgA is missing from the respiratory and urinary tracts, and unless the newborn is breast-fed, is absent from the gastrointestinal tract as well (Srivastava *et al.*, 2007). Although secretory IgA can be detected as early as the first week after birth, significant salivary IgA levels are detected only by 4–6 weeks of life (Ogra, 2010). Neonatal leukocytes are less bactericidal and phagocytic, compared to adult ones. The newborn has also low or non-existent level of antibodies, IgM and IgE and IgG antibodies have been acquired from the mother. However, it is important to note that passive transfer of maternal antibodies does not take place until the 29th week of gestation. This has implications for preterm infants born between 25–29th week of gestation; they are susceptible to infection despite the mother's antibody status. A slow rise of immunoglobulin levels to that of older children occurs after 3 months of age (Srivastava *et al.*, 2007).

Several factors contribute translocation of microorganisms from mucosal surfaces. First, neonates, especially preterms have an increased intestinal permeability, mainly in first two days of life, which may lead to bacterial translocation to systemic organs and tissues (Westerbeek *et al.*, 2006; De La Cochetiere *et al.*, 2007; Ambroise, 2009). Second, natural barriers, such as the acidity of the stomach or the production of pepsin and trypsin that maintain sterility of the small intestine, are not fully developed until 3–4 weeks after birth (Srivastava *et al.*, 2007), especially in enterally fed preterms (De La Cochetiere *et al.*, 2007). Third, in preterm neonates poor gastric empting and immature small intestinal motility may result in NEC (Neu, 2007). Although the development of GIT motility begins in the second trimester, it does not mature until the third trimester, and migrating motor complexes which pass as wavers along the intestines are not present until by about 34 weeks of gestation age (GA) (Lin *et al.*, 2008). Fourth, as mentioned above a newborn infant particularly the preterm, does not have a mature immune system and is often unable to mount an effective immune response (Kelly *et al.*, 2000; Modi *et al.*, 2000; Mussi-Pinhata *et al.*, 2005; Srivastava *et al.*, 2007; Lin *et al.*, 2008; Ambroise, 2009).

2.4.3. The definition and aetiology of early sepsis onset and late onset sepsis in the neonatal intensive care unit

Neonatal sepsis is defined as systemic inflammatory response syndrome occurring in the presence of or as a result of suspected or proven infection in the first 4 weeks of life (Goldstein *et al.*, 2005) and is the most common neonatal infection in the NICU (45–55%) (Borghesi *et al.*, 2008). The reported incidence varies from 3 per 1000 live births in Northen-Europe (Tessin *et al.*, 1990); 6–9 in US (Cohen-Wolkowiez *et al.*, 2009) and 7–23 in Africa and 7–38 in Asia (Vergnano *et al.*, 2005; Thaver *et al.*, 2009). No population-based statistics describing situation in Estonia is available. The nosocomial infection rate in NICU has increased over the past decade; between 2% to 28% of babies admitted to NICU experience at least one episode of bacteraemia (Edwards *et al.*, 2002; Clark *et al.*, 2004; Phillips *et al.*, 2008). Since neonatal sepsis is associated with increased mortality, prolonged length of hospital stay and neurodevelopmental impairment both the human and fiscal costs are high (Edwards *et al.*, 2002; Stoll *et al.*, 2002b; Stoll *et al.*, 2003; Apostolopoulou *et al.*, 2004; Clark *et al.*, 2004; Zaidi *et al.*, 2011).

Neonatal sepsis can be classified into two sub-types depending upon whether the symptoms develop before 72 h of PNA (EOS) or later (LOS) (Karlowicz *et al.*, 2000; Edwards *et al.*, 2002; Stoll *et al.*, 2002a; Clark *et al.*, 2004; Ambroise, 2009; Smith *et al.*, 2010). Infants who develop BSI in the first few days of life typically acquire the agent from their mother during the intrapartum period, while LOS originates from community or nosocomial source (Mussi-Pinhata *et al.*, 2001; Brady, 2005; Mishra *et al.*, 2006; Schrag *et al.*, 2006b). Other time-limits have also been suggested: episodes diagnosed on the day of delivery are

very EOS (Ronnestad *et al.*, 2005b), those diagnosed from day 2 to day 7 of life EOS (Schrag *et al.*, 2002; Ronnestad *et al.*, 2005b), and LOS is defined as microbiologically verified septicaemia occurring after the first week of life (Ronnestad *et al.*, 2005a). Still, even authors supporting the last mentioned LOS classification accept, that in >50% of cases, EOS manifests within 6 h of birth, and most cases occur within 72 h (Schrag *et al.*, 2002).

The major agents of EOS and LOS in different countries are presented on Table 2 and 3. The most frequent isolates in EOS in developed countries are group-B streptococci (GBS) and *E. coli,* although administration of IAP has reduced the rate of GBS sepsis over the last two decades by 50–80% (Moore *et al.*, 2003; Daley *et al.*, 2004; Trijbels-Smeulders *et al.*, 2007). Some surveillance data suggest that this success has been achieved at the cost of increasing cases caused by *E. coli* (Joseph *et al.*, 1998; Towers *et al.*, 1998; Schuchat *et al.*, 2000; Stoll *et al.*, 2002a; Stoll *et al.*, 2003; Cordero *et al.*, 2004; Bizzarro *et al.*, 2005; Vergnano *et al.*, 2010). The most frequent microorganisms involved in LOS are CoNS, *Enterobacteriaceae* and nonfermentative bacteria, including *Acinetobacter* spp and *Pseudomonas* spp.

Since the late 1980s, Gram-positive organisms have replaced Gram-negative ones as the most common bacteria causing sepsis in developed countries (Bizzarro *et al.*, 2005; Hira *et al.*, 2007; Mackenzie *et al.*, 2007; Stoll *et al.*, 2002b; Stoll *et al.*, 2003; Tarnow-Mordi *et al.,* 2010; Vergnano *et al.*, 2010). This change can be welcomed, as the mortality of Gram-negative (26–40%) and *Candida* sepsis (28–32%) is similar and significantly higher than that of Gram-positive sepsis (9–10%) (Bizzarro *et al.*, 2005; Costa *et al.*, 2004; Edwards *et al.*, 2002; Hira *et al.*, 2007; Kayange *et al.*, 2010; Ronnestad *et al.*, 2005a; Ronnestad *et al.*, 2005b; Stoll *et al.*, 2002b; Zaidi *et al.*, 2005). For example, in Australian neonatal units the mortality risk of sepsis caused by Gram-negative bacilli (RR=45.5; 95% 16.8–123.3) or *S. aureus* (RR=36.1; 95% CI 13.0–100.2) was significantly higher than that of sepsis caused by CoNS (Tarnow-Mordi *et al.*, 2010).

Table 2. Causative agents in percentages of EOS in the different countries by the result of articles published within last decade

Study	**Stoll *et al.*, 2002b**	**Bizzarro *et al.*, 2005**	**Cordero *et al.*, 2004**	**Ronnestad *et al.*, 2005b**		**Vergnano *et al.*, 2010**	**Gheibi *et al.*, 2008**	**Mahmood *et al.*, 2002**	**Agnihotri *et al.*, 2004**	**Milledge *et al.*, 2005**
Year	1998–2000	1989–2003	1986–2002	1999–2000		2006–2008	2002–2006	1996–1999		1996–2001
Country	USA	USA	USA	Norway		UK	Iran	Pakistan	India	Malawi
Sepsis onset	<72h	D0–4	<48h	D1	D2–7	≤48h	<D7	<1 week	<D7	<D7
CoNS	11	6	–	–	54	20*	49	–	–	–
S. aureus	–	7	4	–	39	5	7	24	45	15
GBS	11	49	36	21		50	1	2	–	16
Other streptococci	8	–	2	–		6	–	–	–	10
Enterococcus spp	–	5	2	–		2	5	–	5	–
Bacillus	–	1	–	–		4	–	–	–	–
Other Gram-positives	5	–	–	–		–	–	–	–	13
Listeria	2	–	–	7		6	–	–	–	–
E. coli	44	24	36	64		18	10	11	12	11
Klebsiella spp	–	–	3	–	8	–	17	38	19	11
E. cloacae	–	–	–	–		–	–	9	7	–
Serratia spp	–	–	–	–		1	–	–	–	–
H. influenzae	8	5	14	–		3	–	–	–	–
Acinetobacter	–	1	1	–		–	1	11	6	–
Pseudomonas	–	2	3	–		1	7	2	6	–
Other Gram-negatives	8	1	–	7		3	2	3	–	24
Candida spp	2	1	–	1		1	–	–	–	–

* data on CoNS is presented without 2006

In greatest part of developing countries, Gram-negative rods (Agnihotri *et al.*, 2004; Amaya *et al.*, 2010; Asghart *et al.*, 2010; Zaidi *et al.*, 2005), often presenting with a more rapid clinical deterioration commonly associated with endotoxin production, shock and coagulation problems, are the major agents of neonatal sepsis (Clark *et al.*, 2004). A review of 11471 bloodstream samples showed that Gram-negative rods were isolated from at least 60% of positive blood cultures in all the developing regions world-wide, with *K. pneumoniae*, *E. coli*, *Pseudomonas* spp, and *Acinetobacter* spp predominating (Zaidi *et al.*, 2005).

Table 3. Causative agents in percentages of BSI or LOS in the different countries by the result of articles published within last decade

Study	**Stoll *et al.*, 2002b**	**Bizzarro *et al.*, 2005**		**Cordero *et al.*, 2004**	**Ronnestad *et al.*, 2005a**	**Vergnano *et al.*, 2010**	**Sadowska-Krawczenko *et al.*, 2009**	**Milledge *et al.*, 2005**	**Gheibi *et al.*, 2008**	**Mahmood *et al.*, 2002**
Year	1998–2000	1989–2003		1986–2002	1999–2000	2006–2008	2004–2008	1996–2001	2002–2006	1996–1999
Country	USA	USA		USA	Nor-way	UK	Poland	Malawi	Iran	Pakistan
Sepsis onset	>72h	D5–30	D>30	>48h	>D7	>48h	BSI	D8–30	>D7	>D7
CoNS	48	40	25	57	43	54*	47	–	70	–
S. aureus	8	6	12	5	10	18	1	7	5	57
GBS	2	4	4	1	9	8		19	–	–
Other streptococci	–	1	2	–	–	2		23	–	–
Enterococcus spp	3	7	14	5	1	16	1	–	3	–
Bacillus	–	–	1	–	–	8	–	–	–	–
Other Gram-positive	9	–	–	–	2	2	–	6	–	–
E. coli	5	9	7	6	2		2	7	14	7
Klebsiella spp	4	9	10	6	8	9	14	5	6	14
E. cloacae	3	2	3	3	–	9	9	–	–	7
Serratia spp	2	2	6	1	–	2	11	–	–	–
Acinetobacter	–	–	1	–	–	2	4	–	–	12
Pseudomonas	3	5	2	3	–	5	2	–	2	2
Other Gram-negatives	1	3	3	–	10	10	2	–	–	–
Candida spp	12	11	8	12	13	9	7	33	–	–

* data of CoNS is presented without 2006

2.5. Relevance of mucosal surveillance cultures to predict LOS

For decades clinicians and researchers have attempted to use surveillance cultures to predict invasive infections but the issue remains controversial. Two types of studies have been published – those looking at the predictive value of each individual mucosal sample (Evans, 1988; Bertrand *et al.*, 2001; Miranda *et al.*, 2009) and the others comparing colonised patients with non-colonised ones (Harris *et al.*, 1976; Sprunt, 1985; Pierro *et al.*, 1998; Shankar *et al.*, 2001). The first method based on the correlation of mucosal invasive sample pairs is more

precise and scientifically sound as it allows estimating the predictive value of each individual sample.

Three decades ago Harris *et al.* (Harris *et al.*, 1976) and Sprunt *et al.* (1978) reported the clinical relevance of surveillance cultures in neonatal units. They showed that some changes in NP microbiota allow predicting infection occurrence. For example by Harris *et al* (1976) systemic infection developed only in those orotracheally intubated neonates who became colonised with potentially pathogenic microbes either before or after intubation. By Sprunt *et al* (1978) infections occurred only in the group of neonates with "abnormal" pharyngeal colonisation (18 infections in 115 neonates, whose predominating NP coloniser was other than α-hemolytic streptococci in a concentration of 10^4 or greater cfu/ml and made up more than 90% of a sample population). On the other hand, neonates with normal flora (α-hemolytic streptococci predominating) or with cultures showing low or no microbial titres did not become infected. However, the authors pointed out that the surveillance collection strategy was labour intensive and expensive.

The question of whether an intensive surveillance strategy is cost-effective remains unanswered (Blot *et al.*, 2005; Mcginigle *et al.*, 2008), although others have also suggested that mucosal samples taken once or twice a week allow identifying patients at risk of nosocomial infection (Pierro *et al.*, 1998; Silvestri *et al.*, 1999; Shankar *et al.*, 2001). Some previous studies looking at the sensitivity and specificity of surveillance cultures in predicting invasive disease in the NICU setting have found them suboptimal (Evans, 1988; Choi *et al.*, 2008). In these studies, the overall sensitivity, specificity, and positive predictive values (PPV) were 16 – 56%, 38 – 82%, and 5 – 7.7%, respectively. The potential issues of these studies could be the inclusion of all hospitalised neonates the majority of whom were at low risk for nosocomial infection (Evans, 1988) or looking at high number of different species; many of them with low potential of causing invasive disease (Choi *et al.*, 2008). More recently Smith *et al* (2010) evaluated the sensitivity, specificity, and PPV of GIT surveillance cultures to predict BSI caused by gentamicin-nonsusceptible Gram-negative bacteria in VLBW infants and reported sensitivity, specificity and PPV values of 100%, 98%, and 94% respectively. They found, that under these circumstances and considering specific risk factors, surveillance cultures are useful during the second to sixth week of life to guide empiric therapy for LOS.

In adults admitted to intensive care unit (ICU) the sensitivity, specificity, PPV and negative predictive value (NPV) of 53.6%, 90.3%, 27.6% and 96.6%, respectively, has been reported for mucosal samples in the detection of further positive deep site cultures (Bertrand *et al.*, 2001). Surveillance cultures may also provide important information on the drug resistance of Gram-negative pathogens and this information can improve empiric antibiotic therapy for patients subsequently developing BSI (Papadomichelakis *et al.*, 2008; Baba *et al.*, 2011). McGingle *et al* (2008) reviewed the data on morbidity, mortality and costs from sixteen articles regarding the use of active surveillance cultures in

intensive care units (ICU) to reduce MRSA. While the reduction of MRSA infection was proven, the overall quality of the evidence was poor; thus definitive, evidence-based clinical recommendations cannot be made.

2.6. Microbiological methods to study mucosal colonisation in neonates

2.6.1. Sample collection

Studies looking at the mucosal colonisation have collected samples from different sites with variable frequency. More often, depending on the aim of the study, first sample was taken on admission, the last one on discharge, and remaining samples were taken with the frequency of once or twice a week. The differences in timing make it difficult to compare results of one study to the other. The methods applied in different studies are further detailed in Table 4.

Table 4. Different timing of sample collection has been used in studies looking at factors associated with early mucosal colonisation

Reference	R/NP	County	n=	Sample collection
Almuneef *et al.*, 2001	R	USA	239	on admission, once a W and on discharge
Baltimore, 1998	NP	USA	49	within 72h and W 2, 6, 12,18, 26 of PNA
Bennet *et al.*, 1982	R	Sweden	22	3 during the 1st, 6 during the 2nd, 2 during the-3th W; on 22 and 49 D
Blakey *et al.*, 1982	R/NP	Australia	28	whithin 8H of admission, twice a W
Bonnemaison *et al.*, 2003	R	France	30	within 12H and D 3,7 and 10 of PNA
De Man *et al.*, 2000	R/NP	Netherlands	436	on admission and once a W thereafter
Duman *et al.*, 2005	R	Turkey	118	after admission, altough 4 samples every 2 W
Gewolb *et al.*, 1999	R	USA	29	on D 10, 20 and 30 of PNA
Harris *et al.*, 1976	NP	USA	54	within 4H of intubation and daily while intubated
Hallstrom *et al.*, 2004	R	Finland	140	from first stool and twice a W until discharge
Jaureguy *et al.*, 2004	R	France	50	on D3 after birth
Mammina *et al.*, 2007	R	Italy	210	Twice a W (Thursday and Friday)
Mendiratta *et al.*, 2006	R/NP	India	203	within 24H; D 3,5,7; once a W until discharge
Millar *et al.*, 2008	R	UK	221	W 2 of PNA and at discharge
Penders *et al.*, 2006	R	Netherlands	1032	M 1of PNA
Pierro *et al.*, 1998	R/NP	UK	94	at the start of PN and thereafter twice a W
Saiman *et al.*, 2001	R	USA	2157	on admission, once a W until discharge

Reference	R/NP	County	n=	Sample collection
Schwiertz *et al.*, 2003	R	Germany	29	Daily for 2 W and thereafter twice a W
Sprunt *et al.*, 1978	NP	USA	223	twice a W
Tapiainen *et al.*, 2006	R	Finland	32	from each stool in the first 2–7D PNA
Tanaka *et al.*, 2009	R	Japan	44	daily for the first 5D, monthly for the first 2M
Toltzis *et al.*, 2001	R/NP	USA	1180	Monday, Wednesday, Friday

M – month; W – week; D – day; R – rectal samples; NP – nasopharyngeal samples; n= – number of participants

2.6.2. Identification of microorganisms, detection of genetic relatedness between different microbes and microbial diversity

Current knowledge of gut microbial ecology and diversity is almost exclusively based on the use of classic culture techniques (Baltimore *et al.*, 1989; Pierro *et al.*, 1998; Gewolb *et al.*, 1999; De Man *et al.*, 2000; Almuneef *et al.*, 2001; Bonnemaison *et al.*, 2003; Hallstrom *et al.*, 2004; Mammina *et al.*, 2007; Millar *et al.*, 2008;) and the composition of intestinal microbiota of the neonate cannot yet be considered as clearly defined, as many bacterial species living in the gut are unculturable by conventional cultivation techniques (Morowitz *et al.*, 2010; Mshvildadze *et al.*, 2010a). By applying molecular techniques it has been shown that 105 of 325 isolated clones from infant faeces were unknown species (De La Cochetiere *et al.*, 2007), and according to Mshidadze *et al* (Mshvildadze *et al.*, 2010a) even 80% of microbes in the intestinal tract are very difficult if not impossible to culture by current methods. In addition, acquisition of unculturable bacteria expands rapidly after the third day of life (De La Cochetiere *et al.*, 2007; Biasucci *et al.*, 2010).

Molecular approaches that identify microbes from small-submit gene sequences offer advantages over cultivation (Mshvildadze *et al.*, 2010a). For example, an electrophoresis technique, PFGE, made it possible to separate large DNA fragments. This method has remarkable discriminatory power and reproducibility, and has therefore become a widely applicable for comparative typing of almost all bacterial species (Van Belkum *et al.*, 2007). PFGE and multienzyme multiplex polymerase chain reaction (PCR) amplified fragment length polymprphism (AFLP) methods are closer described in chapter 4.3.3.3. For molecular analyses also 16S rRNA gene is typically chosen. The rRNA gene sequences comprise highly conserved sequence domains interspersed with more variable regions and simple profiling approaches such as DGGE, automated ribosomal intergenic spacer analysis (ARISA) and restriction fragment length polymorphism methods (RFLP) allow for an efficient evaluation of overall microbiota diversity. Methods, such as fluorescent in situ hybridization (FISH) and real time PCR are often complemented with quantitative tools (Mshvildadze *et al.*, 2010a). Also, microarray chip technology allows the assessment of

all bacterial species known to inhabit the human colon, but it is still very costly (Enck *et al.*, 2009). Anyway, powerful novel sequencing approaches, including pyrosequencing, allow for an in depth analysis of even minor members of microbiota (Mshvildadze *et al.*, 2010a).

As previously described, molecular methods have been used to study human microbiota for only a decade and the available data are limited. These methods have been mostly applied for monitoring of microbial cross-colonisation (Almuneef *et al.*, 2001; Toltzis *et al.*, 2001; 2002; Cartelle *et al.*, 2004; Mammina *et al.*, 2007; Prelog *et al.*, 2009; Sanchez-Carrillo *et al.*, 2009;), translocation (Cartelle *et al.*, 2004; Miranda *et al.*, 2009) or their diversity (Schwiertz *et al.*, 2003; Tanaka *et al.*, 2009). This may, however, change in the near future, although in the last decade the data obtained by culture-independent techniques (FISH; DGGE; 16S rRNA cloning and sequencing, real time PCR) mostly support the results obtained by using selective cultures (Morelli, 2008).

2.6.3. Antibiotic susceptibility testing

Antibacterial resistance is spreading among a variety of clinically significant bacterial species. Therefore, the microbiology laboratory plays a key role in patient management by providing accurate data on which physicians can rely in treatment decisions (Turnidge *et al.*, 2007). Laboratories can choose from among several conventional or novel methods for performance of routine antimicrobial susceptibility testing: the disk diffusion test, E-tests, broth microdilution, agar gradient, and rapid automated instrument methods (Jorgensen *et al.*, 2005). Also molecular methods, such as *mecA* gene detection for determination of methicillin-resistance of *S. aureus*, are used (Chambers, 1997; Dickinson *et al.*, 2000). All these differ in cost, labour-intensity and accuracy, and are of course of different specificity. For this reason the choice of different methods in studies depends on the aims and amount of surveys, needed and performed.

In colonisation studies where many susceptibility tests for different antibiotics are done mostly breakpoint susceptibility testing on selective medias with antimicrobial agents in certain concentrations (Frebourg *et al.*, 1999; Lidsky *et al.*, 2002; Udo *et al.*, 2008) or disc diffusion testing (Shi *et al.*, 1996; Lindberg *et al.*, 2004; Chiu *et al.*, 2005; Bagattini *et al.*, 2006; Dalben *et al.*, 2008; Mitt *et al.*, 2009; Lima *et al.*, 2010) have been used. In most cases, E-tests are used only as an additional method for detailing minimal inhibiting concentration (MIC) values or for further detecting ESBLs (Ayan *et al.*, 2003; Chiu *et al.*, 2005; Duman *et al.*, 2005; Bagattini *et al.*, 2006). As in the beginning of herein study the claims of Clinical and Laboratory Standards Institute (CLSI) were essential, the other criteria also exist. For example by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) lower MIC values are recommend, and in comparison by CLSI, the higher level of resistance may occur. For example by CLSI *vs* EUCAST MIC values for ampicillin for *Enterobacteriaceae* ≥32 *vs* >8, respectively, have been recommended.

2.7. Summary of the literature

Colonisation of mucosal surfaces with opportunistic microorganisms starts right after birth. On one hand mucosal colonisation by Gram-negative micro-organisms is a normal process occurring over time but on the other hand it could also serve as a source for invasive infection (Cartelle *et al.*, 2004; Graham *et al.*, 2007; Miranda *et al.*, 2009). Therefore a better understanding of the factors associated with the colonisation process might contribute to improved infection control-strategies and eventually improve the outcomes for critically ill neonates (Donskey, 2004; Miranda *et al.*, 2009). Numerous studies have looked at factors associated with early mucosal colonisation and have identified several, like country of origin, maternal microbiota, PROM, route of delivery, GA, surrounding environment, feeding habits, and antibiotic use, as having a role. However, most of these studies have either included only a limited number of infants, have focused on gut colonisation alone, included healthy infants, or looked at only a few microbial species or risk factors at a time despite that the majority of factors are highly interrelated. In addition, studies in critically ill neonates admitted to NICU and especially those, looking at multiple factors and species simultaneously, are scarce.

Fear of infection often leads to early use of empiric broad-spectrum anti-biotics, a strategy that may select for resistant bacteria and *Candida* spp. (Gewolb *et al.*, 1999; Bonnemaison *et al.*, 2003; Fanaro *et al.*, 2003). The use of ampicillin in intrapartum prophylaxis of group B streptococcal infection has been associated with the emergence of *E. coli* as a major causative pathogen of EOS in many countries and probably with an increase in ampicillin-resistance (Laugel *et al.*, 2003; Stoll *et al.*, 2002a) but the data are not conclusive (Jaureguy *et al.*, 2004). On the other hand narrow spectrum penicillins like penicillin G have the least potential of interfering with normal gut colonisation (Bennet *et al.*, 1982). Although, the negative impact of antibiotics to bowel colonisation is widely reported, the number of comparative studies, especially those reflecting the empiric use of the two mostly recommended regimens (penicillin G or ampicillin with gentamicin) for EOS, is still limited.

Due to disturbed integrity of the barriers and immature immune response colonisation may lead to invasive disease in sick neonates; concordance of colonising and invasive strains has been demonstrated previously (Graham *et al.*, 2007). For decades clinicians and researches have attempted to use surveillance cultures to predict invasive infections but the issue remains controversial. Surveillance cultures of the GIT, pharynx, and/or skin have been studied in an effort to identify infants who will develop infections. The surveillance cultures have been found to be relevant but their collection is labour intensive and expensive. Furthermore the sensitivity and specificity of surveillance cultures in predicting invasive disease in the setting of neonatal unit have found to be suboptimal (Evans, 1988; Choi *et al.*, 2008). Still, some experts suggest that mucosal samples taken once or twice a week allow

identifying patients at risk of nosocomial infection (Pierro *et al.*, 1998; Silvestri *et al.*, 1999; Shankar *et al.*, 2001). In addition, surveillance cultures may provide insight into the patients' colonisation status and may guide physicians in antibiotic choice when infection occurs (Glynn *et al.*, 2005). Hence, the role of surveillance cultures in predicting BSI needs to be proven, with special focus on specific high risk populations.

3. AIMS OF THE RESEARCH

The general aim of the study was to compare how antibiotics commonly used for empiric therapy of EOS (ampicillin or penicillin both combined with gentamicin) influence the development of gut and NP microbiota and to define which of the abovementioned regimens should be preferred in terms of mucosal colonisation.

The following specific aims were addressed:

1. to characterize the prevalence and dynamics of mucosal colonisation by aerobic opportunistic bacteria and *Candida* spp. in neonates admitted to NICU with risk factor based suspicion of EOS;
2. to identify independent perinatal, neonatal, and environmental factors influencing the colonisation process;
3. to compare the influence of empiric EOS treatment with ampicillin and penicillin G (both combined with gentamicin) on early gut colonisation by aerobic, facultatively anaerobic and ampicillin resistant bacteria, accounting for other known risk factors interfering with the colonisation process;
4. to identify the relatedness of microorganisms isolated from NP and rectum to isolates form sterile sites and thus characterize their potential for causing invasive disease;
5. to define the value of twice a week collected surveillance cultures in predicting Gram-negative LOS in high risk neonates admitted to NICU.

4. PATIENTS AND METHODS

This thesis is based on a prospective open label two centre cluster randomised study that compared the efficacy of penicillin G and ampicillin (both combined with gentamicin) in the empiric treatment of neonates with risk of EOS and evaluated the impact of these antibiotics on early gut colonisation. The study was conducted between August 2, 2006 and November 30, 2007 in the paediatric and neonatal intensive care units of Tartu University Hospital and Tallinn Children's Hospital. The analyses that were conducted are presented in Table 5.

Table 5. Description of analyses of the thesis

Analyses	Number of subjects	Collection site and number of samples	Publication
Risk factors of gut and NP colonisation	276	Rectal 1242; NP 145; tracheal 60	III
Comparative influence of empiric AB regimen on initial rectal colonisation	276	Rectal 1242; NP 1145; tracheal 60	I
Predictive value of surveillance cultures	278	Rectal 1250; NP 1153; tracheal 60; sterile fluid samples 554	II
Detection of the relatedness between mucosal and invasive strains:			I, II
MRSA	11	Rectal 16; NP 1; tracheal 3; blood 5; breast milk 1; axilla 1; eye 1	
K. pneumoniae	47	Rectal 96; NP 56; tracheal 3; blood 5	
A baumannii	7	Rectal 4; NP 4; blood 6	
E. cloacae	5	Rectal 3; NP 1; blood 5	

4.1. Ethics

The study was approved by the Ethics Committee of the University of Tartu and registered at ClinicalTrials.gov identifier: NCT00487019.

The study design was discussed with the Ethics Committee and an informed consent was not considered necessary because both antibiotic regimens are routinely used in the participating units. Similaer to the study in Netherlands (De Man *et al* 2000) our investigation can also be considered also a quality control of the hospital hygene practices, and not experimentation with human beings. Parents of the newborns were informed of all antibiotic treatments. Furthermore no additional interventions were conducted for study purposes only and for assessment the influence of different antibiotic regimens on the colonisation and the circulation of resistant microorganisms in NICU inclusion

of all affected patients were required. Finally the collection of surveillance cultures from all patients admitted to NICU was introduced by hospital infection control services prior commencing this study.

4.2. Study setting, population and design

4.2.1. Study setting

Both participating units (later referred as unit A and unit B) are third level mixed referral PICUs admitting neonates either directly from the maternity or intermediate care units or from home. About 60–65% of all admissions are neonates cared for in a separate area. Both units have facilities for high frequency ventilation, inhaled nitric oxide therapy and neonatal surgery. The paediatric intensive care unit (PICU) in Tartu University Hospital is divided into four and in Tallinn Childrenś Hospital into five rooms; the number of neonates in a room varies from three to six. The nursing staff/infant ratio in the units is 1:2, but can be 1:3 occasionally. Gloves, gowns, caps and masks are used routinely in all aseptic procedures. In general both units follow similar hospital infection prevention guidelines and strict antibiotic policy, in which narrow spectrum antibiotics and short courses are preferred. According to the infection control guidelines patients colonised with alert microorganisms are isolated in separate rooms and cared for by separate nurses. Both units practice early introduction of enteral feeding with preference given to breast milk. Breast milk is frozen but neither pasteurized nor fortified except in VLBW neonates after reaching enteral volume of 100 ml/kg. Formula if needed is prepared centrally; donor milk is not used.

4.2.2. Study population

Neonates were included in the study if they were younger than 72 hours, needed early empiric antibiotic treatment on clinical suspicion and/or due to risk factors of infection according to the Centers for Disease Control and Prevention (CDC) criteria (i.e. maternal fever, chorionamnionitis, PROM more than 18h, preterm labour of <35 weeks of gestation) (Schrag *et al.*, 2002) and were expected to stay in the unit for >24 hours. Patients who had received a different antibiotic regimen for more than 24h, had suspicion of meningitis, NEC, peritonitis or severe sepsis with a history of isolation of microorganisms resistant to the study regimen from maternal urinary tract or birth canal or had other situations where the treating physician considered a different antibiotic regimen necessary were excluded.

4.2.3. Study design and antibiotic treatment

In the first period of the study (from August 2, 2006 to March 20, 2007) in unit A all patients received ampicillin (25 mg/kg 8–12 hourly) and in unit B penicillin G (25,000 IU /kg 8–12 hourly), both in combination with gentamicin (4–5 mg/kg 24–48 hourly, according to GA) (Metsvaht, 2010). After enrolling half of the patients required to prove clinical equivalence of the two antibiotic regimens the penicillins were switched (from March 21, 2007 to November 30, 2007) so that in unit A penicillin G and in unit B ampicillin was used (Figure 1). Further patients receiving ampicillin with gentamicin or penicillin with gentamicin will be called the ampicillin and penicillin group, respectively. If no clinical or laboratory signs of invasive infection were present and initial blood cultures remained negative, antibiotic therapy was stopped on Day 3.

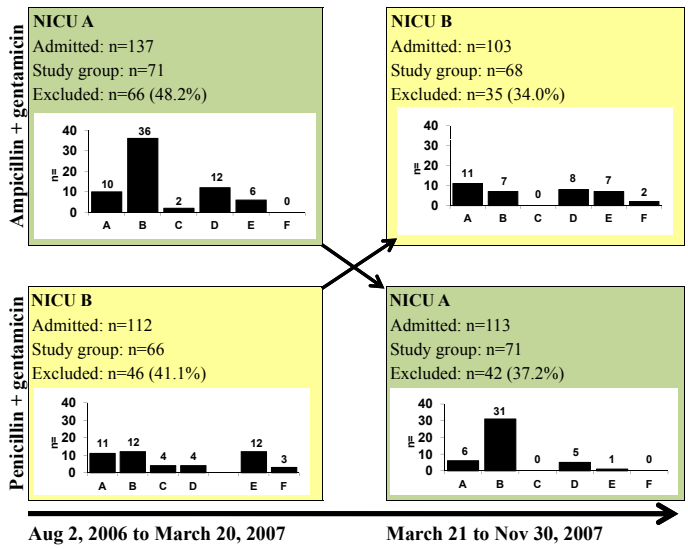

Figure 1. Study outline and reasons for exclusion (A – age on admission more than 72 hours; B – no need for early empirical antibiotic treatment; C – different antibiotic regimen for more than 24 hours; D – need for different antibiotic regimen on admission; E – transfer from neonatal intensive care unit within 24 hours; F – no samples). NICU – neonatal intensive care unit. Number of cases is shown on y-axis.

In case of clinical or culture proven infection initial antibiotic regimen could be continued if susceptible pathogens were involved or changed to a prespecified regimen depending on the antibacterial susceptibility of the isolate. For the empiric therapy of LOS cefuroxime, cefotaxime, ampicillin/sulbactam, or piperacillin/tazobactam alone or in combination with gentamicin was recommended. In neonates with BW below 800 g and central vascular catheter(s) in place vancomycin was added until infection caused by CoNS was ruled out by negative cultures.

4.2.4. Data collection

The following maternal and perinatal characteristics were registered: maternal age, type and time of antibiotic treatment during pregnancy and delivery, presence of PROM for more than 18 h, multiple birth, mode of delivery, including reason for caesarean section.

For each patient the demographic characteristics and clinical data were collected as follows: BW; GA; duration of ALV; age on admission to PICU; ward; study period (divided into first and second) (Figure 1); duration of PICU stay; time, type and duration of initial and subsequent antibiotic regimens (carbapenems, third and fourth generation cephalosporins, and beta-lactamase resistant penicillins), duration of CvC and arterial catheter use.

Feeding regimen was documented on Days 1, 3 and 7 with patients categorized into three groups based on the route of nutrition and the character of enteral feeds: (1) TPN when enteral calories constituted less than 10% of total daily calories; (2) breastfeeding when breast milk constituted more than 10% of enteral feeds and (3) formula feeding when formula constituted more than 89% of enteral feeds.

4.3. Microbiological studies

4.3.1. Pilot study

Prior to commencing the main study (from October 21, 2005 to January 1, 2006) a pilot study aiming to evaluate the influence of freezing on microbial recovery rates was conducted. In this study two rectal swabs (n=70) were collected from each subject (n=26) into a transport medium. One swab was sent directly to the microbiology laboratory for immediate culturing and species identification and the other was frozen at –20^{o}C for a maximum of 22 days (median 14; interquartile range (IQR) 11–15). A total of 35 sample pairs were obtained. The recovery rate of frozen samples was good (86.4%). The sensitivity of 100% (95% CI 79; 100), specificity of 81% (95% CI 54; 95), PPV of 86% (95% CI 64; 96) and NPV of 14% (95% CI 4; 36) were observed. As 2 of the 3 cultures with a difference between frozen and fresh samples had been stored for more than two weeks we concluded that storing for a maximum of two weeks will not significantly influence the outcome.

4.3.2. Sample collection and storage

Rectal and NP specimens were collected with transport swabs (Nuova Aptaca, Canelli, Italy) on admission to PICU and then twice a week until discharge or Day 60 whichever occurred first. From patients receiving ALV tracheal aspirate instead of NP swab was collected. Samples were stored at –20°C for a maximum of two weeks and analysed in batches in the Department of Microbiology, University of Tartu.

Normally sterile body fluids (e.g. 0.5 ml blood, 0.2 ml cerebrospinal fluid if needed) were cultured preferably before administration of antibiotics on admission and then if clinical condition deteriorated and symptoms suggestive of neonatal sepsis, as detailed elsewhere, appeared (Metsvaht *et al.*, 2010). Samples were processed immediately after collection in the microbiology laboratories of the Tartu University Hospital or North Estonia Medical Centre. All mucosal and invasive isolates were stored in skim-milk at –80ºC for further analysis, if needed.

4.3.3. Microbiological analyses

4.3.3.1. Identification of microorganisms

The flowchart of mucosal sample testing is presented on Figure 2. Briefly, after thawing rectal swabs were directly plated onto blood agar, MacConkey agar for isolation of Gram-negative bacteria, and Sabouraud agar for isolation of yeasts. NP specimens were plated onto McConkey agar. The blood and MacConkey agar plates were incubated at 37^{o}C in ambient air for 24 to 48 h and Sabouraud agar plates at 25^{o}C for at least one week. Each morphologically different colony type was identified on species and genus level according to the CLSI criteria (CLSI, 2005). The *Staphylococcus aureus* strains were further tested by PCR for *nuc* genes using sequence specific primers (Brakstad *et al.*, 1992; Khan *et al.*, 2007), as presented in Table 6; detailed PCR procedure is described in chapter 4.3.3.2.

Table 6. Sequences of adapters and primers of the molecular analyses

Primer name	Primer sequences (5' → 3')	Adapter sequences (5' → 3')	Recognition site	Restriction enzyme
Pxn *	AGAGTCTGCCAGT ACTAG	CTAGTACTGGCAGAC TCTGCCAGTA	5' T CTAGA 3' 3' AGATC T 5'	XbaI
Pst-at*	GACTGCGTACATG CAGAT	CTCGTAGACTGCGTA CATGCATGTACGCAG TCTAC	5' CTGCA G 3' 3' G ACGTC 5'	PstI
*nuc*F**	GCGATTGATGGTG ATACGGTT	NA	NA	NA
*nuc*R**	AGCCAAGCCTTGA CGAACTAAAGC	NA	NA	NA
mecA***	AGTTGTAGTTGTC GGGGTTT	NA	NA	NA
mecA***	AGTGGAACGAAGG TATCATC	NA	NA	NA

* For AFLP. Adapted from van der Zee *et al* (2003). All oligonucletides were purchased from 5' end of each oligonucleotide was phosphorylated.
** For identification *nuc* gene (Brakstad *et al.*, 1992; Khan *et al.*, 2007)
*** For identification *mecA* gene (Chambers, 1997; Khan *et al.*, 2007)

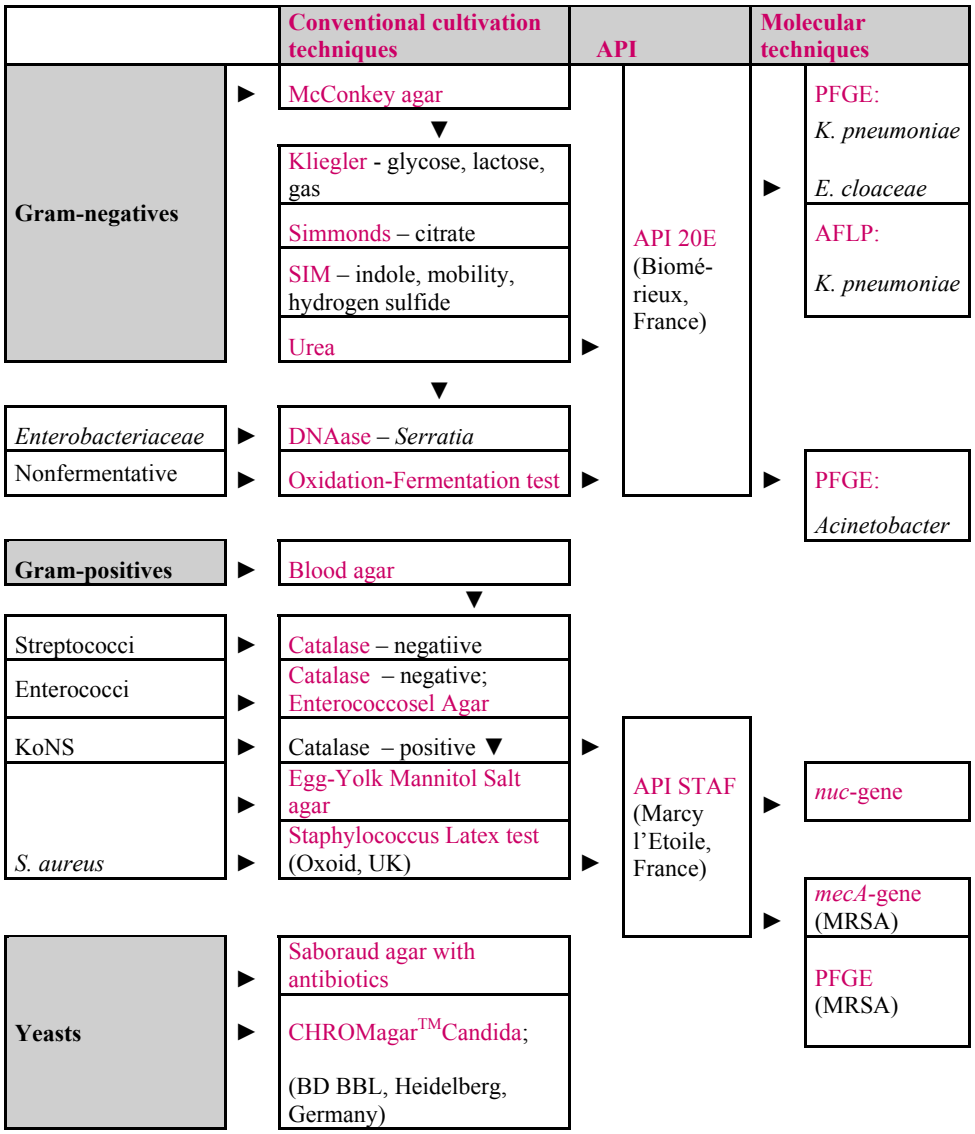

Figure 2. Rectal and NP sample flow

4.3.3.2. Antibiotic susceptibility testing

All samples were also plated onto MacConkey agar with 16 µg/ml of ampicillin for the detection of Gram-negative AR strains. For staphylococci oxacillin-resistance was determined using Mueller-Hinton agar plates containing 4% NaCl with 6 µg/ml of oxacillin for *S. aureus* and 2% NaCl with 0.75 µg/ml of oxacillin for CoNS (Huang *et al.*; 1993; CLSI, 2005). The *S. aureus* strains

were tested for methicillin-resistance by PCR detection of *mecA* gene using sequence specific primers as shown in Table 6 (Chambers, 1997; Khan *et al.*, 2007). PCR conditions comprised a thermal temperature 94°C for 3 minutes, followed by 30 cycles of 94°C for 30 seconds, annealing at 53°C for 20 seconds, followed by a final extension for 5 minutes at 72°C.

The ampicillin and gentamicin MIC values of all Gram-negative invasive isolates and of the first mucosal isolate of the same microorganisms from the same person were determined by E-tests (AB BIODISK; Sweden) on Mueller-Hinton agar plates and evaluated according to the CLSI criteria.

Microbiological culture techniques were not altered during the study.

4.3.3.3. Detection of genetic relatedness between different microorganisms

PFGE

The PFGE analysis was performed for mucosal and invasive strain pairs of *K. pneumoniae, E. cloacae* and MRSA according to the manufacturer's instructions (Genepath, Bio-Rad, France). Briefly, a colony was picked up from the blood agar plate, inoculated in 5 ml trypticase soya broth, incubated overnight at 37^{o} C in ambient air and pelleted at 12,000 rpm for 2 min. DNA was restricted with commercially available PFGE kits using *Sma*I (MRSA) and *Spe*I (*Enterobacteriaceae*) enzyme (Genepath, Bio-Rad, France). Electrophoresis was performed in the CHEF-DR II (Bio-Rad, France) with linear ramped pulse times of 5–35 s and at a voltage of 6 V/cm, at 12ºC for 20 h. The images of the gels were processed with the software Gene Tools (Syngene Ver 1,2, UK). For *A. baumannii* a previously described method using enzyme *Apa*I (Zarrilli *et al.*, 2007; Arpac, 2009) was performed. The results were estimated discriminatory if at least ten-banding-pattern appeared; up to three band differences in PFGE were considered to represent clonally related strains (Tenover *et al.*, 1995; Toltzis *et al.*, 2002).

AFLP

For AFLP from each patient colonised with *K. pneumoniae* the first and last NP and rectal isolate was selected. If the baby was colonised by susceptible and AR strains the selection was performed the same way for both isolates. From those colonised for longer than three weeks an additional isolate was selected between the first and last sample.

Multienzyme multiplex AFLP protocol developed by van der Zee *et al* (Van Der Zee *et al.*, 2003) was used with few modifications. Briefly, bacterial DNA was digested with four different restriction enzymes and adapter oligonucleotides were ligated to fragments simultaneously. After purification of DNA fragments, PCR targeting adapter sequences was performed. The PCR products were separated on agarose gel electrophoresis.

DNA was extracted from bacterial culture using QIAamp DNA minikit (Qiagen, Hilden, Germany) following manufactures instructions. Approximately 300 ng of genomic DNA was subjected to a restriction-ligation reaction in a final volume of 30 μL. Restriction enzymes PstI, NheI, EcoRI and XbaI (20 U each; MBI Fermentas, Vilnius, Lithuania) were used in addition to 1 U of T4 ligase, 3 μL of T4 ligase buffer, 15 nmol of ATP (all MBI Fermentas, Vilnius, Lithuania) and 20 pmol of each of the four ligation adapter oligonucleotides (TAG Copenhagen A/S, Denmark), targeting the overhanging ends of XbaI and PstI restricted fragments. The restriction-ligation mixture was incubated at 37°C for 3 h, followed by DNA precipitation with 2.5 M ammonium acetate in 100 μL cooled absolute ethanol. DNA was washed twice using 70% ethanol and resuspended in 50 μL TE-buffer (pH 8.0).

According to van der Zee *et al* (Van Der Zee *et al.*, 2003), PCR with the combination of two PCR primers, Pxn and Pst-at, has the best discriminatory power and band resolution compared to separate runs with both primers (Pxn or Pst-at). PCR was performed in a total volume of 25 μL, using 0.5 U recombinant *Taq DNA polymerase* (MBI Fermentas, Vilnius, Lithuania), 62.5 nmol $MgCl_2$, 5 nmol of each deoxynucleotide (all from MBI Fermentas, Vilnius, Lithuania), 10 pmol of primers Pxn and Pst-at (Table 6) and 1 μL of DNA. The reaction was carried out in the Eppendorf thermocycler under the following conditions: 4 minutes of preheating at 94°C; 33 cycles of amplification at 94°C for 1 minute, at 60°C for 1 minute and at 72°C for 2.5 minutes; ending by final elongation at 72°C for 7 minutes. Twelve μL of each PCR product was analysed on 1.5% (w/v) agarose gel stained with ethidium bromide. The band patterns on gel were analysed using GeneTools program (Syngene, Cambridge, UK).

4.4. Definitions

Neonatal sepsis (proven or clinical) was diagnosed in the presence of at least two clinical and two laboratory criteria as detailed elsewhere (Metsvaht *et al.*, 2010): hyper- or hypothermia, apnoea or bradycardia spells, increased oxygen requirement, feeding intolerance, abdominal distension, lethargy and hypotonia, hypertension, skin and subcutaneous lesions such as petechial rash, abscess, scleroderma; WBC count <5 or >20 X 10^9 /L; immature to total WBC ratio (I/T) >0.2; platelet count <100 x 10^9/L; CRP >10 mg/L.

Early onset sepsis was defined as sepsis occurring within the first 72 h of life; all other cases were considered **late onset sepsis** (Stoll *et al.*, 2002a;, 2002b; Hira *et al.*, 2007).

Neonates with the GA ≤28 weeks were considered **extremely preterm**, 29–36 weeks **late preterm**, and ≥37 weeks **term infants**.

Organisms were considered **phenotypically similar** if the same species of microorganisms were cultured and detected by similar biochemical properties at

different time points and/or from different sites. Phenotypically similar microorganisms from mucosa and sterile body fluids were called **matching organisms**. If results of PFGE analysis were available they overwrote phenotypic culture data.

Genotypically similar organisms were defined according to PFGE if up to three band differences were considered to represent clonally related strains. For AFLP-PCR only fragments in the range of 200 to 2000 bp were included in the analysis and isolates showing >80% homology in pattern was considered clonally related strains.

Prior mucosal colonisation was defined as the presence of microorganisms on mucosal surfaces for ≥2days but <14 days before the collection of positive normally sterile fluid cultures.

Colonisation density (CD) was defined as the ratio of colonising days per 100 PICU days. The number of colonising days was counted from the first until the last positive culture and extra two days were added to compensate for the sampling interval, which was 3 to 4 days.

Invasive samples included blood and other sterile body fluids.

4.5. Statistical analysis

The software programs Sigma Stat for Windows 2.0 (Jandel Corporation, USA); R 2.6.2 (A Language and Environment, http://www.r-project.org), and EpiMax Table Calculator (Epidemiology and Lab Statistics from Study Counts, http://www.healthstrategy.com) were used for statistical analysis. Differences in proportions were compared using Chi-square test, odds ratios and/or Yates' continuity correlation as appropriate. Normally distributed continuous variables were compared with Student's *t*-test and non-normally distributed data with Mann-Whitney or Wald chi-square tests.

4.5.1. Calculation of risk factors for mucosal colonisation by Gram-negative microorganisms

A separate analysis was performed for each species and for the respective AR strain.

First, an univariate logistic regression analysis was performed. All variables significant at a p-value of ≤0.1 were entered into multiple logistic regression model with backward stepwise removal in order of insignificance. To avoid confounding by simultaneous inclusion of highly interrelated variables only one was included. There was a high correlation between BW and GA (r = 0.933) and between the duration of NICU stay and PNA (r = 0.996). The GA was considered a better determinant of immaturity than BW (Almuneef *et al.*, 2001; Kelly *et al.*, 2000) and the duration of NICU stay more important than PNA in influencing mucosal colonisation.

Continuous variables like use of empirical antibacterial therapy, ALV, central venous and arterial catheters were studied by including binomial and duration characteristics. Wherever feasible continuous variables involving the impact of duration were given a priority.

4.5.2. Comparing influence of penicillin G with gentamicin *vs* ampicillin with gentamicin on gut colonisation

First, two treatment regimens were compared to each other in terms of the number of patients colonised and CD by using mixed models adjusted for participating unit and treatment period, as appropriate for cluster randomized study design. Next, for microorganisms in which empirical antibiotic regimen appeared a significant determinant of colonisation in primary analysis at a p-value of ≤0.1, multivariate mixed effect models adjusted for other factors known to interfere with mucosal colonisation (GA, mode of delivery, maternal chorionamnionitis, PROM for more than 18 h before delivery, use of antenatal steroids and antibiotics, duration of ICU stay, type of feeding, presence of mechanical ventilation and culture proven EOS and use of carbapenems, third and fourth generation cephalosporins and beta-lactamase resistant penicillins) (Mackie *et al.*, 1999; Fanaro *et al.*, 2003; Thompson-Chagoyan *et al.*, 2007) were developed.

4.5.3. Calculation of sensitivity, specificity, positive and negative predictive values of mucosal samples

For each mucosal-invasive sample pair an algorithm was generated as follows: (1) when both samples contained phenotypically similar organisms mucosal sample was considered "true positive" (TP); (2) when both samples were negative it was labelled "true negative" (TN); (3) positive mucosal samples that matched with negative blood culture or with positive blood culture of a different organism were considered "false positive" (FP); and (4) negative mucosal samples with positive blood cultures were considered "false negative" (FN). Separate analyses were conducted for rectal and NP samples as well as for the combined dataset. In the combined analysis if a sample of one site was TP and of the other site FN it was labelled TP or if a sample of one site was FP and of the other site TN it was labelled FP.

The sensitivity, specificity, PPV and NPV were calculated for all Gram-negatives and on species level according to the time frame of sample collection (2–6, 7–10 and 11–14 days prior to sepsis). For the per patient analysis odds ratios (OR) with 95% confidence intervals were calculated similar to per sample analyses.

5. RESULTS AND DISCUSSION

5.1. Study population

A total of 465 neonates were admitted to both units; 142 in unit A and 141 in unit B met the study inclusion criteria and 142 and 134, respectively, qualified for colonisation studies (Figure 1) The study population with half of the neonates having BW <1500g, half born via caesarean section, three quarters artificially ventilated and/or with indwelling catheters and a quarter with culture proven neonatal sepsis is characteristic for a third level NICU being in line with some previous studies (Perlman *et al.*, 2007). With a few exceptions the population characteristics in both treatment groups and study periods were well balanced and similar in both units (Table 7).

The colonisation studies were incorporated in an efficacy study that aimed to compare the efficacy of penicillin to ampicillin in the empiric treatment of EOS (Metsvaht *et al.*, 2010). The cluster randomized design similar to De Man *et al.* (De Man *et al.*, 2000) was chosen to evaluate possible effects of the NICU environment and study period on mucosal colonisation. We appreciate that this design has some limitations. First, not all admitted neonates but only those aged <72 hours and requiring treatment for suspected or proven EOS were included. Thus, we have missed at least 1/3 of admitted patients who could have been a source of microorganism circulating in the NICU. Also, the duration of each study regimen just over eight months is likely too short to trigger changes in the circulating microflora within a unit. Another disadvantage was not having a control group of antibiotic-naïve subjects. Such a group of neonates in a third level NICU would have been extremely difficult to recruit as almost all critically ill neonates are exposed to antibiotics (Lass *et al*. 2011). Recruiting controls with very different disease severity may have biased the comparisons.

On the other hand the study was one of largest conducted on the topic with more than 1250 rectal and 1153 NP samples collected from 276 critically ill neonates. Also, collection of multiple data allowed investigation of 22 potential colonisation determinants in a multivariate manner in order to distinguish independent effects. We believe that the advantages of the study design outweigh its few limitations.

Table 7. Demographic and clinical characteristics of the study population

	Ampicillin + gentamicin	Penicillin + gentamicin	Unit A	Unit B	All
Number of patients	139	137	142	134	276
Neonatal factors					
Duration of NICU days; median (IQR)	6.0 (3.0; 15.8)	7.5 (4.0; 17.2)	9.4 (4.2; 20.4)	5.1 (3.0; 13.0)	6.7 (3.8; 17.0)
GA (week); mean, SD (min; max)	31.2; 5.1 (23; 41)	31.5; 5.1 (22; 42)	30.2; 5.2 (22; 42)	32.6; 4.7 (24; 42)	31.3; 5.1 (22; 42)
GA <28 weeks; n (%)	47 (33.8)	45 (32.8)	63 (44.4)	29 (21.6)	92 (33.3)
GA ≥37 weeks; n (%)	27 (19.4)	28 (20.4)	24 (16.9)	31 (23.1)	55 (19.9)
BW (g) mean (SD)	1842.9 (1111.8)	1807.8 (1012.5)	1637.5 (1045.0)	2024.6; (1047.0)	1825.5 (1061.9)
≤1000g; n (%)	36 (25.9)	39 (28.5)	53 (37.3)	22 (16.4)	75 (27.2)
≤1500g; n (%)	72 (51.8)	70 (52.0)	87 (61.3)	55 (41.1)	142 (51.5)
≥2500g; n (%)	36 (25.9)	31 (22.6)	27 (19.0)	40 (29.9)	67 (24.3)
Male; n	76	81	76	81	157
Nutrition regimen; n (%)					
TPN	28 (20.1)	30 (21.9)	52 (36.6)	6 (4.5)	58 (21.0)
Breast milk	32 (23.0)	39 (28.5)	14 (9.9)	57 (42.5)	71 (25.7)
Formula	79 (56.8)	67 (48.9)	75 (52.8)	71 (53.0)	146 (52.9)
Additional AB, n (%)					
Beta-lactam + betalactamase inhibitors	23 (16.6)	25 (18.3)	37 (26.6)	11 (8.2)	48 (17.4)
III and IV generation cephalosporines	7 (5.0)	12 (8.8)	1 (0.7)	18 (13.4)	19 (6.9)
Carbapenems	13 (9.4)	18 (13.1)	15 (10.6)	16 (11.9)	31 (11.2)
Neonates with sepsis; n (%)	31 (22.3)	36 (26.3)	43 (30.3)	24 (17.9)	67 (24.3)
EOS	6 (4.3)	8 (5.8)	5 (3.5)	9 (6.7)	14 (5.1)
LOS	25 (18.0)	28 (20.4)	38 (26.8)	15 (11.2)	53 (19.2)
ALV; n (%)	99 (71.2)	111 (81.0)	131 (92.3)	79 (60.0)	210 (76.1)
Central venous catheters; n (%)	104 (74.8)	106 (77.4)	127 (89.4)	83 (61.9)	210 (76.1)
Arterial catheters; n (%)	108 (77.7)	111 (81.0)	113 (79.6)	106 (79.1)	219 (79.4)

	Ampicillin + gentamicin	Penicillin + gentamicin	Unit A	Unit B	All
Maternal factors					
Multiple birth; n (%)	28 (20.1)	23 (16.8)	23 (16.2)	28 (20.9)	51 (18.5)
Caesarian section; n (%)	79 (56.8)	78 (56.9)	77 (54.2)	80 (59.7)	157 (56.9)
Antenatal steroids; n (%)	85 (61.2)	70 (51.1)	85 (59.9)	70 (52.2)	115 (56.2)
Antenatal antibiotics; n (%)	36 (25.9)	25 (18.2)	25 (17.6)	36 (26.9)	61 (22.1)
Maternal chorioamnionitis; n (%)	21 (15.1)	29 (21.2)	25 (17.6)	25 (18.7)	55 (19.9)
PROM >18 h; n (%)	24 (17.3)	27 (19.7)	24 (16.9)	27 (20.2)	51 (18.4)
Mother's age; median (min; max)	28 (16; 44)	29 (17; 42)	28 (17; 43)	27 (16; 44)	28 (16; 44)

SD – standard deviation

Statistically significant differences between units:

Unit A *vs* unit B:

BW ≤1000g – OR=3.03; 95% CI 1.72 – 5.36;

GA<28 weeks – OR=2.89; 95% CI 1.70 – 4.90;

Additional beta-lactam antibiotics – OR=3.94; 95% CI 1.91 – 8.11;

Cases of BSI – OR=1.99; 95% CI 1.13 – 3.51

Use of ALV – OR=8.29; 95% CI 4.10 – 16.78;

* BW < 2500g – OR=2.27; 95% CI 1.40 – 3.69;

* TPN during the first week of life – OR=12.33; 95% CI 5.08 – 29.93;

* Cases of LOS – OR=2.90; 95% CI 1.51 – 5.57;

* Use of CvC – OR=5.20; 95% CI 7.75 – 9.85.

Unit B *vs* unit A:

BW ≤2500g – OR=1.81; 95% CI 1.04 – 3.17;

Breast milk feeds within the first week of life – OR = 6.77; 95% CI 3.54 – 12.69;

Additional use of cephalosporins – OR=21.88; 95% CI 2.88 – 166.36.

5.2. Prevalence of mucosal colonisation by aerobic and facultatively anaerobic microorganisms

Throughout the study the most frequent rectal colonisers were Gram-positive bacteria (77.8%), among which CoNS, accounting for 69.8%, clearly predominated (Table 8). These findings are in line with other recent studies showing that in modern era of neonatal care and especially in industrialized countries staphylococci are becoming more abundant than enterobacteria, the latter used to predominate prior to 1980ies (Adlerberth *et al.*, 2006; Kelly *et al.*, 2007; Morelli, 2008). About half of the patients developed rectal (55.8%) or NP (42.8%) and 38.8% both sites' colonisation with Gram-negative microorganisms (Table 8). Except for *E. coli* Gram-negative colonisation was very much driven by the presence of AR strains; 73.4% of positive rectal and 60.2% of NP samples contained AR strains.

As shown on Figure 3 the colonisation pattern was quite similar for a given bacterial species in the two studied mucosal sites. Similarly to previous studies (Lidsky *et al.*, 2002) *Enterobacteriaceae* except *Serratia* spp. were more frequently colonising rectum than NP but *Acinetobacter* spp. were more commonly recovered in the NP. The most frequent Gram-negative coloniser including AR strains was *E. cloacae* followed by *K. pneumoniae, K. oxytoca* and *Acinetobacter* spp.

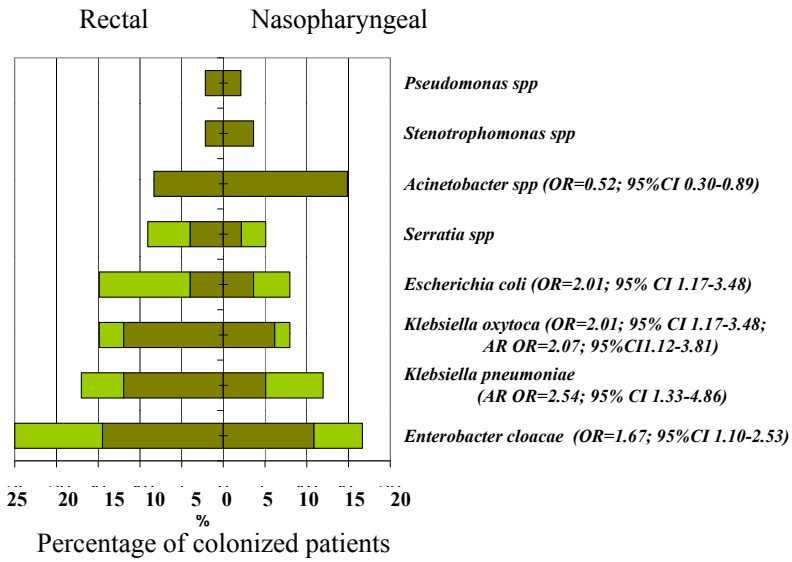

Figure 3. Rectal and NP colonisation with opportunistic Gram-negative microbes throughout the study. AR *Enterobacteriaceae* are shown in dark green bars and ampicillin-susceptible *Enterobacteriaceae* in light green bars. ORs with 95% CIs indicate significant differences between rectal and NP colonisation.

Table 8. Cumulative percentage of patients with rectal colonisation by various opportunistic microorganisms throughout the study

Microbes	Ampicillin		Penicillin		Unit A		Unit B		Period I		Period II		All	
	n%	R%	n%	R%	n%	R%	n%	R%	n%	R%	n%	R%	n%	R%
GRAM-NEGATIVE	56.1	41.7	55.5	40.1	55.6	40.8	56.0	41.0	59.1	47.4	52.5	34.5	55.8	40.9
K. pneumonia	21.6	15.1	12.4	8.8	26.8	19.0	6.7	4.5	22.6	15.3	11.5	8.6	17.0	12.0
K. oxytoca	13.7	12.2	16.1	11.7	16.2	13.4	13.4	10.4	12.4	10.9	17.3	12.9	14.9	12.0
E. cloacae	22.3	15.1	27.7	13.9	31.0	19.7	18.7	9.0	27.7	16.8	22.3	12.2	25.0	14.5
E. coli	12.2	2.9	17.5	5.1	9.9	2.1	20.1	6.0	16.1	2.9	13.7	5.0	14.9	4.0
Serratia spp	7.9	5.0	10.2	2.9	3.5	0.7	14.9	7.5	6.6	2.2	11.5	5.8	9.1	4.0
Acinetobacter spp	6.5	0.0	10.2	5.8	9.2	0.7	7.5	5.2	10.9	5.1	5.8	0.7	8.3	2.9
Stenotrophomonas spp	1.4	0.0	2.9	0.0	2.1	0.0	2.2	0.0	0.7	0.0	3.6	0.0	2.2	0.0
Pseudomonas spp	0.7	0.7	2.2	0.7	0.7	1.4	2.2	0.0	1.5	0.7	1.4	0.7	1.4	0.4
GRAM-POSITIVE	77.7	ND	83.2	ND	83.1	ND	77.6	ND	80.3	ND	80.6	ND	80.4	ND
S. aureus	4.3	3.6	11.7	5.1	4.9	0.7	11.2	8.2	9.5	5.8	6.5	2.9	8.0	4.3
CoNS in total	69.8	ND	78.1	ND	78.2	ND	69.4	ND	74.5	ND	73.4	ND	73.9	ND
S. haemolyticus	30.9	27.3	18.2	16.8	40.1	38.0	8.2	5.2	30.7	27.0	18.7	17.3	24.6	22.1
S. epidermidis	41.0	32.4	50.4	43.8	44.4	37.3	47.0	38.8	42.3	31.4	48.9	44.6	45.7	38.0
S. hominis	15.1	14.4	2.9	2.2	13.4	13.4	4.5	3.0	15.3	14.6	2.9	2.2	9.1	8.3
Streptococcus spp	8.6	ND	8.0	ND	9.9	ND	6.7	ND	9.5	ND	7.2	ND	8.3	ND
Enterococcus spp	25.9	ND	40.1	ND	37.3	ND	28.4	ND	30.7	ND	35.3	ND	33.0	ND
Candida albicans	14.4	ND	12.4	ND	18.3	ND	8.2	ND	16.8	ND	10.1	ND	13.4	ND
Other *Candida* spp.	7.2	ND	3.6	ND	5.6	ND	5.2	ND	6.6	ND	4.3	ND	5.4	ND

n% – percentage of neonates colonised with the respective microbe; R% – percentage of neonates colonised with resistant strains: Gram-negatives – AR; staphylococci – oxacillin-resistance

Significant differences

Unit B *vs* unit A: *E. coli* (OR = 2.31; 95% CI 1.15 – 4.62); *Serratia* spp (OR = 4.81; 95% CI 1.75 – 13.21; AR *Serratia* spp (OR=24.74; 95% CI 3.27 – 187.13); *E. cloacae* (OR = 0.51; 95% CI 0.29 – 0.90); AR *E. cloacae* (OR=0.40; 95% CI 0.19 – 0.83); *K. pneumoniae* (OR = 0.20; 95% CI 0.09 – 0.43); AR *K. pneumoniae* (OR=0.20; 95% CI 0.08–0.50); *S. haemolyticus* (OR = 0.13; 95% CI 0.06 – 0.26); *S. hominis* (OR = 0.28; 95% CI 0.11 – 0.71).

First *vs* second period: *K. pneumoniae* (OR = 2.25; 95% CI 1.17 – 4.34); *S. haemolyticus* (OR=1.92; 95% CI 1.10 – 3.36); *S. hominis* (OR = 6.46; 95% CI 2.16).

Ampicillin *vs* penicillin containing regimen: *S. aureus* (OR=0.34; 95% CI 0.13 – 0.91); *Enterococcus* spp. (OR=0.5; 95% CI 0.3 – 0.83); *S.haemolyticus* (OR=2.22; 95% CI 1.2 – 4.12; *S.hominis* (OR=6.46; 95% CI 2.12 – 19.67); AR *Acinetobacter* spp. (p=0.008).

5.3. Dynamics of mucosal colonisation

On admission to NICU on the first day of life about 1/3 of neonates were already colonised with Gram-positive microorganisms (mainly CoNS) and their predominance continued throughout the first 16 days (Figure 4). By Day 6 to 9 more than 90% had colonisation by Gram-positive and about 30% with Gram-negative AR microorganisms. These findings are very similar to previous studies conducted in NICUs in industrial countries although linear rather than sigmoid type increase has been shown by Almuneef *et al.* (Almuneef *et al.*, 2001) and Smith *et al* (Smith *et al.*, 2010).

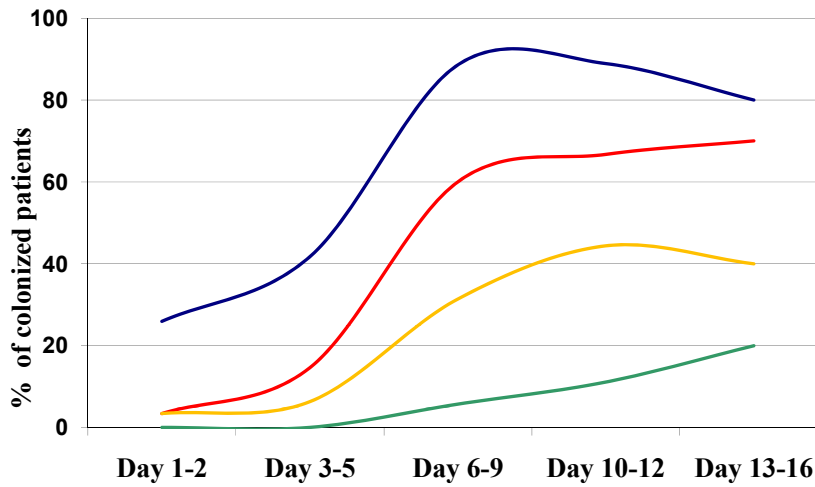

Figure 4. Rectal colonisation in first 16 days of life. Gram-positives are presented in blue, Gram-negative es in red, Gram-negative AR in yellow, and yeasts in green line.

In terms of individual species a steady increase in prevalence was observed for all enterobacterial species up to the 4th week of life, with the exception of *Serratia* spp and *E.coli*, the prevalence of which remained constantly around 5% and 10%, respectively (Figure 5).

In the first week of life *K. pneumoniae* and *E. coli* more often colonised rectum than NP (OR = 2.55; 95% CI 1.08 – 6.03 and OR = 2.19; 95% CI 1.11 – 4.31, respectively) but later on no relevant differences between the two sites except for *E. cloacae* (OR = 2.28; 95% CI 1.26–4.13) in the second week were observed. AR *E. cloacae* and AR *Klebsiella* spp. increased steadily from Day 1 levelling off at 15% (Haanpera *et al.*) to 20% (rectal) by week 4 to 5. These findings confirm the previous understanding that *E.coli* and *Serratia* spp. most likely originate from the mother while the other members of *Enterobacteriacae* arise from the hospital environment (Rotimi *et al.*, 1977; Pietzak, 2004; Prelog *et al.*, 2009).

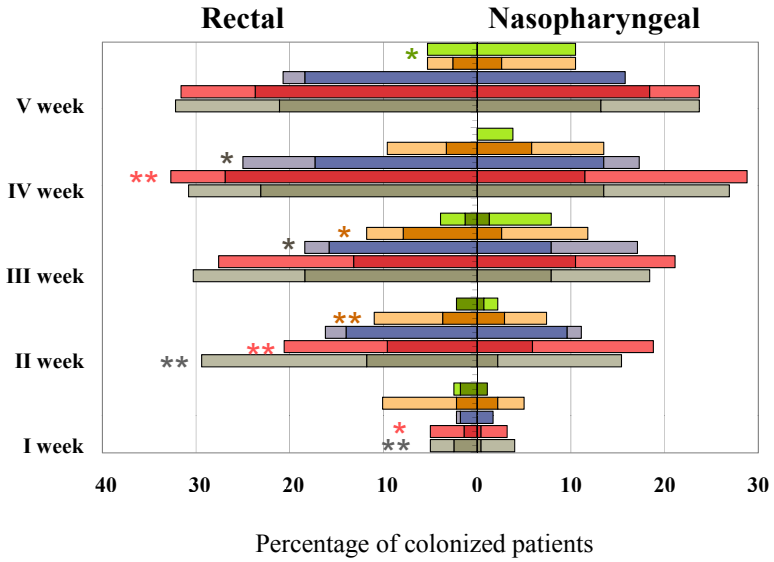

Figure 5. The percentage of patients colonised by different species of *Enterobacteriaceae* during the first five weeks of the NICU stay. X-axis represents percentage of colonised patients. The rectal colonisation is shown on the left and NP on the right. Colonisation by AR strains is presented in darker and ampicillin susceptible strains in lighter colours. *E. cloacae* is shown in grey, *K. pneumoniae* in red, *K. oxytoca* in blue, *E. coli* in brown and *Serratia* spp in green bars. Asterisks indicate each case of LOS.

Among nonfermentative microorganisms (Figure 6) no association with the duration of NICU stay was observed. On the second and third week *Acinetobacter* tended to colonise NP more often than the rectum and on the first week *Pseudomonas* was more frequent coloniser of the NP than rectum.

Rectal colonisation by *Candida* spp. was observed in 49 (17.8%) patients led by *C. albicans* (n = 37) and followed by *C. parapsilosis* (n = 10), *C. lusitaniae* (n = 5) and *C. tropicalis* (n = 1). Four patients had *C. albicans* and *C. parapsilosis* concomitantly. Colonisation by *C. albicans* started at birth and increased steadily thereafter whereas a sharp rise in non-albicans *Candida* spp. was seen at the fifth week of NICU stay (Figure 3; publication III) confirming that *C. albicans* most likely is of maternal and non-albicans *Candida* spp. of hospital origin (Miranda *et al.*, 2009; Saiman *et al.*, 2001).

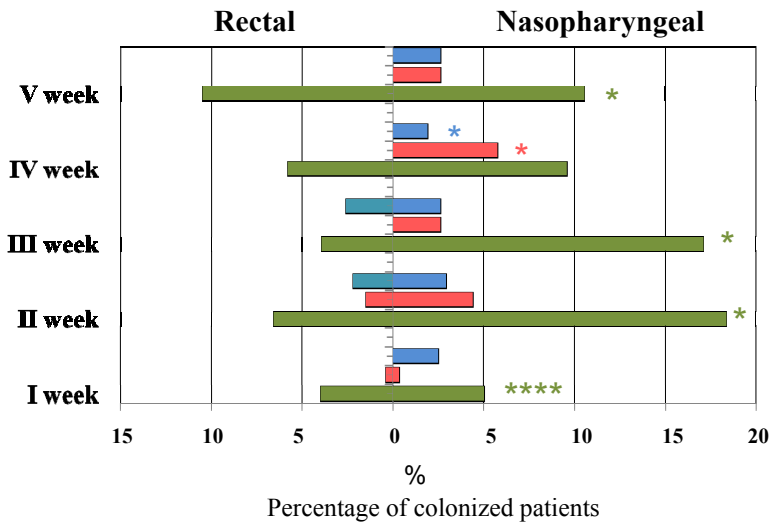

Figure 6. Timing of rectal and NP colonisation with nonfermentive Gram-negative microorganisms during the first 5 weeks of life. X-axis represents percentage of colonised patients. *Pseudomonas* is shown in green, *Stenotrophomonas* in blue and *Acinetobacter* in red bars. The significant differences between NP *and* rectal colonisation are as follows: *Pseudomonas* – first week p=0.022; *Acinetobacter* – second week OR = 3.18; 95% CI 1.42 – 7.10, and third week OR = 5.02; 95% CI 1.37 – 18.42. Asterisks indicate cases of LOS and the causative agents are represented in the same colour as colonisers.

5.4. Risk factors associated with mucosal colonisation

In univariate regression analysis a total of 22 factors (3 environmental, 5 maternal and 14 neonatal) were associated with the colonisation by various opportunistic microorganisms at a p value of ≤0.1 (Table in Appendix; publication III). Further only the factors that remained associated with colonisation in multiple regression analysis are presented and discussed.

5.4.1. Environmental factors – participating unit, treatment period and duration of stay in neonatal intensive care unit

The duration of NICU stay was the most important factor influencing mucosal colonisation. It was associated with rectal and NP colonisation of *Acinetobacter* spp. and all *Enterobacteriaceae* except *E. coli* (Table 2 in publication III) and rectal colonisation of CoNS, *S. aureus*, *Enterococcus* spp. and yeasts (Table 9).

Table 9. Factors influencing rectal colonisation by various Gram-positive microorganisms and yeasts – results of multiple logistic regression analyses.

Influencing factor	CONS		*Enterococcus*		*S. aureus*		*C. albicans*		Non-albicans *Candida*	
	OR	95% CI	OR	95% CI	OR	95% CI	OR	95% CI	OR	95% CI
NICU stay days	1.28	1.17–1.40	1.11	1.07–1.14	1.07	1.04–1.10	1.05	1.03–1.07	1.07	1.04–1.10
Unit B *vs* unit A					3.27	1.14–9.38				
Vaginal delivery *vs* Cesarean section							2.27	1.06–4.86		
GA ≥37 *vs* 29–36 weeks	2.35	1.01–5.45								
Multiple birth					5.07	1.72–14.93				
Parenteral *vs* breast milk							2.91	1.02–8.26		
Ampicillin *vs* penicillin			0.38	0.19–0.73	0.29	0.10–0.84				
CvC			5.12	1.70–17.80						
Duration of CvC days									1.06	1.00–1.13
Duration of ALV days			0.95	0.92–0.99						

Depending on the species each day in NICU increased colonisation risk by 3% to 8%. Indeed PNA almost completely overlapping with the NICU stay in this study could also have influenced mucosal colonisation. When comparing NP carriage rates in healthy and age-matched hospitalized infants over the first 6 months of life Baltimore *et al* (1989) suggested PNA's primary effect in colonisation with Gram-negative microorganisms. However, we were unable to distinguish between their roles as due to strong correlation between the two only NICU stay was entered into the multiple regression model. We considered the duration of hospital stay a more important determinant of mucosal colonisation than PNA.

We also observed some differences between rectal colonisation pattern between treatment periods and participating units (Table 8). As shown in Figure 7 *K. pneumoniae* was extensively circulating in unit A, especially during the 1st period whereas only a few patients were colonised in unit B. Heavy colonisation in unit A was accompanied by an endemic occurrence of *K. pneumoniae* BSI lasting for more than 6 months. During this period altogether 23 neonates became colonised with genotypically related strains and five developed invasive disease. The clonal relatedness of invasive and colonising strains was confirmed by AFLP (Figure 7) and PFGE (Figure 8). Also a smaller outbreak of MRSA infection involving three patients occurred in unit B during the period of November 30 – December 28, 2006. These data further underline the role of hospital environment in mucosal colonisation process.

Our results are not surprising as also previous studies have recognised hospital environment as one of the most important sources for colonisation by several Gram-negative organisms, including antibiotic-resistant strains, and MRSA and yeasts (Almuneef *et al.*, 2001; Cartelle *et al.*, 2004; Beggs *et al.*, 2006; Gregory *et al.*, 2009; Heinrich *et al.*, 2011). Microbes may spread via hands of health-care workers, air and/or contaminated water and/or environmental surfaces (Huang *et al.*, 1998b; De Man *et al.*, 2001; Gupta *et al.*, 2004; Bagattini *et al.*, 2006; Amaya *et al.*, 2010). Medical devices such as CvCs, urinary catheters and endotracheal tubes frequently used in the NICU are also well-known sources of mucosal colonisation and BSIs (Frebourg *et al.*, 1999; Mahieu *et al.*, 2001; O'grady *et al.*, 2002; Srivastava *et al.*, 2007). To study the role of the above mentioned environmental factors was clearly out of the scope of this study.

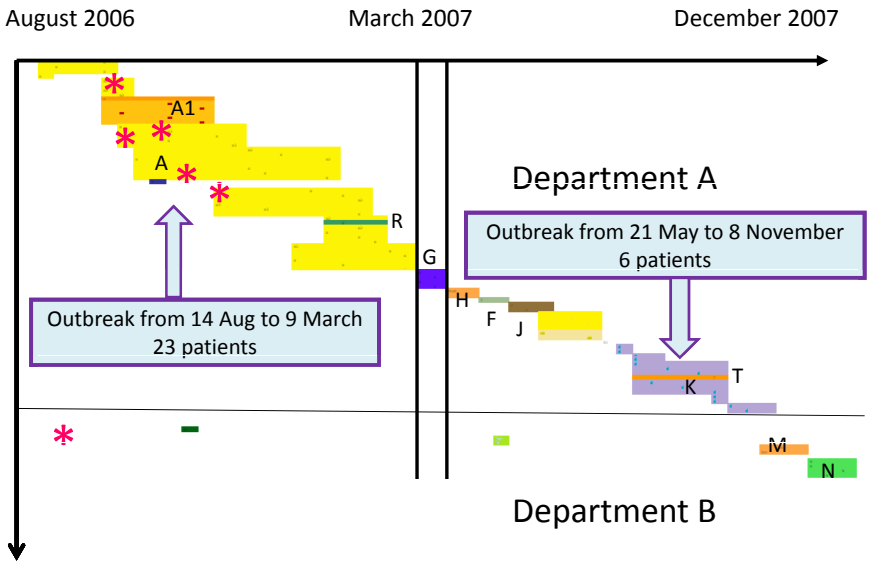

Figure 7. Clonal dissemination of *K. pneumoniae* in unit A. The horizontal arrow represents the progression of the study and vertical arrow represents the cumulation of patients colonised by *K. pneumoniae* in the corresponding study period. Above the red horizontal line the situation in unit A and below the line in unit B is presented. In March 2007 the antibiotic regimens were changed between the units. All different genotypes are presented by different colours and letters. Genotypically related strains during the first outbreak are presented in yellow and those during the second outbreak in violet. Asterisks indicate BSIs caused by *K. pneumoniae*.

These findings support the previous recommendations that NICU hygiene oriented interventions may enable to avoid or reduce colonisation by potentially pathogenic microorganisms and could eventually result in diminished numbers of invasive disease (Almuneef *et al.*, 2001; Edwards *et al.*, 2002; Lidsky *et al.*, 2002; Cartelle *et al.*, 2004; Mammina *et al.*, 2007; Millar *et al.*, 2008; Phillips *et al.*, 2008). For example, in US after a collaborative quality improvement process wherein among the other activities improved hand cleaning and education of the entire staff to encourage maximal compliance with infection control practices, reduced nosocomial infection rate in the first year by 26% and in the second and third year by another 29% (Schelonka *et al.*, 2006). Still, this study measured only reduction in infection rates, and did not monitor the mucosal colonisation.

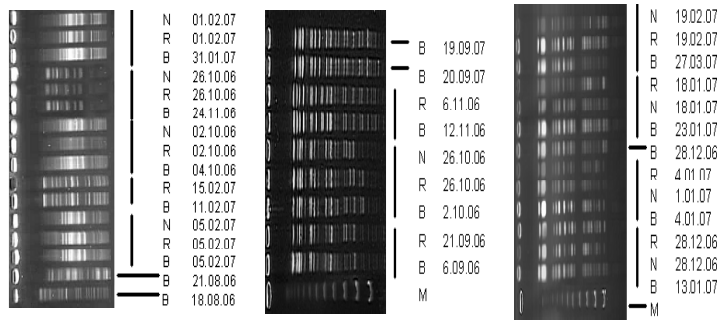

Figure 8. Relatedness between of *A. baumannii*, *K. pneumoniae,* and *E. cloacae* strains isolated from rectal (R), nasopharyngeal (N) and blood (B) samples using PFGE analysis. M indicates marker. Each line shows different patient.

Another measure suggested to reduce the number of hospital acquired infections is isolation and cohorting of patients, although some authors have pointed out, that evidence from randomized controlled trials is lacking (Traore *et al.*, 2002; Cooper *et al.*, 2004; Mohan *et al.*, 2007). However, if the spread of a single strain is responsible for the outbreak of MRSA or MRSE, cohorting of infected and/or colonised patients might be useful as suggested (Mussi-Pinhata *et al.*, 2001; Traore *et al.*, 2002; Mcdonald *et al.*, 2007; Van Rossem *et al.*, 2007; Gregory *et al.*, 2009; Navarro *et al.*, 2011).

5.4.2. Perinatal factors – gestational age, mode of delivery, premature rupture of membranes

We have shown that the GA played an important role in colonisation process. Term neonates were more likely colonised by *E. coli* (Table 2 in publication III) and CoNS (Table 9) compared with late preterm but not with the extremely premature babies. In terms of Gram-positives and yeasts the colonisation pattern of extremely preterm neonates was similar to late preterms, but the first mentioned group had higher risk of developing NP colonisation by *K. pneumoniae* as compared with both other age categories and by *E. cloacae* compared with term neonates.

In terms of colonisation term neonates had almost four times greater chance to be colonised on Day 1–2 compared with late preterms (OR=4.27; 95% CI 1.80–10.16). As term babies are more likely born via vaginal delivery than preterms this suggests that a vaginal tract could be a potential source of microorganisms (Bettelheim *et al.*, 1974a; Bettelheim *et al.*, 1974b; Bettelheim *et al.*, 1974c; Goldmann, 1981; Tannock *et al.*, 1990; Penders *et al.*, 2006; Ogra,

2010). This is further supported by the fact that compared with neonates born via Caesarean section; those delivered vaginally had increased risk of mucosal colonisation by *E. coli* (Table 2 in publication III) and *C. albicans* (Table 9).

Previously suggested reasons for later colonisation in preterms compared with term neonates include reduced maternal contact, different dietary intake, peristalsis and glandular secretions as well as the impaired immune response of intestinal mucosa (Kelly *et al.*, 2000; Mshvildadze *et al.*, 2010a; Wolfs *et al.*, 2010). Diminished host cell gene expression resulting in lower total counts of bacteria and limited numbers of species has also been proposed (Gewolb *et al.*, 1999; Thompson-Chagoyan *et al.*, 2007).

Similar to us previous studies showed that term neonates are more likely colonised with *E. coli* (Fanaro *et al.*, 2003), and extremely preterms have greater risk of colonisation by *K. pneumoniae, E. cloacae* (Goldmann, 1981; Schwiertz *et al.*, 2003) and *Candida* spp, especially non-albicans *Candida* spp (Saiman *et al.*, 2001; Mendiratta *et al.*, 2006; Manzoni *et al.*, 2008). These data suggest that in extremely preterm infants colonisation depends rather on the endemic microorganisms present in NICU, while in term infants it is more affected by the mothers' microbiota (Almuneef *et al.*, 2001; Mammina *et al.*, 2007; Millar *et al.*, 2008). However, in the present study the association between GA and *Candida* colonisation was not significant in multivariate analysis indicating that low GA explains the greater colonisation by *Candida* spp. of preterms only partly.

PROM was associated with increased risk of rectal colonisation by *K. pneumoniae* and NP colonisation by *K. oxytoca, E. coli* and *Stenotrophomonas* spp (OR=19.00; 95% CI 1.39; 260.21) (Table 2 in publication III). It is presumed that contact with intraamniotic fluid is associated first with NP colonisation. All above mentioned microbes (*E. coli, K. pneumoniae* and other *Enterobacter* spp) have been found in patients with PROM rising most likely from the ascending infection from perineum and vagina (Kayange *et al.*, 2010). As PROM-related colonisation may be followed by a serious threat of immediate postnatal infection and intraamniotic bacterial infections may cause preterm birth (Asindi *et al.*, 2002; Kenyon *et al.*, 2003; Simhan *et al.*, 2005; Kirchner *et al.*, 2007; Levy, 2007; Lafeber *et al.*, 2008; Veleminsky *et al.*, 2008), avoidance of PROM ≥18 h is essential.

5.4.3. Feeding regimen

The dynamics of mucosal colonisation depending on the feeding habits is presented on Figure 9. Not surprisingly and similarly to previous studies we demonstrated that enteral feeding is an important determinant of colonisation process and that the use of TPN delays colonisation by Gram-negative bacilli (Smith *et al.*, 2010). As shown, by Days 6–9 and 10–12 neonates fed with breast milk-containing regimen had six and nine times greater risk of colonisation with Gram-negative bacteria and six and seven times greater risk of colonisation with

Gram-negative AR strains compared to those on TPN, respectively. Differences between breast milk and formula feeding were minor; there was a greater colonisation risk by Gram-negative AR strains by Day 10–12 (OR=3.1; 95% CI 1.08 – 8.86) in breast milk as compared with formula fed babies.

Not surprisingly and consistent with previous studies feeding regimen affected rectal but not NP colonisation. Neonates receiving TPN were less likely colonised with *K. oxytoca* and *E. coli* (Table 2 in publication III), but more likely with *C. albicans* (Table 9) than those fed with breast milk, suggesting the likely maternal origin of these microorganisms (Berthelot *et al.*, 2001; Martin *et al.*, 2007). The association between TPN (more specifically intravenous lipids) and colonisation by *C. albicans* has been shown previously (Huang *et al.*, 1998a; Manzoni *et al.*, 2008; Saiman *et al.*, 2001). In one hand, TPN solutions are considered to be relatively good growth mediums (Fox *et al.*, 1999; Kuwahara *et al.*2010), but on the other hand, it is not easy to explain how these microorganisms get into the GI tract. It may be explained by the fact that neonates receiving TPN have limited numbers of Gram-negatives (Smith *et al.*, 2010) leading to overgrowth of *Candida* spp obtained from their mothers or health-care workers.

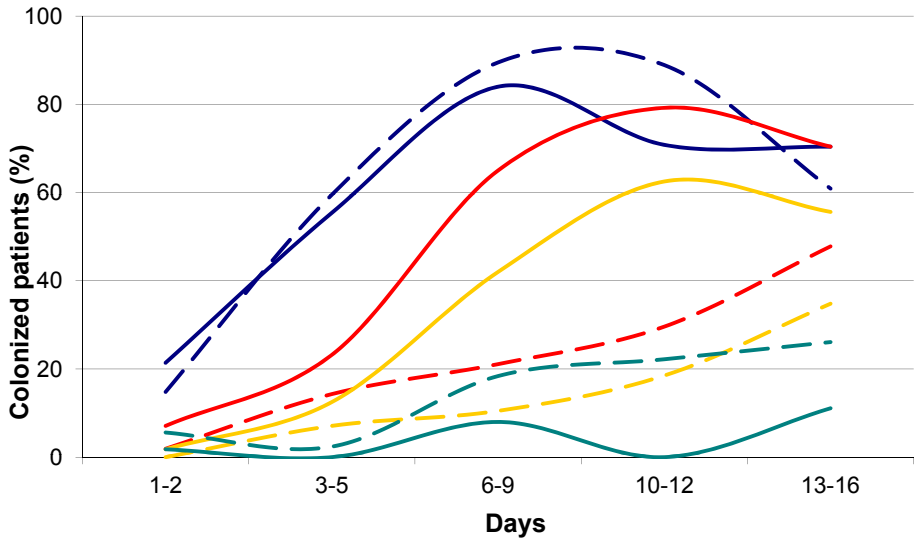

Figure 9. Comparison of dynamics of rectal colonisation between groups fed with the breast milk (presented by continuous lines) *vs* TPN (presented by dotted lines). The colonisation with Gram-positives is presented in blue, Gram-negatives in red, Gram-negative ARs in yellow and with yeasts in green lines. The following significant differences were observed: the rate of patients colonised with Gram-negatives and Gram-negative AR strains is greater in breast milk-fed group *vs* TPN group on Days 6–9 (OR=6.12; 95% CI 2.33–16.09 and OR=6.16; 95% CI 1.89–20.00, respectively), and Days 10–12 (OR=9.03; 95% CI 2.50–32.64 and OR=7.33; 95% CI 2.05–26.25, respectively).

However, in contrast to some other studies (Orrhage *et al.*, 1999) we did not observe differences in gut colonisation between breast milk and exclusively formula fed neonates (Table 2 in publication III) suggesting that rather the route and not the content of food plays a critical role in the colonisation process. These results affirm that the spread of colonising microorganisms likely occurs via cross-colonisation including contamination of nasogastric tubes as demonstrated recently by Hurrell *et al* (2009) and underline once again the potential of hospital hygiene oriented interventions in preventing hospital acquired infections (Edwards *et al.*, 2002; Cohen *et al.*, 2003; Brady, 2005; Larson *et al.*, 2005; Mcdonald *et al.*, 2007; Borghesi *et al.*, 2008; Phillips *et al.*, 2008; Gregory *et al.*, 2009; Jarvis, 2010).

The association between mucosal colonisation and invasive infection will be discussed in chapter 5.6.

5.4.4. Medical devices (artificial lung ventilation and indwelling catheters)

The presence of arterial lines was associated with increased risk of rectal and NP colonisation by *K. pneumoniae* and the presence of CvC increased the risk of rectal colonisation by *Acinetobacter* spp (Table 2 in publication III), enterococci, and non-albicans *Candida* spp (Table 9). The mechanisms how indwelling catheters are associated with mucosal colonisation are likely multifactorial. It may occur via horizontal transmission by the hands of NICU care givers (Saiman *et al.*, 2000). Previous studies have shown that direct and indirect procedures in neonates are all associated with significant increase in hand colony count. For example, skin contact, respiratory tract care, contact with equipment and CvC device manipulation increased bacterial count of ungloved healthcare workers' hands during 135 secons of care among neonates in the NICU of 21.2 CFU/min ($p = 0.08$), 20.4 CFU/min ($p < 0.001$), 10.0 CFU/min ($p = 0.004$) and 10.0 CFU/min ($p = 0.015$), respectively. Altogether 13.8% and 1.8% of these microorganisms were *Enterobacteriaceae* and fungi, respectively (Pessoa-Silva *et al.*, 2004). Even plain soaps may become contaminated and lead to hand colonisation by Gram-negatives (Sartor *et al.*, 2000). On the other hand, colonisation by these microbes is facilitated by immaturity; serious general condition and prolonged hospital stay all associated with the use of indwelling catheters.

The duration of ALV (in median of 80.6 hours; IQR 36.5–164 h) was associated with increased risk of NP colonisation by *K. pneumoniae* (Table 2 in publication III), and decreased risk of rectal colonisation by enterococci (Table 9). The relationship between tracheal intubation and NP colonisation remains presumptive, as an association between intubation, especially for more than 72 hours, and frequent NP colonisation by *Entrobacteriaceae* in NICU has been shown previously (Harris *et al.*, 1976). Also, the presence of intubation has been identified as an independent risk factor for colonisation/infection by *K. pneumoniae* during an outbreak (OR=27.0; 95% CI 5.39 – 135.14) (Cartelle *et al.*, 2004).

5.4.5. Influence of antibiotics used for the empiric treatment of early onset sepsis

Both antibiotic regimens (penicillin G + gentamicin and ampicillin + gentamicin) had similar effect on the dynamics of early gut colonisation with Gram-negative microorganisms and *Candida* spp. except for the greater number of patients colonised by *Acinetobacter* spp on Days 13–16 in the penicillin compared with the ampicillin group (Figure 2B in publication 1). In terms of Gram-positive organisms, however, the two antibiotic regimens differed – penicillin treatment resulted in significantly greater number of patients colonised with *Enterococcus* spp. at all time-points from Day 6 to 16 and by *S. aureus* between Days 3 to 5 compared to ampicillin (Figure 2A in publication 1).

In the mixed effect model analysis the number of patients colonised with all Gram-negative organisms was similar in both treatment arms except of AR *Acinetobacter* spp., that was found only in the penicillin arm (0 *vs* 8; $p = 0.008$). Again, differences among Gram-positive colonisation were observed – compared with penicillin ampicillin treatment was associated with twofold greater odds of colonisation by *S. haemolyticus* and six fold that of *S. hominis*, whereas the odds of colonisation by *Enterococcus* spp. and *S. aureus* were about a half and one third of that seen in the penicillin arm, respectively (Table 10).

In terms of CD the penicillin and ampicillin treatments were more distinct than in cumulative per patient colonisation – the mean CDs in multivariate analyses of *K. pneumoniae* (difference +6.6) and AR *Serratia* spp. (+3.7) were greater in ampicillin as compared with penicillin arm and those of AR *Acinetobacter* spp. (–2.5) in the penicillin as compared with the ampicillin arm. Among Gram-positive bacteria the CDs followed similar trends as in the per patient analysis; ampicillin was associated with higher mean CDs of *S. haemolyticus* (difference +11.4) and *S. hominis* (+6.4) but lower CDs of *Enterococcus* spp. (–13). In univariate analysis ampicillin was also associated with lower CDs of *S. epidermidis* (–8.5) but in multivariate analyses this remained of borderline significance ($p = 0.0725$). In addition, in multivariate analysis ampicillin treatment was associated with higher CD of *Candida* spp. ($p = 0.02$). Although colonisation by *S. aureus* was different between treatment arms in per patients analysis, in CD analysis this did not reach significance at the p value of < 0.05 ($p = 0.054$) (Table 10).

Table 10. Significant impact of ampicillin and penicillin regimen on gut colonisation – results of univariate hierarchical model correlated for participating unit and treatment period, and multifactorial mixed effect model analysis.

	Univariate analysis				**Multivariate analysis**			
	Per subject	p=	CD	p=	Per subject	p=	CD	p=
Ampicillin treatment associated with increased colonisation			*K. pneumonia*	0.004			*K. pneumoniae*	0.012
			AR *Serratia* spp	0.011			AR *Serratia* spp	0.012
	S. haemolyticus	0.01	*S. haemolyticus*	0.001	*S. haemolyticus*	0.039	*S. haemolyticus*	0.001
	S. hominis	0.001	*S. hominis*	0.001	*S. hominis*	0.009	*S. hominis*	0.001
							Candida spp	0.02
Penicillin treatment associated with increased colonisation	AR *Acinetobacter*	0.008	AR *Acinetobacter*	0.004			AR *Acinetobacter*	0.001
	Enterococcus spp	0.01	*Enterococcus spp*	0.001	*Enterococcus* spp	<0.001	*Enterococcus* spp	0.001
	S. aureus	0.03	*S. epidermidis*	0.039	*S. aureus*	0.006	*S. aureus*	0.052

The multifactorial model was not conducted if the p value in univariate hierarchical model was >0.1.
CD – colonisation density – the ratio of colonising days per 100 PICU days

In contrast to a previous EOS study (De Man *et al.*, 2000), comparing amoxicillin plus cefotaxime regimen to penicillin plus tobramycin, as well as some studies on intrapartum antibiotic prophylaxis (Mcduffie *et al.*, 1993; Joseph *et al.*, 1998; Levine *et al.*, 1999; Almuneef *et al.*, 2001; Stoll *et al.*, 2002a; Laugel *et al.*, 2003; Cordero *et al.*, 2004; Bizzarro *et al.*, 2008), we failed to prove our main hypothesis that ampicillin as an antibiotic with broader Gram-negative coverage than penicillin selects for AR *Enterobacteriaceae* except for the greater CD of AR *Serratia* spp. However, our findings support those of Jauréguy *et al.* (Jaureguy *et al.*, 2004) who in an intrapartum prophylaxis study failed to demonstrate that amoxicillin selects for beta-lactam resistant enterobacteria. At present in most countries all *Enterobacteriaceae* other than *E. coli* have become uniformly resistant to ampicillin (Bennet *et al.*, 2002; Jain *et al.*, 2003; Mitt *et al.*, 2009) and thus it is not surprising that ampicillin and penicillin have similar influence on gut colonisation with Gram-negative bacteria.

A more serious problem emerging during penicillin containing regimen was the greater number of patients colonised with *S. aureus*, a well-recognized and frequent opportunistic pathogen in NICU (Milledge *et al.*, 2005; Elwan *et al.*, 2009). It has been shown previously that compared with untreated subjects ampicillin in combination with gentamicin reduces faecal colonisation of *S. aureus* (Bennet *et al.*, 1982). The results of herein study are not surprising as the vast majority of *S. aureus* isolates are penicillin resistant nowadays (Livermore, 2000; Hawkey, 2008) but may still be susceptible to ampicillin.

The shift in colonisation by CoNS species so that penicillin treatment is associated with greater colonisation of *S. epidermidis* and lower colonisation by *S. haemolyticus* and *S. hominis* than ampicillin is more difficult to explain and to our knowledge has not been described before. It is likely that these different species of staphylococci compete for the colonisation niche with each other. The different colonisation pattern may still have clinical implications as mucosal colonisation is an important source of CoNS in invasive infections (Costa *et al.*, 2004). In addition, differences in the pathogenicity between CoNS species have been demonstrated so that 50% to 71% of *S. epidermidis* but only 35% of *S. haemolyticus* and 26% of *S. hominis* strains are capable of slime production, the latter being associated with invasive disease (Hall *et al.*, 1990; Drozenova et al., 2000; Arslan *et al.*, 2007; Koksal *et al.*, 2009). The relevance of differences in gut colonisation observed in this study is further supported by the clinical findings showing a trend towards greater prevalence of LOS caused by *S. epidermidis* in the penicillin as compared with the ampicillin arm (7.6 vs 2.7 per 100 patient days, respectively). See section 5.6.1.

5.4.6. Influence of other antibiotics

In an univariate analysis shown in Appendix Table in publication III all additionally administrated antibiotic classes were associated with increased rectal colonisation by non-albicans *Candida* spp. In addition, carbapenems predisposed

to colonisation by *K. pneumoniae* and NP colonisation by *K. oxytoca* and broad spectrum beta-lactams by *K. oxytoca* and *E. cloacae.* However, none of these associations remained significant in risk factor adjusted multivariate analysis.

These findings contrast some previous studies showing that the use of broad spectrum antibiotics leads to outselection of specific organisms like *Candida* spp. and antibiotic resistant strains (Garcia-Rodriguez *et al.*, 2002; Jain *et al.*, 2003; Chiu *et al.*, 2005). However, one should note that this controversy could in part be explained by different study conditions and analyses used. So studies monitoring antibiotic use as single factor (Burman *et al.*, 1993), or monitoring multiple factors but not applying multivariate analyses (De Man *et al.*, 2000; Duman *et al.*, 2005), or looking at the influence of antibiotics in an outbreak period (Cassettari *et al.*, 2009) have reported an association between antibiotic therapy and resistance occurrence. On the other hand, studies applying multivariate analyses have not confirmed this association (Mammina *et al.*, 2007; Millar *et al.*, 2008). The selection of resistant strains is a lengthy process likely not observed in studies with short duration or using antibiotics for a limited number of days as ours (Jaureguy *et al.*, 2004; Millar *et al.*, 2008; Auriti *et al.*, 2009; Puopolo *et al.*, 2010).

5.5. Factors associated with colonisation by ampicillin resistant strains

As shown in Table 3 in publication III, the risk factors of colonisation with AR microorganisms were largely similar to those seen in all strains. While rectal colonisation by AR *K. oxytoca* more likely occurred in term babies AR *K. pneumoniae* was three times more often isolated in extremely immature as compared with late preterm babies. Breast milk feeding increased the risk of colonisation by AR *K. oxytoca.* Early empiric ampicillin therapy was associated with increased risk of NP colonisation by AR *E. cloacae*. Mode of delivery did not influence colonisation by AR strains, but PROM > 18h increased risk of NP colonisation by AR *K. pneumoniae, K. oxytoca,* and *E. coli.* Intrapartum antibiotic use favored rectal colonisation by AR *K. pneumoniae.*

The role of antibacterial therapy in inducing colonisation by resistant *Enterobacteriaceae* has been shown in several studies (Garcia-Rodriguez *et al.*, 2002; Jain *et al.*, 2003; Chiu *et al.*, 2005). However, more recently this effect has been questioned (Karami *et al.*, 2006; Millar *et al.*, 2008). As discussed above overinterpreting associations between short-term antibiotic use and resistance development may rise from analyses including only a limited number of risk factors and not considering others with major impact, like duration of NICU stay, maternal antibiotic use during pregnancy and/or invasive procedures or population characteristics. For example, the AR rates of microbes in the intestinal flora of healthy subjects are much greater in developing than in the developed countries – 96.8% in India or South Africa (Amyes *et al.*, 1992;

Shanahan *et al.*, 1995) *vs* 42% in the UK (Shanahan *et al.*, 1994). This could explain the relationship between antibiotic use and resistance development in a Brasilian study (Cassettari *et al.*, 2009). Similar to general colonisation early mucosal colonisation by resistant strains is more likely associated with transmission of microbiota from other patients than reflection of the emergence of resistance due to antibiotics; the NICU environment itself is a reservoir of resistant Gram-negative bacilli (Toltzis *et al.*, 2002; Mammina *et al.*, 2007; Millar *et al.*, 2008).

5.6. Associations between mucosal colonisation and development of LOS

5.6.1. Prevalence and aetiology of LOS

A total of 75 culture proven LOS cases in 53 neonates occurred during the study. Similar to other studies in industrialised countries (De Man *et al.*, 2000; Stoll *et al.*, 2002b; Bizzarro *et al.*, 2005; Ronnestad *et al.*, 2005a) the majority of cases (59%) were caused by Gram-positive organisms. Gram-negatives and *Candida* spp. represented 36% and 6%, respectively (Figure 10). A greater number of LOS cases caused by *S. epidermidis* occurred in the penicillin compared with the ampicillin group (2.7 *vs* 7.6 per 1000 patients days, RR 0.32; 95% CI 0.19–0.55). However, in multivariate model adjusted for other risk factors of infection the difference between the two treatments became non-significant (p = 0.08).

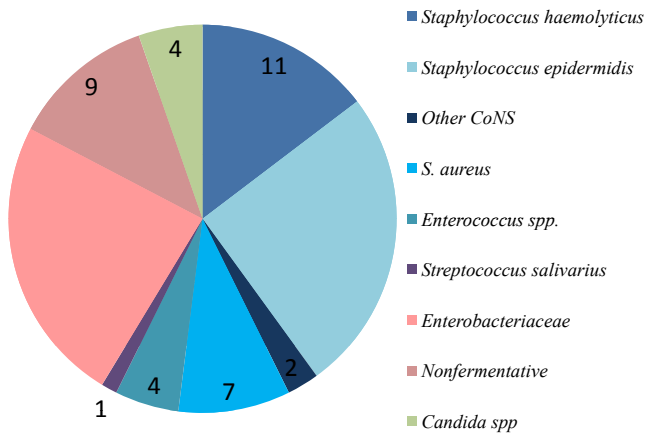

Figure 10. Distribution of causative agents of LOS. Numbers in the chart indicate the cases. Gram-negative sepsis occurred at a median on Day 13 (IQR 9 – 21); *E. cloacae* sepsis was the earliest (median on Day 8; IQR 5 – 14) and *K. oxytoca* the latest (median on Day 30; IQR 22.5 – 39). Sepsis caused by CoNS occurred at a median on Day 8 (IQR 5 – 13), by *S. aureus* on Day 7 (IQR 5 – 10.5), and by *Candida* spp on Day 21 (IQR 13.75 – 49.5). Further only Gram-negative organisms are analysed and discussed.

5.6.2. Relatedness of colonising and invasive strains

In 22 of 27 Gram-negative LOS cases mucosal and invasive strains were phenotypically similar and in 16 of those 22 colonisation with the respective strain preceded invasive disease. The median duration of prior colonisation with a matching strain was 10.5 days (range 2 to 36) for *Enterobacteriacae* and seven days (range 2 to 29) for nonfermentative microorganisms. Of 16 patients colonised with the phenotypically similar microorganism prior to LOS nine were colonised with several GN strains (number ranging from 2 to 6). All invasive infections were monoinfections. Patients with mucosal colonisation, except with *E. cloacae* and *Serratia* spp., were more likely to develop invasive infection than those without (Table 11).

Table 11. Association between mucosal colonisation by a specific microorganism and LOS caused by the same organism

	Total N of pt colonised	N of pt with LOS	N of pt colonised at any time and with LOS	OR / 95% CI *	N of pt colonised prior to LOS	OR / 95% CI **
E. cloacae	76	5	3	4.11 / 0.55–35.96	1	0.66 / 0.28–6.15
K. pneumonia	51	6	5	24.57 / 2.69–587.93	4	9.57 / 1.45–77.74
E. coli	49	3	3	34.55 / 2.03–20474.48	3	34.55 / 2.03–20474.48
Acinetobacter spp	47	7	5	13.63 / 2.41–105.29	2	2.01 / 0.26–12.20
K. oxytoca	46	3	3	37.41 / 2.20–22188.59	3	37.41 / 2.20–22188.59
Serratia spp	31	1	1	24.34 / 0.81–16921.25	1	24.34 / 0.81–16921.25
Stenotrophomonas spp	12	1	1	69.52 / 2.19–49681.68	1	69.52 / 2.18–49681.68
P. aeruginosa	7	1	1	125.31 / 3.69–92781.90	1	125.31 / 3.69–92781.90

* chance to be infected in patients who had colonisation by particular microbe during hospital stay *vs* noncolonised neonates

** chance to be infected in patients who had colonisation by particular microbe prior LOS

The genetic relatedness between mucosal and colonising strains was studied for *K. pneumoniae* (4 patients), *E. cloacae* (3 patients) and *A. baumannii* (5 patients); all studied sample pairs were genetically related (Figure 8). This affirms

the findings of previous studies suggesting that the large intestine and NP are important reservoirs for *Enterobacteriaceae* and other Gram-negative bacilli causing invasive disease (Foca *et al.*, 2000; Cartelle *et al.*, 2004; Gupta *et al.*, 2004; Graham *et al.*, 2007).

5.6.3. Value of mucosal site cultures in predicting invasive disease

Two analyses – per sample and per patient – were conducted. The results of per sample analysis are presented in Table 2 in publication II. As shown the overall sensitivity (37% *vs* 11%) and PPV (3.5% *vs* 0.8%) in per sample analyses were greater for *Enterobacteriaceae* compared to nonfermentative organisms, respectively and at Days 2–6 compared to Day 7–10 and 11–14 prior to invasive disease (sensitivity: 2–6 Days 50% *vs* 16%; 7–10 Days 26% *vs* 7%, 11–14 Days 31% *vs* 8%, and PPV: 2–6 Days 24% *vs* 1%, 7–10 Days 3% *vs* 0.5% and 11–14 Days 3.5% *vs* 1%, respectively).

In the per patient analyses overall sensitivity, specificity, PPV and NPV of 59%, 42%, 10% and 91%, respectively were greater than the ones in an individual sample analysis. The by species and per samples analysis indicated the best sensitivity (above 60%) in *K. oxytoca*, *E. coli* and *P. aeruginosa* (Table 12). Still, most likely due to the small sample size, the 95% CI intervals varied widely although the specificity exceeded 92% for all organisms. In addition, high NPV (above 98% for all species) enabling to identify patients with low risk of LOS, was observed.

Our results resemble the findings reported in adult ICUs indicating that if colonised patients and specific organisms instead of a single sample and all organisms are considered, the sensitivity and specificity of mucosal cultures in predicting invasive disease is acceptable and might aid in guiding selection of empiric antimicrobial treatment. At least as important should be considered the prevention of the spread of difficult to manage or multidrug resistant organisms such as *P. aeruginosa* (Bertrand *et al.*, 2001; Blot *et al.*, 2005; Smith *et al.*, 2010). However, it is important to emphasize that during NICU stay (at least in this study) most patients are colonised with several microorganisms and this method does not allow discriminating which colonising strain would become invasive.

Table 12. Sensitivity, specificity, positive and negative predictive value of mucosal-invasive sample pairs and per patient calculation by individual species. Both mucosal sampling sites and all time points (within 2–14 days prior to invasive sample) are included

Microbes / per samples	Sensitivity		Specificity		PPV		NPV	
	%	95% CI	%	95% CI	%	95% CI	%	95% CI
K. oxytoca	75.0	43.3–93.3	92.8	92.5–93.0	10.3	6.0–12.9	99.7	99.3–99.9
P. aeruginosa	66.7	12.7–98.2	99.0	98.8–99.1	15.4	2.9–22.7	99.9	99.8–100
E. coli	62.5	26.2–89.7	94.8	94.5–95.0	8.1	3.4–11.6	99.7	99.4–99.9
K. pneumonia	35.7	14.1–63.8	94.0	93.8–94.4	7.1	2.8–12.8	99.1	98.9–99.5
Serratia spp	33.3	1.8–87.3	98.6	98.5–98.8	6.3	0.3–16.4	99.8	99.7–100
Stenotrophomonas spp	33.3	1.8–87.3	97.9	97.8–98.0	4.2	0.2–10.9	99.8	99.7–100
E. cloacae	7.7	0.4–37.2	93.2	93.1–93.5	1.3	0.1–6.5	98.8	98.7–99.2
Acinetobacter spp	4.8	0.2–25.1	93.8	93.7–94.2	1.5	0.1–7.7	98.1	98.0–98.5
Microbes / per patient								
K. oxytoca	100	31.0–100	84.4	79.4–88.3	6.5	1.7–18.9	100	98.0–100
P. aeruginosa	100	5.5–100	97.8	95.1–99.1	14.3	0.7–58.0	100	98.3–100
E. coli	100	31.0–100	83.3	78.2–87.4	6.1	1.6–17.9	100	97.9–100
Serratia spp	100	5.4–100	89.2	84.2–92.5	3.2	0.2–18.5	100	98.1–100
Stenotrophomonas spp	100	5.4–100	96.0	92.8–97.9	8.3	0.4–40.2	100	98.2–100
K. pneumonia	66.7	24.1–94.0	82.7	77.6–86.9	7.8	2.5–19.7	99.1	96.5–99.8
Acinetobacter spp	28.6	51.1–69.7	83.4	78.3–87.5	4.3	0.7–15.7	97.8	94.7–99.2
E. cloacae	20	1.0–70.1	72.5	66.8–77.7	1.3	0.07–8.1	98.0	94.7–99.4

In multivariate mixed model analysis we demonstrated that greater number of true positive samples prior to invasive disease and lower PNA improve the accuracy of prediction – OR 4.66; 95% CI 2.28–9.55; $p<0.0001$ and OR 0.94; 95% CI 0.88–1.00; $p=0.047$, respectively.

Our results suggest that in high risk patients admitted to 3rd level NICU the value of surveillance cultures in predicting invasive disease is suboptimal. Fairly similar data have been reported in general NICU patients with a sensitivity of 16% and 56%, specificity of 38% and 82%, and PPV of 5% and 7.5% shown by Choi *et al* (2008) and Evans *et al*. (1998), respectively. Similar to these authors we also showed that the predictive value of surveillance cultures collected shortly prior to invasive disease is better (2–6 days in our study) than those taken at more distant times (Evans, 1988; Choi *et al.*, 2008).

Still, the predictive value of mucosal cultures was especially poor in infections caused by nonfermentative organisms and more specifically in cases of *A. baumannii* (Table 12). Although here the ratio of infection to colonisation was 2:7 (Table 11) in most cases invasive disease preceded mucosal colonisation. *A. baumannii* is a known hospital acquired microorganism with which outbreaks are not uncommon (Zarrilli *et al.*, 2007; Curtis, 2008; Shete *et al.*, 2009). The most compromising group are patients with prior risk factors; the fact that mucosal colonisation does not precede infection suggest that infection develops immediately after transmission. Thus, in *A. baumannii* infection surveillance cultures are of little value in selecting appropriate antibacterial agents but may be relevant in recognising potential cross-colonisation and avoiding further spread of infection.

6. GENERAL DISCUSSION

To our best knowledge this is the first prospective study comparing the effect of two widely used antibiotic regimens – penicillin and ampicillin, both combined with gentamicin – on initial mucosal colonisation in neonates with suspected EOS. The role of other potentially interfering factors was also explored.

6.1. Complexities of conducting studies on mucosal colonisation in neonates

One of the most essential issues when studying mucosal colonisation is the selection of laboratory methods. Variations in methodology also partly explain the different results obtained in previously conducted studies.

6.1.1. Selection of laboratory methods

The laboratory work in colonisation studies is labour consuming and needs a significant amount of coordination between laboratories. In this study several methods were used in order to simplify laboratory work with minimal loss in quality. First, the samples were processed in one laboratory in batches that required their transport and freezing for a maximum of two weeks at –20° C. We appreciate that freezing may influence survival of microorganisms but according to our pilot data the rate of potentially lost organisms for the storage period used, did not exceed 14%. In the circumstances of limited human and laboratory resources the processing of swabs in batches once a week was considered a reasonable alternative to simplify laboratory work.

Second, when measuring antibacterial susceptibility for the resource saving reasons we used screening for resistant organisms by plating samples on the culture media with antibiotic concentration one dilution lower than CLSI resistance breakpoint. Again we may have lost some organisms with borderline resistance but if that happened it should have been seen in both study arms and the number of such organisms was most likely not very high.

Third, antibiotic susceptibility testing were performed according to the CSLI criteria in which *Enterobacteriaceae* are considered resistant if the MIC was >32 mg/L. More recently the EUCAST criteria with the resistance breakpoint of 8 for *Enterobacteracae* were established. This may change interpretation and increase rates of antibiotic resistance in the future. Although comparative studies on the topic are still limited.

Fourth, only classic culture based techniques were employed as at the time of initiating these studies they were the only readily available methods. However, we appreciate that in recent decade various molecular methods like RT-PCR, pyrosequencing, microarray chip technology, FISH etc, have emerged and have revealed the presence of uncultivable microorganisms like *Akkermansia*

muciniphila and *Ruminococcus obeum* and others in human GIT microbiome (Derrien *et al.*, 2004; Zoetendal *et al.*, 2002). We believe that with molecular methods becoming more affordable to researches (which is already happening), future studies looking into the development of neonatal microbiota should either be complimented by or relay entirely on the PCR or sequence based methods.

6.1.2. Statistical approach

When studying complex issues like the development of mucosal colonisation and factors that influence it the selection of most appropriate statistical methods is of utmost importance. Numerous previous studies on the topic have drawn their conclusions by using simple statistical approach comparing different categorical values to each other although those factors are interrelated (Baltimore *et al.*, 1989; Pierro *et al.*, 1998; Toltzis *et al.*, 2001; Bennet *et al.*, 2002; Hallstrom *et al.*, 2004; Jaureguy *et al.*, 2004; Mendiratta *et al.*, 2006). Even if multivariate analysis has been employed the studies have looked at only one or two microorganisms or included a small number of risk factors in the analysis (Cartelle *et al.*, 2004; Cassettari *et al.*, 2009; Penders *et al.*, 2006; Saiman *et al.*, 2001). Therefore it is not surprising that conflicting results on influence of antibiotics on mucosal colonisation have been reported.

For example, in studies monitoring antibiotics as a single factor (Burman *et al.*, 1993), or monitoring multiple factors but not applying multivariate analysis (De Man *et al.*, 2000; Toltzis *et al.*, 2001; Duman *et al.*, 2005) an association between antibiotic therapy and resistance occurrence has been reported. On the other hand, in studies applying multivariate analysis, this association has not been confirmed (Cartelle *et al.*, 2004; Mammina *et al.*, 2007; Millar *et al.*, 2008). Similarly, in a study by Schrag *et al* (2006a) univariate analysis found a significant association between the use of antibiotic prophylaxis and infection caused by AR *E. coli;* but this association was not confirmed in multivariate analysis adjusted for other risk factors. Analogous results were shown by Hufnagel *et al* (2007), who compared the results of Chi-square test and multivariate logistic regression in monitoring the association between prepartum antibiotic treatment and neonatal colonisation by multidrug-resistant enterococci. These data affirm the understanding that colonisation in neonates is influenced by multiple factors and this fact needs to be taken into account.

We believe that one of the strengths of our study is the complex statistical approach. In order to evaluate the influence of different antibiotic regimens on mucosal colonisation by aerobic and facultatively anaerobic microorganisms during NICU stay we included a total of more than 2300 Gram-negative and almost 1200 Gram-positive isolates and evaluated a total of 22 factors that have been described to influence mucosal colonisation in previous studies (Fanaro *et al.*, 2003; Westerbeek *et al.*, 2006; Thompson-Chagoyan *et al.*, 2007). Due to the cluster-randomised study design all analyses were first adjusted for study centre and treatment period and multivariate approach avoiding inclusion of

highly interrelated factors was employed in all analyses. Thus, our conclusion that differences between penicillin and ampicillin containing regimens do not involve *Enterobacteriaceae* but affect colonisation with Gram-positive microbes adequately reflects the true differences between the studied antibiotic regimens. Furthermore, we believe that given the wide variety of potential risk factors entered into our mixed model analysis, a comprehensive description of their interplay has been achieved. However, one should still appreciate that these conclusions are valid for the population under study and may not be relevant to all neonates admitted to NICU.

6.2. What are the most important factors influencing mucosal colonisation by opportunistic microorganisms in neonates?

The following independent colonisation risk factors were identified: participating unit, duration of NICU stay, GA, feeding regimen, mode of delivery, PROM >18 h and use of indwelling catheters. Our results suggest that in neonates admitted to NICU right after birth the predominant microbiota is acquired from the hospital environment, although host characteristics (like GA) also play a role. In contrast, healthy neonates spending only few days in the hospital become colonised predominantly by *E. coli* and *Candida albicans*, microorganisms known to be acquired from the mother (Saiman *et al.*, 2001; Eggesbo *et al.*, 2010; Mendiratta *et al.*, 2006).

With some exceptions, such as nonfermentative organisms more commonly colonising NP mucosa and rectal cultures more frequently yielding *Enterobacteriaceae* or feeding habits affecting colonisation of gut but not NP, we found that the colonisation pattern and the interfering risk factors for gut and NP are quite similar for a given bacterial species. This suggests that when performing colonisation studies extrapolations from one site to the other are feasible and in the interest of saving resources only one site (most likely gut as more abundant site of microbial colonisation) could be studied (Goldmann, 1981; Ogra, 2010).

The NICUs environment as a source of colonisation is not surprising since opportunistic microorganisms *K. pneumoniae, E. cloacae, Serratia* spp., *Acinetobacter* spp., *P. aeruginosa*, and MRSA are known to circulate in hospitals and cross-colonise via contaminated equipment and/or hands of medical personnel (Hira *et al.*, 2007; Mcdonald *et al.*, 2007; Srivastava *et al.*, 2007). As in our study the patients were admitted to NICU during the first hours after birth, and first days of PNA are likely critical for the development of GI tract microbiota, it is important to avoid as possible the colonisation by microbes got from the hospital (Srivastava *et al.*, 2007; Mshvildadze *et al.*, 2010a).

In line with others we showed that gut and NP mucosa are potential sources of invasive disease; patients colonised with *Klebsiella* spp., *Serratia* spp.,

Stenotrophomonas spp., *Pseudomonas* spp., and *E. coli* had greater odds of developing invasive disease as compared to those without colonisation. In order to avoid colonisation with opportunistic organisms with greater pathogenic potential (e.g. MRSA, *Klebsiella* spp., *E. coli*, *E. cloacae*, *Pseudomonas* spp., *Candida* spp.) specific measures should be targeted in preventing cross-colonisation and spread of these microorganisms. However, while environmental factors like nutrition habits and hygienic conditions in NICU are easier to change, little can be done to host related ones (e.g. GA and BW).

Although the duration of NICU stay is felt to be an immutable factor, early discharge can be achievable for a number of infants by avoiding nosocomial infections, a known risk factor prolonging hospital stay (Merritt *et al.*, 2003; Hira *et al.*, 2007). Furthermore longer hospital stay is associated with higher likelihood of colonisation by resistant microbes (Toltzis *et al.*; 2001Mammina *et al.*, 2007). The findings, that the duration of NICU stay was the most common factor influencing mucosal colonisation and outbreaks of *K. pneumoniae* and MRSA were unit-dependent, definitely support the recommendations that hygiene oriented interventions may avoid or reduce colonisation by potentially pathogenic microorganisms and could eventually result in diminished numbers of invasive disease (Saiman, 2002; Schelonka *et al.*, 2006; Srivastava *et al.*, 2007).

Another important factor in colonisation process, though hard to prevent, is too low GA. Colonisation with microorganisms that are also the main causative agents of LOS in neonates, like *E. coli, K. pneumoniae, E. cloacae* and CoNS (Stoll *et al.*, 2002b; Bizzarro *et al.*, 2005; Vergnano *et al.*, 2010), was influenced by GA. Reasons why GA plays a crucial role in mucosal colonisation are multiple. It has been speculated that preterms are separated from the mother for prolonged period allowing colonisation by environmental rather than by maternal microflora (Schwiertz *et al.*, 2003; Sanders *et al.*, 2010). Also preterm neonates have higher exposure to other risk factors such as prolonged hospital stay, indwelling catheters, and prolonged use of antibiotics (Stoll *et al.*, 2002b; Stoll *et al.*, 2003; Curtis *et al.*, 2008). So, to avoid colonisation by bacteria circulating in NICU maternal-infant contact could be reinforced, however, this may not be feasible in critically ill ventilated neonates. One should also bear in mind that mothers, especially those hospitalized during pregnancy may carry more virulent or antibiotic resistant strains (Toltzis 2001).

The fact that the route of feeding as well as the character of enteral feeds are essential for early gut but not NP colonisation is not surprising. We like others have shown that preterm infants receiving TPN have delayed colonisation with almost all microorganisms but especially with Gram-negative bacilli (Smith *et al.*, 2010). However, the differences between breast milk and formula feeding were minor, including only greater colonisation risk by Gram-negative AR strains by Day 10–12 among breast milk compared with formula fed babies. Compared to those, receiving TPN, breast milk fed babies were more likely colonised by *E. coli* and *K. oxytoca,* likely of maternal origin. However, in

feeding process possible contamination of milk bank or equipment used for enteral feeding should be considered. Outbreaks, caused by *Enterobacteriaceae* (including *K. oxytoca* and *E. coli*) or *Pseudomonas aeruginosa* (Donowitz *et al.*, 1981; Berthelot *et al.*, 2001; Gras-Le Guen *et al.*, 2003; Hurrell *et al.*, 2009; Sanchez-Carrillo *et al.*, 2009) transmitted via contaminated feeding equipment, have been described. Whether avoidance of enteral feeding will affect the rates of invasive disease is less clear. Although the protective role of early human milk feeding in avoiding episodes of neonatal sepsis in preterm infants has been demonstrated in large studies (El-Mohandes *et al.*, 1997; Hylander *et al.*, 1998; Ronnestad *et al.*, 2005a; Neu, 2007) the results of the review of nine studies from various countries indicated that the advantage of breast milk over formula is not conclusively proven (De Silva *et al.*, 2004). Drawing any firm conclusions of the issue remains also beyond the scope of the current study.

The finding that route of delivery influenced colonisation by opportunistic microorganisms was expected. Our results confirm that colonisation by *E. coli* and *C. albicans* is mostly of maternal origin as vaginally delivered neonates are more likely colonised than those born via Caesarean section. On one hand, vaginal delivery is the normal route of birth, but on the other hand colonisation by *C. albicans* could lead to invasive infections. Especially poor outcome of these infections in extremely premature babies have been shown (Stoll *et al.*, 2002b; Pappas *et al.*, 2009), although vaginal delivery in this group is not frequent. However, we suggest that prevention of *Candida* infection should include multiple measures and not be limited to fluconazole prophylaxis alone in extremely preterm babies. So, colonisation monitoring during pregnancy is essential.

Even more serious problems may be associated with intraamniotic bacterial infections related to PROM that may cause preterm birth followed by a serious threat of immediate postnatal infections. We showed an association between PROM and rectal colonisation with *K. pneumoniae* but not with other microorganisms. More importantly PROM was the premier predictor for colonisation by almost all AR resistant *Enterobacteriaceae,* studied, suggesting that when EOS or LOS develops in a neonate born to a mother with PROM, empiric antibiotic treatment should also cover AR organisms.

6.3. Do antibiotics promote colonisation by potentially pathogenic members of *Enterobacteriaceae* and *Candida* spp. or ampicillin-resistant strains?

Previous studies have shown that broad spectrum antibiotics (e.g. ampicillin, cephalosporins) when used in the empiric treatment of EOS lead to increased rates of colonisation by potentially pathogenic members of *Enterobacteriaceae* and *Candida* spp. and will select for antibiotic resistant strains (Wong-Beringer *et al.*, 2002; Bonnemaison *et al.*, 2003; Duman *et al.*, 2005; Glynn *et al.*, 2005).

We showed that ampicillin as a broader spectrum antibiotic than penicillin influenced gut colonisation, but not in the way we hypothesized when planning the study. In terms of Gram-negative microorganisms and *Candida* spp. the number of colonised patients in both treatment arms was similar but greater CDs of *K. pneumoniae,* AR *Serratia* spp. and *Candida* spp. were seen in the ampicillin and that of AR *Acinetobacter* spp. in the penicillin arm.

The issue whether short courses of antibiotics induce colonisation by resistant *Enterobacteriaceae* or other resistant organisms has been recently questioned (Karami *et al.*, 2006; Millar *et al.*, 2008; Hall *et al.*, 2011). Likely colonisation by resistant strains is rather accompanied by long-term antibiotic strategies in a specific unit, as usually resistant strains are isolated from numerous patients on antibacterials and they become part of the flora of the unit (Baltimore, 1998). It is likely that the selection of resistant strains is a lengthy process not observed in studies of short duration or using antibiotics for a limited number of days as in ours (Jaureguy *et al.*, 2004; Millar *et al.*, 2008; Auriti *et al.*, 2009; Puopolo *et al.*, 2010). At present in most countries all enterobacterial species other than *E. coli* have become uniformly resistant to ampicillin and the resistance of *E. coli* is also increasing (Cordero *et al.*, 2004; Kayange *et al.*, 2010). Still, one should bear in mind that our study was conducted in a country with low antibiotic resistance (Lõivukene *et al.*, 2006) including low prevalence of ESBL producing microorganisms in the NICU setting (approximately 2%) (Mitt *et al.*, 2009). Future studies should identify whether our data are applicable to countries with higher resistance rates, as a recent study from Brazil found that 87% of patients colonised with ESBL producing *K. pneumoniae* had received previously a penicillin containing regimen, whereas the rate among non-colonised patients was 29%; the respective results for ampicillin receiving patients were 13% and 9%. Unfortunately the study was too small to be conclusive (Cassettari *et al.*, 2009).

We found only limited associations between mucosal colonisation and the use of additional broad spectrum antibiotics or its duration in multivariate analyses, although associations between the administration of carbapenems, betalactamases with β-lactamase inhibitors, and III and IV generation cephalosporins and rectal colonisation by nonalbicans *Candida*, and administration of cephalosporins and betalactamases with β-lactamase inhibitors and NP colonisation mostly by both susceptible and resistant strains of *K. oxytoca* were observed in univariate analysis. Of note the influence of other antibiotics on gut colonisation remained beyond the primary scope of this study and the finding could be biased by small numbers of patients receiving each individual antibiotic.

Another controversial issue was mucosal colonisation by *Candida* spp. Earlier studies have shown that broad spectrum antibiotics including ampicillin but not penicillin outselect *Candida* spp. which then could lead to invasive disease, especially in ELBW infants (Bennet *et al.*, 2002; Bonnemaison *et al.*, 2003). We found similar numbers of patients colonised with *Candida* spp. in

both treatment arms but ampicillin treated patients had greater CD than those receiving penicillin indicating that when colonisation occurs its duration after ampicillin treatment is longer than after the use of penicillin. Still, the association between antibiotic treatment and *Candida* colonisation/infection remains a controversial issue as demonstrated in some though not in all studies (Bonnemaison *et al.*, 2003; Clark *et al.*, 2004; Donskey, 2004; Brecht *et al.*, 2009). Nosocomial spread and cross colonisation especially in ELBW babies cannot be excluded.

Although we did not demonstrate significant differences in AR Gram-negative or *Candida* colonisation between the two empiric antibiotic regimens one should bear in mind that the relatively short duration of the study (each regimen lasted for 8 months) may have been too limited to trigger changes in the circulating microflora, especially as the selection of resistant strains is likely a lengthy process. We hypothesize that not antibacterial therapy itself but poor hospital hygiene will enable the circulation and transmission of multiresistant strains which then require the use of broad spectrum antibacterial agents and result in longer NICU stay and in turn greater potential for colonisation by resistant organisms. Early colonisation by resistant strains seen in about 40% of subjects in this study is likely another proof of cross colonisation that can be avoided by improving hospital hygiene rather than changing antibacterial guidelines.

6.4. Which empiric antibiotic regimen should be preferred in terms of mucosal colonisation?

The most significant argument in the decision whether to prefer ampicillin or penicillin is based on preferring either enterococci and *S. aureus* or *S. haemolyticus* and *S. hominis*. Considering translocation and subsequent BSI, CoNS infection is often indolent rather than fulminant and the mortality rate is low, although fatalities have been reported. In a recent study conducted in England and Wales (Depani *et al.*, 2011), staphylococcal infections, primarily CoNS, were more commonly noted among deaths in extremely preterm neonates. Still, the mortality rates of BSI caused by *S. aureus* remain much higher, although not as high as caused by Gram-negative bacilli (Tarnow-Mordi *et al.*, 2010).

The fact that ampicillin is associated with greater colonisation by enterococci than penicillin has been shown previously (Miedema *et al.*, 2000; Bennet *et al.*, 2002). The role of this difference, however, is less clear. Enterococci are rarely associated with EOS or LOS (1 – 16% of LOS in previous studies and 1.4% in our study) (Cordero *et al.*, 2004; Bizzarro *et al.*, 2005; Ronnestad *et al.*, 2005a; Vergnano *et al.*, 2010). More interestingly recent in vitro data indicate that some strains of *E. faecalis* could suppress the proliferation of intestinal pathogens and thus have a potential to prevent infection and induction of

inflammation (Wang *et al.*, 2008) suggesting potential benefit rather than harm of enterococcal colonisation.

A more serious problem is larger colonisation by *S. aureus,* a well-known agent of HAIs with a potential of inducing allergies (Lundell *et al.*, 2007). However, *S. aureus* infections in NICU are not very common (Depani *et al.*, 2011). In this study they were as infrequent as enterococcal infections (1.4%). Still, clonal transmission of MRSA has become an increasing problem in NICUs around the world (Shiojima *et al.*, 2003; Singh *et al.*, 2003; Regev-Yochay *et al.*, 2005; Mcdonald *et al.*, 2007; Gregory *et al.*, 2009; Heinrich *et al.*, 2011). Although during this study an epidemic of MRSA infection occurred in one but not in the other unit, the association between penicillin and greater colonisation by *S. aureus* remained significant after adjusting for participating unit and study period. Whether ampicillin treatment has an advantage over penicillin in preventing colonisation by MRSA needs further studies.

The shift in colonisation by CoNS so that ampicillin treatment was associated with greater colonisation of *S. haemolyticus* and *S. hominis* than penicillin is more difficult to explain and to our knowledge has not been described before. It is likely that different species of staphylococci compete for the colonisation niche but we are not aware of any studies on the subject. The relevance of differences in gut colonisation observed in this study is further supported by the trend towards higher prevalence of LOS caused by *S. haemolyticus* in the ampicillin compared with penicillin arm (7 *vs* 4). Differences in the virulence between CoNS species have been demonstrated with 71% of *S. epidermidis* but only 35% of *S. haemolyticus* and 26% of *S. hominis* strains being capable of slime production, the latter being associated with invasive disease (Drozenova *et al.*, 2000; Arslan *et al.*, 2007; Koksal *et al.*, 2009).

Thus, the interference with the initial mucosal colonisation should not be a limiting factor in choosing between ampicillin and penicillin for the empiric treatment of EOS although one should bear in mind that some clinically less important differences between these two agents occurred. We suggest that the selection should be made mainly according to the local distribution of EOS causing microorganisms and their antibiotic susceptibility. The equal effectiveness and safety of two these antibiotic regimens was previously published by Metsvaht *et al* (2010). Anyway, ampicillin for extremely preterms is more recommended, as in this group higher mortality rates in penicillin arm occurred.

6.5. When if at all are surveillance cultures useful?

In this study, conducted in neonates at high risk of health care associated infections we demonstrated that prior mucosal colonisation with Gram-negative microorganisms except *E. cloacae*, *Serratia* spp and *Acinetobacter* spp. increases the risk for invasive disease. Thus it will be tempting to use the results of mucosal cultures in guiding the selection of empiric antibiotic therapy and

due to extra pain and distress avoid taking blood cultures in critically as much as possible. Surveillance cultures, in turn, are labour intensive and require significant amount of laboratory resources.

In concordance with other studies (Evans, 1988; Choi *et al.*, 2008) we showed that the predictive value of surveillance cultures in general is sub-optimal – the sensitivity on sample level was 27% and on patient level 59%; the specificity being only slightly better, 66% and 42%, respectively. Furthermore, a study in adult ICU has shown that regular screening sample collection has also a downside as therapy guided by these may result in overuse of antibiotics with consequent increase in costs, antibiotic resistance and probably superinfections (Warren *et al.*, 2005).

Colleting rectal or NP swabs may have some value in cohorting colonised patients (Friedman *et al.*, 2008) or selecting appropriate antibacterial therapy during outbreaks of hospital acquired infections. Monitoring of alert micro-organisms and reinforcing infection control measures like isolation of colonised patients may help to interrupt horizontal spread and avoid outbreaks of hospital acquired infections (Bertrand *et al.*, 2001; Regev-Yochay *et al.*, 2005; Mammina *et al.*, 2007; Mcdonald *et al.*, 2007; Anderson *et al.*, 2008; Heinrich *et al.*, 2011).

The issue which patients should be isolated and cohorted is less clear. In principle, isolation needs should be determined by the mode of transmission and by the pathogen involved. Newborns are nonmobile and often cared for in incubators, so there is no direct contact between patients. Under these circumstances, for contact transmission, involved in most nosocomial Gram-negative and –positive pathogens as well as *Candida* spp., care measures focusing on prevention of vector-related (i.e. contaminated hand or equipment) transmission have likely the greatest potential. Some previous studies have questioned the clinical and economical value of surveillance cultures as part of such strategies. For example, to prevent the spread of MRSA, Gregory *et al* (2009) cultured samples from nares and rectum weekly over 7 years and cohorted colonised or infected patients. As a results MRSA in the NICU was not permanently eradicated although $ 1 500 000 was spent. One potential explanation for this failure was the inability to locate and eliminate the source of MRSA. So, to ensure success, a more complex approach is probably needed. Also, no evidence has been found to either support or refute the use of patient isolation measures in neonates with *Candida* colonisation or infection (Mohan *et al.*, 2007).

Thus mucosal surfaces are an important source of infection but routine and non-targeted surveillance cultures are not efficient in predicting LOS in NICU patients. However, mucosal sampling targeted for specific organisms (e.g *Klebsiella* spp., *E. coli, Stenotrophomonas* and *Pseudomonas*) may offer an opportunity to improve infection control measures and selection of empiric antibiotic regimen.

6.6. Limitations of the study

Several limitations which we believe did not significantly interfere with our conclusions should be noted. First, involvement of two units only (although these cover the entire 3rd level NICU population in Estonia) did not allow accounting for the variability potentially occurring in multicentre settings. Second, not all admitted patients but only those require empiric antibiotic treatment with ampicillin or penicillin plus gentamicin were recruited, excluding 40% of NICU admissions. Third, the short duration of the study and each antibiotic regimen did not allow addressing long term changes in circulating microbiota. Fourth, the lack of antibiotic treatment free control group made it impossible to assess the influence of any antibacterial treatment on development of gut microbiota; although one should mention that antibiotic naive group in third level NICU is almost non-existent (Lass *et al.*, 2011). Fifth, environmental cultures as well as maternal colonisation were not evaluated.

In addition, in both arms 35% neonates received additional broad spectrum antibiotics which could interfere with gut colonisation process and may have affected our conclusions on the impact of penicillin and ampicillin on mucosal colonisation. Still, our population closely mirrored the one admitted to NICU where the majority of infected neonates receive more than one antibiotic regimen during their stay. Exclusion of these patients from the analysis would have introduced a major population bias with the sickest babies at highest risk of adverse colonisation being left out. We believe that the use of other antibiotics did not influence the overall conclusions as in both treatment arms their frequency as well as the type of drugs used, was similar. Also, risk factor adjusted multivariate analysis allowing correction for possible confounders, including broad spectrum antibiotics, was applied.

6.7. Future research

In this study we focused on the collection of potentially pathogenic opportunistic organisms and did not look at the non-pathogenic bacteria like *Lactobacilli, Bifidobacteria* and *Bacteroides*. However, we appreciate their enormous role in mucosal colonisation that may be interfered by all risk factors identified in this study. Therefore, further detailed studies are needed. Also, quantitative cultures which may reveal additional information, when development of invasive disease is concerned, were not performed. The stool samples from ELBW babies were collected and are analysed separately by using qRT-PCR (Drell *et al.*, 2009).

The results of herein study showed that neonates acquire opportunistic microbes frequently from the hospital environment but collection of cultures from environment or hospital staff was beyond the scope of our study. A better understanding of the circulation of microorganisms in the NICU may offer the possibility to improve infection control measures.

As in our study one antibiotic regimen was given only over a maximum of 8 months, this period may have been too short to trigger changes in the circulating microbiota. Long term effects of various antibiotics should be addressed in future surveillance studies. Also further research should clarify our understanding of the clinical relevance of differences in early gut colonisation by various CoNS species and enterococci. Better knowledge about the sources of CoNS BSI is of ultimate importance, as it remains the most frequently encountered HAI in preterm neonates, associated with significant cost in both human and material resources.

Several studies but not all provide compelling evidence that prophylactic administration of certain *Lactobacillus* and *Bifidobacterium* probiotics reduces LOS (Dani *et al.*, 2002; Bin-Nun *et al.*, 2005; Lin *et al.*, 2005). Thus, operating with probiotics in infant nutrition can only be justified once we have developed a detailed understanding of their action, not only short but also in the longer term (Salminen *et al.*, 2006; Kelly *et al.*, 2007; Sanders *et al.*, 2010).

We also performed an analysis to identify risk factors of invasive disease. However, most likely due to relatively small numbers of Gram-negative infections in this study (n = 27) the 95% CI of associated risk factors were extremely wide. Larger studies should be conducted to identify risk factors of invasive disease caused by opportunistic microorganisms.

7. CONCLUSIONS

1. The first colonisers in neonates admitted to NICU with risk factors of EOS were Gram-positive bacteria, among which CoNS clearly predominated throughout the study. By the end of first week more than 90% of patients were colonised with Gram-positive and about 30% with Gram-negative AR micro-organisms. Colonisation by Gram-negatives in NP and rectal area were similar, but *Enterobacteriaceae* except *Serratia* spp. were more frequently colonising rectum than NP but *Acinetobacter* spp. were more commonly recovered in the NP. The most frequent Gram-negative coloniser including AR strains was *E. cloacae* followed by *K. pneumoniae, K. oxytoca* and *Acinetobacter* spp. The frequent opportunistic colonisers were also frequent causative agents of LOS.

2. The mucosal colonisation by *E. coli, K. oxytoca* and *C. albicans* is mainly influenced by maternal and early perinatal factors, while *K. pneumoniae, E. cloacae,* MRSA and *non-albicans Candida* spp. are predominantly affected by the hospital environment and prematurity. Risk factors (e.g. duration of NICU stay, unit, GA, route of delivery) influencing NP and rectal colonisation including AR strains are similar and species-specific and are closely inter-related making extrapolations from one site to the other feasible.

3. In patients with risk factors for EOS the impact of ampicillin (a broader spectrum antibacterial agent compared with penicillin) on mucosal colonisation with Gram-negative microorganisms including AR strains may have been over-estimated. The main differences between these two agents are as follows:

a. Patients receiving ampicillin containing regimen are less frequently colonised with *S. aureus* and *Enterococcus* spp. but more often colonised with *S. haemolyticus* and *S. hominis* than those treated with penicillin.
b. Patients receiving ampicillin have greater CDs of *K. pneumoniae,* AR *Serratia* spp. and *Candida* spp. but lower CDs of AR *Acinetobacter* spp. than those receiving penicillin.
c. The colonisation of *Candida* spp. and all other Gram-negatives are similar in both groups.

Thus, the avoidance of unwanted initial gut colonisation should not be a limiting factor in choosing between ampicillin and penicillin for the empiric treatment of EOS. We suggest that the selection should be made mainly according to the local distribution of EOS causing microorganisms and their antibiotic susceptibility. Host-related factors like the degree of prematurity should also be considered.

4. Prior mucosal colonisation with Gram-negative organisms other than *E. cloacae*, *Serratia* spp., *Acinetobacter* spp. and MRSA may lead to invasive disease. However, the sensitivity, specificity and PPVs of mucosal surveillance

samples in predicting invasive disease is moderate for *Enterobacteriaceae* and suboptimal for non-fermentative micro-organisms.

5. Routine and non-targeted surveillance cultures are not efficient in predicting LOS in NICU as they are of low diagnostic accuracy, expensive and time consuming. Targeted for specific organisms (e.g MRSA, *Klebsiella* spp.), surveillance cultures may prove to be useful especially during outbreaks of nosocomial infections. They may offer an opportunity to improve infection control measures, to cohort patients and to select the most appropriate empiric antibiotic regimen.

8. REFERENCES

Adam, B., Baillie, G. S., and Douglas, L. J. (2002). Mixed species biofilms of Candida albicans and Staphylococcus epidermidis. *J Med Microbiol* **51**(4), 344–349.

Adlerberth, I., Carlsson, B., de Man, P., Jalil, F., Khan, S. R., Larsson, P., Mellander, L., Svanborg, C., Wold, A. E., and Hanson, L. A. (1991). Intestinal colonization with Enterobacteriaceae in Pakistani and Swedish hospital-delivered infants. *Acta Paediatr Scand* **80**(6–7), 602–610.

Adlerberth, I., Lindberg, E., Aberg, N., Hesselmar, B., Saalman, R., Strannegard, I. L., and Wold, A. E. (2006). Reduced enterobacterial and increased staphylococcal colonization of the infantile bowel: an effect of hygienic lifestyle? *Pediatr Res* **59**(1), 96–101.

Adlerberth, I., and Wold, A. E. (2009). Establishment of the gut microbiota in Western infants. *Acta Paediatr* **98**(2), 229–238.

Agnihotri, N., Kaistha, N., and Gupta, V. (2004). Antimicrobial susceptibility of isolates from neonatal septicemia. *Jpn J Infect Dis* **57**(6), 273–275.

Almuneef, M. A., Baltimore, R. S., Farrel, P. A., Reagan-Cirincione, P., and Dembry, L. M. (2001). Molecular typing demonstrating transmission of gram-negative rods in a neonatal intensive care unit in the absence of a recognized epidemic. *Clin Infect Dis* **32**(2), 220–227.

Amaya, E., Caceres, M., Fanc, H., Torres Ramirez, A., Palmgren, A. C., Nord, C. E., and Weintraub, A. (2010). Antibiotic resistance patterns in gram-negative and gram-positive bacteria causing septicemia in newborns in Leon, Nicaragua: correlation with environmental samples. *J Chemother* **22**(1), 25–29.

Ambroise, M. (2009). Emerging Trends in Antibiotic Use in Neonates: New or Not-so-New Drugs for New Bugs. *Newborn & Infant Nursing Reviews* **9**, 48–52.

Amyes, S. G., Tait, S., Thomson, C. J., Payne, D. J., Nandivada, L. S., Jesudason, M. V., Mukundan, U. D., and Young, H. K. (1992). The incidence of antibiotic resistance in aerobic faecal flora in south India. *J Antimicrob Chemother* **29**(4), 415–425.

Anderson, B., Nicholas, S., Sprague, B., Campos, J., Short, B., and Singh, N. (2008). Molecular and descriptive epidemiologyof multidrug-resistant Enterobacteriaceae in hospitalized infants. *Infect Control Hosp Epidemiol* **29**(3), 250–255.

Aniansson, G., Alm, B., Andersson, B., Larsson, P., Nylen, O., Peterson, H., Rigner, P., Svanborg, M., and Svanborg, C. (1992). Nasopharyngeal colonization during the first year of life. *J Infect Dis* **165 Suppl 1**, S38–42.

Apostolopoulou, E., Lambridou, M., and Lambadaridis, I. (2004). Nosocomial bloodstream infections in a neonatal intensive care unit. *Br J Nurs* **13**(13), 806–812.

Arlet, G., Rouveau, M., Casin, I., Bouvet, P. J., Lagrange, P. H., and Philippon, A. (1994). Molecular epidemiology of Klebsiella pneumoniae strains that produce SHV-4 beta-lactamase and which were isolated in 14 French hospitals. *J Clin Microbiol* **32**(10), 2553–2558.

ARPAC (2009). PFGE Typing Protocol Recommended by ARPAC for Acinetobacter baumannii.

Arslan, S., and Ozkardes, F. (2007). Slime production and antibiotic susceptibility in staphylococci isolated from clinical samples. *Mem Inst Oswaldo Cruz* **102**(1), 29–33.

Asghart, M., Samaee, H., Hossein, P. M., and Amir, V. A. (2010). Neonatal Late-Onset Sepsis in NICU: Analysis of Causative Organisms and Antimicrobial Susceptibility

Ali Asghar Children Hospital from (2004/5–2007/5), Teheran, Iran. *Research Journal of Biological Sciences* **5**(5), 376–379.

Asindi, A. A., Archibong, E. I., and Mannan, N. B. (2002). Mother-infant colonization and neonatal sepsis in prelabor rupture of membranes. *Saudi Med J* **23**(10), 1270–1274.

Askin, D. F., and Diehl-Jones, W. L. (2005). Improving on Perfection: Breast milk and Breast-milk Additives for Preterm Neonates. *Newborn & Infant Nursing Reviews* **5**(1), 10–18.

Auriti, C., Rava, L., di Ciommo, V., Ronchetti, M., and Orzalesi, M. (2009). Short antibiotic prophylaxis for bacterial infections in a neonatal intensive care unit: a randomized controlled trial. *The Journal of hospital infection* **59**(4), 292–298.

Ayan, M., Kuzucu, C., Durmaz, R., Aktas, E., and Cizmeci, Z. (2003). Analysis of three outbreaks due to Klebsiella species in a neonatal intensive care unit. *Infect Control Hosp Epidemiol* **24**(7), 495–500.

Baba, H., Nimmo, G. R., Allworth, A. M., Boots, R. J., Hayashi, Y., Lipman, J., and Paterson, D. L. (2011). The role of surveillance cultures in the prediction of susceptibility patterns of Gram-negative bacilli in the intensive care unit. *Eur J Clin Microbiol Infect Dis* **30**(6), 739–744.

Babazono, A., Kitajima, H., Nishimaki, S., Nakamura, T., Shiga, S., Hayakawa, M., Tanaka, T., Sato, K., Nakayama, H., Ibara, S., Une, H., and Doi, H. (2008). Risk factors for nosocomial infection in the neonatal intensive care unit by the Japanese Nosocomial Infection Surveillance (JANIS). *Acta Med Okayama* **62**(4), 261–268.

Bagattini, M., Crivaro, V., Di Popolo, A., Gentile, F., Scarcella, A., Triassi, M., Villari, P., and Zarrilli, R. (2006). Molecular epidemiology of extended-spectrum beta-lactamase-producing Klebsiella pneumoniae in a neonatal intensive care unit. *J Antimicrob Chemother* **57**(5), 979–982.

Baltimore, R. S., Duncan, R. L., Shapiro, E. D., and Edberg, S. C. (1989). Epidemiology of pharyngeal colonization of infants with aerobic gram-negative rod bacteria. *J Clin Microbiol* **27**(1), 91–95.

Baltimore, R. S. (1998). Neonatal nosocomial infections. *Semin Perinatol* **22**(1), 25–32.

Beggs, C. B., Kerr, K. G., Snelling, A. M., and Sleigh, P. A. (2006). Acinetobacter spp. and the Clinical Environment. *Indoor and Built Environment* **15**(1), 19–24.

Behari, P., Englund, J., Alcasid, G., Garcia-Houchins, S., and Weber, S. G. (2004). Transmission of methicillin-resistant Staphylococcus aureus to preterm infants through breast milk. *Infect Control Hosp Epidemiol* **25**(9), 778–780.

Ben, X. M. (2008). Nutritional management of newborn infants: practical guidelines. *World J Gastroenterol* **14**(40), 6133–6139.

Bendel, C. M. (2003). Colonization and epithelial adhesion in the pathogenesis of neonatal candidiasis. *Semin Perinatol* **27**(5), 357–364.

Bennet, R., Eriksson, M., Nord, C. E., and Zetterstrom, R. (1982). Suppression of aerobic and anaerobic faecal flora in newborns receiving parenteral gentamicin and ampicillin. *Acta Paediatr Scand* **71**(4), 559–562.

Bennet, R., Eriksson, M., Nord, C. E., and Zetterstrom, R. (1986). Fecal bacterial microflora of newborn infants during intensive care management and treatment with five antibiotic regimens. *Pediatr Infect Dis* **5**(5), 533–539.

Bennet, R., Eriksson, M., and Nord, C. E. (2002). The fecal microflora of 1–3-month-old infants during treatment with eight oral antibiotics. *Infection* **30**(3), 158–160.

Berthelot, P., Grattard, F., Patural, H., Ros, A., Jelassi-Saoudin, H., Pozzetto, B., Teyssier, G., and Lucht, F. (2001). Nosocomial colonization of premature babies with

Klebsiella oxytoca: probable role of enteral feeding procedure in transmission and control of the outbreak with the use of gloves. *Infect Control Hosp Epidemiol* **22**(3), 148–151.

Bertrand, X., Thouverez, M., Patry, C., Balvay, P., and Talon, D. (2001). Pseudomonas aeruginosa: antibiotic susceptibility and genotypic characterization of strains isolated in the intensive care unit. *Clin Microbiol Infect* **7**(12), 706–708.

Betremieux, P., Donnio, P. Y., and Pladys, P. (1995). Use of ribotyping to investigate tracheal colonisation by Staphylococcus epidermidis as a source of bacteremia in ventilated newborns. *Eur J Clin Microbiol Infect Dis* **14**(4), 342–346.

Bettelheim, K. A., Breadon, A., Faiers, M. C., O'Farrell, S. M., and Shooter, R. A. (1974a). The origin of O serotypes of Escherichia coli in babies after normal delivery. *J Hyg (Lond)* **72**(1), 67–70.

Bettelheim, K. A., Teoh-Chan, C. H., Chandler, M. E., O'Farrell, S. M., Rahamim, L., Shaw, E. J., and Shooter, R. A. (1974b). Spread of Escherichia coli colonizing newborn babies and their mothers. *J Hyg (Lond)* **73**(3), 383–387.

Bettelheim, K. A., Teoh-Chan, C. H., Chandler, M. E., O'Farrell, S. M., Rahamin, L., Shaw, E. J., and Shooter, R. A. (1974c). Further studies of Escherichia coli in babies after normal delivery. *J Hyg (Lond)* **73**(2), 277–285.

Bettelheim, K. A., and Lennox-King, S. M. (1976). The acquisition of Escherichia coli by new-born babies. *Infection* **4**(3), 174–179.

Biasucci, G., Benenati, B., Morelli, L., Bessi, E., and Boehm, G. (2008). Cesarean delivery may affect the early biodiversity of intestinal bacteria. *J Nutr* **138**(9), 1796S-1800S.

Biasucci, G., Rubini, M., Riboni, S., Morelli, L., Bessi, E., and Retetangos, C. (2010). Mode of delivery affects the bacterial community in the newborn gut. *Early Hum Dev*.

Bin-Nun, A., Bromiker, R., Wilschanski, M., Kaplan, M., Rudensky, B., Caplan, M., and Hammerman, C. (2005). Oral probiotics prevent necrotizing enterocolitis in very low birth weight neonates. *J Pediatr* **147**(2), 192–196.

Bizzarro, M. J., Raskind, C., Baltimore, R. S., and Gallagher, P. G. (2005). Seventy-five years of neonatal sepsis at Yale: 1928–2003. *Pediatrics* **116**(3), 595–602.

Bizzarro, M. J., Dembry, L. M., Baltimore, R. S., and Gallagher, P. G. (2008). Changing patterns in neonatal Escherichia coli sepsis and ampicillin resistance in the era of intrapartum antibiotic prophylaxis. *Pediatrics* **121**(4), 689–696.

Björksten, B., Naaber, P., Sepp, E., and Mikelsaar, M. (1999). The intestinal microflora in allergic Estonian and Swedish 2-year-old children. *Clin Exp Allergy* **29**(3), 342–346.

Björksten, B. (2001). The gastrointestinal flora and the skin – Is there a link? *Pediatr Allergy Immunol* **12 Suppl 14**, 51–55.

Bjorksten, B. (2006). The gut microbiota: a complex ecosystem. *Clin Exp Allergy* **36**(10), 1215–1217.

Blakey, J. L., Lubitz, L., Barnes, G. L., Bishop, R. F., Campbell, N. T., and Gillam, G. L. (1982). Development of gut colonisation in pre-term neonates. *J Med Microbiol* **15**(4), 519–529.

Blot, S., Depuydt, P., Vogelaers, D., Decruyenaere, J., De Waele, J., Hoste, E., Peleman, R., Claeys, G., Verschraegen, G., Colardyn, F., and Vandewoude, K. (2005). Colonization status and appropriate antibiotic therapy for nosocomial bacteremia caused by antibiotic-resistant gram-negative bacteria in an intensive care unit. *Infect Control Hosp Epidemiol* **26**(6), 575–579.

Bonnemaison, E., Lanotte, P., Cantagrel, S., Thionois, S., Quentin, R., Chamboux, C., and Laugier, J. (2003). Comparison of fecal flora following administration of two antibiotic protocols for suspected maternofetal infection. *Biol Neonate* **84**(4), 304–310.

Borghesi, A., and Stronati, M. (2008). Strategies for the prevention of hospital-acquired infections in the neonatal intensive care unit. *J Hosp Infect* **68**(4), 293–300.

Boyce, J. M., Opal, S. M., Potter-Bynoe, G., and Medeiros, A. A. (1993). Spread of methicillin-resistant Staphylococcus aureus in a hospital after exposure to a health care worker with chronic sinusitis. *Clin Infect Dis* **17**(3), 496–504.

Brady, M. T. (2005). Health care-associated infections in the neonatal intensive care unit. *Am J Infect Control* **33**(5), 268–275.

Brakstad, O. G., Aasbakk, K., and Maeland, J. A. (1992). Detection of Staphylococcus aureus by polymerase chain reaction amplification of the nuc gene. *J Clin Microbiol* **30**(7), 1654–1660.

Brecht, M., Clerihew, L., and McGuire, W. (2009). Prevention and treatment of invasive fungal infection in very low birthweight infants. *Arch Dis Child Fetal Neonatal Ed* **94**(1), F65–69.

Burman, L. G., Berglund, B., Huovinen, P., and Tullus, K. (1993). Effect of ampicillin versus cefuroxime on the emergence of beta-lactam resistance in faecal Enterobacter cloacae isolates from neonates. *J Antimicrob Chemother* **31**(1), 111–116.

Caicedo, R. A., Schanler, R. J., Li, N., and Neu, J. (2005). The developing intestinal ecosystem: implications for the neonate. *Pediatr Res* **58**(4), 625–628.

Cappuccino, J., and Sherman, N. (2000). "Microbiology. A Laboratory Manual." Sixth edition ed. Normal Microbial Flora of the Throat and Skin. Benjamin Cummings, San Francsisco, Boston, New York, Capetown, Hong Kong, London, Madrid, Mexico City, Montreal, Munich, Paris, Singapore, Sidney, Tokio, Toronto.

Cardozo, D. M., Nascimento-Carvalho, C. M., Souza, F. R., and Silva, N. M. (2006). Nasopharyngeal colonization and penicillin resistance among pneumococcal strains: a worldwide 2004 update. *Braz J Infect Dis* **10**(4), 293–304.

Cartelle, M., del Mar Tomas, M., Pertega, S., Beceiro, A., Dominguez, M. A., Velasco, D., Molina, F., Villanueva, R., and Bou, G. (2004). Risk factors for colonization and infection in a hospital outbreak caused by a strain of Klebsiella pneumoniae with reduced susceptibility to expanded-spectrum cephalosporins. *J Clin Microbiol* **42**(9), 4242–4249.

Cassettari, V. C., da Silveira, I. R., Dropa, M., Lincopan, N., Mamizuka, E. M., Matte, M. H., Matte, G. R., and Menezes, P. R. (2009). Risk factors for colonisation of newborn infants during an outbreak of extended-spectrum beta-lactamase-producing Klebsiella pneumoniae in an intermediate-risk neonatal unit. *J Hosp Infect* **71**(4), 340–347.

CDC (2002). Guidlines for the Preventention of Intravascular Catheter-Related Infections (D. o. H. a. H. Services, Ed.), Vol. 51, pp. 1–33.

Chambers, H. F. (1997). Methicillin resistance in staphylococci: molecular and biochemical basis and clinical implications. *Clin Microbiol Rev* **10**(4), 781–791.

Chiu, S., Huang, Y. C., Lien, R. I., Chou, Y. H., and Lin, T. Y. (2005). Clinical features of nosocomial infections by extended-spectrum beta-lactamase-producing Enterobacteriaceae in neonatal intensive care units. *Acta Paediatr* **94**(11), 1644–1649.

Choi, Y., Saha, S. K., Ahmed, A. S., Law, P. A., Chowdhury, M. A., Islam, M., and Darmstadt, G. L. (2008). Routine skin cultures in predicting sepsis pathogens among hospitalized preterm neonates in Bangladesh. *Neonatology* **94**(2), 123–131.

Clark, R., Powers, R., White, R., Bloom, B., Sanchez, P., and Benjamin, D. K., Jr. (2004). Nosocomial infection in the NICU: a medical complication or unavoidable problem? *J Perinatol* **24**(6), 382–388.

CLSI (2005). Performance Standards for Antimicrobial Susceptibility Testing; Fifteenth Informational Supplement. This document provides updated tables for the CLSI/ NCCLS antimicrobial susceptibility testing standards M2-A8 and M7-A6.

Cohen-Wolkowiez, M., Moran, C., Benjamin, D. K., Cotten, C. M., Clark, R. H., Benjamin, D. K., Jr., and Smith, P. B. (2009). Early and late onset sepsis in late preterm infants. *Pediatr Infect Dis J* **28**(12), 1052–1056.

Cohen, B., Saiman, L., Cimiotti, J., and Larson, E. (2003). Factors associated with hand hygiene practices in two neonatal intensive care units. *Pediatr Infect Dis J* **22**(6), 494–499.

Cooper, B. S., Stone, S. P., Kibbler, C. C., Cookson, B. D., Roberts, J. A., Medley, G. F., Duckworth, G., Lai, R., and Ebrahim, S. (2004). Isolation measures in the hospital management of methicillin resistant Staphylococcus aureus (MRSA): systematic review of the literature. *Bmj* **329**(7465), 533.

Cordero, L., Rau, R., Taylor, D., and Ayers, L. W. (2004). Enteric gram-negative bacilli bloodstream infections: 17 years' experience in a neonatal intensive care unit. *Am J Infect Control* **32**(4), 189–195.

Costa, S. F., Miceli, M. H., and Anaissie, E. J. (2004). Mucosa or skin as source of coagulase-negative staphylococcal bacteraemia? *Lancet Infect Dis* **4**(5), 278–286.

Cotten, C. M., Taylor, S., Stoll, B., Goldberg, R. N., Hansen, N. I., Sanchez, P. J., Ambalavanan, N., and Benjamin, D. K., Jr. (2009). Prolonged duration of initial empirical antibiotic treatment is associated with increased rates of necrotizing enterocolitis and death for extremely low birth weight infants. *Pediatrics* **123**(1), 58–66.

Crivaro, V., Bagattini, M., Salza, M. F., Raimondi, F., Rossano, F., Triassi, M., and Zarrilli, R. (2007). Risk factors for extended-spectrum beta-lactamase-producing Serratia marcescens and Klebsiella pneumoniae acquisition in a neonatal intensive care unit. *J Hosp Infect* **67**(2), 135–141.

Crivaro, V., Di Popolo, A., Caprio, A., Lambiase, A., Di Resta, M., Borriello, T., Scarcella, A., Triassi, M., and Zarrilli, R. (2009). Pseudomonas aeruginosa in a neonatal intensive care unit: molecular epidemiology and infection control measures. *BMC Infect Dis* **9**, 70.

Curtis, C., and Shetty, N. (2008). Recent trends and prevention of infection in the neonatal intensive care unit. *Curr Opin Infect Dis* **21**(4), 350–356.

Curtis, L. (2008). Prevention of hospital-acquired infections: review of non-pharmacological interventions. *Journal of Hospital Infection* **69**, 204–219.

D'Angio, C. T., McGowan, K. L., Baumgart, S., St Geme, J., and Harris, M. C. (1989). Surface colonization with coagulase-negative staphylococci in premature neonates. *J Pediatr* **114**(6), 1029–1034.

Dalben, M., Varkulja, G., Basso, M., Krebs, V. L., Gibelli, M. A., van der Heijden, I., Rossi, F., Duboc, G., Levin, A. S., and Costa, S. F. (2008). Investigation of an outbreak of Enterobacter cloacae in a neonatal unit and review of the literature. *J Hosp Infect* **70**(1), 7–14.

Daley, A. J., and Isaacs, D. (2004). Ten-year study on the effect of intrapartum antibiotic prophylaxis on early onset group B streptococcal and Escherichia coli neonatal sepsis in Australasia. *Pediatr Infect Dis J* **23**(7), 630–634.

Dani, C., Biadaioli, R., Bertini, G., Martelli, E., and Rubaltelli, F. F. (2002). Probiotics feeding in prevention of urinary tract infection, bacterial sepsis and necrotizing enterocolitis in preterm infants. A prospective double-blind study. *Biol Neonate* **82**(2), 103–108.

Davies, M. W., Mehr, S., Garland, S. T., and Morley, C. J. (2000). Bacterial colonization of toys in neonatal intensive care cots. *Pediatrics* **106**(2), E18.

de Hoog, M., Mouton, J. W., and van den Anker, J. N. (2005). New dosing strategies for antibacterial agents in the neonate. *Semin Fetal Neonatal Med* **10**(2), 185–94.

de La Cochetiere, M., Rouge, C., Darmaun, D., Roze, J., Potel, G., and Leguen, C. (2007). Intestinal Microbiota in Neonates and Preterm Infants: A Review. *Current Pediatric Reviews* **3**, 21–34.

de Man, P., Verhoeven, B. A., Verbrugh, H. A., Vos, M. C., and van den Anker, J. N. (2000). An antibiotic policy to prevent emergence of resistant bacilli. *Lancet* **355**(9208), 973–978.

de Man, P., van Der Veeke, E., Leemreijze, M., van Leeuwen, W., Vos, G., van Den Anker, J., Verbrugh, H., and van Belkum, A. (2001). Enterobacter species in a pediatric hospital: horizontal transfer or selection in individual patients? *J Infect Dis* **184**(2), 211–214.

de Silva, A., Jones, P. W., and Spencer, S. A. (2004). Does human milk reduce infection rates in preterm infants? A systematic review. *Arch Dis Child Fetal Neonatal Ed* **89**(6), F509–13.

Depani, S. J., Ladhani, S., Heath, P. T., Lamagni, T. L., Johnson, A. P., Pebody, R. G., Ramsay, M. E., and Sharland, M. (2011). The contribution of infections to neonatal deaths in England and Wales. *Pediatr Infect Dis J* **30**(4), 345–347.

Derrien, M., Adawi, D., Ahrne, S., Jeppsson, B., Molin, G., Osman, N., Stsepetova, J., Vaughan, E. E., de Vos, W. M., and Ouwehand, A. C. (2004). The intestinal mucosa as habit of the gut microbiota and a rational target for probiotic functionality and safety. *Microbial Ecology in Health and Disease*.

Dickinson, T. M., and Archer, G. L. (2000). Phenotypic expression of oxacillin resistance in Staphylococcus epidermidis: roles of mecA transcriptional regulation and resistant-subpopulation selection. *Antimicrob Agents Chemother* **44**(6), 1616–1623.

Dominguez-Bello, M. G., Costello, E. K., Contreras, M., Magris, M., Hidalgo, G., Fierer, N., and Knight, R. (2010). Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns. *Proc Natl Acad Sci U S A* **107**(26), 11971–11975.

Donowitz, L. G., Marsik, F. J., Fisher, K. A., and Wenzel, R. P. (1981). Contaminated breast milk: A source of Klebsiella bacteremia in a newborn intensive care unit. *Rev Infect Dis* **3**(4), 716–720.

Donskey, C. J. (2004). The role of the intestinal tract as a reservoir and source for transmission of nosocomial pathogens. *Clin Infect Dis* **39**(2), 219–226.

Drell, T., Stsepetova, J., Lutsar, I., Metsvaht, T., Parm, Ü., Julge, K., Ilmoja, M.-L., Jürisson, M., and Sepp, E. (2009). *Well-known infections-the hottest features of diagnostics and treatment: VIII Nordic-Baltic Congress on infectious diseases., Saint Petersburg, Russia.*

Drozenova, J., and Petras, P. (2000). Characteristics of coagulase-negative staphylococci isolated from hemocultures. *Epidemiol Mikrobiol Imunol* **49**(2), 51–58.

Duman, M., Abacioglu, H., Karaman, M., Duman, N., and Ozkan, H. (2005). Beta-lactam antibiotic resistance in aerobic commensal fecal flora of newborns. *Pediatr Int* **47**(3), 267–273.

Eckhardt, C., Halvosa, J. S., Ray, S. M., and Blumberg, H. M. (2003). Transmission of methicillin-resistant Staphylococcus aureus in the neonatal intensive care unit from a patient with community-acquired disease. *Infect Control Hosp Epidemiol* **24**(6), 460–461.

Edwards, R. K., Clark, P., Sistrom, C. L., and Duff, P. (2002). Intrapartum antibiotic prophylaxis 1: relative effects of recommended antibiotics on gram-negative pathogens. *Obstet Gynecol* **100**(3), 534–539.

Eggesbo, M., Moen, B., Peddada, S., Baird, D., Rugtveit, J., Midtvedt, T., Bushel, P. R., Sekelja, M., and Rudi, K. (2010). Development of gut microbiota in infants not exposed to medical interventions. *Apmis* **119**(1), 17–35.

el-Mohandes, A. E., Picard, M. B., Simmens, S. J., and Keiser, J. F. (1997). Use of human milk in the intensive care nursery decreases the incidence of nosocomial sepsis. *J Perinatol* **17**(2), 130–134.

Elwan, A., and Zarouk, W. (2009). Diagnosis of Neonatal Bacterial Sepsis by Polymerase Chain Reaction. *Journal of Biological Sciences* **6**, 533–540.

Enck, P., Zimmermann, K., Rusch, K., Schwiertz, A., Klosterhalfen, S., and Frick, J. S. (2009). The effects of maturation on the colonic microflora in infancy and childhood. *Gastroenterol Res Pract* **2009**, 752401.

Evans, M. S., W.;Federspiel, C; Cotton, RB; McKee, KT; Stratton, CW (1988). Sensitivity, Specificity, and Predictive Value of Body Surface Cultures in a Neonatal Intensive Care Unit. *JAMA* **259**, 248–252.

Fanaro, S., Chierici, R., Guerrini, P., and Vigi, V. (2003). Intestinal microflora in early infancy: composition and development. *Acta Paediatr Suppl* **91**(441), 48–55.

Feja, K. N., Wu, F., Roberts, K., Loughrey, M., Nesin, M., Larson, E., Della-Latta, P., Haas, J., Cimiotti, J., and Saiman, L. (2005). Risk factors for candidemia in critically ill infants: a matched case-control study. *J Pediatr* **147**(2), 156–161.

Foca, M., Jakob, K., Whittier, S., Della Latta, P., Factor, S., Rubenstein, D., and Saiman, L. (2000). Endemic Pseudomonas aeruginosa infection in a neonatal intensive care unit. *N Engl J Med* **343**(10), 695–700.

Forno, E., Onderdonk, A. B., McCracken, J., Litonjua, A. A., Laskey, D., Delaney, M. L., Dubois, A. M., Gold, D. R., Ryan, L. M., Weiss, S. T., and Celedon, J. C. (2008). Diversity of the gut microbiota and eczema in early life. *Clin Mol Allergy* **6**, 11.

Fox, M., Molesky, M., Van Aerde, J. E., and Muttitt, S. (1999). Changing parenteral nutrition administration sets every 24 h versus every 48 h in newborn infants. *Can J Gastroenterol* **13**(2), 147–151.

Frebourg, N. B., Cauliez, B., and Lemeland, J. F. (1999). Evidence for nasal carriage of methicillin-resistant staphylococci colonizing intravascular devices. *J Clin Microbiol* **37**(4), 1182–1185.

Friedman, N. D., Kotsanas, D., Brett, J., Billah, B., and Korman, T. M. (2008). Investigation of an outbreak of Serratia marcescens in a neonatal unit via a case-control study and molecular typing. *Am J Infect Control* **36**(1), 22–28.

Fryklund, B., Tullus, K., Berglund, B., and Burman, L. G. (1992). Importance of the environment and the faecal flora of infants, nursing staff and parents as sources of gram-negative bacteria colonizing newborns in three neonatal wards. *Infection* **20**(5), 253–257.

Fujimura, S., Kato, S., Hashimoto, M., Takeda, H., Maki, F., and Watanabe, A. (2004). Survey of methicillin-resistant Staphylococcus aureus from neonates and the environment in the NICU. *J Infect Chemother* **10**(2), 131–132.

Garcia-Rodriguez, J. A., and Fresnadillo Martinez, M. J. (2002). Dynamics of nasopharyngeal colonization by potential respiratory pathogens. *J Antimicrob Chemother* **50 Suppl S2**, 59–73.

Gewolb, I. H., Schwalbe, R. S., Taciak, V. L., Harrison, T. S., and Panigrahi, P. (1999). Stool microflora in extremely low birthweight infants. *Arch Dis Child Fetal Neonatal Ed* **80**(3), F167–173.

Gheibi, S., Fakoor, Z., aramyyar, M., Khashabi, J., Ilkhanizadeh, B., Asghari-Sana, F., Mahmoodzadeh, H., and Majlesi, A. (2008). Coagulase Negative Staphylococcus; the Most Common Cause of Neonatal Septicemia in Urmia, Iran. *Iran J Pediatr* **18**(3), 237–243.

Glynn, C., and Azadian, B. (2005). Empiric antimicrobial therapy for severe sepsis in the intensive care unit: In early, hit hard, out early. *Current Anaesthesia & Critical Care* **16**(Critical care: antibiotics and ICU), 221–230.

Goldmann, D. A. (1981). Bacterial Colonization and Infection in the Neonate. *The American Journal of Medicine* **70**, 417–422.

Goldstein, B., Giroir, B., and Randolph, A. (2005). International pediatric sepsis consensus conference: definitions for sepsis and organ dysfunction in pediatrics. *Pediatr Crit Care Med* **6**(1), 2–8.

Graham, P. L., 3rd, Della-Latta, P., Wu, F., Zhou, J., and Saiman, L. (2007). The gastrointestinal tract serves as the reservoir for Gram-negative pathogens in very low birth weight infants. *Pediatr Infect Dis J* **26**(12), 1153–1156.

Gras-Le Guen, C., Lepelletier, D., Debillon, T., Gournay, V., Espaze, E., and Roze, J. C. (2003). Contamination of a milk bank pasteuriser causing a Pseudomonas aeruginosa outbreak in a neonatal intensive care unit. *Arch Dis Child Fetal Neonatal Ed* **88**(5), F434–435.

Gregory, M. L., Eichenwald, E. C., and Puopolo, K. M. (2009). Seven-year experience with a surveillance program to reduce methicillin-resistant Staphylococcus aureus colonization in a neonatal intensive care unit. *Pediatrics* **123**(5), e790–796.

Gupta, A., Della-Latta, P., Todd, B., San Gabriel, P., Haas, J., Wu, F., Rubenstein, D., and Saiman, L. (2004). Outbreak of extended-spectrum beta-lactamase-producing Klebsiella pneumoniae in a neonatal intensive care unit linked to artificial nails. *Infect Control Hosp Epidemiol* **25**(3), 210–215.

Haanpera, M., Forssten, S. D., Huovinen, P., and Jalava, J. (2008). Typing of SHV extended-spectrum beta-lactamases by pyrosequencing in Klebsiella pneumoniae strains with chromosomal SHV beta-lactamase. *Antimicrob Agents Chemother* **52**(7), 2632–2635.

Haenel, H., and Bendig, J. (1975). Intestinal flora in health and disease. *Prog Food Nutr Sci* **1**(1), 21–64.

Hakalehto, E. (2006). Semmelweis'present day follow-up: Updating bacterial sampling and enrichment in clinical hygiene. *Pathophysiology* **13**, 257–267.

Hall, M. A., Wain, S., Pallett, A., and Faust, S. N. (2011). Empirical antibiotics for suspected early neonatal sepsis. *Arch Dis Child Fetal Neonatal Ed*.

Hall, S. L., Riddell, S. W., Barnes, W. G., Meng, L., and Hall, R. T. (1990). Evaluation of coagulase-negative staphylococcal isolates from serial nasopharyngeal cultures of premature infants. *Diagn Microbiol Infect Dis* **13**(1), 17–23.

Hallström, M., Eerola, E., Vuento, R., Janas, M., and Tammela, O. (2004). Effects of mode of delivery and necrotising enterocolitis on the intestinal microflora in preterm infants. *Eur J Clin Microbiol Infect Dis* **23**(6), 463–470.

Harris, H., Wirtschafter, D., and Cassady, G. (1976). Endotracheal intubation and its relationship to bacterial colonization and systemic infection of newborn infants. *Pediatrics* **58**(6), 816–823.

Hawkey, P. M. (2008). The growing burden of antimicrobial resistance. *J Antimicrob Chemother* **62 Suppl 1**, i1–9.

Heikkilä, M. P., and Saris, P. E. (2003). Inhibition of Staphylococcus aureus by the commensal bacteria of human milk. *J Appl Microbiol* **95**(3), 471–478.

Heinrich, N., Mueller, A., Bartmann, P., Simon, A., Bierbaum, G., and Engelhart, S. (2011). Successful management of an MRSA outbreak in a neonatal intensive care unit. *Eur J Clin Microbiol Infect Dis* **30**(7), 909–913.

Hessle, C., Andersson, B., and Wold, A. E. (2000). Gram-positive bacteria are potent inducers of monocytic interleukin-12 (IL-12) while gram-negative bacteria preferentially stimulate IL-10 production. *Infect Immun* **68**(6), 3581–3586.

Hira, V., Sluijter, M., Estevao, S., Horst-Kreft, D., Ott, A., de Groot, R., Hermans, P. W., and Kornelisse, R. F. (2007). Clinical and molecular epidemiologic characteristics of coagulase-negative staphylococcal bloodstream infections in intensive care neonates. *Pediatr Infect Dis J* **26**(7), 607–612.

Huang, M. B., Gay, T. E., Baker, C. N., Banerjee, S. N., and Tenover, F. C. (1993). Two percent sodium chloride is required for susceptibility testing of staphylococci with oxacillin when using agar-based dilution methods. *J Clin Microbiol* **31**(10), 2683–2688.

Huang, Y. C., Li, C. C., Lin, T. Y., Lien, R. I., Chou, Y. H., Wu, J. L., and Hsueh, C. (1998a). Association of fungal colonization and invasive disease in very low birth weight infants. *Pediatr Infect Dis J* **17**(9), 819–822.

Huang, Y. C., Lin, T. Y., Peng, H. L., Wu, J. H., Chang, H. Y., and Leu, H. S. (1998b). Outbreak of Candida albicans fungaemia in a neonatal intensive care unit. *Scand J Infect Dis* **30**(2), 137–142.

Huang, Y. C., Su, L. H., Wu, T. L., Liu, C. E., Young, T. G., Chen, P. Y., Hseuh, P. R., and Lin, T. Y. (2004). Molecular epidemiology of clinical isolates of methicillin-resistant Staphylococcus aureus in Taiwan. *J Clin Microbiol* **42**(1), 307–310.

Huang, Y. C., Chou, Y. H., Su, L. H., Lien, R. I., and Lin, T. Y. (2006a). Methicillin-resistant Staphylococcus aureus colonization and its association with infection among infants hospitalized in neonatal intensive care units. *Pediatrics* **118**(2), 469–474.

Huang, Y. C., Wang, Y. H., Su, L. H., Chou, Y. H., Lien, R. I., and Lin, T. Y. (2006b). Determining the significance of coagulase-negative staphylococci identified in cultures of paired blood specimens from neonates by species identification and strain clonality. *Infect Control Hosp Epidemiol* **27**(1), 70–73.

Hufnagel, M., Liese, C., Loescher, C., Kunze, M., Proempeler, H., Berner, R., and Krueger, M. (2007). Enterococcal colonization of infants in a neonatal intensive care unit: associated predictors, risk factors and seasonal patterns. *BMC Infect Dis* **7**, 107.

Hurrell, E., Kucerova, E., Loughlin, M., Caubilla-Barron, J., Hilton, A., Armstrong, R., Smith, C., Grant, J., Shoo, S., and Forsythe, S. (2009). Neonatal enteral feeding tubes as loci for colonisation by members of the Enterobacteriaceae. *BMC Infect Dis* **9**, 146.

Huurre, A., Kalliomaki, M., Rautava, S., Rinne, M., Salminen, S., and Isolauri, E. (2008). Mode of delivery – effects on gut microbiota and humoral immunity. *Neonatology* **93**(4), 236–240.

Hylander, M. A., Strobino, D. M., and Dhanireddy, R. (1998). Human milk feedings and infection among very low birth weight infants. *Pediatrics* **102**(3), E38.

Ibrahim, E. H., Sherman, G., Ward, S., Fraser, V. J., and Kollef, M. H. (2000). The influence of inadequate antimicrobial treatment of bloodstream infections on patient outcomes in the ICU setting. *Chest* **118**(1), 146–155.

Inglis, G. D., Jardine, L. A., and Davies, M. W. (2007). Prophylactic antibiotics to reduce morbidity and mortality in ventilated newborn infants. *Cochrane Database Syst Rev*(3), CD004338.

Jain, A., Roy, I., Gupta, M. K., Kumar, M., and Agarwal, S. K. (2003). Prevalence of extended-spectrum beta-lactamase-producing Gram-negative bacteria in septicaemic neonates in a tertiary care hospital. *J Med Microbiol* **52**(Pt 5), 421–425.

Jarvis, W. R. (2010). Prevention and control of methicillin-resistant Staphylococcus aureus: dealing with reality, resistance, and resistance to reality. *Clin Infect Dis* **50**(2), 218–220.

Jaureguy, F., Carton, M., Panel, P., Foucaud, P., Butel, M. J., and Doucet-Populaire, F. (2004). Effects of intrapartum penicillin prophylaxis on intestinal bacterial colonization in infants. *J Clin Microbiol* **42**(11), 5184–5188.

Jones, B. L., Gorman, L. J., Simpson, J., Curran, E. T., McNamee, S., Lucas, C., Michie, J., Platt, D. J., and Thakker, B. (2000). An outbreak of Serratia marcescens in two neonatal intensive care units. *J Hosp Infect* **46**(4), 314–319.

Jorgensen, J. H., and Sahm, D. F. (2005). Antimicrobial Susceptibility Testing: General Considerations. Sixth edition ed. *In* "Manual of Clinical Microbiology" (P. R. Murray, E. J. Baron, M. A. Pfaller, F. C. Tenover, and R. H. Yolken, Eds.), Vol. 1, pp. 1277–1280. ASM PRESS Washington, D.C. .

Joseph, T. A., Pyati, S. P., and Jacobs, N. (1998). Neonatal early-onset Escherichia coli disease. The effect of intrapartum ampicillin. *Arch Pediatr Adolesc Med* **152**(1), 35–40.

Joung, T., Ed. (2008). Neofax: Manual of Drugs Used in Neonatal Care.: USA: Phisican's Desk Reference Inc.

Kaleida, P. H., Nativio, D. G., Chao, H. P., and Cowden, S. N. (1993). Prevalence of bacterial respiratory pathogens in the nasopharynx in breast-fed versus formula-fed infants. *J Clin Microbiol* **31**(10), 2674–2678.

Kalliomäki, M., Kirjavainen, P., Eerola, E., Kero, P., Salminen, S., and Isolauri, E. (2001a). Distinct patterns of neonatal gut microflora in infants in whom atopy was and was not developing. *J Allergy Clin Immunol* **107**(1), 129–134.

Kalliomäki, M., Salminen, S., Arvilommi, H., Kero, P., Koskinen, P., and Isolauri, E. (2001b). Probiotics in primary prevention of atopic disease: a randomised placebo-controlled trial. *Lancet* **357**(9262), 1076–1079.

Karami, N., Nowrouzian, F., Adlerberth, I., and Wold, A. E. (2006). Tetracycline resistance in Escherichia coli and persistence in the infantile colonic microbiota. *Antimicrob Agents Chemother* **50**(1), 156–161.

Karlowicz, M. G., Buescher, E. S., and Surka, A. E. (2000). Fulminant late-onset sepsis in a neonatal intensive care unit, 1988–1997, and the impact of avoiding empiric vancomycin therapy. *Pediatrics* **106**(6), 1387–1390.

Kayange, N., Kamugisha, E., Mwizamholya, D. L., Jeremiah, S., and Mshana, S. E. (2010). Predictors of positive blood culture and deaths among neonates with suspected neonatal sepsis in a tertiary hospital, Mwanza-Tanzania. *BMC Pediatr* **10**, 39.

Kelly, D., and Coutts, A. G. P. (2000). Development of digestive and immunological function in neonates: role of early nutrition. *Livestock Production Science* **66**, 161–167.

Kelly, D., King, T., and Aminov, R. (2007). Importance of microbial colonization of the gut in early life to the development of immunity. *Mutat Res* **622**(1–2), 58–69.

Kenyon, S., Boulvain, M., and Neilson, J. (2003). Antibiotics for preterm rupture of membranes. *Cochrane Database Syst Rev*(2), CD001058.

Khan, A. U., Sultan, A., Tyagi, A., Zahoor, S., Akram, M., Kaur, S., Shahid, M., and Vaishnavi, C. V. (2007). Amplification of mecA gene in multi-drug resistant Staphylococcus aureus strains from hospital personnel. *J Infect Dev Ctries* **1**(3), 289–295.

Kirchner, L., Helmer, H., Heinze, G., Wald, M., Brunbauer, M., Weninger, M., and Zaknun, D. (2007). Amnionitis with Ureaplasma urealyticum or other microbes leads to increased morbidity and prolonged hospitalization in very low birth weight infants. *Eur J Obstet Gynecol Reprod Biol* **134**(1), 44–50.

Koksal, F., Yasar, H., and Samasti, M. (2009). Antibiotic resistance patterns of coagulase-negative staphylococcus strains isolated from blood cultures of septicemic patients in Turkey. *Microbiol Res* **164**(4), 404–410.

Kononen, E. (2005). Anaerobes in the upper respiratory tract in infancy. *Anaerobe* **11**(3), 131–136.

Kuwahara, T., Shimono, K., Kaneda, S., Tamura, T., Ichihara, M., and Nakashima, Y. Growth of microorganisms in total parenteral nutrition solutions containing lipid. *Int J Med Sci* **7**(3), 101–109.

Lafeber, H. N., Westerbeek, E. A., van den Berg, A., Fetter, W. P., and van Elburg, R. M. (2008). Nutritional factors influencing infections in preterm infants. *J Nutr* **138**(9), 1813S-1817S.

Larson, E. L., Cimiotti, J., Haas, J., Parides, M., Nesin, M., Della-Latta, P., and Saiman, L. (2005). Effect of antiseptic handwashing vs alcohol sanitizer on health care-associated infections in neonatal intensive care units. *Arch Pediatr Adolesc Med* **159**(4), 377–383.

Lass, J., Kaar, R., Jõgi, K., Varendi, H., Metsvaht, T., and Lutsar, I. (2011). Drug utilisation pattern and off-label use of medicines in Estonian neonatal units. *Eur J Clin Pharmacol* **67**(12), 1263–1271.

Laugel, V., Kuhn, P., Beladdale, J., Donato, L., Escande, B., Astruc, D., and Messer, J. (2003). Effects of antenatal antibiotics on the incidence and bacteriological profile of early-onset neonatal sepsis. A retrospective study over five years. *Biol Neonate* **84**(1), 24–30.

Lee, S. O., Lee, E. S., Park, S. Y., Kim, S. Y., Seo, Y. H., and Cho, Y. K. (2004). Reduced use of third-generation cephalosporins decreases the acquisition of extended-spectrum beta-lactamase-producing Klebsiella pneumoniae. *Infect Control Hosp Epidemiol* **25**(10), 832–837.

Levine, E. M., Ghai, V., Barton, J. J., and Strom, C. M. (1999). Intrapartum antibiotic prophylaxis increases the incidence of gram-negative neonatal sepsis. *Infect Dis Obstet Gynecol* **7**(4), 210–213.

Levy, O. (2007). Innate immunity of the newborn: basic mechanisms and clinical correlates. *Nat Rev Immunol* **7**(5), 379–390.

Lidsky, K., Hoyen, C., Salvator, A., Rice, L. B., and Toltzis, P. (2002). Antibiotic-resistant gram-negative organisms in pediatric chronic-care facilities. *Clin Infect Dis* **34**(6), 760–766.

Lievin, V., Peiffer, I., Hudault, S., Rochat, F., Brassart, D., Neeser, J. R., and Servin, A. L. (2000). Bifidobacterium strains from resident infant human gastrointestinal microflora exert antimicrobial activity. *Gut* **47**(5), 646–652.

Lima, A. B. M., Leao, L. S. N. O., Oliviera, L. S. C., and Pimenta, F. C. (2010). Nasopharyngeal Gram-negative bacilli colonization inh Brazilian children attending day-care centers. *Brazilian Journal of Microbiology* **41**, 24–27.

Lin, H. C., Su, B. H., Chen, A. C., Lin, T. W., Tsai, C. H., Yeh, T. F., and Oh, W. (2005). Oral probiotics reduce the incidence and severity of necrotizing enterocolitis in very low birth weight infants. *Pediatrics* **115**(1), 1–4.

Lin, P. W., Nasr, T. R., and Stoll, B. J. (2008). Necrotizing enterocolitis: recent scientific advances in pathophysiology and prevention. *Semin Perinatol* **32**(2), 70–82.

Lindberg, E., Adlerberth, I., and Wold, A. E. (2004). Antibiotic resistance in Staphylococcus aureus colonising the intestines of Swedish infants. *Clin Microbiol Infect* **10**(10), 890–894.

Livermore, D. M. (2000). Antibiotic resistance in staphylococci. *Int J Antimicrob Agents* **16 Suppl 1**, S3–10.

Ljubovic, A., Bekic, D., Hukic, M., and Sarajevo, B. (2007). *17th European Congress of Clinical Microbiology and Infectious Diseases and 25th International Congress of Chemotherapy, Munich/Germany, 31 March – 3 April 2007*.

Lõivukene, K., Kermes, K., Sepp, E., Adamson, V., Mitt, P., Jürna, M., Mägi, H., Kallandi, U., Otter, K., and Naaber, P. (2006). The comparison of susceptibility patterns of Gram-negative invasive and non-invasive pathogens in Estonian hospitals. *Antonie Van Leeuwenhoek* **89**(3–4), 367–371.

Lundell, A. C., Adlerberth, I., Lindberg, E., Karlsson, H., Ekberg, S., Aberg, N., Saalman, R., Hock, B., Steinkasserer, A., Hesselmar, B., Wold, A. E., and Rudin, A. (2007). Increased levels of circulating soluble CD14 but not CD83 in infants are associated with early intestinal colonization with Staphylococcus aureus. *Clin Exp Allergy* **37**(1), 62–71.

Mackenzie, G. A., Leach, A. J., Carapetis, J. R., Fisher, J., and Morris, P. S. (2010). Epidemiology of nasopharyngeal carriage of respiratory bacterial pathogens in children and adults: cross-sectional surveys in a population with high rates of pneumococcal disease. *BMC Infect Dis* **10**, 304.

Mackenzie, I., and Lever, A. (2007). Management of sepsis. *Bmj* **335**(7626), 929–932.

Mackie, R. I., Sghir, A., and Gaskins, H. R. (1999). Developmental microbial ecology of the neonatal gastrointestinal tract. *Am J Clin Nutr* **69**(5), 1035S-1045S.

Mahieu, L. M., De Dooy, J. J., Lenaerts, A. E., Ieven, M. M., and De Muynck, A. O. (2001). Catheter manipulations and the risk of catheter-associated bloodstream infection in neonatal intensive care unit patients. *J Hosp Infect* **48**(1), 20–26.

Mahmood, A., Karamat, K. A., and Butt, T. (2002). Neonatal sepsis: high antibiotic resistance of the bacterial pathogens in a neonatal intensive care unit in Karachi. *J Pak Med Assoc* **52**(8), 348–350.

Mammina, C., Di Carlo, P., Cipolla, D., Giuffre, M., Casuccio, A., Di Gaetano, V., Plano, M. R., D'Angelo, E., Titone, L., and Corsello, G. (2007). Surveillance of multidrug-resistant gram-negative bacilli in a neonatal intensive care unit: prominent role of cross transmission. *Am J Infect Control* **35**(4), 222–230.

Manzoni, P., Kaufman, D., Mostert, M., and Farina, D. (2008). Neonatal Candida spp. infections: an update. *Pediartic Health* **2**(1), 79–88.

Maraqa, N. F., Aigbivbalu, L., Masnita-Iusan, C., Wludyka, P., Shareef, Z., Bailey, C., and Rathore, M. H. (2011). Prevalence of and risk factors for methicillin-resistant

Staphylococcus aureus colonization and infection among infants at a level III neonatal intensive care unit. *Am J Infect Control* **39**(1), 35–41.

Marchisio, P., Gironi, S., Esposito, S., Schito, G. C., Mannelli, S., and Principi, N. (2001). Seasonal variations in nasopharyngeal carriage of respiratory pathogens in healthy Italian children attending day-care centres or schools. *J Med Microbiol* **50**(12), 1095–1099.

Martin, R., Heilig, H. G., Zoetendal, E. G., Jimenez, E., Fernandez, L., Smidt, H., and Rodriguez, J. M. (2007). Cultivation-independent assessment of the bacterial diversity of breast milk among healthy women. *Res Microbiol* **158**(1), 31–37.

McDonald, J. R., Carriker, C. M., Pien, B. C., Trinh, J. V., Engemann, J. J., Harrell, L. J., Oden, M. A., Tanaka, D. T., Goldberg, R. N., Sexton, D. J., and Kaye, K. S. (2007). Methicillin-resistant Staphylococcus aureus outbreak in an intensive care nursery: potential for interinstitutional spread. *Pediatr Infect Dis J* **26**(8), 678–683.

McDuffie, R. S., Jr., McGregor, J. A., and Gibbs, R. S. (1993). Adverse perinatal outcome and resistant Enterobacteriaceae after antibiotic usage for premature rupture of the membranes and group B streptococcus carriage. *Obstet Gynecol* **82**(4 Pt 1), 487–489.

McGinigle, K. L., Gourlay, M. L., and Buchanan, I. B. (2008). The use of active surveillance cultures in adult intensive care units to reduce methicillin-resistant Staphylococcus aureus-related morbidity, mortality, and costs: a systematic review. *Clin Infect Dis* **46**(11), 1717–1725.

Mears, A., White, A., Cookson, B., Devine, M., Sedgwick, J., Phillips, E., Jenkinson, H., and Bardsley, M. (2009). Healthcare-associated infection in acute hospitals: which interventions are effective? *J Hosp Infect* **71**(4), 307–313.

Mehta, D., Ed. (2005). BNF49. British National Formulary. Wallingford: Pharmaceutical Press.

Mendiratta, D. K., Rawat, V., Thamke, D., Chaturvedi, P., Chhabra, S., and Narang, P. (2006). Candida colonization in preterm babies admitted to neonatal intensive care unit in the rural setting. *Indian J Med Microbiol* **24**(4), 263–267.

Merritt, T. A., Pillers, D., and Prows, S. L. (2003). Early NICU discharge of very low birth weight infants: a critical review and analysis. *Semin Neonatol* **8**(2), 95–115.

Metha, D. (2005). "BNF49: British National Formulary." Pharmacolagical Press, Wallingford.

Metsvaht, T., Pisarev, H., Ilmoja, M. L., Parm, Ü., Maipuu, L., Merila, M., Müürsepp, P., and Lutsar, I. (2009). Clinical parameters predicting failure of empirical antibacterial therapy in early onset neonatal sepsis, identified by classification and regression tree analysis. *BMC Pediatr* **9**, 72.

Metsvaht, T. (2010). Optimal antibacterial therapy of neonates at risk of early onset sepsis. Dissertation. University of Tartu, Tartu. pp 193.

Metsvaht, T., Ilmoja, M. L., Parm, Ü., Maipuu, L., Merila, M., and Lutsar, I. (2010). Comparison of ampicillin plus gentamicin vs. penicillin plus gentamicin in empiric treatment of neonates at risk of early onset sepsis. *Acta Paediatr* **99**(5), 665–672.

Miedema, C. J., Kerkhof, M., Arends, J. P., Bergman, K. A., and Kimpen, J. L. (2000). Risk factors for colonization with enterococci in a neonatal intensive care unit. *Clin Microbiol Infect* **6**(1), 53.

Mikelsaar, M. (1992). Evaluation of gastrointestinal microbial ecosystem in health and disease. Dissertation. University of Tartu.

Mikelsaar, M., and Zilmer, M. (2009). Lactobacillus fermentum ME-3 – an antimicrobial and antioxidative probiotic. *Microb Ecol Health Dis* **21**(1), 1–27.

Mikelsaar M, Mändar R, Sepp E, Annuk H. (2004). Human lactic acid microflora and its role in the welfare of the host. In: Lactic Acid Bacteria. Microbiological and Functional Aspects (Eds. S Salminen, A von Wright, A Ouwehand). New York, Marcel Dekker, Inc., p. 453–506.

Millar, M., Philpott, A., Wilks, M., Whiley, A., Warwick, S., Hennessy, E., Coen, P., Kempley, S., Stacey, F., and Costeloe, K. (2008). Colonization and persistence of antibiotic-resistant Enterobacteriaceae strains in infants nursed in two neonatal intensive care units in East London, United Kingdom. *J Clin Microbiol* **46**(2), 560–567.

Milledge, J., Calis, J. C., Graham, S. M., Phiri, A., Wilson, L. K., Soko, D., Mbvwinji, M., Walsh, A. L., Rogerson, S. R., Molyneux, M. E., and Molyneux, E. M. (2005). Aetiology of neonatal sepsis in Blantyre, Malawi: 1996–2001. *Ann Trop Paediatr* **25**(2), 101–110.

Mims, C., Dockrell, H., Goering, R., Roitt, I., Wakllin, D., and Zukerman, M. (2004). "Medical Microbiology." Third edition ed. The host-parasite relationship Mosby, Edinburgh, London, New York, Oxford, Philadelphia, St. Lows, Sydney, Toronto.

Miranda, L. N., van der Heijden, I. M., Costa, S. F., Sousa, A. P., Sienra, R. A., Gobara, S., Santos, C. R., Lobo, R. D., Pessoa, V. P., Jr., and Levin, A. S. (2009). Candida colonisation as a source for candidaemia. *J Hosp Infect* **72**(1), 9–16.

Mishra, U. K., Jacobs, S. E., Doyle, L. W., and Garland, S. M. (2006). Newer approaches to the diagnosis of early onset neonatal sepsis. *Arch Dis Child Fetal Neonatal Ed* **91**(3), F208–212.

Mitsou, E. K., Kirtzalidou, E., Oikonomou, I., Liosis, G., and Kyriacou, A. (2008). Fecal microflora of Greek healthy neonates. *Anaerobe* **14**(2), 94–101.

Mitt, P., Adamson, V., Lõivukene, K., Lang, K., Telling, K., Päro, K., Rõõm, A., Naaber, P., and Maimets, M. (2009). Epidemiology of nosocomial bloodstream infections in Estonia. *J Hosp Infect* **71**(4), 365–370.

Modi, N., and Carr, R. (2000). Promising stratagems for reducing the burden of neonatal sepsis. *Arch Dis Child Fetal Neonatal Ed* **83**(2), F150–153.

Mohan, P., Eddama, O., and Weisman, L. E. (2007). Patient isolation measures for infants with candida colonization or infection for preventing or reducing transmission of candida in neonatal units. *Cochrane Database Syst Rev*(3), CD006068.

Moore, M. R., Schrag, S. J., and Schuchat, A. (2003). Effects of intrapartum antimicrobial prophylaxis for prevention of group-B-streptococcal disease on the incidence and ecology of early-onset neonatal sepsis. *Lancet Infect Dis* **3**(4), 201–213.

Morel, A. S., Wu, F., Della-Latta, P., Cronquist, A., Rubenstein, D., and Saiman, L. (2002). Nosocomial transmission of methicillin-resistant Staphylococcus aureus from a mother to her preterm quadruplet infants. *Am J Infect Control* **30**(3), 170–173.

Morelli, L. (2008). Postnatal development of intestinal microflora as influenced by infant nutrition. *J Nutr* **138**(9), 1791S-1795S.

Morowitz, M. J., Poroyko, V., Caplan, M., Alverdy, J., and Liu, D. C. (2010). Redefining the role of intestinal microbes in the pathogenesis of necrotizing enterocolitis. *Pediatrics* **125**(4), 777–785.

Mshvildadze, M., Neu, J., and Mai, V. (2008). Intestinal microbiota development in the premature neonate: establishment of a lasting commensal relationship? *Nutr Rev* **66**(11), 658–663.

Mshvildadze, M., and Neu, J. (2010a). The infant intestinal microbiome: friend or foe? *Early Hum Dev* **86 Suppl 1**, 67–71.

Mshvildadze, M., Neu, J., Shuster, J., Theriaque, D., Li, N., and Mai, V. (2010b). Intestinal microbial ecology in premature infants assessed with non-culture-based techniques. *J Pediatr* **156**(1), 20–25.

Mussi-Pinhata, M. M., and Nascimento, S. D. (2001). Neonatal nosocomial infections. *J Pediatr (Rio J)* **77 Suppl 1**, S81–96.

Mussi-Pinhata, M. M., and Rego, M. A. (2005). Immunological peculiarities of extremely preterm infants: a challenge for the prevention of nosocomial sepsis. *J Pediatr (Rio J)* **81**(1 Suppl), S59–68.

Mändar, R., and Mikelsaar, M. (1996). Transmission of mother's microflora to the newborn at birth. *Biol Neonate* **69**, 30–35.

Mändar, R., K, L. i., Ehrenberg, A., Smidt, I., Raukas, E., Kask, V., and Mikelsaar, M. (2001). Amniotic fluid microflora in asymptomatic women at mid-gestation. *Scand J Infect Dis* **33**(1), 60–62.

Naaber, P., Klaus, K., Sepp, E., Björksten, B., and Mikelsaar, M. (1997). Colonization of infants and hospitalized patients with Clostridium difficile and lactobacilli. *Clin Infect Dis* **25 Suppl 2**, S189–190.

Navarro, L. R., Pekelharing-Berghuis, M., de Waal, W. J., and Thijsen, S. F. (2011). Bacterial colonization patterns in neonates transferred from neonatal intensive care units. *Int J Hyg Environ Health* **214**(2), 167–171.

Neu, J. (2007). Gastrointestinal development and meeting the nutritional needs of premature infants. *Am J Clin Nutr* **85**(2), 629S-634S.

Noverr, M. C., and Huffnagle, G. B. (2004). Does the microbiota regulate immune responses outside the gut? *Trends Microbiol* **12**(12), 562–568.

O'Grady, N. P., Alexander, M., Dellinger, E. P., Gerberding, J. L., Heard, S. O., Maki, D. G., Masur, H., McCormick, R. D., Mermel, L. A., Pearson, M. L., Raad, II, Randolph, A., and Weinstein, R. A. (2002). Guidelines for the prevention of intravascular catheter-related infections. *Am J Infect Control* **30**(8), 476–489.

Ogra, P. L. (2010). Ageing and its possible impact on mucosal immune responses. *Ageing Res Rev* **9**(2), 101–106.

Onderdonk, A. B., Hecht, J. L., McElrath, T. F., Delaney, M. L., Allred, E. N., and Leviton, A. (2008). Colonization of second-trimester placenta parenchyma. *Am J Obstet Gynecol* **199**(1), 52 e1–52 e10.

Orrhage, K., and Nord, C. E. (1999). Factors controlling the bacterial colonization of the intestine in breastfed infants. *Acta Paediatr Suppl* **88**(430), 47–57.

Papadomichelakis, E., Kontopidou, F., Antoniadou, A., Poulakou, G., Koratzanis, E., Kopterides, P., Mavrou, I., Armaganidis, A., and Giamarellou, H. (2008). Screening for resistant gram-negative microorganisms to guide empiric therapy of subsequent infection. *Intensive Care Med* **34**(12), 2169–2175.

Pappas, P. G., Kauffman, C. A., Andes, D., Benjamin, D. K., Jr., Calandra, T. F., Edwards, J. E., Jr., Filler, S. G., Fisher, J. F., Kullberg, B. J., Ostrosky-Zeichner, L., Reboli, A. C., Rex, J. H., Walsh, T. J., and Sobel, J. D. (2009). Clinical practice guidelines for the management of candidiasis: 2009 update by the Infectious Diseases Society of America. *Clin Infect Dis* **48**(5), 503–535.

Park, H. K., Shim, S. S., Kim, S. Y., Park, J. H., Park, S. E., Kim, H. J., Kang, B. C., and Kim, C. M. (2005). Molecular analysis of colonized bacteria in a human newborn infant gut. *J Microbiol* **43**(4), 345–353.

Penders, J., Thijs, C., Vink, C., Stelma, F. F., Snijders, B., Kummeling, I., van den Brandt, P. A., and Stobberingh, E. E. (2006). Factors influencing the composition of the intestinal microbiota in early infancy. *Pediatrics* **118**(2), 511–521.

Perez, P. F., Dore, J., Leclerc, M., Levenez, F., Benyacoub, J., Serrant, P., Segura-Roggero, I., Schiffrin, E. J., and Donnet-Hughes, A. (2007). Bacterial imprinting of the neonatal immune system: lessons from maternal cells? *Pediatrics* **119**(3), e724–732.

Perlman, S. E., Saiman, L., and Larson, E. L. (2007). Risk factors for late-onset health care-associated bloodstream infections in patients in neonatal intensive care units. *Am J Infect Control* **35**(3), 177–182.

Pessoa-Silva, C. L., Dharan, S., Hugonnet, S., Touveneau, S., Posfay-Barbe, K., Pfister, R., and Pittet, D. (2004). Dynamics of bacterial hand contamination during routine neonatal care. *Infect Control Hosp Epidemiol* **25**(3), 192–197.

Phillips, P., Cortina-Borja, M., Millar, M., and Gilbert, R. (2008). Risk-adjusted surveillance of hospital-acquired infections in neonatal intensive care units: a systematic review. *J Hosp Infect* **70**(3), 203–211.

Pierro, A., van Saene, H. K., Jones, M. O., Brown, D., Nunn, A. J., and Lloyd, D. A. (1998). Clinical impact of abnormal gut flora in infants receiving parenteral nutrition. *Ann Surg* **227**(4), 547–552.

Pietzak, M. (2004). Bacterial colonization of the neonatal gut. *J Pediatr Gastroenterol Nutr* **38**(4), 389–391.

Pillay, T., Pillay, D. G., Adhikari, M., Pillay, A., and Sturm, A. W. (1999). An outbreak of neonatal infection with Acinetobacter linked to contaminated suction catheters. *J Hosp Infect* **43**(4), 299–304.

Prelog, M., Grif, K., Decristoforo, C., Wurzner, R., Kiechl-Kohlendorfer, U., Brunner, A., Zimmerhackl, L. B., and Orth, D. (2009). Tetracycline-resistant Escherichia coli strains are inherited from parents and persist in the infant's intestines in the absence of selective pressure. *Eur J Pediatr* **168**(10), 1181–1187.

Puopolo, K. M., and Eichenwald, E. C. (2010). No change in the incidence of ampicillin-resistant, neonatal, early-onset sepsis over 18 years. *Pediatrics* **125**(5), e1031–1038.

Rautava, S., and Isolauri, E. (2002). The development of gut immune responses and gut microbiota: effects of probiotics in prevention and treatment of allergic disease. *Curr Issues Intest Microbiol* **3**(1), 15–22.

Regev-Yochay, G., Rubinstein, E., Barzilai, A., Carmeli, Y., Kuint, J., Etienne, J., Blech, M., Smollen, G., Maayan-Metzger, A., Leavitt, A., Rahav, G., and Keller, N. (2005). Methicillin-resistant Staphylococcus aureus in neonatal intensive care unit. *Emerg Infect Dis* **11**(3), 453–456.

Reid, G., Younes, J. A., Van der Mei, H. C., Gloor, G. B., Knight, R., and Busscher, H. J. (2011). Microbiota restoration: natural and supplemented recovery of human microbial communities. *Nat Rev Microbiol* **9**(1), 27–38.

Rice, L. B., Willey, S. H., Papanicolaou, G. A., Medeiros, A. A., Eliopoulos, G. M., Moellering, R. C., Jr., and Jacoby, G. A. (1990). Outbreak of ceftazidime resistance caused by extended-spectrum beta-lactamases at a Massachusetts chronic-care facility. *Antimicrob Agents Chemother* **34**(11), 2193–2199.

Roilides, E., Farmaki, E., Evdoridou, J., Dotis, J., Hatziioannidis, E., Tsivitanidou, M., Bibashi, E., Filioti, I., Sofianou, D., Gil-Lamaignere, C., Mueller, F. M., and Kremenopoulos, G. (2004). Neonatal candidiasis: analysis of epidemiology, drug susceptibility, and molecular typing of causative isolates. *Eur J Clin Microbiol Infect Dis* **23**(10), 745–750.

Ronnestad, A., Abrahamsen, T. G., Medbo, S., Reigstad, H., Lossius, K., Kaaresen, P. I., Egeland, T., Engelund, I. E., Irgens, L. M., and Markestad, T. (2005a). Late-onset

septicemia in a Norwegian national cohort of extremely premature infants receiving very early full human milk feeding. *Pediatrics* **115**(3), e269–276.

Ronnestad, A., Abrahamsen, T. G., Medbo, S., Reigstad, H., Lossius, K., Kaaresen, P. I., Engelund, I. E., Irgens, L. M., and Markestad, T. (2005b). Septicemia in the first week of life in a Norwegian national cohort of extremely premature infants. *Pediatrics* **115**(3), e262–268.

Rotimi, V. O., and Duerden, B. (1977). The development of anaerobic fecal flora in healthy newborn infants. *J Pediatr* **91**, 298–301.

Rotimi, V. O., and Duerden, B. I. (1981). The development of the bacterial flora in normal neonates. *J Med Microbiol* **14**(1), 51–62.

Sadowska-Krawczenko, I., and Korbal, P. (2009). Healtcare-associated bloodstream infection in neonatal intensive care unit. *Archives of Perinatal Medicine* **15**(1), 26–30.

Saiman, L., Ludington, E., Pfaller, M., Rangel-Frausto, S., Wiblin, R. T., Dawson, J., Blumberg, H. M., Patterson, J. E., Rinaldi, M., Edwards, J. E., Wenzel, R. P., and Jarvis, W. (2000). Risk factors for candidemia in Neonatal Intensive Care Unit patients. The National Epidemiology of Mycosis Survey study group. *Pediatr Infect Dis J* **19**(4), 319–324.

Saiman, L., Ludington, E., Dawson, J. D., Patterson, J. E., Rangel-Frausto, S., Wiblin, R. T., Blumberg, H. M., Pfaller, M., Rinaldi, M., Edwards, J. E., Wenzel, R. P., and Jarvis, W. (2001). Risk factors for Candida species colonization of neonatal intensive care unit patients. *Pediatr Infect Dis J* **20**(12), 1119–1124.

Saiman, L. (2002). Risk factors for hospital-acquired infections in the neonatal intensive care unit. *Semin Perinatol* **26**(5), 315–321.

Sakata, H., Yoshioka, H., and Fujita, K. (1985). Development of the intestinal flora in very low birth weight infants compared to normal full-term newborns. *Eur J Pediatr* **144**(2), 186–190.

Salminen, S., Benno, Y., and de Vos, W. (2006). Intestinal colonisation, microbiota and future probiotics? *Asia Pac J Clin Nutr* **15**(4), 558–562.

Samanta, S., Farrer, K., Breathnach, A., and Heath, P. T. (2011). Risk factors for late onset gram-negative infections: a case-control study. *Arch Dis Child Fetal Neonatal Ed* **96**(1), F15–18.

Sanchez-Carrillo, C., Padilla, B., Marin, M., Rivera, M., Cercenado, E., Vigil, D., Sanchez-Luna, M., and Bouza, E. (2009). Contaminated feeding bottles: the source of an outbreak of Pseudomonas aeruginosa infections in a neonatal intensive care unit. *Am J Infect Control* **37**(2), 150–154.

Sanders, M. E., Akkermans, L. M., Haller, D., Hammerman, C., Heimbach, J., Hormannsperger, G., Huys, G., Levy, D. D., Lutgendorff, F., Mack, D., Phothirath, P., Solano-Aguilar, G., and Vaughan, E. (2010). Safety assessment of probiotics for human use. *Gut Microbes* **1**(3), 164–185.

Sartor, C., Jacomo, V., Duvivier, C., Tissot-Dupont, H., Sambuc, R., and Drancourt, M. (2000). Nosocomial Serratia marcescens infections associated with extrinsic contamination of a liquid nonmedicated soap. *Infect Control Hosp Epidemiol* **21**(3), 196–199.

Schelonka, R. L., Scruggs, S., Nichols, K., Dimmitt, R. A., and Carlo, W. A. (2006). Sustained reductions in neonatal nosocomial infection rates following a comprehensive infection control intervention. *J Perinatol* **26**(3), 176–179.

Schrag, S., Gorwitz, R., Fultz-Butts, K., and Schuchat, A. (2002). Prevention of perinatal group B streptococcal disease. Revised guidelines from CDC. *MMWR Recomm Rep* **51**(RR-11), 1–22.

Schrag, S. J., Hadler, J. L., Arnold, K. E., Martell-Cleary, P., Reingold, A., and Schuchat, A. (2006a). Risk factors for invasive, early-onset Escherichia coli infections in the era of widespread intrapartum antibiotic use. *Pediatrics* **118**(2), 570–576.

Schrag, S. J., and Stoll, B. J. (2006b). Early-onset neonatal sepsis in the era of widespread intrapartum chemoprophylaxis. *Pediatr Infect Dis J* **25**(10), 939–940.

Schuchat, A., Zywicki, S. S., Dinsmoor, M. J., Mercer, B., Romaguera, J., O'Sullivan, M. J., Patel, D., Peters, M. T., Stoll, B., and Levine, O. S. (2000). Risk factors and opportunities for prevention of early-onset neonatal sepsis: a multicenter case-control study. *Pediatrics* **105**(1 Pt 1), 21–26.

Schwiertz, A., Gruhl, B., Lobnitz, M., Michel, P., Radke, M., and Blaut, M. (2003). Development of the intestinal bacterial composition in hospitalized preterm infants in comparison with breast-fed, full-term infants. *Pediatr Res* **54**(3), 393–399.

Sepp, E., Julge, K., Vasar, M., Naaber, P., Björksten, B., and Mikelsaar, M. (1997). Intestinal microflora of Estonian and Swedish infants. *Acta Paediatr* **86**(9), 956–961.

Sepp, E. (1998). Formation of intestinal microbial ecosystem in children. Dissertation. University of Tartu.

Sepp, E., Naaber, P., Voor, T., Mikelsaar, M., and Björksten, B. (2000). Development of intestinal microflora during the first month of life in Estonian and Swedish infants. *Microbial Ecology in Health and Disease* **12**, 22–26.

Sepp, E., Stsepetova, J., Lõivukene, K., Truusalu, K., Kõljalg, S., Naaber, P., and Mikelsaar, M. (2009). The occurrence of antimicrobial resistance and class 1 integrons among commensal Escherichia coli isolates from infants and elderly persons. *Ann Clin Microbiol Antimicrob* **8**, 34.

Shanahan, P. M., Thomson, C. J., and Amyes, S. G. (1994). Beta-lactam resistance in aerobic faecal flora from general practice patients in the UK. *Eur J Clin Microbiol Infect Dis* **13**(9), 760–763.

Shanahan, P. M., Thomson, C. J., and Amyes, S. G. (1995). Beta-lactam resistance in normal faecal flora from South Africa. *Epidemiol Infect* **115**(2), 243–253.

Shankar, K. R., Brown, D., Hughes, J., Lamont, G. L., Losty, P. D., Lloyd, D. A., and van Saene, H. K. (2001). Classification and risk-factor analysis of infections in a surgical neonatal unit. *J Pediatr Surg* **36**(2), 276–281.

Shete, V. B., Ghadage, D. P., Muley, V. A., and Bhore, A. V. (2009). Acinetobacter septicemia in neonates admitted to intensive care units. *Journal of Laboratory Physicans* **1**(2), 73–76.

Shi, Z. Y., Liu, P. Y., Lau, Y. J., Lin, Y. H., and Hu, B. S. (1996). Epidemiological typing of isolates from an outbreak of infection with multidrug-resistant Enterobacter cloacae by repetitive extragenic palindromic unit b1-primed PCR and pulsed-field gel electrophoresis. *J Clin Microbiol* **34**(11), 2784–2790.

Shiojima, T., Ohki, Y., Nako, Y., Morikawa, A., Okubo, T., and Iyobe, S. (2003). Immediate control of a methicillin-resistant Staphylococcus aureus outbreak in a neonatal intensive care unit. *J Infect Chemother* **9**(3), 243–247.

Silvestri, L., Monti Bragadin, C., Milanese, M., Gregori, D., Consales, C., Gullo, A., and van Saene, H. K. (1999). Are most ICU infections really nosocomial? A prospective observational cohort study in mechanically ventilated patients. *J Hosp Infect* **42**(2), 125–133.

Simhan, H. N., and Canavan, T. P. (2005). Preterm premature rupture of membranes: diagnosis, evaluation and management strategies. *Bjog* **112 Suppl 1**, 32–37.

Simmonds, A., Munoz, J., Aguero-Rosenfeld, M., Carbonaro, C., Montecalvo, M., Clones, B., and LaGamma, E. F. (2009). Outbreak of Acinetobacter infection in extremely low birth weight neonates. *Pediatr Infect Dis J* **28**(3), 210–214.

Singh, K., Gavin, P. J., Vescio, T., Thomson Jr, R. B., Jr., Deddish, R. B., Fisher, A., Noskin, G. A., and Peterson, L. R. (2003). Microbiologic surveillance using nasal cultures alone is sufficient for detection of methicillin-resistant Staphylococcus aureus isolates in neonates. *J Clin Microbiol* **41**(6), 2755–2757.

Smith, A., Saiman, L., Zhou, J., Della-Latta, P., Jia, H., and Graham, P. L., 3rd (2010). Concordance of Gastrointestinal Tract Colonization and Subsequent Bloodstream Infections With Gram-negative Bacilli in Very Low Birth Weight Infants in the Neonatal Intensive Care Unit. *Pediatr Infect Dis J* **29**(9), 831–835.

Sprunt, K., Leidy, G., and Redman, W. (1978). Abnormal colonization of neonates in an intensive care unit: means of identifying neonates at risk of infection. *Pediatr Res* **12**(10), 998–1002.

Sprunt, K. (1985). Practical use of surveillance for prevention of nosocomial infection. *Semin Perinatol* **9**(1), 47–50.

Srivastava, S., and Shetty, N. (2007). Healthcare-associated infections in neonatal units: lessons from contrasting worlds. *J Hosp Infect* **65**(4), 292–306.

Stecher, B., and Hardt, W. D. (2011). Mechanisms controlling pathogen colonization of the gut. *Curr Opin Microbiol* **14**(1), 82–91.

Stoll, B. J., Hansen, N., Fanaroff, A. A., Wright, L. L., Carlo, W. A., Ehrenkranz, R. A., Lemons, J. A., Donovan, E. F., Stark, A. R., Tyson, J. E., Oh, W., Bauer, C. R., Korones, S. B., Shankaran, S., Laptook, A. R., Stevenson, D. K., Papile, L. A., and Poole, W. K. (2002a). Changes in pathogens causing early-onset sepsis in very-low-birth-weight infants. *N Engl J Med* **347**(4), 240–247.

Stoll, B. J., Hansen, N., Fanaroff, A. A., Wright, L. L., Carlo, W. A., Ehrenkranz, R. A., Lemons, J. A., Donovan, E. F., Stark, A. R., Tyson, J. E., Oh, W., Bauer, C. R., Korones, S. B., Shankaran, S., Laptook, A. R., Stevenson, D. K., Papile, L. A., and Poole, W. K. (2002b). Late-onset sepsis in very low birth weight neonates: the experience of the NICHD Neonatal Research Network. *Pediatrics* **110**(2 Pt 1), 285–291.

Stoll, B. J., and Hansen, N. (2003). Infections in VLBW infants: studies from the NICHD Neonatal Research Network. *Semin Perinatol* **27**(4), 293–301.

Stsepetova, J., Sepp, E., Julge, K., Vaughan, E., Mikelsaar, M., and de Vos, W. M. (2007). Molecularly assessed shifts of Bifidobacterium ssp. and less diverse microbial communities are characteristic of 5-year-old allergic children. *FEMS Immunol Med Microbiol* **51**(2), 260–269.

Sullivan, A., Edlund, C., and Nord, C. E. (2001). Effect of antimicrobial agents on the ecological balance of human microflora. *Lancet Infect Dis* **1**(2), 101–114.

Zaidi, A. K., Huskins, W. C., Thaver, D., Bhutta, Z. A., Abbas, Z., and Goldmann, D. A. (2005). Hospital-acquired neonatal infections in developing countries. *Lancet* **365**(9465), 1175–1188.

Zaidi, A. K., Ganatra, H. A., Syed, S., Cousens, S., Lee, A. C., Black, R., Bhutta, Z. A., and Lawn, J. E. (2011). Effect of case management on neonatal mortality due to sepsis and pneumonia. *BMC Public Health* **11 Suppl 3**, S13.

Zarrilli, R., Casillo, R., Di Popolo, A., Tripodi, M. F., Bagattini, M., Cuccurullo, S., Crivaro, V., Ragone, E., Mattei, A., Galdieri, N., Triassi, M., and Utili, R. (2007). Molecular epidemiology of a clonal outbreak of multidrug-resistant Acinetobacter baumannii in a university hospital in Italy. *Clin Microbiol Infect* **13**(5), 481–489.

Zoetendal, E. G., Ben-Amor, K., Harmsen, H. J., Schut, F., Akkermans, A. D., and de Vos, W. M. (2002). Quantification of uncultured Ruminococcus obeum-like bacteria in human fecal samples by fluorescent in situ hybridization and flow cytometry using 16S rRNA-targeted probes. *Appl Environ Microbiol* **68**(9), 4225–4232.

Tallur, S. S., Kasturi, A. V., Nadgir, S. D., and Krishna, B. V. (2000). Clinico-bacteriological study of neonatal septicemia in Hubli. *Indian J Pediatr* **67**(3), 169–174.

Tanaka, S., Kobayashi, T., Songjinda, P., Tateyama, A., Tsubouchi, M., Kiyohara, C., Shirakawa, T., Sonomoto, K., and Nakayama, J. (2009). Influence of antibiotic exposure in the early postnatal period on the development of intestinal microbiota. *FEMS Immunol Med Microbiol* **56**(1), 80–87.

Tannock, G. W., Fuller, R., Smith, S. L., and Hall, M. A. (1990). Plasmid profiling of members of the family Enterobacteriaceae, lactobacilli, and bifidobacteria to study the transmission of bacteria from mother to infant. *J Clin Microbiol* **28**(6), 1225–1228.

Tapiainen, T., Ylitalo, S., Eerola, E., and Uhari, M. (2006). Dynamics of gut colonization and source of intestinal flora in healthy newborn infants. *Apmis* **114**(11), 812–817.

Tarnow-Mordi, W., Isaacs, D., and Dutta, S. Adjunctive immunologic interventions in neonatal sepsis. *Clin Perinatol* **37**(2), 481–499.

Tarnow-Mordi, W., Isaacs, D., and Dutta, S. (2010). Adjunctive immunologic interventions in neonatal sepsis. *Clin Perinatol* **37**(2), 481–499.

Tenover, F. C., Arbeit, R. D., Goering, R. V., Mickelsen, P. A., Murray, B. E., Persing, D. H., and Swaminathan, B. (1995). Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *J Clin Microbiol* **33**(9), 2233–2239.

Tessin, I., Trollfors, B., and Thiringer, K. (1990). Incidence and etiology of neonatal septicaemia and meningitis in western Sweden 1975–1986. *Acta Paediatr Scand* **79**(11), 1023–1030.

Thaver, D., and Zaidi, A. K. (2009). Burden of neonatal infections in developing countries: a review of evidence from community-based studies. *Pediatr Infect Dis J* **28**(1 Suppl), S3–9.

Thompson-Chagoyan, O. C., Maldonado, J., and Gil, A. (2007). Colonization and impact of disease and other factors on intestinal microbiota. *Dig Dis Sci* **52**(9), 2069–2077.

Tlaskalova-Hogenova, H., Stepankova, R., Hudcovic, T., Tuckova, L., Cukrowska, B., Lodinova-Zadnikova, R., Kozakova, H., Rossmann, P., Bartova, J., Sokol, D., Funda, D. P., Borovska, D., Rehakova, Z., Sinkora, J., Hofman, J., Drastich, P., and Kokesova, A. (2004). Commensal bacteria (normal microflora), mucosal immunity and chronic inflammatory and autoimmune diseases. *Immunol Lett* **93**(2–3), 97–108.

Toltzis, P., Dul, M. J., Hoyen, C., Salvator, A., Walsh, M., Zetts, L., and Toltzis, H. (2001). Molecular epidemiology of antibiotic-resistant gram-negative bacilli in a neonatal intensive care unit during a nonoutbreak period. *Pediatrics* **108**(5), 1143–1148.

Toltzis, P., Dul, M. J., Hoyen, C., Salvator, A., Walsh, M., Zetts, L., and Toltzis, H. (2002). The effect of antibiotic rotation on colonization with antibiotic-resistant bacilli in a neonatal intensive care unit. *Pediatrics* **110**(4), 707–11.

Top, K. A., Huard, R. C., Fox, Z., Wu, F., Whittier, S., Della-Latta, P., Saiman, L., and Ratner, A. J. Trends in methicillin-resistant Staphylococcus aureus anovaginal

colonization in pregnant women in 2005 versus 2009. *J Clin Microbiol* **48**(10), 3675–3680.

Towers, C. V., Carr, M. H., Padilla, G., and Asrat, T. (1998). Potential consequences of widespread antepartal use of ampicillin. *Am J Obstet Gynecol* **179**(4), 879–883.

Traore, O., Springthorpe, V. S., and Sattar, S. A. (2002). A quantitative study of the survival of two species of Candida on porous and non-porous environmental surfaces and hands. *J Appl Microbiol* **92**(3), 549–555.

Trijbels-Smeulders, M., de Jonge, G. A., Pasker-de Jong, P. C., Gerards, L. J., Adriaanse, A. H., van Lingen, R. A., and Kollee, L. A. (2007). Epidemiology of neonatal group B streptococcal disease in the Netherlands before and after introduction of guidelines for prevention. *Arch Dis Child Fetal Neonatal Ed* **92**(4), F271–276.

Turnidge, J. D., Ferraro, M. J., and Jorgensen, J. H. (2007). Susceptibility Test Method: General Considerations 9th edition ed. *In* "Manual of Clinical Microbiology" (P. R. Murray, E. J. Baron, J. H. Jorgensen, M. L. Landry, and M. Pfaller, Eds.), Vol. 1, pp. 1146 – 1151., Washington, D.C.

Udo, E. E., Al-Sweih, N., Dhar, R., Dimitrov, T. S., Mokaddas, E. M., Johny, M., Al-Obaid, I. A., Gomaa, H. H., Mobasher, L. A., Rotimi, V. O., and Al-Asar, A. (2008). Surveillance of antibacterial resistance in Staphylococcus aureus isolated in Kuwaiti hospitals. *Med Princ Pract* **17**(1), 71–75.

Vaishampayan, P. A., Kuehl, J. V., Froula, J. L., Morgan, J. L., Ochman, H., and Francino, M. P. (2010). Comparative metagenomics and population dynamics of the gut microbiota in mother and infant. *Genome Biol Evol* **2**, 53–66.

Wall, R., Ross, R. P., Ryan, C. A., Hussey, S., Murphy, B., Fitzgerald, G. F., and Stanton, C. (2009). Role of gut microbiota in early infant development. *Clinical Medicine Pediatrics* **3**, 45–54.

Valvano, M. A., Hartstein, A. I., Morthland, V. H., Dragoon, M. E., Potter, S. A., Reynolds, J. W., and Crosa, J. H. (1988). Plasmid DNA analysis of Staphylococcus epidermidis isolated from blood and colonization cultures in very low birth weight neonates. *Pediatr Infect Dis J* **7**(2), 116–120.

van Belkum, A., Tassios, P. T., Dijkshoorn, L., Haeggman, S., Cookson, B., Fry, N. K., Fussing, V., Green, J., Feil, E., Gerner-Smidt, P., Brisse, S., and Struelens, M. (2007). Guidelines for the validation and application of typing methods for use in bacterial epidemiology. *Clin Microbiol Infect* **13 Suppl 3**, 1–46.

van den Berg, R. W., Claahsen, H. L., Niessen, M., Muytjens, H. L., Liem, K., and Voss, A. (2000). Enterobacter cloacae outbreak in the NICU related to disinfected thermometers. *J Hosp Infect* **45**(1), 29–34.

van der Zee, A., Steer, N., Thijssen, E., Nelson, J., van't Veen, A., and Buiting, A. (2003). Use of multienzyme multiplex PCR amplified fragment length polymorphism typing in analysis of outbreaks of multiresistant Klebsiella pneumoniae in an intensive care unit. *J Clin Microbiol* **41**(2), 798–802.

van der Zwet, W. C., Parlevliet, G. A., Savelkoul, P. H., Stoof, J., Kaiser, A. M., Koeleman, J. G., and Vandenbroucke-Grauls, C. M. (1999). Nosocomial outbreak of gentamicin-resistant Klebsiella pneumoniae in a neonatal intensive care unit controlled by a change in antibiotic policy. *J Hosp Infect* **42**(4), 295–302.

van Rossem, M. C., de Waal, W. J., van Hannen, E. J., Verboon-Maciolek, M. A., van Wieringen, H., Van de Vijver, D. A., van Dyk, Y., and Thijsen, S. F. (2007). Enterobacter colonisation in newborn infants: predictors, follow-up and implications for infection control. *J Hosp Infect* **67**(2), 142–148.

Wang, S., Ng, L. H., Chow, W. L., and Lee, Y. K. (2008). Infant intestinal Enterococcus faecalis down-regulates inflammatory responses in human intestinal cell lines. *World J Gastroenterol* **14**(7), 1067–1076.

Warren, M. M., Gibb, A. P., and Walsh, T. S. (2005). Antibiotic prescription practice in an intensive care unit using twice-weekly collection of screening specimens: a prospective audit in a large UK teaching hospital. *J Hosp Infect* **59**(2), 90–95.

Veleminsky, M., and Tosner, J. (2008). Relationship of vaginal microflora to PROM, pPROM and the risk of early-onset neonatal sepsis. *Neuro Endocrinol Lett* **29**(2), 205–221.

Vergnano, S., Sharland, M., Kazembe, P., Mwansambo, C., and Heath, P. T. (2005). Neonatal sepsis: an international perspective. *Arch Dis Child Fetal Neonatal Ed* **90**(3), F220–224.

Vergnano, S., Menson, E., Kennea, N., Embleton, N., Russell, A. B., Watts, T., Robinson, M. J., Collinson, A., and Heath, P. T. (2010). Neonatal infections in England: the NeonIN surveillance network. *Arch Dis Child Fetal Neonatal Ed* **96**(1), F9–F14.

Verweij, P. E., Van Belkum, A., Melchers, W. J., Voss, A., Hoogkamp-Korstanje, J. A., and Meis, J. F. (1995). Interrepeat fingerprinting of third-generation cephalosporin-resistant Enterobacter cloacae isolated during an outbreak in a neonatal intensive care unit. *Infect Control Hosp Epidemiol* **16**(1), 25–29.

Westerbeek, E. A., van den Berg, A., Lafeber, H. N., Knol, J., Fetter, W. P., and van Elburg, R. M. (2006). The intestinal bacterial colonisation in preterm infants: a review of the literature. *Clin Nutr* **25**(3), 361–368.

Willing, B. P., Russell, S. L., and Finlay, B. B. (2011). Shifting the balance: antibiotic effects on host-microbiota mutualism. *Nat Rev Microbiol* **9**(4), 233–243.

Wolfs, T. G., Derikx, J. P., Hodin, C. M., Vanderlocht, J., Driessen, A., de Bruine, A. P., Bevins, C. L., Lasitschka, F., Gassler, N., van Gemert, W. G., and Buurman, W. A. (2010). Localization of the lipopolysaccharide recognition complex in the human healthy and inflamed premature and adult gut. *Inflamm Bowel Dis* **16**(1), 68–75.

von Eiff, C., Peters, G., and Heilmann, C. (2002). Pathogenesis of infections due to coagulase-negative staphylococci. *Lancet Infect Dis* **2**(11), 677–685.

Wong-Beringer, A., Hindler, J., Loeloff, M., Queenan, A. M., Lee, N., Pegues, D. A., Quinn, J. P., and Bush, K. (2002). Molecular correlation for the treatment outcomes in bloodstream infections caused by Escherichia coli and Klebsiella pneumoniae with reduced susceptibility to ceftazidime. *Clin Infect Dis* **34**(2), 135–146.

Voor, T., Julge, K., Bottcher, M. F., Jenmalm, M. C., Duchen, K., and Bjorksten, B. (2005). Atopic sensitization and atopic dermatitis in Estonian and Swedish infants. *Clin Exp Allergy* **35**(2), 153–159.

Wynn, J. L., Neu, J., Moldawer, L. L., and Levy, O. (2009). Potential of immunomodulatory agents for prevention and treatment of neonatal sepsis. *J Perinatol* **29**(2), 79–88.

Xu, R. J. (1996). Development of the newborn GI tract and its relation to colostrum/milk intake: a review. *Reprod Fertil Dev* **8**(1), 35–48.

Yurdakok, M. (1998). Antibiotic use in neonatal sepsis. *Turk J Pediatr* **40**(1), 17–33.

9. SUMMARY IN ESTONIAN

Varase sepsise riskiga vastsündinute limaskestade kolonisatsioon ja selle osatähtsus invasiivse infektsiooni prognoosimisel

Inimese seederakti mikrofloora on mitmekesise koostisega dünaamiline ja tasakaalustatud ökosüsteem (Mackie *et al*.,1999; Morelli, 2008; Mshvildadze *et al*., 2010a). Üsasiseselt on loote seedetrakt mikroobivaba ning esmane limaskestade kokkupuude mikroobidega toimub sünnituse ajal ja vahetult peale sündi (Srivastava *et al*. 2007; Mshvildadze *et al*., 2008). Tavaliselt on esmased koloniseerijad oportunistlikud aeroobsed ja fakultatiivselt aeroobsed mikroobid (Almuneef *et al*., 2001; Rautava *et al*., 2002; Hallstrom *et al.* 2004; Morelli, 2008; Enck *et al.,* 2009; Ogra, 2010). Ühest küljest valmistavad nad soole ette nn kasulikele anaeroobsetele mikroobidele nagu *Bacteroides* spp*., Bifidobacterium* spp. ja *Clostridium* spp. (Morelli, 2008; Srivastava *et al*., 2007; Thompson-Chagoyan *et al*., 2007) ning neil on positiivne mõju immuunsuse väljakujunemisel. Samuti mõjutavad nad lapse organismi arengut ning ka üldist tervislikku seisundit hilisemas eas. Teisest küljest võivad needsamad mikroobid translotseeruda keha steriilsetesse piirkondadesse ja põhjustada infektsiooni, seda eriti enneaegsetel ja immuunkomprimeeritud lastel (Cartelle *et al*., 2004; Donskey, 2004; Graham *et al.,* 2007; Miranda *et al*., 2009).

Limaskestade kolonisatsiooni mikroobidega mõjutavad mitmed erinevad sünnieelsed, -aegsed ja -järgsed tegurid, nagu ema tervis, sünniviis, gestatsiooni aeg, sünnikaal, antibakteriaalne ravi, toit, keskkond jne. Kui ajaliste tervete laste esmane kolonisatsioon pärineb peamiselt ema mikrofloorast (Mackie *et al.,* 1999; Morelli, 2008), siis intensiivravi osakondades viibijatel on selle allikaks suures osas haigla keskkond (Saiman, 2002; Srivastava *et al.*2007). Samas on haiglas ringlevad tüved sageli virulentsemad ja ka ravimresistentsed (Ayan *et al.,* 2003; Bagattini *et al*., 2006; Mears *et al*., 2009, Millar *et al.,* 2008; Simmonds *et al.,* 2009).

Siiski on teadmised oportunistlike mikroobide varast kolonisatsiooni mõjutavate faktorite mõjust ja nende omavahelistest seostest ebapiisavad, kuna varasemates uuringutes on peamiselt jälgitud kas üksiku konkreetse mikroobi kolonisatsiooni või vaid mõnede riskifaktorite mõju (DeMan *et al.,* 2000; Bernard *et al.,* 2001; Asindi *et al.*, 2002; Cartelle *et al.,* 2004; Hallstrom *et al,* 2004). Kolonisatsiooni mõjutavate faktorite omavahelisi seoste ja nende olulisuse uurimist takistab enamasti väike uuritavate arv (Bennet *et al.,* 1982; Gewolb et al., 1999; Bennet *et al.,* 2002; Bonnemaison et al, 2003; Taipiainen et al., 2006) või vaid univariantse statistika kasutamine (Baltimore *et al.*, 1989; Bennet *et al.*, 2002; Jaureguy *et al.*, 2004). Täpsemad teadmised mikrofloora kujunemisest ja seda mõjutavatest teguritest intensiivravi osakondades aitaksid parandada infektsioonikontrolli strateegiaid ja mõjutada kolonisatsiooni teket lapse tervisest lähtuvalt (Donskey, 2004; Miranda *et al.,* 2009).

Kuna enneaegsetel ja nõrgestatud immuunsüsteemiga vastsündinutel on suur oht mikroobide translokatsiooniks ja infektsiooni kujunemiseks, on empiiriline antibakteriaalne ravi laialt kasutusel. Käesoleval ajal on peamiselt soovitatavad kaks ravirežiimi: ampitsilliin või penitsilliin koos gentamütsiiniga (Schrag *et al*., 2002; Metha, 2005; Joung, 2008). On leitud, et ampitsilliin, kui laiema toimespektriga antibiootikum mõjutab küll enam Gram-negatiivset mikrofloorat, kuid samas soodustab ravimresistentsete enterobakterite teket ning kolonisatsiooni pärmseentega (DeMan *et al.,* 2000; Bonnemaison *et al*., 2003; Stoll *et al*., 2005). Teisest küljest on penitsilliinil normaalsele mikrofloorale säästvam toime (Bennet *et al*., 1982). Ampitsilliini + gentamütsiini ja penitsilliini + gentamütsiini kliinilist efektiivsust jälgiv uuring tõestas, et mõlemad ravimi kombinatsioonid on sarnase efektiivsuse ja ohutusega (Metsvaht *et al.,* 2010). Samas vastavad kaht režiimi võrdlevad uuringud mikrofloora seisukohast puuduvad.

Aastakümneid on klinitsistid ja teadlased püüdnud prognoosida sooletrakti, neelu või naha järelvalvekülvide järgi sepsise teket. Need katsed on andnud enamasti statistiliselt ebarahuldavaid tulemusi, kuid teatud olukordades osutunud siiski kasulikuks. Kolm aastakümmet tagasi hindasid Harris (1976) ja Sprunt (1978) kaasautoritega sel otstarbel võetud järelevalve külve kulukaks ja aeganõudvaks, kuid puudub ülevaade nende kulukusest käesoleval ajal (Blot *et al*., 2005; Mcginigle *et al.,* 2008). On teadlasi, kes soovitavad kord või kaks nädalas koguda järelevalvekülve haiglainfektsiooniriskiga patsientide väljaselgitamiseks (Pierro *et al* 1998; Silvestri *et al.,* 1999; Shankar *et al*., 2001). Kahe varasema vastsündinute intensiivravi osakonnas läbiviidud uuringu tulemuste järgi olid siin kogutud järelvalvekülvidel madal sensitiivsus (16% – 56%), spetsiifilisus (38% – 82%) ja positiivne prognostiline väärtus (PPV) 5% – 7,7%) (Evans, 1988; Choi *et al.*, 2008). Hiljuti Smith kaasautoritega (2010) poolt läbiviidud uuringus, millega püüti prognoosida gentamütsiin-resistentsete Gram-negatiivsete mikroobide poolt põhjustatud sepsist väikese sünnikaaluga lastel, olid samad arvtulemused tunduvalt paremad; vastavalt 100%, 98%, and 90%. Nii kõrged väärtused lubvad arvata, et kasutati patsiendi-, mitte aga külvidepõhist analüüsi. Seega on järelevalvekülvide sepsist prognoosiv väärtus siiani ebaselge.

Uurimistöö eesmärk

Uurimistöö peamiseks eesmärgiks oli selgitada, kuidas vastsündinute varase sepsise empiiriliseks raviks kasutatavad ravirežiimid (ampitsilliin ja penitsilliin kombinatsioonis gentamütsiiniga) mõjutavad varast laste rektaalset ja ninaleelu kolonisatsiooni oportunistlike mikroobidega ja kumba nest seoses eelnenuga tuleks eelistada.

Konkreetsed eesmärgid:

- iseloomustada vastsündinute intensiivravi osakonnas viibivate vastsündinute limaskestade varast kolonisatsiooni aeroobsete oportunistlike bakterite ja *Candida spp*. esindajatega;
- selgitada, millised perinataalsed, neonataalsed ja keskkonna faktorid seda eeskätt mõjutavad;
- võrrelda kahe varase sepsise empiiriliseks raviks kasutatava ravirežiimi (penitsilliin või ampitsilliin koos gentamütsiiniga) mõju aeroobse, fakultatiivselt aeroobse ja ampitsilliin-resistentse rektaalse mikrofloora kujunemisele teiste võimalike kolonisatsiooni mõjutavate faktorite foonil;
- hinnata seost kolonisatsiooni ja infektsioone põhjustavate mikroobitüvede vahel;
- hinnata kaks korda nädalas kogutud järelvalvekülvide prognostilist väärtust sepsisesse haigestumisel.

Materjal ja meetodid

Uuring viidi läbi prospektiivse, kahekeskuselise, klaster-randomiseeritud avatud uuringuna Tallinna Lastehaigla ja Tartu Ülikooli Kliinikumi laste- ja vastsündinute intensiivravi osakondades 02.08.2006 kuni 30.11.2007. Uuringu esimesel perioodil kasutati ühes keskuses ampitsilliini koos gentamütsiiniga ja teises penitsilliini koos gentamütsiiniga. Peale seda, kui mõlemas osakonnas oli materjal kogutud pooltelt vastava ravirežiimiga lastelt (vajalik laste arv saadi võimsusanalüüsi alusel), ravirežiimid osakondades vahetati. Uuringusse kaasati 276 (prognoosi uuringusse 278) vastsündinut, kes olid osakonda saabudes nooremad kui 72 tundi, vajasid sepsise kahtluse või riskifaktorite olemasolu tõttu empiirilist antibakteriaalset ravi ning viibisid osakonnas vähemalt 24 tundi.

Limaskestade kolonisatsiooni hindamiseks koguti nina-neelu ja perirektaalne kaabe patsiendi osakonda saabumisel ja edaspidi kaks korda nädalas (esmaspäev ja neljapäev) kuni osakonnas viibimise lõpuni või kuni 60-da ravipäevani. Tampoone materjaliga säilitati –20 °C juures ja külvati kord nädalas veri-, MacConkey ja Sabouraud agarile ning MacConkey agarile, kuhu oli lisatud 16 µg/ml ampitsilliini. Veri- ja MacConkey agarit inkubeeriti 37 °C juures 24–48 tundi ja Sabouraud agarit 25 °C juures kuni nädal. Igast morfoloogiliselt erinevalt kasvanud pesast saadud mikroobid värviti Grami järgi ja identifitseeriti kasutades erinevaid söötmeid ja biokeemilisi meetodeid (API 20E ja API STAF). *S. aureus* ja MRSA määrati *nuc*- ja *mecA*-geeni olemasolu järgi. Veri võeti haiglasse saabumisel ja edasi kahtlusel invasiivsele infekstioonile ning selle uuringud viidi läbi TÜ Kliinikumi laboris. Translokatsiooni (*K. pneumoniae, E. cloacae, Acinetobacter* spp., MRSA) ja puhangutüvede (*K. pneumoniae*) klonaalsuse selgitamiseks kasutati molekulaarseid meetodeid, vastavalt pulssvälja geel-elektoforeesi (PFGE) ja amplifitseeritud fragmendi pikkuse polümorfismi (AFLP).

Kolonisatsiooni riskifaktorite selgitamiseks registreeriti järgmised perinataalsed parameetrid: ema vanus, antibakteriaalne ravi raseduse ja sünnituse ajal, lootevete puhkemine varem kui 18 tundi enne sünnitust, mitmiksünd ja sünniviis. Igalt patsiendilt koguti järgmised andmed: sünnikaal, gestatsiooni vanus, kopsude kunstliku ventilatsiooni rakendamine ja selle kestvus, intensiivravi osakonda saabumise vanus ja seal viibimise aeg, osakond, uuringu periood, empiiriline antibakteriaalne ravi ja selle kestvus, lisavajadus laia toimespektriga antibakteriaalne ravi järele ja selle kestvus (karbapeneemid, kolmanda ja neljanda põlvkonna tsefalosporiinid ja beeta-laktamaas resistentsed penitsilliinid), tsentraalsete intravenoossete veeni- ja intraarteriaalste kanüülide kasutamine ja selle kestvus ning lapse toitmise viis (täielik parenteraalne toitmine, rinnapiim, piimasegu).

Kolonisatsiooni riskifaktorite selgitamiseks kasutati ühest ja mitmest logistilist regressiooni. Selgitamaks kahe erineva empiirilise ravirežiimi mõju kolonisatsiooni tekkele ja kolonisatsioonitihedusele (kolonisatsioonipäevi 100 voodipäeva kohta) kasutati osakonnale ja uuringuperioodile kohandatud mitmest multifaktoriaalset hierarhilist segamudelit. Järelevalvekülvide väärtuse hindamiseks sepsise prognoosimisel arvutati eraldi nii külvipõhine kui ka patsiendipõhine sensitiivsus, spetsiifilisus ning PPV negatiivne prognostiline väärtus (NPV).

Tulemused

Varase empiirilise antibakteriaalse ravi mõju seedetrakti kolonisatsioonile

Esmased seedetrakti koloniseerijad mõlemas ravigrupis olid Gram-positiivsed mikroobid, neile järgnesid Gram-negatiivsed ja pärmid. Kahe erineva ravigrupi (ampitsilliin koos gentamütsiiniga *vs* penitsilliin koos gentamütsiiniga) vahel esinesid mõningad erinevused. Nimelt oli penitsilliini saanud grupis rohkem:

(1) enterokokkidega koloniseerunud vastsündinuid nii 6.–9. elupäeval (OR = 2,99; 95% CI 1,23–7,25) kui ka 13.–16. elupäeval (OR = 3,33; 95 % CI 1,29–8,63);
(2) *S. aureus*'ga koloniseerunuid 3.–5. elupäeval (p = 0,012) ja
(3) *Acinetobacter* spp-ga koloniseerunuid 13.–16. elupäeval (6 *vs* 0; p=0,009) ning sama mikroobi ampitsilliin-resistentsete tüvedega koloniseerunuid 10.–12. (6 *vs* 0; p=0,045) ja 13.–16. (4 *vs* 0; p=0,046) elupäeval.

Vastupidiselt meie hüpoteesile kolonisatsioon ampitsilliin-resistentsete tüvedega kahe ravirežiimi vahel oluliselt ei erinenud.

Kahe erineva ravirežiimi ja kolonisatsiooni tekke vahelise seose jälgimisel selgus, et ampitsilliinravi grupis võrreldes penitsilliinraviga oli enam koloniseerunuid *S. haemolyticus'*e (erinevus +0,7; p=0,039) ja *S. hominis'*ga (+1,9; p=0,003) kuid vähem enterokokkide (–1,2; p<0,001) ja *S. aureus*'ga (–1,9; p=0,006).

Ampitsilliini grupis oli statistiliselt suurem koloniseeritud päevade arv 100 voodipäeva kohta *K. pneumoniae* (erinevus +6,6 päeva; p = 0.012), ampitsilliin-resistentsete *Serratia* spp (+3,6; p = 0,012), *S. haemolyticus'*e (+11,4; p = 0,001), *S. hominis'*e (+6,3; p = 0,001) ja *Candida* spp'ga (+6,1; p=0,020), kuid väiksem *S. epidermidis*'e (–7,1; p = 0,073), *Enterococcus* spp (–13,0; p = 0,001), ampitsilliin-resistentse *Acinetobacter* spp (–2,5; p = 0,001) ja piiripealselt ka *S. aureus'ga* (–2,8; p=0,052).

Limaskestade kolonisatsiooni mõjutavad riskifaktorid

Uuringu käigus koguti 276lt antud uuringusse kaasatud patsiendilt 1250 rektaalset ja 1205 ninaneelu kaabet, milledes vastavalt 55,8% ja 42,8% esinesid Gram-negatiivsed mikroobid. Rektaalsetest isolaatidest 73,4% ja nasofarüngeaalsetest 60,2% olid ampitsilliin-resistentsed. 49-l (17,8%) patsiendil ilmnes rektaalne kolonisatsioon *Candida* spp-ga.

Kasutades mitmest logistilist regressiooni hinnati 22 riskifaktori (millest 3 olid seotud keskkonnaga, 5 emaga ja 14 vastsündinuga) mõju rektaalse ja ninaneelu mikrofloora kujunemisele. Leiti, et erinevatel faktoritel on sarnane mikroobispetsiifiline mõju kahele erinevale piirkonnale. Siiski esines ka kahe erineva piirkonna (ninaneel, *rectum*) valikuline mõju mikroobsele kolonisatsioonile. See väljendus statistiliselt 1,5–2,5 korda suuremas šansis rektaalseks (võrreldes ninaneeluga) kolonisatsiooniks enterobakteritega (va *Serratia* spp) ja väiksemas šansis (OR=0,52; 95% CI 0,30–0,89) kolonisatsiooniks *Acinetobacter* spp'ga.

Kõige rohkem mõjutas oportunistlike mikroobidega kolonisatsiooni patsiendi haiglasviibimise kestus. Olenevalt mikroobiliigist suurendas iga haiglasveedetud päev kolonisatsiooniriski 5–8% võrra. *E. coli*, *K. oxytoca* ja *C. albicans*'i kolonisatsiooni mõjutasid peamiselt emaga seotud või varased neonataalsed riskifaktorid. Näiteks oli rinnapiimaga toidetutel võrreldes parenteraalset toitmist saajatega üheksa korda suurem šanss rektaalseks kolonisatsiooniks *E. coli* (OR=8,90; 95% CI 2,27–34,70) ning vastavalt viis ja kuus korda suurem šanss *K. oxytoca* nii tundlike kui ampitsilliin-resistentsete tüvedega (vastavalt OR=5,00; 95% CI 1,57–15,92 ja OR=5,81; 95% CI 1,65–20,44). Vastupidiselt omas parenteraalne toitmine võrreldes rinnapiimaga suuremat riski kolonisatsiooniks *C. albicans*'ga (OR=2,91; 95% CI 1,02–8,26).

Ka oli vaginaalse sünnituse järgselt võrreldes keisrilõikega üle kahe korra suurem šanss kolonisatsiooniks *C. albicans*'ga (OR=2,27; 95% CI 1,06–4,86) ja samuti soodustas vaginaalne sünd nii rektaalset kui nasofarüngeaalselt kolonisatsiooni *E. coli*'ga (OR= 4,44; 95% CI 1,97–10,00 ja OR=2,69; 95% CI 1,01–7,18). Lootevete varase puhkemise korral oli vastavalt neli ja viis korda suurem šanss ninaneelu kolonisatsiooniks *K. oxytoca* nii tundlike kui ka ampitsilliin-resistentsete tüvedega (OR=4,33; 95% CI 1,54–12,21 ja OR=4,83; 95% CI 1,44–16,18) ning neli ja kuus korda suurem šanss *E. coli* nii tundlike kui ka ampitsilliin-resistentsete tüvedega (OR=4,43; 95% CI 1,66–11,78 ja OR=5,94;

95% CI 1,58–22,42). Ajalised lapsed (gestatsiooniaeg ≥37) võrreldes enneaegsetega (gestatsiooniaeg 29–36 nädalat) koloniseerusid rektaalselt sagedamini *E. coli* (OR=3,13; 95% CI 1,18–8,27) ja *K. oxytoca* resistentsete tüvedega (OR=3,13; 95% CI 1.02–9,57).

Kolonisatsioon *K. pneumoniae*, *E. cloacae* ja mitte-albicans-*Candida spp.* pärines peamiselt haigla keskkonnast ja/või oli seotud lapse enneaegsusega. Näiteks oli suurem šanss koloniseeruda *K. pneumoniae* ja *E. cloaceae*'ga nii rektaalselt kui ka nasofarüngeaalselt nii tundlike kui ka ampitsillin-resistentsete tüvedega peamiselt ühes osakonnas. Samuti soodustas *E. cloacae* (OR=8,48; 95% CI 1,04–68,89) ja *K. pneumoniae* (OR=9,00; 95% CI 1,93–42,08) nasofarüngeaalset kolonisatsiooni sügav enneaegsus (gestatsiooniaeg ≤28 *vs* ≥37nädalat). Päevade arv, mil kasutati tsentraalseid veenikateetreid, tõstis rektaalse kolonisatsiooni šanssi *K. pneumoniae* (OR=1,06; 95% CI 1,01–1,12) ja mitte-albicans-*Candida*'ga (OR=1,06; 95% CI 1.00–1,13).

Järelvalvekülvide prognostiline väärtus vastsündinute intensiivravi osakondades

Järelevalvekülvide väärtust Gram-negatiivsete mikroobide poolt tekitatud hilise sepsise tekke ennustamiseks hinnati 278-lt patsiendilt kogutud 1250 rektaal-, 1153 ninaneelu ja neile lisatud 60 mikroobse kasvuga trahhea materjali alusel. Neid võrreldi 555 steriilsetest kehavedelikest (514 verest, 36 liikvorist, 5 mujalt) võetud materjaliga. Kokku analüüsiti 2108 limaskesta ja invasiivsete külvide paari. Gram-negatiivseid mikroobe isoleeriti 171 (62%) patsiendil; kusjuures kasv esines 55,4% rektaal-, 48,4% nina-neelu ja 5,8% invasiivsetes külvides. Erinevate hinnatud liikide hulk ühel patsiendil oli 1 – 8 (mediaan 3).

Kolonisatsiooni ja infektsiooni seoste leidmiseks jälgiti 27-l Gram-negatiivsete sepsisega patsiendil (mediaan tekkeks oli 13.-elupäev) identsete mikroobide esinemist limaskestadel. 22-l juhul koloniseerusid hilist sepsit põdevad lapsed fenotüüpiliselt sarnaste tekitajatega, kusjuures kolonisatsioon esines 2–36 päeva enne sepsist. Genotüüpiline koloniseeruvate ja sepsist põhjustavate tüvede sarnasus (*K. pneumoniae, E. cloaceae, A. baumannii*) kinnitati pulssvälja geelelektroforeesiga.

Üldine järelvalvekülvide prognostiline väärtus oli madal – sensitiivsus, spetsiifilisus, NPP ja PPV vastavalt 27%, 66%, 4% ja 94%. Võrreldes mittefermenteerivate mikroobidega olid enterobakteritel kõrgemad sensitiivsuse (37% *vs* 11%) ja PPV (3,5% *vs* 0,8%) näitajad. Samuti olid enterobakterite väärtused kõrgemad kõigil uuritud ajahetkedel. Parim oli sensitiivsus kui kolonisatsioon tekkis 2–6 päeva enne sepsist. Mitmese segamudelite analüüsi kasutamisel selgus, et sepsise prognoosimist aitab parandada positiivsete järelvalvekülvide suurem arv enne infektsiooni (iga positiivse külvi kohta OR= 4.66; 95% CI 2.28–9.55; p=<0,0001) ja patsiendi väiksem eluiga (iga elupäeva kohta OR=0,94; 95% CI 0,88–1.00; p=0.047).

Klonaalne seos limaskestade kolonisatsiooni põhjustavate ja infektsioonitüvede vahel leidis eriti kinnitust ühes osakonnas esinenud *K. pneumoniae* ja teises osakonnas esinenud kahe väiksema MRSA puhangu ajal. Esimesena mainitud mikroobi poolt tingitud puhang kestis rohkem kui kuus kuud. Sel perioodil koloniseerus sama mikroobitüvega 23 patsienti, kellest viiel ilmnes vereringe infektsioon.

Järeldused

- Esmased varase sepsise kahtlusega intensiivravi osakonnas viibivate vastsündinute limaskestade koloniseerijad olid Gram-positiivsed bakterid, milledest kogu uuringu vältel domineeris KoNS. Esimese elunädala lõpuks oli üle 90%-i uuritavatest koloniseerunud Gram-positiivsete ja ligi kolmandik Gram-negatiivsete ampitsilliin-resistentsete mikroobidega. Rektaalne ja nina-neelu kolonisatsioon Gram-negatiivsete mikroobidega oli sarnane, kuid enterobaktereid, va *Serratia* spp., leidus rohkem rektaalselt ja *Acinetobacter* spp. esindajaid isoleeriti rohkem nina-neelust. Sagedasemad nii tundlikud kui ka ampitsilliin-resistentsed Gram-negatiivsed mõlema limaskesta koloniseerijad olid *E. cloaceae, K. pneumonia, K. oxytoca* ja *Acinetobacter* spp. Sagedasemad oportunistlikud koloniseerijad olid ka sagedasemad vereringe infektsiooni põhjustajad.
- Limaskestade kolonisatsiooni *E. coli, K. oxytoca* ja *C. albicans*'ga mõjutavad peamiselt emapoolsed ja perinataalsed faktorid ning kolonisatsioon *K. pneumoniae, E. cloaceae,* MRSA ja mitte-albicans-*Candida*'ga on peamiselt tingitud haigla keskkonnast ja lapse enneaegsusest. Riskifaktorite mõju (näiteks haiglas viibimise aeg, osakond, gestatsiooni vanus, sünniviis) sooletrakti ja nina-neelu kolonisatsioonile nii antibiootikum-tundlike kui ka ravimresistentsete tüvede osas on sarnane, omavahel tihedalt seotud ja mikroobispetsiifiline ning seetõttu on neid võimalik üldistada mõlemale piirkonnale.
- Võrreldes ampitsilliini kui laiema toimespektriga antibiootikumi mõju penitsilliiniga, kui neid kasutatakse empiiriliselt varase sepsise ohu korral, võib väita, et esmamainitu toimet Gram-negatiivsele mikrofloorale ja ravimresistentsete tüvede tekkele on ilmselt ülehinnatud. Peamised erinevused nende režiimide osas olid järgmised:
  a. Ampitsilliinravi korral võrreldes penitsilliiniga koloniseeruti rohkem *S. haemolyticus*'e ja *S. hominis*'ga, kuid vähem *S. aureus*'e ja enterokokkidega.
  b. Ampitsilliinravi saavad patsiendid võrreldes penitsilliinravi saavatega omasid suuremat kolonisatsioonitihedust *K. pneumoniae,* ampitsilliin-resistentse *Serratia* spp. ja *Candid*a spp.'ga, kuid neil oli väiksem kolonisatsioonitihedus ravimresistentse *Acinetobacter spp*.'ga.
  c. Kolonisatsioon *Candida* spp. ja eelpool mainimata Gram-negatiivstete mikroobide osas oli mõlemas ravigrupis sarnane.

Seega empiirilise ravi mõju uurimisel soovimatu mikrofloora kujunemisele ei tohiks keskenduda vaid kahe erineva ravirežiimi (ampitsilliin ja penitsilliin koos gentamütsiiniga) eelistamise tõestamisele. Ravimrežiimi eelistus peaks pigem baseeruma lokaalsel olukorral: millised on vastava osakonna sagedasemad infektsioonitekitajad ja nende ravimtundlikkus. Samuti tuleks arvestada enneaegsust ja muid lapsest sõltuvaid faktoreid.

- Eelnev limaskestade kolonisatsioon Gram-negatiivsete mikroobidega (va *E. cloacae, Serratia* spp., *Acinetobacter* spp.) ja MRSA-ga võib viia invasiivse infektsiooni väljakujunemisele. Siiski on järelevalvekülvide sensitiivsus, spetsiifilisus ja PPV ennustamaks invasiivse infektsiooni teket enterobakterite korral mõõdukas, kuid mittefermenteerivate mikroobide jaoks suboptimaalne.
- Regulaarselt kogutud järelevalvekülvid on väheefektiivsed, samas ka küllalt kulukad ja aeganõudvad ennustamaks intensiivravi osakondades viibivatel vastsündinutel sepsise teket. Järelvalvekülve on mõttekas koguda teatud spetsiifiliste mikroobide poolt põhjustatud haiglainfektsioonide puhangute korral (nt. MRSA, *Klebsiella pneumoniae*). See aitaks parandada infektsioonikontrolli meetmeid ja oleks abiks empiirilise ravi valikul.

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PUBLICATIONS

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2005–2012	University of Tartu, Faculty of Medicine, PhD student

Professional employment

1983–	Tartu Health Care College (Tartu Medical School) – lecturer of pathology, infectious diseases, enternal diseases and hospital infections
2009–2011	University of Tartu, Institute of Microbiology – laboratory assistant

Scientific work

The mean subject of research work has been development of rectal and nasopharyngeal microbiota in neonates who needs intensive care. Several publications and presentations in international scientific conferences.

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Member of Estonian Society for Infection Control (ESIC).

Publications

Parm, Ü.; Metsvaht, T.; Sepp, E.; Ilmoja, ML.; Pisarev, H.; Pauskar, M.; Lutsar, I. (2011). Risk factors associated with gut and nasopharyngeal

colonisation by common Gram-negative species and yeasts in neonatal intensive unit patients. Early Human Development, 87: 391–399.
Parm, Ü.; Metsvaht, T.; Sepp, E.; Ilmoja, ML.; Pisarev, H.; Pauskar, M.; Lutsar, I. (2010). Impact of empiric antibiotic regimen on bowel colonisation in neonates with suspected early onset sepsis. European Journal of Clinical Microbiology & Infectious Diseases, 29: 807–816.
Parm, Ü.; Metsvaht, T.; Sepp, E.; Ilmoja, ML.; Pisarev, H.; Pauskar, M.; Lutsar, I. (2011). Mucosal surveillance cultures in predicting Gram-negative late onset sepsis in neonatal intensive care units. Journal of Hospital Infection, 78: 327–332.
Metsvaht, T.; Ilmoja, ML.; Parm, Ü.; Maipuu, L.; Merila, M.; Lutsar, I. (2010). Comparison of ampicillin plus gentamicin vs. penicillin plus gentamicin in empiric treatment of neonates at risk of early onset sepsis. Acta Paediatrica, 99: 665–672.
Metsvaht, T.; Pisarev, H.; Ilmoja, ML.; Parm, U.; Maipuu, L.; Merila, M.; Muursepp, P.; Lutsar, I. (2009). Clinical parameters predicting failure of empirical antibacterial therapy in early onset neonatal sepsis, identified by classification and regression tree analysis. BMC Pediatrics, 9: 72.
Metsvaht, T.; Ilmoja, ML.; Parm, Ü.; Merila, M.; Maipuu, L.; Müürsepp, P.; Julge, K.; Sepp, E.; Lutsar, I. (2011) Ampicillin versus penicillin in the empiric therapy of ELBW neonates at risk of early onset sepsis. Pediatrics International 2011 (*accepted*).

Published textbooks for students of health care college and analogous material

Parv, V; Lutsar, I; Parm, Ü; Alliksoo, S.; Ehin, R. 2009. Gripp (32 pp)
Parm, Ü.; Parv, V. 2006. Nakkushaigused ja epidemioloogia. (239 pp)
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Parm, Ü.; Parv, V. 2002. Nakkushaigused. (228 pp)
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colonisation by common Gram-negative species and yeasts in neonatal intensive unit patients. Early Human Development, 87: 391–399.
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Parv, V; Lutsar, I; Parm, Ü; Alliksoo, S.; Ehin, R. 2009. Gripp. (32 lk)
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