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Department of Microbiology, Institute of Biomedicine and Translational Medicine, Faculty of Medicine, University of Tartu, Estonia

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Supervisor: Professor Irja Lutsar, MD, PhD
Department of Microbiology
Institute of Biomedicine and Translational Medicine
University of Tartu, Tartu, Estonia

Reviewers: Professor Tanel Tenson, PhD
Institute of Technology
University of Tartu, Tartu, Estonia

Associate Professor Heili Varendi, MD, PhD
Department of Pediatrics
Institute of Clinical Medicine
University of Tartu, Tartu, Estonia

Opponent: Associate Professor Micael Widerström, MD, PhD
Department of Clinical Microbiology
Umeå University, Umeå, Sweden

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IV Soeorg H, Metsvaht T, Eelmäe I, Merila M, Treumuth S, Huik K, Jürna-Ellam M, Ilmoja ML, Lutsar I. The role of breast milk in the colonization of neonatal gut and skin with coagulase-negative staphylococci. Pediatr Res. 2017 Jun 30. [Epub ahead of print]

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- Soeorg H, Metsvaht T, Eelmäe I, Treumuth S, Merila M, Ilmoja ML, Lutsar I. *Staphylococcus epidermidis* nosocomial strain in breast milk of mothers of preterm neonates. The 11th International Meeting on Microbial Epidemiological Markers, March 9–12, 2016, Estoril, Portugal.
- Soeorg H, Metsvaht T, Eelmäe I, Treumuth S, Merila M, Ilmoja ML, Lutsar I. Molecular characterization of *Staphylococcus epidermidis* colonizing skin and gut of preterm neonates. The 26th European Congress of Clinical Microbiology and Infectious Diseases, April 9–12, 2016, Amsterdam, the Netherlands.
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- Soeorg H, Metsvaht T, Eelmäe I, Treumuth S, Merila M, Ilmoja ML, Lutsar I. Genetic relatedness of *Staphylococcus epidermidis* colonizing gut and skin of neonates and breast milk. The 35th Annual Meeting of the ESPID, May 23–27, 2017, Madrid, Spain.
- Soeorg H, Metsvaht T, Eelmäe I, Treumuth S, Merila M, Ilmoja ML, Lutsar I. Mother's own breast milk is a source of *mecA*-positive *Staphylococcus epidermidis*. The 35th Annual Meeting of the ESPID, May 23–27, 2017, Madrid, Spain.

ABBREVIATIONS

ACME	–	arginine catabolic mobile element
agr	–	accessory gene regulator
BM	–	breast milk
BW	–	birth weight
CC	–	clonal complex
CI	–	confidence interval
CoNS	–	coagulase-negative staphylococci
CVC	–	central venous catheter
EOS	–	early-onset sepsis
GA	–	gestational age
GC	–	genetic cluster
IQR	–	interquartile range
IS*256*	–	insertion sequence 256
IVC	–	intravascular catheter
LOS	–	late-onset sepsis
MALDI-TOF	–	matrix-assisted laser desorption/ionisation time-of-flight
MIC	–	minimum inhibitory concentration
MLST	–	multilocus sequence typing
MLVA	–	multilocus variable-number tandem-repeat analysis
MT	–	MLVA-type
NEC	–	necrotizing enterocolitis
NICU	–	neonatal intensive care unit
OR	–	odds ratio
PCR	–	polymerase chain reaction
PFGE	–	pulsed field gel electrophoresis
PSM	–	phenol-soluble modulin
SCC*mec*	–	staphylococcus cassette chromosome *mec*
SID	–	Simpson's index of diversity
SLV	–	single locus variant
ST	–	sequence type
VLBW	–	very low birth weight

1 INTRODUCTION

Coagulase-negative staphylococci (CoNS) are one of the most important colonizers of human skin (Grice and Segre 2011). Although considered as harmless bacteria for healthy people, increasing number of immunocompromised patients and use of invasive foreign devices have resulted in the emergence of CoNS as the commonest causative agents of nosocomial bloodstream infections (ECDC 2013). The majority of infections are caused by strains adapted to hospital environment (Tolo et al. 2016) that colonize patients soon after hospitalization (Widerström et al. 2016). The endogenous nature of CoNS infections, the adaptation of infection-causing strains to hospital environment and the ubiquity of CoNS complicate the prevention of infections and warrant understanding of their characteristics, but also of the colonization with and the spread of potentially invasive strains.

Preterm neonates due to immature skin (Evans and Rutter 1986) and gut barriers (Weaver et al. 1984), undeveloped immune system (Björkqvist et al. 2004; Granslo et al. 2013) and requirement for invasive devices are among those particularly vulnerable to infections caused by CoNS. Approximately half of late-onset sepsis (LOS) cases, i.e. sepsis occurring at age of >72 hours of life, are caused by CoNS (Mitt et al. 2014; Gowda et al. 2017). Although LOS caused by CoNS is associated with low mortality (1–2%) (Bizzarro et al. 2015; Gowda et al. 2017), it increases the risk of long-term consequences such as chronic lung disease (Anderson-Berry et al. 2011) and neurodevelopmental impairment (Alshaikh et al. 2014).

As skin is considered to be the main source of invasive CoNS strains, prevention of LOS has focused mostly on the care of intravascular catheters (IVC) that has been successful in decreasing the incidence of LOS in some centres (Kaplan et al. 2011). Molecular epidemiological studies have revealed that CoNS infections are mostly caused by a few predominant strains indicating transmission of infection-causing strains (Klingenberg et al. 2007; Chong et al. 2016). As neonatal intensive care unit (NICU) staff carries such strains on their hands, hand hygiene is an important infection prevention measure (Hira et al. 2010). Still, CoNS remain the commonest causative agents of healthcare-associated infections in neonates (Zingg et al. 2017). As a result, the hypothesis that CoNS may invade bloodstream by translocating from gut (Costa et al. 2004), where they are common in preterm neonates (Hira et al. 2013), has gained more recognition in recent years (Cossey et al. 2014; Shaw et al. 2015; Butin et al. 2017b), but the gut colonization with CoNS and its relationship with LOS have been poorly studied in preterm neonates. Therefore, a call to improve the knowledge of pathophysiology of bloodstream infections in neonates has been made (Decousser 2017).

Mother's own breast milk (BM) is considered to be the best feeding option for neonates, because of its several short- and long-term benefits (Eidelman et al. 2012), including the reduced incidence of LOS (Corpeleijn et al. 2012). The

latter could be attributable, at least in part, to bacteria in BM that are less pathogenic compared with those causing infections in NICU (Cossey et al. 2013). Indeed, gut of healthy term neonates becomes colonized with CoNS strains with low pathogenic potential present in BM (Jiménez et al. 2008b), but this has not been studied in preterm neonates. On the other hand, BM may be a source of infection, exemplified by LOS cases due to *S. aureus* strains colonizing mother's BM (Kayıran et al. 2014). To improve clinical practice such that benefits of BM-feeding are increased and risks decreased, it is warranted to understand the colonization of BM with CoNS and its influence on preterm neonates.

Although outcome of very preterm neonates has improved during recent decades in Estonia (Toome et al. 2012), overall rate of bloodstream infections in NICU remains high (Mitt et al. 2014). To improve the treatment of preterm neonates, researchers at the University of Tartu have performed studies on optimized antibacterial treatment in neonates (Metsvaht et al. 2010), control of outbreaks (Adamson et al. 2012), surveillance of bloodstream infections in NICUs (Mitt et al. 2014) and relationship between gut colonization and LOS caused by Gram-negative microorganisms (Parm et al. 2011). By determining the relationship between colonization and development of LOS caused by CoNS in neonates hospitalized in NICU, this thesis is built upon and continues the above-described research on neonates.

2 REVIEW OF THE LITERATURE

2.1 Overview of CoNS

Staphylococcus spp. are Gram-positive cocci and as of 2017, this genus is represented by 52 different species (http://www.bacterio.net/index.html, last accessed January 18, 2017) (Euzéby 1997). In human medicine, classification of staphylococci is based on clinical and diagnostic importance. Staphylococci are divided into coagulase-positive staphylococci and CoNS and both of these groups further into human- and animal-associated staphylococci (Becker et al. 2014). In humans, coagulase-positive staphylococci are almost exclusively confined to *S. aureus* that is one of the most important pathogen. Human-associated CoNS are divided into three groups. *S. epidermidis*-like group contains the most commonly present species, such as *S. epidermidis*, *S. haemolyticus*, *S. hominis*, *S. capitis*. *S. saprophyticus* and *S. lugdunensis* form the second and third group, respectively, because the former mostly causes acute urethritis and the latter due to its high pathogenicity resembles *S. aureus* rather than other CoNS.

CoNS colonize skin of all healthy people, whereas the commonest species is *S. epidermidis*, followed by *S. hominis*, *S. capitis*, *S. haemolyticus*, *S. lugdunensis* (Cavanagh et al. 2016). They also frequently colonize gut (Vitali et al. 2014), particularly in neonates in Western countries (Adlerberth et al. 2006). On the other hand, since 1980s CoNS have emerged as important pathogens, mostly in immunocompromised patients and in patients with foreign devices (Ahlstrand et al. 2014). The species most commonly causing infections are the same as the commonest colonizing ones, i.e. *S. epidermidis* predominates and is followed by *S. haemolyticus*, *S. hominis*, *S. capitis* (Argemi et al. 2015). In preterm neonates (Mitt et al. 2014; Gowda et al. 2017) and hemato-oncological patients (Weisser et al. 2017) CoNS cause up to half of all infections. Foreign-body related infections include those associated with IVCs (Lepainteur et al. 2013), cerebrospinal fluid shunts (Conen et al. 2008), prosthetic joints (Hellmark et al. 2013), prosthetic heart valves (García de la Mària et al. 2015) and cardiac devices (Gordon et al. 2012). In addition, CoNS can cause ocular infections, such as conjunctivitis and endophtalmitis (Flores-Páez et al. 2015), native heart valve infective endocarditis (Petti et al. 2008), and skin and soft tissue infections, particularly *S. lugdunesis* (Böcher et al. 2009).

2.2 Colonization and infection by CoNS in neonates

2.2.1 CoNS as colonizers of gut and skin of neonates

CoNS are one of the most ubiquitous bacteria colonizing skin and gut of neonates with prevalence up to 100% in both body sites already within the first few days of life (Carr and Kloos 1977; Adlerberth et al. 2006; Hira et al. 2013). The most common species is *S. epidermidis* that can be isolated from the majority of

neonates, while other species occur less frequently (Table 1). More than half of neonates are colonized with at least 2–3 different species (Center et al. 2003; Aujoulat et al. 2014). The species distribution is generally similar in different skin areas (Carr and Kloos 1977; Keyworth et al. 1992; Marchini et al. 2005) as well as body sites, such as skin and gut (Center et al. 2003; Hira et al. 2013). In addition to being the most commonly isolated species, *S. epidermidis* and *S. haemolyticus* are the most persistent colonizers, i.e. present for longer period of time, and can be isolated from multiple samples from one neonate, whereas other species, e.g. *S. warneri*, *S. hominis*, are transient colonizers and can be isolated only from few samples (Parm et al. 2010; Aujoulat et al. 2014).

Table 1. Prevalence of colonization of gut and skin of neonates with different CoNS species

	Preterm neonates hospitalized in the NICU		Healthy term neonates	
Gut		% of neonates colonized[1]		% of neonates colonized[2]
	S. epidermidis	49…100%	***S. epidermidis***	86%
	S. haemolyticus	30–35…87%	***S. haemolyticus***	–
	S. hominis	9…20%	***S. hominis***	55%
	S. capitis	–	***S. capitis***	–
	S. warneri	6–7…53%	***S. warneri***	–
Skin		% of all CoNS isolates[3]		% of samples[4]
	S. epidermidis	33…82%	***S. epidermidis***	44…88%
	S. haemolyticus	5…19%	***S. haemolyticus***	40…50%
	S. hominis	2…6%	***S. hominis***	4…55%
	S. capitis	<1…15%	***S. capitis***	5%
	S. warneri	4…24%	***S. warneri***	4%

–, the presence of this species has not been reported
Data from
[1]Gewolb et al. (1999), Moles et al. (2013), Aujoulat et al. (2014), Said et al. (2014)
[2]Jiménez et al. (2008b), Martín et al. (2012)
[3]Keyworth et al. (1992), Szewczyk et al. (2000), de Silva et al. (2001), Hira et al. (2013)
[4]Carr and Kloos (1977), Marchini et al. (2005)

The distribution of CoNS species is influenced in part by feeding regimen and surrounding environment. The proportion of stool samples containing *S. epidermidis*, the commonest CoNS in mother's own BM, is significantly higher in BM-fed compared with formula-fed neonates (el-Mohandes et al. 1993; Jiménez et al. 2008b). In contrast, *S. haemolyticus* that is a rare species in BM colonizes more frequently formula-fed compared with BM-fed neonates (39–61% vs 8–13%) (Gewolb et al. 1999; Harmsen et al. 2000; Parm et al. 2015). If CoNS species other than *S. epidermidis* cause outbreak or endemic spread in NICU, they also colonize hospitalized neonates more commonly than usually reported, as has been described for *S. caprae* or *S. haemolyticus* (Jain et al. 2004; Ross et al. 2005; Kornienko et al. 2016).

2.2.1.1 Origin of CoNS colonizing neonates

Colonization of neonate with CoNS is initiated *in utero*. Viable CoNS can be cultured from up to 14% of amniotic fluid (Zbinden et al. 2011; Collado et al. 2016) and 28% of placenta samples (Onderdonk et al. 2008; Collado et al. 2016). As a result, skin sampled within the first few minutes of life or at Caesarean section is already colonized with staphylococci (Dominguez-Bello et al. 2010; Zbinden et al. 2011). Amniotic fluid swallowed by foetus is one of the first inoculum for gut microbiota, because microbial communities in meconium resemble those in amniotic fluid and placenta rather than those in maternal colostrum, faeces, vaginal or oral cavity (Ardissone et al. 2014; Collado et al. 2016). Despite relatively low abundance of staphylococci in amniotic fluid (<1% of all microbial communities in pooled amniotic fluid samples) (Collado et al. 2016), more than half of meconium samples from preterm and term neonates contain CoNS (Jiménez et al. 2008c; Moles et al. 2013).

Colonization process continues during birth. Vaginally delivered neonates are exposed to staphylococci in vaginal cavity (Stokholm et al. 2014), while those born by Caesarean section to staphylococci in the operating room environment, most likely shed from the skin of surgeons or nurses (Shin et al. 2015). Due to the lack of exposure to vaginal microbiota, the microbial communities colonizing neonates delivered by Caesarean section resemble those on human skin (Dominguez-Bello et al. 2010). Indeed, gut of neonates delivered by Caesarean section is often enriched with CoNS in contrast to neonates delivered vaginally (Bäckhed et al. 2015; Madan et al. 2016), although this has not been unequivocally demonstrated (Martin et al. 2016; Stokholm et al. 2016).

After delivery, mother's own BM serves as one of the most important sources of CoNS for gut of neonates enriching it with staphylococci (Balmer and Wharton 1989; Martin et al. 2016), although no difference in the abundance of staphylococci between formula- and BM-fed neonates has been observed (Cossey et al. 2014; Madan et al. 2016). Strain-level studies have confirmed neonatal gut colonization with CoNS strains present in BM in at least half of healthy term BM-fed neonates (Jiménez et al. 2008b; Martín et al. 2012). Mother's skin is also a considerable source of colonizing CoNS for neonates as a result of skin-to-skin contact between parents and neonates (Lindberg et al. 2004; Lamy Filho et al. 2015).

2.2.1.2 Differences in colonization between preterm and term neonates

Despite the commonality of such major sources of colonizing CoNS, healthy term and preterm neonates differ in terms of the development of colonization with CoNS in several aspects. First, soon after birth, preterm neonates are often hospitalized in the NICU, where they are exposed to numerous potential sources of CoNS: toys placed in cots or incubators (Davies et al. 2000), the inner walls of incubators (de Goffau et al. 2011; Bokulich et al. 2013), medical equipment

that comes to contact with neonate, such as tubings of ventilators, diaphragms of stethoscopes, nasal suction catheters, pacifiers (Bokulich et al. 2013), and air (Krediet et al. 2001; Botelho et al. 2012). Such ubiquity of staphylococci in NICU environment is eventually reflected in the microbiota composition of hospitalized neonates (Brooks 2014). Second, in preterm neonates, skin-to-skin contact with their parents may last only few hours a day (Blomqvist et al. 2013), whereas 19% do not experience skin-to-skin contact at all (Catherine et al. 2016). Instead, they are exposed to and become colonized with CoNS on the hands of NICU staff (Krediet et al. 2001) that differ from strains on the hands of healthy people with no contact to hospital environment (Aiello et al. 2003; Hira et al. 2010) and mothers of hospitalized neonates (Hira et al. 2013). Third, in preterm neonates enteral feeding may be delayed (Klingenberg et al. 2012) and 8.5–12% do not receive mother's BM (Corpeleijn et al. 2012; Corpeleijn et al. 2016). Thus, their exposure to CoNS in mother's own BM is considerably reduced. Finally, preterm neonates often require antibacterial treatment that may decrease the abundance of staphylococci in gut (Greenwood et al. 2014; Arboleya et al. 2015), although not all studies have shown this (Tanaka et al. 2009; Chernikova et al. 2016; Itani et al. 2016) or instead have found enrichment of gut with CoNS after antibiotic treatment (Gibson et al. 2016).

The many characteristics of preterm neonates potentially influencing colonization with CoNS probably contribute to the controversial studies demonstrating equal abundance of staphylococci in preterm and term neonates (Arboleya et al. 2015), higher abundance in preterm (Costello et al. 2013) or, on the contrary, in term neonates (Arboleya et al. 2012). Geographical variation of the abundance of staphylococci colonizing gut of neonates (Echarri et al. 2011), temporal shifts in the prevalence of staphylococci in NICU as well as differences between NICUs (Taft et al. 2014) even further complicate identification of the factors influencing colonization.

2.2.1.3 Diversity and dynamics of CoNS colonization in neonates

The numerous sources and influencing factors may contribute to the strain-level characteristics of CoNS colonization. Multiple distinct strains of one CoNS species colonize one body site at the same time (Bialkowska-Hobrzanska et al. 1993; Eastick et al. 1996; Jiménez et al. 2008b), indicating genetic diversity. On the other hand, colonization is clonal, as one strain may colonize several body sites simultaneously (Bialkowska-Hobrzanska et al. 1993; Eastick et al. 1996). Finally, the failure to isolate the same strain from multiple samples from one body site (Hall et al. 1990) and replacement of initially found CoNS with distinct strains (Björkqvist et al. 2010; Ternes et al. 2013), indicate dynamic colonization at the strain level. Although the apparent changes may result from genetic diversity that complicates characterization of all the strains present in one sample, wide fluctuations in the count of CoNS, particularly on skin (Keyworth et al. 1992; Eastick et al. 1996), may indicate exposure to various sources and thus diversification of colonizing CoNS. Rapid decline of one

S. epidermidis strain and simultaneously occurring proliferation of another (Sharon et al. 2013) corroborates the changing nature of staphylococcal colonization.

2.2.2 CoNS as colonizers of BM

Staphylococci also belong to the core microbiome of BM being the most abundant species (Hunt et al. 2011; Boix-Amorós et al. 2016) and present in 80–100% of BM samples from healthy lactating women (Heikkilä and Saris 2003; Jiménez et al. 2008b; Boix-Amorós et al. 2016), whereas their prevalence is as high as 92% already in colostrum sampled within 6 hours after delivery (Jiménez et al. 2008a). As in other body sites, the predominant CoNS species is *S. epidermidis*, mostly followed by *S. hominis* and *S. lugdunensis* or *S. capitis* (Table 2). Interestingly, *S. haemolyticus* is rare colonizer of BM, often constituting only small proportion of all CoNS isolates in BM (<5%) (Heikkilä and Saris 2003; Marín et al. 2009; Jost et al. 2013; Filleron et al. 2014).

Table 2. Prevalence of colonization of BM with different CoNS species

	Healthy lactating mothers	
BM		% of samples[1]
	S. epidermidis	73…100%
	S. hominis	11…28%
	S. capitis	3…20%
	S. lugdunensis	5…16%
	S. haemolyticus	3%
	S. warneri	3%

[1] Data from Heikkilä and Saris (2003), Jiménez et al. (2008a), Jiménez et al. (2008b), Delgado et al. (2009), Boix-Amorós et al. (2016)

2.2.2.1 Factors influencing colonization of BM with CoNS

The composition of BM between mothers and between samples from one mother is varying in terms of the abundance and the presence of staphylococci (Hunt et al. 2011; Boix-Amorós et al. 2016; Cabrera-Rubio et al. 2016), exemplified by the count of staphylococci differing more than 10,000 times between individuals (Obermajer et al. 2014; Almutawif et al. 2017). Of various factors potentially influencing the composition of BM, gestational age (GA) has not been demonstrated to have impact on the count or abundance of staphylococci (Khodayar-Pardo et al. 2014; Urbaniak et al. 2016), ranging from $10^{2.4}$ to $10^{3.4}$ cfu/mL in mothers of healthy term as well as preterm neonates (Delgado et al. 2009; Martín et al. 2012; Khodayar-Pardo et al. 2014; Moles et al. 2015b). Controversial results have been obtained about the influence of delivery mode (Khodayar-Pardo et al. 2014; Cabrera-Rubio et al. 2016; Urbaniak et al. 2016) and lactation stage (Jost et al. 2013; Khodayar-Pardo et al.

2014; Moles et al. 2015b) on the count of staphylococci, but one study group has reported higher count, abundance and the prevalence of staphylococci in overweight mothers (Cabrera-Rubio et al. 2012; Collado et al. 2012). Although CoNS may contaminate BM from maternal skin after or during milk expression, the hygiene regimen (strict or standard) or the method of expressing BM (by pump or manually) do not influence the count of CoNS or other Gram-positive bacteria (Boo et al. 2001; Haiden et al. 2016). Even discarding the first few millilitres does not affect the count of skin commensals in BM (Carroll et al. 1980). Thus, currently the factors influencing the composition of BM are poorly studied arising in part from the scarcity of studies on mothers of preterm neonates. Furthermore, considering high genetic diversity of CoNS in BM, exemplified by the presence of up to eight different strains in one sample (Jiménez et al. 2008b; Delgado et al. 2009), the influencing factors of colonization may be evident only at the strain level.

2.2.2.2 Origin of CoNS in BM

The colonization of BM with CoNS has prompted question about their origin in BM. To prevent contamination from skin, BM is mostly collected aseptically with no bacteria present on the nipple areola before BM collection, but staphylococci still predominate in BM (Perez et al. 2007), arguing against contamination from skin. Furthermore, while propionibacteria are common and streptococci rare colonizers of skin, the scarcity of the first and the abundance of the latter in BM suggest other sources of BM-colonizing bacteria than skin (Hunt et al. 2011). Retrograde flow of milk in the mammary gland during suckling (Ramsay et al. 2004) suggests infant's oral microbiota as a source of bacteria in BM, but such transfer of bacteria during breastfeeding does not explain the composition of BM in mothers of preterm neonates because they are not able to suckle from breast. The presence of anaerobic bacteria, such as bifidobacteria and clostridia, that are typical for human gut, in BM (Boix-Amorós et al. 2016) suggests that bacteria in mammary gland and subsequently in BM may originate from mother's gut. Indeed, transfer of bacteria from mother's gut to mammary gland by peripheral blood mononuclear cells, so-called enteromammary pathway, has been demonstrated (Perez et al. 2007). However, staphylococci in maternal gut are subdominant and genotyping revealed that *S. epidermidis* in BM was distinct from that in maternal gut (Jost et al. 2014). Nevertheless, staphylococci are also among the most dominant bacteria in breast tissue in non-lactating women undergoing breast surgery (Urbaniak et al. 2014), suggesting their native presence in the microbiota of BM.

2.2.3 CoNS as causative agents of LOS

LOS is most frequently defined as neonatal sepsis occurring later than 72 hours after birth and is associated with microorganisms acquired postnatally from hospital or community (Dong and Speer 2015). The incidence of LOS is inversely associated with GA as well as birth weight (BW), occurring in 51.2%, 32.5%, 10.2% and 2.2% of neonates with BW of 501–750 g, 751–1000 g, 1000–1499 g, 1500–2500 g, respectively (Vergnano et al. 2011; Boghossian et al. 2013). CoNS are the most common causative agents of LOS in preterm neonates, accounting for 39–49% of all cases in developed countries (Mitt et al. 2014; Gowda et al. 2017). The majority of LOS caused by CoNS occur in the second week of life with median and the highest incidence at the age of 8–10 days (Hemels et al. 2011; Jean-Baptiste et al. 2011; Vergnano et al. 2011).

The most common CoNS species causing LOS is *S. epidermidis* accounting for approximately two thirds of CoNS LOS cases, followed by *S. haemolyticus*, *S. hominis, S. capitis* and less commonly *S. warneri* (Figure 1). Similar distribution of CoNS species among blood culture isolates from neonates and infants of age less than 90 days was also demonstrated in years 2010–2012 in Tartu University Hospital (Padari et al. 2016). Nevertheless, occasionally CoNS species such as *S. haemolyticus* (Jain et al. 2004; Pereira et al. 2014), *S. capitis* (Rasigade et al. 2012; Ben Said et al. 2016), *S. hominis* (Chaves et al. 2005) and even *S. caprae* (Ross et al. 2005) may cause uncommonly many LOS cases.

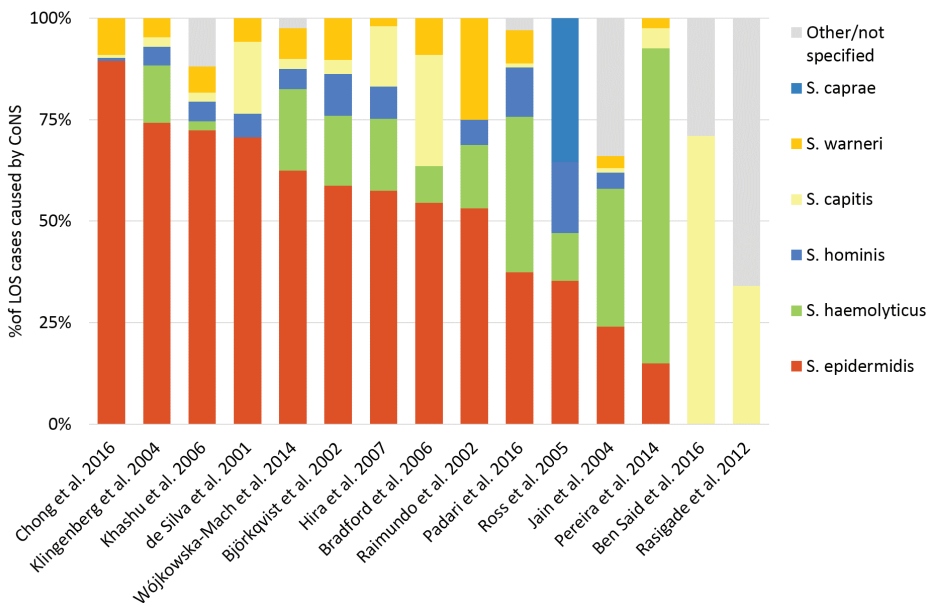


Figure 1. Species distribution among CoNS isolates causing LOS in neonates in different studies

2.2.3.1 Clinical relevance of different CoNS species

Differences in the pathogenicity of different CoNS species probably have not been conclusively determined, because of frequent lack of identification of CoNS to the species level (Shaw et al. 2015) and small number of LOS cases due to species other than *S. epidermidis*, but overall, CoNS species most likely have similar potential to cause infection. This is supported by the similar distribution of CoNS species among invasive (Figure 1) and colonizing isolates (Table 1) and blood culture isolates considered as contaminants (de Silva et al. 2002; Klingenberg et al. 2004; Rasigade et al. 2012), suggesting that probability to invade bloodstream reflects the extent of dissemination rather than the potential to cause infection. Second, no association has been observed between the species of CoNS causing LOS and the host response measured as the value of C-reactive protein (Klingenberg et al. 2005) or the duration of LOS (Dimitriou et al. 2011). On the other hand, in a NICU where 71% of LOS due to CoNS were caused by *S. capitis*, death or severe morbidity was more common in neonates with LOS caused by *S. capitis* compared with other CoNS (57% vs 32%, respectively) (Ben Said et al. 2016). These differences might have been due to a spread of a particular *S. capitis* clone (Ben Said et al. 2016), highlighting that pathogenicity could be attributable to the characteristics of genotype rather than species.

Interestingly, *S. haemolyticus* has been reported to be more common among invasive isolates and blood culture contaminants compared with other CoNS species in more premature neonates (median GA 26–27.5 weeks vs 29–35 weeks, respectively) (Björkqvist et al. 2002; Klingenberg et al. 2007). Similarly, prevalence of colonization with *S. haemolyticus* was higher in neonates with GA <31 weeks compared with GA >30 weeks (Kazembe et al. 1993). The higher rate of infection and colonization in the most premature neonates could be attributable to simultaneously occurring spread of a *S. haemolyticus* strain adapted to hospital environment and increase in the number of very low birth weight (BW <1500 g) (VLBW) neonates (Björkqvist et al. 2002). In addition, more frequent use of antibiotics in VLBW compared with less premature neonates (Neubert et al. 2010) could favour colonization with *S. haemolyticus* that is usually the most resistant CoNS species (Hira et al. 2007).

2.3 Virulence, resistance and molecular epidemiology of CoNS

Characterization of the infecting and colonizing CoNS isolates beyond the species level has revealed several virulence-related factors probably contributing to the development of infection and antimicrobial resistance mechanisms conferring survival in the presence of antibiotics. Predominance of certain genetic lineage among infection-causing isolates and transmission of strains causing the

majority of LOS cases between neonates highlight the role of infection control measures in the prevention of infections. Such knowledge could help to reduce the burden of LOS caused by CoNS and promote less harmful colonization in preterm neonates.

2.3.1 Virulence factors and -related genes

CoNS possess several factors that enable them to adhere to and persist on skin and mucosal surfaces. These characteristics are also likely involved in the development of infection by conferring attachment to the host or invasive devices, immune evasion and the regulation of gene expression.

2.3.1.1 Biofilm formation

Biofilm consists of agglomerated bacteria in extracellular material composed of proteins, polysaccharides, extracellular DNA and is required for adhesion to host tissues, protection from human immune system and resistance to anti-bacterial agents (Otto 2012). The majority of CoNS are capable of producing biofilm under some growth conditions (Bradford et al. 2011) and most of the genes encoding various proteins necessary for biofilm production are present in infection-causing as well as colonizing CoNS (Jiménez et al. 2008a; Hell et al. 2013; Salgueiro et al. 2017). Still, the *icaA* gene located in the *ica*-operon encoding one of the main component of the extracellular matrix in biofilm, polysaccharide intercellular adhesion (Heilmann et al. 1996), is associated with strains spreading in hospital and thereby potentially contributing to the infection development (Tolo et al. 2016). The carriage rate of the *icaA* gene is 31–75% in invasive CoNS (Hira et al. 2010; Dimitriou et al. 2011), 31–53% in isolates colonizing hospitalized neonates (de Silva et al. 2002; Salgueiro et al. 2017), 29–44% in those colonizing healthy term neonates (Jiménez et al. 2008b; Hell et al. 2013) and 11–23% in isolates from BM of healthy lactating mothers (Jiménez et al. 2008a; Jiménez et al. 2008b; Delgado et al. 2009).

After formation of biofilm its disruption and detachment of bacterial cells by enzymes (proteases, nucleases) and surfactants called phenol-soluble modulins (PSMs) results in dissemination of bacterial cells and thereby infection to other body sites (Otto 2012). The expression of extracellular degradative enzymes and PSMs is regulated by the accessory gene regulator (*agr*) (Yao et al. 2006). The *agr* system in *S. epidermidis* is represented by three distinct types (Dufour et al. 2002) and due to its role in the regulation of virulence, it has been suggested that the type of *agr* may influence the development of infection. Indeed, in adults, nearly 90% of infection-causing isolates were *agr* type I and none type III, but of commensals from healthy volunteers type I constituted only half and type III was represented by quarter of isolates (Carmody and Otto 2004).

2.3.1.2 Arginine catabolic mobile element (ACME)

ACME is a genetic island containing the *arc* gene cluster (ACME type I), the *opp3* gene cluster (type III) or both (type III) (Diep et al. 2006; Miragaia et al. 2009). The *arc* gene cluster encodes enzymes for arginine deiminase pathway that catalyse conversion of arginine to carbon dioxide, adenosine triphosphate and ammonia, conferring resistance to acidic conditions, such as on human skin (Lindgren et al. 2014). The *opp3* gene cluster encodes oligopeptide permease that has many functions, including uptake of nutrients, expression of virulence-related factors and sensing of environmental conditions (Borezée-Durant et al. 2009). Such functions of ACME elements could contribute to advantage for colonization and transmission (Miragaia et al. 2009). The carriage rate of ACME in *S. epidermidis* causing bloodstream infections in neonates is 23–43%, whereas the most common is type I (29–84% of ACME elements), followed by type II (15–57%) and type III (0–14%) (Granslo et al. 2010; Svensson et al. 2011; Salgueiro et al. 2017). Higher prevalence of ACME among blood culture contaminants compared with invasive isolates suggests that ACME is an indicator of benign commensal flora (Granslo et al. 2010), but it was present only in 5% of *S. epidermidis* from nares of neonates hospitalized in the NICU (Salgueiro et al. 2017).

2.3.1.3 Insertion sequence IS256

IS*256* is a transposable element considered to contribute to adaptation and thereby successful spread in hospital environment by conferring genome flexibility (Schoenfelder et al. 2010). IS*256* can regulate the expression of biofilm by inserting itself into the genes in the *ica*-operon (Ziebuhr et al. 1999), may have role in regulating antibiotic-resistance genes (Ziebuhr et al. 2000; Weisser et al. 2010), spontaneous chromosomal deletions and homologous recombination events (Schoenfelder et al. 2010). The carriage rate of the element in CoNS from blood cultures of neonates is 73–94% in *S. epidermidis* and it is also found in *S. haemolyticus*, *S. capitis* and *S. warneri* (Bradford et al. 2006; Foka et al. 2006; Hell et al. 2013). In contrast, the element was not present in *S. epidermidis* from skin of healthy neonates (Hell et al. 2013), but no data about the presence of IS*256* in isolates colonizing preterm neonates or mother's BM are available.

2.3.1.4 Clinical relevance of virulence-related genes

The higher carriage rate of the *icaA* gene and IS*256* in invasive compared with CoNS isolates from healthy term neonates and/or healthy lactating mothers, suggests the role of these factors in pathogenicity. However, the contribution of the *icaA* gene or IS*256* to the development of LOS is still arguable. First, the CoNS isolates contaminating blood cultures carry the *icaA* gene or IS*256* at similar rate compared with those causing LOS (de Silva et al. 2002;

Klingenberg et al. 2005; Bradford et al. 2006). Second, higher carriage rate of the *icaA* gene in skin-colonizing CoNS does not increase the risk of CoNS infection in hospitalized neonates (de Silva et al. 2002). Finally, the carriage rate of the *icaA* gene are similar in CoNS causing persistent or non-persistent bacteremia (Eftekhar and Speert 2009; Dimitriou et al. 2011). In line with ACME being more common in commensals rather than invasive strains, there is no difference in the C-reactive protein value, cytokine level or granulocyte or monocyte burst between neonates with LOS caused by ACME-positive or ACME-negative *S. epidermidis* isolates (Granslo et al. 2010).

The lack of association between clinical significance and the virulence-related factors may be attributable in part to the same source, probably NICU environment, of invasive strains, blood culture contaminants and those colonizing preterm neonates (Krediet et al. 2001). In line with this, recent analysis of *S. epidermidis* genetic markers concluded that the *icaA* gene, IS*256* and ACME were associated with the source of isolates rather than prediction of the development of infection – the *icaA* gene and IS*256* are more common in hospital isolates, while ACME in carriage isolates (Tolo et al. 2016). Nevertheless, even if infecting isolates are derived at random these are more likely to be selected from strains adapted to hospital setting, i.e. carrying the virulence-related genes (Tolo et al. 2016).

2.3.2 Antimicrobial resistance of CoNS

Frequent use of antibiotics may have contributed to high rate of resistance, particularly to beta-lactams and aminoglycosides in CoNS causing infections in NICU (Table 3). CoNS colonizing neonates hospitalized in the NICU also exhibit high rate of resistance, but on the contrary, isolates from healthy term neonates and healthy lactating mothers are mostly susceptible, reflecting low resistance in CoNS in community (Cavanagh et al. 2016).

2.3.2.1 Resistance to beta-lactams

Resistance to beta-lactam antibiotics is mediated by the *mecA* gene that encodes penicillin-binding protein 2a with low affinity for beta-lactams conferring resistance to nearly all antibiotics of this class. In accordance with resistance rates to oxacillin (Table 3), the carriage rate of the *mecA* gene is 81–98% in invasive CoNS (Klingenberg et al. 2005; Hira et al. 2007; Hira et al. 2010; Dimitriou et al. 2011), 76–95% in isolates colonizing preterm neonates (Krediet et al. 2001; Hira et al. 2013; Ternes et al. 2013), 33% in those colonizing healthy term neonates (Jiménez et al. 2008b), 13–44% in isolates from BM of healthy lactating mothers (Jiménez et al. 2008a; Jiménez et al. 2008b; Delgado et al. 2009).

Table 3. Antimicrobial resistance rates of CoNS from various sources

	Blood cultures from neonates with LOS[1]	Gut/skin of neonates hospitalized in NICU[2]	Gut/skin of healthy term neonates[3]	BM of healthy mothers[4]
Penicillin	94–100%	96–99%	46%	35–75%
Oxacillin	81–94%	75–97%	11–43%	<10–39%
Gentamicin	61–94%	43–88%	0–15%	12–13%
Clindamycin	11–27%	87%	–	8–16%
Ciprofloxacin	0–58%	4–63%	–	4–6%
Rifampicin	0–21%	12%	15%	<10%
Vancomycin	0–8%	0%	0%	0%

–, data not available
Data from
[1]Vermont et al. (1998), Villari et al. (2000), de Silva et al. (2001), Jain et al. (2004), Klingenberg et al. (2005), Hira et al. (2007), Qu et al. (2010), van den Hoogen et al. (2010), Lutsar et al. (2014), Pereira et al. (2014)
[2]Scheifele and Bjornson (1988), Bialkowska-Hobrzanska et al. (1993), Eastick et al. (1996), de Silva et al. (2001), Jain et al. (2004)
[3]Scheifele and Bjornson (1988), Jiménez et al. (2008b), Hell et al. (2013), El-Kersh et al. (2016)
[4]Jiménez et al. (2008a), Jiménez et al. (2008b), Delgado et al. (2009)

Table 4. The composition and the prevalence of the most common SCC*mec* types in CoNS from various sources

	***ccr* gene complex**	***mec* gene complex**	**Blood cultures from neonates with LOS[1]**	**Gut/skin of neonates hospitalized in NICU[2]**	**Gut/skin of healthy term neonates[3]**	**BM of healthy mothers[4]**
Type I	*ccrA1, ccrB1*	class B	17–25%	34%	–	–
Type II	*ccrA2, ccrB2*	class A	4–33%	1%	–	–
Type III	*ccrA3, ccrB3*	class A	8–57%	4%	–	13%
Type IV	*ccrA2, ccrB2*	class B	14–33%	7%	100%	87–100%
Type V	*ccrC*	class C2	8–15%	1–17%	–	–
NT			17–90%	54–86%	–	–

NT, non-typeable; –, this type has not been reported
Data from
[1]Svensson et al. (2011), Pereira and Cunha (2013), Salgueiro et al. (2014), Saffari et al. (2016), Salgueiro et al. (2017)
[2]Ternes et al. (2013), Salgueiro et al. (2017)
[3]Jiménez et al. (2008b)
[4]Jiménez et al. (2008a), Jiménez et al. (2008b), Delgado et al. (2009)

The *mecA* gene is carried on mobile genetic element termed staphylococcus cassette chromosome *mec* (SCC*mec*) that contains the *mec* gene complex

(consists of the *mecA* and regulatory genes) and the *ccr* gene complex (encodes recombinases that integrate or excise SCC*mec* from the chromosome). Based on the *mec* and *ccr* gene complex SCC*mec* elements are divided into types (IWG-SCC 2009), of which the commonest in CoNS isolates from invasive infections or colonization sites are I to V (Table 4). However, non-typeability of SCC*mec* types in CoNS is common, mostly due to the presence of multiple different *mec* or *ccr* gene complexes (Ibrahem et al. 2008).

2.3.2.2 Factors influencing colonization with resistant strains

Preterm neonates hospitalized in the NICU are colonized with CoNS strains with high resistance rate that are most likely acquired from NICU. The proportion of multidrug-resistant CoNS among skin-colonizing isolates rises from 40% at the age of 24 hours to 90% at the end of the first week of life (Hira et al. 2013), whereas the risk is increased by longer NICU stay (Ternes et al. 2013). Such strains could originate in part from skin of NICU staff, considering high resistance rate in CoNS from the hands of NICU staff exceeding that in CoNS from healthy volunteers (Aiello et al. 2003; Hira et al. 2010) and being comparable to infection-causing strains (Cimiotti et al. 2007). Frequent antibacterial treatment may also contribute to the colonization of neonate with resistant strains, suggested by the increase in the abundance of the *mecA* gene in gut (Gibson et al. 2016) and the risk of colonization with CoNS with reduced susceptibility to vancomycin (Center et al. 2003) after administration of antibiotics.

On the other hand, another study found no effect of antibacterial treatment on skin colonization with antibiotic-resistant CoNS (Keyworth et al. 1992). Such controversial results may be explained by skin-to-skin contact with mothers who are colonized with CoNS exhibiting low resistance rate (Hira et al. 2013) and thereby may enrich the microflora of preterm neonates with susceptible strains (Lamy Filho et al. 2015).

Colonization of healthy term neonates and their mothers with resistant staphylococci may also be influenced by hospitalization as well as antibacterial treatment. Mothers may acquire resistant staphylococcal strains during hospitalization for delivery that may subsequently appear in BM (Novak et al. 2000) and colonize gut of their healthy term neonates (Benito et al. 2015). Intrapartum antibacterial treatment of mothers may increase the prevalence of colonization of BM (Roca et al. 2016) or gut of their healthy term neonates (Jauréguy et al. 2004) with resistant staphylococcal strains.

2.3.2.3 Clinical relevance of antimicrobial resistance

Recent analysis of *S. epidermidis* strains showed that the *mecA* gene is one of the few genetic markers that is associated with the risk of infection development (Tolo et al. 2016). This could be in part related to production of PSM-mec, a PSM toxin encoded within SCC*mec* elements in about 65% of methicillin-resistant *S. epidermidis*, that increases pathogenicity by contributing to

bacteremia, mortality, cytokine storm, evasion of neutrophil killing and resistance to bactericidal properties of human blood (Qin et al. 2017). In line with this, in preterm neonates CoNS causing LOS compared with blood culture contaminants are more often resistant to oxacillin and carry more frequently the *mecA* gene (Klingenberg et al. 2005), although such differences were not corroborated in other studies (de Silva et al. 2001; Qu et al. 2010). In addition, *mecA*-positive CoNS isolates elicited higher C-reactive protein values compared with *mecA*-negative isolates, although it was suggested to result from delay in adequate antibacterial treatment (Klingenberg et al. 2005). Still, skin colonization with CoNS resistant to larger number of antibiotics increases the risk of development of LOS (de Silva et al. 2001).

2.3.3 Molecular epidemiology of CoNS

CoNS from blood cultures of neonates with LOS often belong to certain strains that cause the majority of LOS cases (Klingenberg et al. 2007; Chong et al. 2016), hereafter designated as predominant strains. Such predominant strains spreading simultaneously in one NICU can be confined to one major clone or several less prevalent genotypes (Figure 2A). Predominant strains may constitute different proportion of blood culture isolates, varying from equally low prevalence of several strains to the majority of all cases caused by a single clone (Figure 2A). In addition, strains indistinguishable by typing method may constitute different proportion of blood culture isolates in different NICUs (Figure 2B).

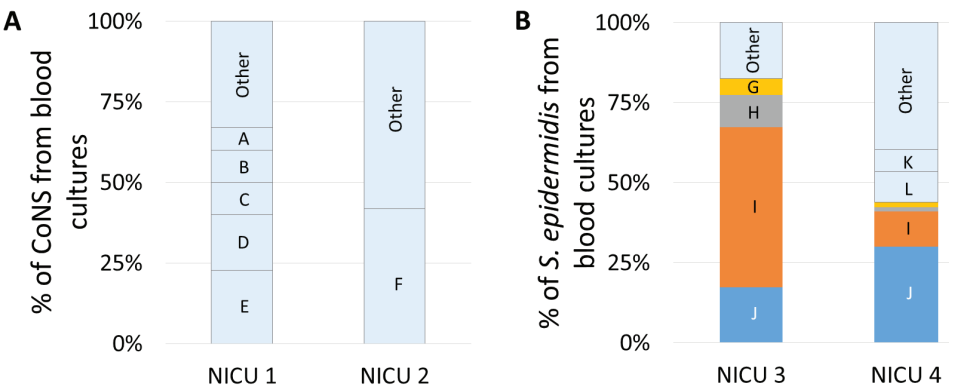


Figure 2. Different frequency distribution of CoNS strains causing LOS in NICU

Frequency distribution of predominant strains, designated by letters (different letter for different strains), and sporadic strains (Other) in (A) two NICUs, located 55 km apart, during the same time period of 1 year (data from Vermont et al. (1998)) and (B) two NICUs, located 275 km apart, during the same time period of 4.5 years (data from Chong et al. (2016)). Black and grey colours highlight that same strains were isolated from different units.

One predominant strain can spread in one unit up to 11–13 years (Krediet et al. 2004; Klingenberg et al. 2007), although the proportion it constitutes of blood culture isolates may vary from year to year (Figure 3A). The predominant strain may spread slowly and cause only small number of cases over a long period of time or after introduction to the NICU rapidly expand and cause many LOS cases (Figure 3B). On the other hand, all the strains causing LOS can be totally different when comparing time periods as few as two years apart (Raimundo et al. 2002) and there could be no evidence of clonal spread (Burnie et al. 1997). Thus, the molecular epidemiology of CoNS in NICU is complex and probably reflects the impact of the source, transmission routes and/or the characteristics of such strains.

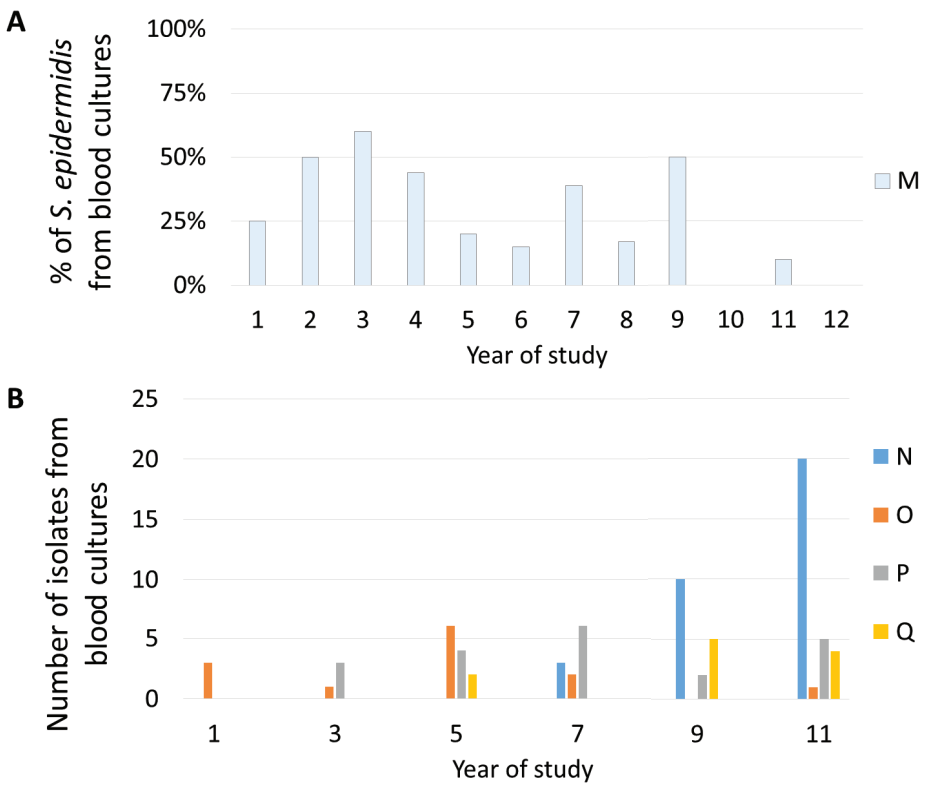


Figure 3. Different dynamics of CoNS strains causing LOS in NICU.

Dynamics of predominant strains, designated by letters (different letter for different strains), causing LOS in (A) NICU where one the most dominant strain persisted for 11 years (data from Klingenberg et al. (2007)) and (B) NICU experiencing introduction and subsequent rapid expansion of a clone (data from Krediet et al. (2004)). Black and grey colours highlight that same strains were isolated in several years.

Although mostly described in *S. epidermidis*, predominant strains can be also found among *S. haemolyticus* constituting up to 63–68% (Foka et al. 2006; Klingenberg et al. 2007; Dimitriou et al. 2011) and in *S. capitis* up to 57–100% of isolates of this species (de Silva et al. 2001; Rasigade et al. 2012; Cui et al. 2013). Other, less commonly occurring species may also exhibit clonal spread during an outbreak, such as *S. warneri* (Cimiotti et al. 2007), *S. hominis* (Chaves et al. 2005) and even *S. caprae* (Ross et al. 2005).

The presence of predominant strains suggests probable transmission, such as from NICU staff to neonates or between neonates, although this remains elusive when only blood cultures are available. Changes in the hand hygiene policy after identification of the presence of predominant strains on the hands of NICU staff may reduce transmission and result in decline in the number of infections caused by the predominant strain (Burnie et al. 1997). Similarly, elimination of disinfectant (Ben Saida et al. 2009) or almond oil bottles (Gras-Le Guen et al. 2007) contaminated with the predominant strains significantly reduced the incidence of infection. Accordingly, limiting horizontal transmission of microorganisms is considered to be the cornerstone to prevent infections in NICU (Cantey et al. 2015a).

2.3.3.1 Characteristics of predominant strains

The spread of predominant strains may be facilitated by ecological advantage over other strains, conferred partly by antibiotic resistance, biofilm production and genome flexibility. Predominant strains of various CoNS species have been demonstrated to exhibit higher rate of resistance or higher minimum inhibitory concentrations (MIC) to commonly used antibiotics, such as oxacillin or gentamicin, compared with less commonly occurring sporadic genotypes (Tabe et al. 1998; Klingenberg et al. 2007). Particularly worrisome is that successfully spreading clones of *S. haemolyticus*, *S. capitis* or *S. warneri* may even have reduced susceptibility to vancomycin in terms of higher MIC or proportion of heteroresistant strains (Tabe et al. 1998; Center et al. 2003; Rasigade et al. 2012). In addition, predominant *S. epidermidis* strains compared with sporadic ones may more frequently produce biofilm, harbour the *ica*-operon (Foka et al. 2006; Klingenberg et al. 2007) or IS*256* (Foka et al. 2006) and carry less often ACME (Granslo et al. 2010).

However, the above-mentioned characteristics are not prerequisites for successful spread, exemplified by predominant strains being less frequently multidrug-resistant (Hira et al. 2007) or having lower carriage rate of the *ica*-operon (de Silva et al. 2002) compared with sporadic strains. Population structure studies have revealed that some genetic lineages are more common among infection-causing isolates (Thomas et al. 2014; Tolo et al. 2016), suggesting that genetic background is important for invasion. According to multilocus sequence typing (MLST), the majority *S. epidermidis* causing infections belong to a successful clonal complex 2 (CC2), of which the most common strains are sequence type (ST) 2 and ST5 that have been reported to cause neonatal

infections across the world (Ibrahem et al. 2008; Chong et al. 2016; Saffari et al. 2016; Soroush et al. 2016; Salgueiro et al. 2017) (Figure 4).

Despite the predominance of ST2 and ST5, the scattering of infection-causing strains across CC2 indicates that pathogenicity is not confined to a limited number of genetic backgrounds but to a wider variety of strains. As of 2017, a total of 626 distinct STs have been described in *S. epidermidis* (https://pubmlst.org/sepidermidis/, last accessed January 26, 2017) that can be grouped in addition to CCs into six different genetic clusters (GC) of which GC5 (comprising also ST2) and GC6 (ST5) are the commonest in hospital environment (Tolo et al. 2016).

Successful and widespread genetic lineages have been also characterized in *S. haemolyticus*, disseminated in several Europe countries, such as Norway, Switzerland, United Kingdom, Belgium and Germany (Cavanagh et al. 2014), and *S. capitis* that causes infections in France, Belgium, United Kingdom, Australia (Butin et al. 2016) and Sweden (Ehlersson et al. 2017).

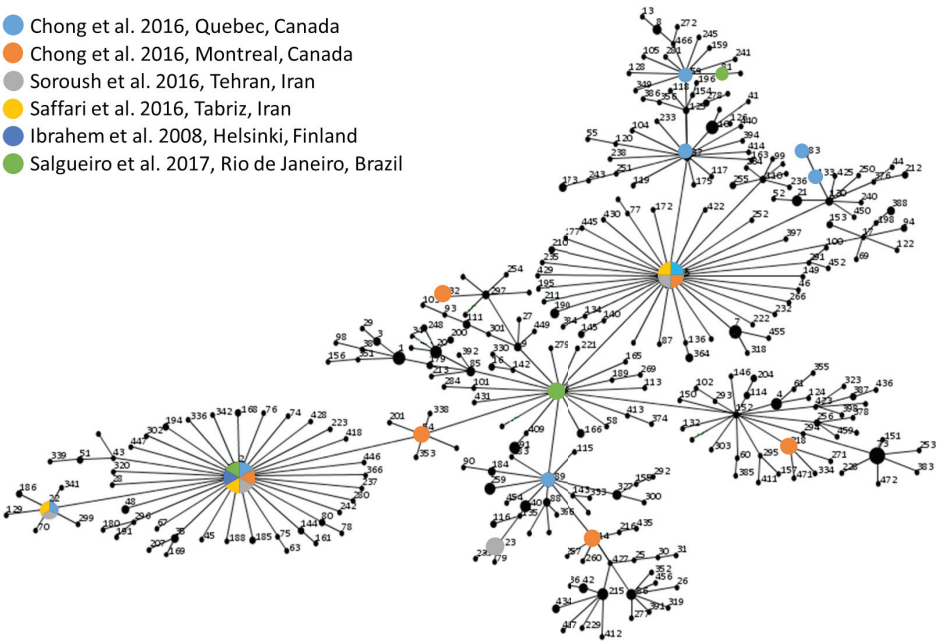


Figure 4. *S. epidermidis* CC2 strains causing infections in NICUs in different studies worldwide

All STs in the database of MLST (as of August 7, 2016) are included. Nodes represent STs, links indicate single locus variants (SLV), i.e. STs differing in one of seven loci, and numbers indicate STs. STs causing infections in NICUs in different studies are indicated with respective colours as shown in the legend.

2.3.3.2 Sources of predominant strains

Specific sources of predominant strains, such as almond oil or disinfectant bottles (Gras-Le Guen et al. 2007; Ben Saida et al. 2009), are only rarely detected, raising questions about the origin of the strains. The predominant strains in NICU may result from larger in-hospital or between-hospital spread of certain genotypes. Indeed, predominant strains in NICU have been found among blood culture CoNS isolates in pediatric as well as adult haematology unit (Ibrahem et al. 2008), intensive care unit (Bogado et al. 2002) and even in other hospitals in the same country (Saffari et al. 2016). The spread may occur through air, suggested by the isolation of the same predominant strain from air cultures from different units in one hospital (Botelho et al. 2012). Additionally, colonization of neonate in NICU with predominant strain and subsequent transfer of the neonate to another NICU can disseminate the strain between units (Milisavljevic et al. 2005). However, none of *S. epidermidis* genotypes from blood cultures of neonates hospitalized in a NICU were found in other wards of the hospital or in other NICUs nearby (Sung et al. 1999; Foka et al. 2006), suggesting sources other than hospital.

An influx of predominant strains into NICU may occur from community. This could be particularly true for CoNS strains with advantageous genetic background, such as *S. epidermidis* ST2 and ST5 (Thomas et al. 2014; Tolo et al. 2016). Indeed, although no transfer of neonates occurred between two NICUs located about 275 km apart, *S. epidermidis* predominant strains were indistinguishable by typing method in the units, but they belonged to widespread ST2 and ST5 (Chong et al. 2016). Moreover, *S. epidermidis* strain ST5 from blood culture in NICU in Iran also spread recently in Northern Europe (Widerström et al. 2006; Widerström et al. 2009; Saffari et al. 2016). The widespread dissemination of successful genetic lineages of *S. haemolyticus* (Cavanagh et al. 2014) and *S. capitis* (Butin et al. 2016), as described above, also suggests that their spread may occur, at least in part, through community. Still, comparative studies have not detected *S. epidermidis* genotypes from blood cultures of neonates hospitalized in the NICU on skin of healthy term neonates (Hell et al. 2013) or on the hands of healthy volunteers (Milisavljevic et al. 2005), arguing against influx from community. However, *S. epidermidis* exhibits extensive genetic diversity (Rolo et al. 2012) and thus small studies may not be appropriate to disprove the community as a reservoir of infection-causing strains.

2.3.3.3 Clinical relevance of predominant strains

In addition to their high prevalence, large proportion of infections caused by predominant strains may be due to their increased invasive capacity. Indeed, the proportion of predominant strains among CoNS isolates causing infections is higher compared with blood culture contaminants (Björkqvist et al. 2002). Moreover, while the majority of genotypes detected in NICU can be found

among infection-causing isolates as well as blood cultures contaminants, some predominant strains can be isolated only from infections (Villari et al. 2000). Finally, comparison of neonatal characteristics (such as BW, GA, length of NICU stay, clinical signs and laboratory values) revealed that neonates infected with the predominant strains compared with those infected with less common genotypes had higher pCO_2 value in the blood and lower blood pH (Vermont et al. 1998).

On the contrary, the proportion of predominant genotypes may be similar among infection causing isolates compared with contaminants (de Silva et al. 2001; Klingenberg et al. 2007) and among persistent and non-persistent LOS cases (Khashu et al. 2006; Dimitriou et al. 2011). Colonization with predominant strains also does not increase the risk of invasive infection, because such strains are similarly frequent among skin colonizing and infection-causing CoNS isolates (de Silva et al. 2001).

2.3.3.4 Colonization of neonates and mothers with predominant strains

The spread of predominant strain may merely reflect the extent of dissemination. Indeed, predominant strains of different CoNS species (*S. epidermidis*, *S. haemolyticus* or *S. caprae*) may colonize up to 19–40% of hospitalized neonates during their NICU stay (Low et al. 1992; Kazembe et al. 1993; Sloos et al. 1996; Sloos et al. 1998; Ross et al. 2005; Kornienko et al. 2016). Predominant strains can be found in one neonate on various body sites, such as ear, nares, axilla, arms, legs and gut (Bialkowska-Hobrzanska et al. 1993; Eastick et al. 1996), and constitute up to 58–70% of all CoNS isolates colonizing neonates (Bialkowska-Hobrzanska et al. 1993; Krediet et al. 2001). The strains are acquired rapidly after hospitalization (Sloos et al. 1998), as the prevalence of colonization increases from 0% at admission to NICU to as high as 84% in the fifth day (Kazembe et al. 1993).

Molecular epidemiological studies are scarce in healthy term neonates and mothers of neonates, but in line with possible spread of predominant strains in community, a strain of *S. epidermidis* was isolated from BM of multiple mothers (Begović et al. 2013).

2.4 Transition from colonization to infection

Although CoNS species distribution on skin of neonates with or without CoNS bacteremia is overall similar (de Silva et al. 2001; Center et al. 2003), *S. epidermidis* may colonize more frequently neonates with than without CoNS bacteremia (Hall et al. 1987). Similarly, colonization with *S. epidermidis* or higher abundance of staphylococci in gut may increase the risk of development of LOS caused by *S. epidermidis* or CoNS (el-Mohandes et al. 1993; Shaw et al. 2015), although such findings have not been corroborated in other studies (Johnson-Robbins et al. 1996; Mai et al. 2013). Controversial results may be

attributable, at least in part, to different characteristics of isolates and may require strain-level studies to determine the sources of subsequently invasive strains.

2.4.1 Skin as a source of CoNS causing LOS

CoNS causing LOS have been mainly considered to originate from skin and enter the bloodstream through IVCs. However, the role of IVCs as the only entry route of invasive strains has been questioned for several reasons. LOS caused by CoNS may develop in the absence of IVC at the onset of LOS or even without ever having IVC during NICU stay (Figure 5). Even if IVC is present, it may not be colonized with CoNS or at least not with the same strain as that causing LOS. Finally, CoNS bacteremia may persist even up to 22–44 days after removal of IVC (Khashu et al. 2006; Anderson-Berry et al. 2011).

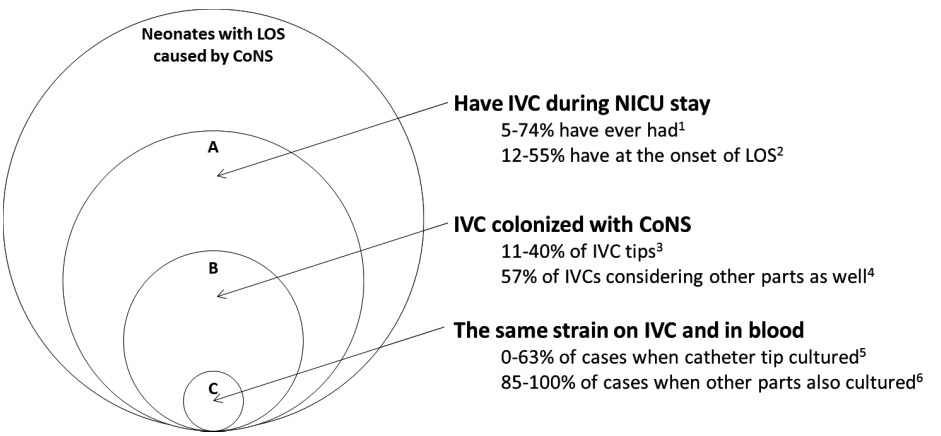


Figure 5. The presence and colonization of IVCs in neonates with LOS caused by CoNS

The figure illustrates that (A) only some neonates with LOS caused by CoNS have IVC during NICU stay and (B) only some IVCs are colonized with CoNS, but (C) not necessarily colonized with strains isolated from blood cultures.
Data from
[1]Johnson-Robbins et al. (1996), Vermont et al. (1998), Klingenberg et al. (2005), Khashu et al. (2006), Hira et al. (2007), Dimitriou et al. (2011)
[2]Klingenberg et al. (2005), Klingenberg et al. (2007), Björkqvist et al. (2010), Anderson-Berry et al. (2011), Hemels et al. (2011)
[3]Vermont et al. (1998) Garland et al. (2001)
[4]Mueller-Premru et al. (1999)
[5]Valvano et al. (1988), Garland et al. (2001), Garland et al. (2008), Brito et al. (2014)
[6]Mueller-Premru et al. (1999), Garland et al. (2008), Riboli et al. (2014)

The role of skin around the catheter entry site as a source of invasive strains has been also questioned, because CoNS may be cultured from the skin surrounding IVC in as few as 0–22% of LOS episodes (Garland et al. 2008; de Brito et al. 2010; Brito et al. 2014).

2.4.2 Gut as a source of CoNS causing LOS

In addition to failure to identify IVC or skin around its entry site as sources, several aspects support the hypothesis that invasive CoNS strains may enter the bloodstream from gut. First, gut colonization with CoNS is frequent, reaching the prevalence of up to 100% by the end of the first week of life (Parm et al. 2010; Moles et al. 2013), when also the count of CoNS in stool samples is highest (Eastick et al. 1996; Moles et al. 2013). This coincides with the postnatal age when the incidence of LOS caused by CoNS is highest (Hemels et al. 2011; Jean-Baptiste et al. 2011; Vergnano et al. 2011). Second, studies with neonatal rats have demonstrated that staphylococci can translocate from gut to mesenteric lymph nodes (Yajima et al. 2001; Nakayama et al. 2003). Third, in 8–50% of neonates with LOS caused by CoNS strains indistinguishable by typing method from gut and blood cultures have been isolated (Bialkowska-Hobrzanska et al. 1990; Eastick et al. 1996; Cossey et al. 2014; Shaw et al. 2015). Importantly, the presence of strains indistinguishable by typing method in gut and on IVC (Eastick et al. 1996) support the hypothesis of haematogenous seeding of IVCs with CoNS from gut (Mueller-Premru et al. 1999; Garland et al. 2008; Lepainteur et al. 2013). Thus, although translocation from gut has not been unequivocally proven in human neonates, the current evidence suggests that gut nevertheless serves as a reservoir of subsequently invasive strains.

2.4.3 BM as a source of CoNS causing LOS

Mother's own BM decreases the risk of LOS in preterm neonates (Corpeleijn et al. 2012). On the other hand, development of LOS due to pathogens in mother's own BM, such as *S. aureus* (Kayıran et al. 2014), has been reported. Moreover, development of necrotizing enterocolitis (NEC) and *S. epidermidis* bacteraemia in two twins was suggested to be due to heavy growth of *S. epidermidis* in BM with the same antimicrobial susceptibility pattern as the strain in blood cultures (Ng et al. 1995). Thus, to prevent any potential harm, some NICUs routinely perform bacteriological cultures (Bonet et al. 2015) or pasteurize (Dicky et al. 2017) mother's own BM.

However, studies conducted so far suggest that the risk of LOS is not increased due to bacteria in BM. First, although pasteurized mother's BM does not contain bacteria, it may not decrease the risk of LOS compared with raw BM (Cossey et al. 2013). Second, feeding intolerance was not associated with CoNS count in BM, being mean of $10^{5.6}$ cfu/mL in milk immediately prior to

the feeding intolerance episode and $10^{5.5}$ cfu/mL before tolerated feeding (Law et al. 1989). Third, routine BM cultures could not predict the development of infection in preterm neonates (Schanler et al. 2011). Finally, although eight episodes of LOS caused by CoNS occurred in BM-fed neonates, none of these were considered to be due to CoNS in BM due to different antimicrobial susceptibility profile of strains causing infection and those colonizing BM (Law et al. 1989). Instead, because mother's own raw BM decreased the incidence of LOS more than pasteurized BM, the reduction of the risk of LOS was suggested to be partly attributable to the introduction of non-pathogenic CoNS strains from BM to neonatal gut (Cossey et al. 2013).

2.5 Methods to study genetic relatedness of colonizing and invasive CoNS strains

The majority of studies determining genetic relatedness of invasive or colonizing strains have used pulsed field gel electrophoresis (PFGE), because of its high discriminatory power, including in *S. epidermidis* (Miragaia et al. 2008) and *S. haemolyticus* (Cavanagh et al. 2012). However, PFGE has multiple disadvantages, such as being laborious and costly, the interpretation of the results is somewhat subjective and between-laboratory comparisons are not straightforward. As an alternative, antibiotyping that is based on antimicrobial susceptibility profiles have been used, particularly when only differentiation of multidrug-resistant from highly susceptible strains is required (Law et al. 1989). However, it could be difficult to distinguish between highly resistant strains spreading in NICU and subsequent typing by PFGE may confute the majority of similarities between isolates identified by antibiotyping (Cossey et al. 2014). On the other hand, antimicrobial susceptibility may vary due to the regulation of gene expression by IS*256*, thus differences in the resistance pattern may not exclude genetic relatedness (Weisser et al. 2010).

Therefore, alternative typing methodologies have been developed. MLST is based on sequencing of seven housekeeping genes and has been developed also for *S. epidermidis* (Thomas et al. 2007). Although MLST is costly and with low discriminatory power, it is useful for characterization of global and long-term epidemiology and detection of predominant genetic lineages, such as CC2 in *S. epidermidis* (Miragaia et al. 2007). For local and short-term epidemiology, multilocus variable-number tandem-repeats analysis (MLVA) can be useful that differentiates isolates based on the copy number of repeated sequences in several genomic loci (Johansson et al. 2006). It is polymerase chain reaction (PCR)-based methodology that yields numerical data about the isolate and thus the interpretation of the results is less subjective compared with PFGE. Although, the disadvantage of MLVA could be its low discriminatory power, MLVA-scheme for *S. epidermidis* has similar discriminatory power to that of PFGE (Johansson et al. 2006) and inclusion of additional loci into MLVA-scheme (Cremniter et al. 2013) may overcome the problem of low discriminatory

power. Recently, MLST and MLVA were also developed for *S. haemolyticus* (Cavanagh et al. 2012). Nowadays, whole genome sequencing has been used to determine the genetic relatedness of colonizing and infecting bacterial strains (Carl et al. 2014), but due to its high cost, its use is still limited.

In addition to the typing methodology, the characteristics of CoNS colonization should be considered. First, as described above, skin or gut can be colonized with multiple strains at the same time (Valvano et al. 1988; Bialkowska-Hobrzanska et al. 1993; Eastick et al. 1996) and typing of additional colonies may reveal even more strains (Valvano et al. 1988). To overcome the genetic diversity, colony morphology has been used to predict the genotype of isolates. Although all morphologically similar colonies from the skin of hospitalized neonates may indeed be the representatives of the same strain (de Silva et al. 2001), even different species may exhibit similar colony morphology (Bialkowska-Hobrzanska et al. 1993). Second, thorough characterization of colonizing genotypes is further complicated by genetic diversity (Bialkowska-Hobrzanska et al. 1993) that may render identification of all simultaneously colonizing strains difficult and rapid changes among colonizing CoNS strains (Sharon et al. 2013). Thus, to determine genetic similarity of CoNS from multiple body sites as well as changes in colonization, characterization of several CoNS isolates with similar morphology and from multiple samples is warranted, precluding the use of too costly and laborious typing methodologies.

2.6 Summary of literature

CoNS are common colonizers of skin in preterm neonates and thus are considered to invade bloodstream from skin, but several studies have shown that invasive strains may not be present on skin or on the tips of IVCs (Garland et al. 2008; Lepainteur et al. 2013; Brito et al. 2014). Although CoNS are also frequent colonizers of gut in preterm neonates, only few studies in limited number of patients have investigated genetic relatedness of gut-colonizing and invasive CoNS strains (Bialkowska-Hobrzanska et al. 1990; Eastick et al. 1996; Cossey et al. 2014; Shaw et al. 2015). Thus, the role of gut in development of LOS has remained inconclusive. Moreover, the characteristics of gut-colonizing strains beyond antimicrobial resistance (Scheifele and Bjornson 1988; Hira et al. 2013) and thus the extent of the role of the gut as a reservoir of potentially invasive strains is still poorly described. Finally, comparison of molecular epidemiology and the presence of virulence-related genes between CoNS colonizing skin and gut of neonates has not been performed. Thus, skin cannot be definitely considered as the only source. Clarifying the role of gut in the development of LOS could contribute to the design of additional infection prevention measures.

One of the most important sources of staphylococci for neonatal gut is mother's BM, demonstrated by colonization of gut of healthy term neonates with the same bacterial strains as those in BM (Jiménez et al. 2008b; Martín et

al. 2012). Preterm neonates are exposed to and thus become colonized mostly with NICU strains, but their mothers are colonized with less resistant bacteria (Hira et al. 2013), most likely representing community strains that are less commonly associated with infection development (Tolo et al. 2016). Introduction of such less pathogenic strains into gut of preterm neonates has been hypothesized to be a mechanism by which BM reduces the risk of LOS (Cossey et al. 2014). However, it is unknown to what extent bacteria in BM are capable of colonizing gut of preterm neonates that is exposed to hospital-adapted strains and is influenced by antibacterial treatment. Understanding colonization of gut of preterm neonates could contribute to design of measures preventing colonization of gut with pathogenic strains and thereby decreasing the risk of LOS.

Despite the beneficial role of BM, mother's own unpasteurized BM may itself be a source of LOS causing strains. Infections caused by bacteria colonizing mother's BM, such as *S. aureus* (Kayıran et al. 2014) and even *S. epidermidis* (Ng et al. 1995), have been reported. Mothers of preterm neonates often require antibacterial treatment and longer hospitalization and thus may become colonized with resistant and potentially pathogenic bacteria from hospital environment. Visiting neonate in NICU may result in colonization of mother's BM with NICU-spreading strains, as described for outbreak-causing *Klebsiella pneumoniae* strain (Rettedal et al. 2012), and thus BM may become additional source of potentially invasive strains. Therefore, understanding of colonization of mother's BM is warranted to design measures for prevention of infections due to bacteria in BM.

We hypothesized that, first, gut of preterm neonates can be colonized with CoNS strains subsequently causing LOS. Second, in contrast to mothers of term neonates, BM of mothers of preterm neonates may harbour CoNS strains carrying virulence-related genes and causing LOS in NICU. Third, early colonization of gut of preterm neonates with less pathogenic strains indistinguishable from those in mother's BM is less common than in term neonates, but eventually occurs during the first month of life.

3 AIMS OF THE RESEARCH

The general aim of this thesis was to assess the relationship between colonization of gut and skin of preterm neonates and BM of their mothers with CoNS and evaluate relationship between mucosal colonization and development of LOS. Healthy term neonates were included as a control group considered to have normal colonization to reveal and determine the extent of deviation of colonization of preterm neonates from the healthy process in terms of the dynamics and the characteristics of colonizing staphylococci.

The study had the following specific objectives:

1. To describe CoNS species distribution in gut and on skin of preterm neonates and BM of their mothers.
2. To describe the presence of virulence- and resistance-related genes in *S. epidermidis* colonizing gut and skin of preterm neonates.
3. To describe the presence of virulence- and resistance-related genes of *S. epidermidis* and *S. haemolyticus* in BM of mothers of preterm neonates.
4. To characterize molecular epidemiology of *S. epidermidis* colonizing gut and skin of preterm neonates.
5. To characterize molecular epidemiology of *S. epidermidis* and *S. haemolyticus* in BM of mothers of preterm neonates.
6. To determine genetic relatedness of *S. epidermidis* colonizing gut and skin of preterm neonates and BM of their mothers.
7. To determine genetic relatedness of CoNS colonizing gut and skin of preterm neonates and BM of their mothers and those subsequently causing LOS.

4 MATERIALS AND METHODS

This thesis is based on two studies conducted in neonates hospitalized in the third-level NICUs of Tallinn Children's Hospital (Unit A) and Tartu University Hospital (Unit B) as presented in Table 5.

Table 5. Description of the studies of the thesis

Study	Timing	Study population	Primary aim	Paper
Clinical trial of empiric treatment of early-onset sepsis (Metsvaht et al. 2010) (Study A)	02.08.2006–30.11.2007	Neonates (n=276) with available stool samples	To determine genetic relatedness between invasive and gut-colonizing CoNS	I
			To characterize *S. epidermidis* colonizing gut of hospitalized neonates	II
Neonatal CoNS colonization study (Study B)	16.01.2014–15.12.2015	Term (n=20) and preterm neonates (n=49) and their mothers	To characterize CoNS colonizing BM of mothers	III
			To determine genetic relatedness between *S. epidermidis* colonizing neonates and mother's BM	IV

4.1 Ethics

The Research Ethics Committee of the University of Tartu approved the protocols of both studies. In Study A, signed informed consent from parents was not considered necessary by the ethics committee because treatment practice was not changed in the units and additional samples from neonates were not required. Still, parents were informed about the participation of the neonate in the study. In Study B, mothers signed informed consent on behalf of herself and her neonate prior to enrolment to the study.

4.2 Design and population of the studies

Study design. Study A was a prospective cluster-randomized study that compared the efficacy of penicillin and ampicillin both combined with gentamicin in the empiric treatment of neonates at risk of early-onset sepsis (EOS) and the effect of antibiotics on the colonization of gut within the first 60 days of life or until discharge from NICU. The study design is detailed elsewhere (Metsvaht et al. 2010; Parm et al. 2010).

Study B was a prospective longitudinal two-group comparative study that characterized the colonization of skin and gut with CoNS of BM-fed healthy

term and BM-fed hospitalized preterm neonates and BM of their mothers within the first month after delivery.

Inclusion/exclusion criteria. The inclusion criteria of neonates to Study A were as follows:

- admission to the NICU within 72 hours of life,
- need of early empiric antibacterial therapy for EOS or due to the risk factors of EOS,
- duration of NICU stay at least 24 hours.

The exclusion criteria from the study were as follows:

- prior antibacterial treatment different from study drugs for at least 24 hours,
- requirement of antibiotics different from study drugs.

Neonates were divided into preterm (GA <37 weeks) and term neonates (GA ≥37 weeks).

The inclusion and exclusion criteria of neonates and mothers in Study B are presented in Table 6.

Table 6. Inclusion and exclusion criteria of neonates and mothers in Study B

	Preterm neonates and their mothers	**Term neonates and their mothers**
Inclusion criteria	• GA <37 weeks • Admission to the NICU within the first week of life • Started receiving mother's own unpasteurized BM within the first week of life	• GA ≥37 weeks and BW ≥2500 g • Neonate exclusively BM-fed • Neonate and mother healthy and without any perinatal problems and need of hospitalization
Exclusion criteria	• Contraindications to feeding with mother's own unpasteurized BM • Predicted not to survive for more than 72 hours • Use of probiotics in mother or neonate	• Contraindications to feeding with mother's own unpasteurized BM • Perinatal administration of antibiotics to mother or neonate • Use of probiotics in mother or neonate

In Study B, a neonate-mother pair was excluded from the analysis if they were sampled for less than 3 weeks or any of the following had occurred:

- administration of mother's own unpasteurized BM to preterm neonate decreased to <10% of entire volume of daily enteral feeding in more than 7 consecutive days,
- term neonate no longer exclusively BM-fed,
- administration of antibiotics to or hospitalization of a term neonate and/or the mother,
- neonate and/or mother consumed probiotics during study period,
- death of neonate and/or mother.

Sample size calculation. The number of neonates enrolled in Study A was based on the calculation of sample size for detecting difference in the treatment failure between the two study groups with acceptable confidence level (Metsvaht et al. 2010). Study B was a pilot study and thus the sample size was not formally calculated.

Diagnostic criteria. In both studies, LOS was considered to be caused by CoNS if after the first 72 hours of life at least two clinical (abdominal distension, apnoea or bradycardia spells, feeding intolerance, hyper- or hypothermia, hypotension, increased oxygen requirement, lethargy and hypotonia, skin and subcutaneous lesions, e.g. petechial rash, abscesses, sclerema) and two laboratory criteria (C-reative protein >10 mg/L, immature to total neutrophil count ratio >0.2, platelet count $<100 \times 10^9$ cells/L, white blood cell count <5 or $>20 \times 10^9$ cells/L) were present in addition to the same species of CoNS growing in at least two blood cultures drawn within 72 hours or CoNS growing in one blood culture and administration of adequate antibacterial treatment for >72 hours.

Data collection. In Study A, the following neonatal and maternal characteristics were recorded: GA, BW, date of birth, gender, Apgar scores, delivery mode; multiple birth; age at admission to NICU, age at and duration of invasive respiratory support; antibacterial agents administered and dates; clinical diagnoses; outcome (dead/alive); the presence of and duration of CVC and arterial catheter; maternal age, smoking, chronic diseases, invasive procedures during pregnancy, antibacterial agents administered during delivery, premature rupture of membranes, chorioamnionitis. Feeding regimen (total parenteral nutrition, BM containing regimen or formula feeding) of neonate was recorded on the 1st, 3rd and 7th day of life and weekly thereafter.

In Study B, the following neonatal characteristics were recorded from medical charts: date of birth; gender; GA; BW; delivery mode; multiple birth; Apgar scores; congenital malformations; date of admission to NICU, transfer to neonatology unit and discharge from hospital; dates of feeding with and daily amounts (in mL) of BM, donor milk and formula (for preterm neonates only); dates of parenteral nutrition (amino acids, lipids); antibacterial agents administered, daily dose (mg/kg) and dates of administration; dates of invasive respiratory support, CVC, arterial catheter and feeding tube; clinical diagnoses and dates; surgeries performed and dates; additional factors for calculation of CRIB score (worst base excess, minimum and maximum appropriate fraction of inspired oxygen (FiO2) during the first 12 h of life); in case of proven or suspected LOS caused by CoNS clinical (absence/presence) and laboratory signs (value) as described in definition of proven LOS caused by CoNS; blood and CVC cultures – dates, microbes growing and interpretation of the growth (contamination/infection); weight of neonate at the end of the study. Maternal age, parity and hospitalization as a caretaker of neonate (i.e. hospitalization in the Children's Clinic of Tartu University Hospital or Tallinn Children's Hospital) were recorded from medical charts. Administration of antibacterial agents

to and hospitalization of mother within 3 months prior to and one month after delivery and dates were asked from mother.

4.3 Sample collection

In Study A, rectal swabs were collected with transport swabs (Nuova Aptaca, Canelli, Italy) at admission to the NICU and twice weekly thereafter until the age of 60 days or discharge, whichever occurred earlier. Swabs were stored at –20 °C until culturing.

In Study B, samples were collected once a week in the first month of life. A sterile container with a sterile spatula was used for collecting stool samples from diapers. For BM collection the breast was washed with soap and water, wiped with a clean towel and finally cleaned with disinfectant available in the unit. After drying, approximately 3 mL of BM was collected by manual expression into a sterile container after discarding the first drops. Prior to the transport to –80 °C within 96 h, stool and BM samples were stored at –20 °C. A transport swab (Copan Italia spa, Brescia, Italy) moistened in normal saline was used for neonatal skin sampling by rubbing an area of approximately 1 cm^2 of the right axilla five times vertically and five times horizontally with the swab. Skin swabs were stored at +4 °C until culturing.

4.4 Processing of samples and identification of staphylococci

Rectal swabs in Study A were cultured onto blood agar plates. After incubation at +37 °C for 24–48 hours, one of each morphologically distinct colonies typical of staphylococcus was identified to the species level by API® Staph (bioMerieux S.A., Marcy-l'Etoile, France) according to the manufacturers' instructions (Figure 6A). Bloodstream isolates were available only from Unit A and were isolated from blood cultures from neonates with LOS and identified to the species level by VITEK® 2 (bioMerieux S.A., Marcy-l'Etoile, France) in Laboratory of Microbiology, Division of Diagnostics, North Estonia Medical Centre, Tallinn, Estonia.

The species of the following isolates from Study A was confirmed by sequencing of the *tuf* gene (Heikens et al. 2005):

- isolates from blood cultures,
- isolates colonizing gut prior to the onset of LOS in neonates with LOS caused by CoNS.

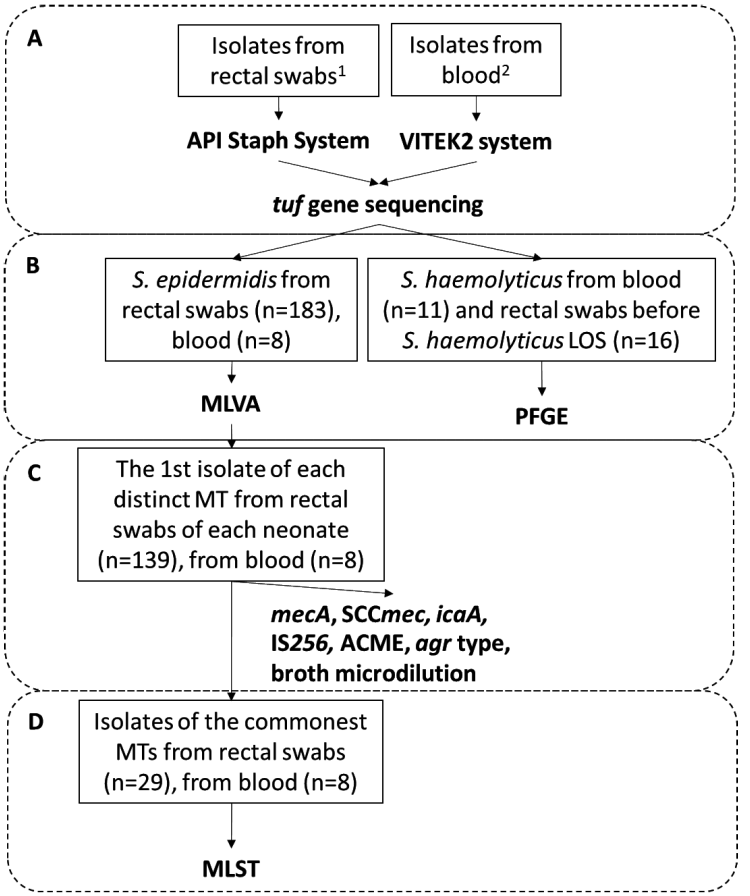


Figure 6. Identification and characterization of CoNS isolates in Study A

The flowchart shows the methodologies for (A) the identification of species, (B) molecular typing, (C) the determination of the presence of the virulence-related genes and antimicrobial susceptibility, (D) multilocus sequence typing and number of isolates analysed in each step.
[1, 2]Isolation of staphylococci from rectal swabs and blood cultures was performed and detailed in previous studies (Metsvaht et al. 2010; Parm et al. 2010).

In Study B, all colonization samples were cultured quantitatively onto mannitol salt agar by plating 10-fold dilutions in normal saline of about 100 µg of stool, 100 µL of BM and skin swab solutions (for that purpose, transport swab was vortexed for 1 min in 1 mL of normal saline). After incubation at +37 °C for 48 h, the total number of colonies was counted and five colonies with morphology typical of staphylococcus, including each morphologically distinct, were identified to the species level by a matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) mass-spectrometry (Bruker Daltonics, Bremen, Germany) (Figure 7A). Bloodstream isolates were obtained from neonates with LOS and identified to the species level by MALDI-TOF mass-spectrometry in Laboratory of Microbiology, Division of Diagnostics, North Estonia Medical

Centre, Tallinn, Estonia or Laboratory of Clinical Microbiology, United Laboratories, Tartu University Hospital, Tartu, Estonia.

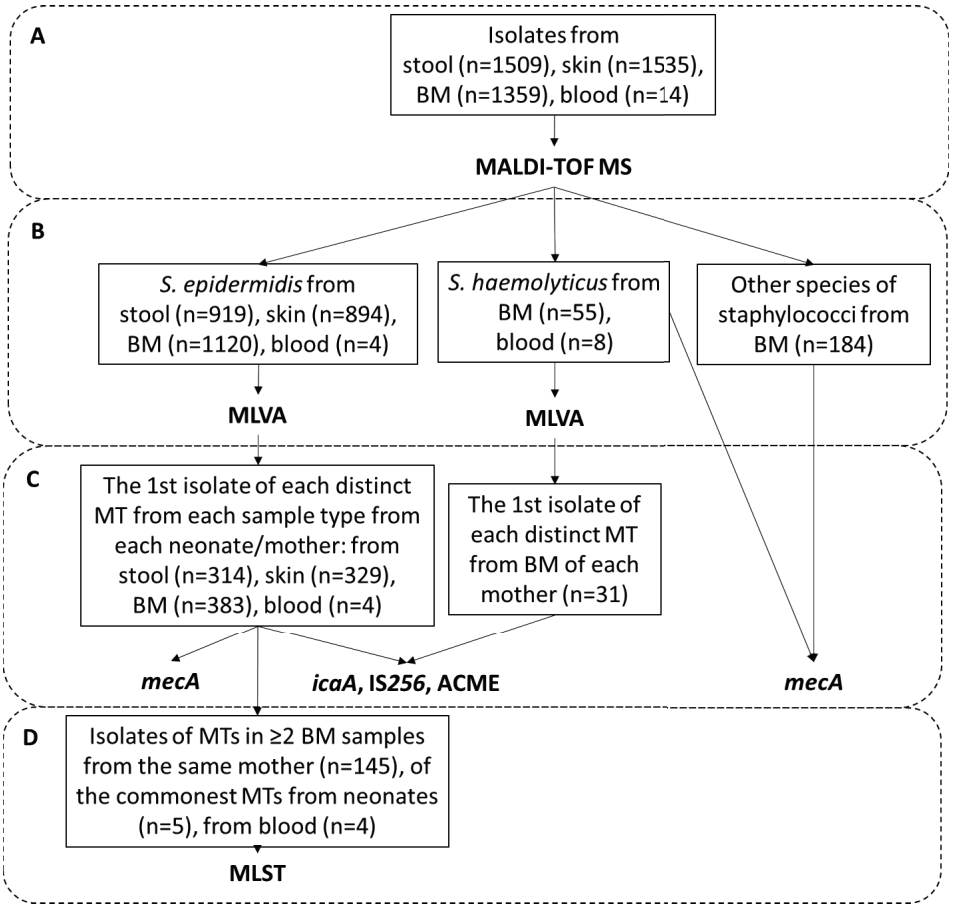


Figure 7. Identification and characterization of CoNS isolates in Study B

The flowchart shows the methodologies for (A) the identification of species, (B) molecular typing, (C) the determination of the presence of the virulence- and resistance-related genes, (D) multilocus sequence typing and number of isolates analysed in each step.

All staphylococcal isolates were stored in skimmed milk at –80 °C for further analyses.

4.5 Molecular characterization of isolates

For molecular characterization (molecular typing, the detection of the virulence- and resistance-related genes), DNA was extracted with QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions from the following isolates from Study A:

- isolates from blood cultures,
- isolates colonizing gut prior to the onset of LOS in neonates with LOS caused by CoNS.

Boiling method, as described elsewhere (Zhang et al. 2004), was used for extracting DNA from all other CoNS isolates.

4.5.1 MLVA

S. epidermidis isolates were typed by MLVA that consists of five loci (Se1, Se2, Se3, Se4, Se5) in the original scheme (Johansson et al. 2006) and additional three (Se6, Se, Se8) in the improved scheme (Cremniter et al. 2013) with some modifications. Due to no repeats observed in Se5 this locus was excluded from the MLVA-scheme. Thus, all *S. epidermidis* in Study A were typed by 7-loci scheme (original five loci, excluding Se5, and additional three loci) (Figure 6B). In Study B, all *S. epidermidis* were typed by 5-loci scheme (derived from the 7-loci scheme by excluding loci Se4 and Se8 due to too long PCR products to be distinguishable by agarose gel electrophoresis or low typability, respectively) (Figure 7B).

In Study B, *S. haemolyticus* from BM and blood cultures was typed by 5-loci MLVA scheme (Cavanagh et al. 2012) (Figure 7B).

Distinct MLVA profiles were designated as separate MLVA-types (MTs) and were arbitrarily assigned an integer, whereas the numbering system is different for *S. epidermidis* isolates from Study A and for *S. epidermidis* and *S. haemolyticus* isolates from Study B.

4.5.2 MLST

The following *S. epidermidis* isolates were additional typed by MLST as described by Thomas *et al*. elsewhere (Thomas et al. 2007) (Figure 6D, Figure 7D):

- all isolates from blood cultures,
- isolates of the most common MLVA-types in Study A,
- isolates of MLVA-types present in at least two BM samples from the same mother and the commonest MLVA-types colonizing neonates in Study B.

STs were assigned according to *S. epidermidis* MLST database (http://sepidermidis.mlst.net/). Alleles of MLST loci and STs that had not been previously described were submitted to the MLST database.

4.5.3 PFGE

In Study A, *S. haemolyticus* was typed by PFGE as described Villari *et al*. (Villari et al. 2000) (Figure 6B). Briefly, from a colony grown overnight on blood agar plate DNA was extracted using QIAGEN DNA extraction kit

(QIAGEN, Hilden, Germany). Subsequently, restriction of DNA was performed with SmaI restriction enzyme (Fermentas, Vilnius, Lithuania). Electrophoresis was performed in the CHEF-DR II System (Bio-Rad, Marnes-la-coquette, France) with linear ramped pulse times of 5–60 s and voltage of 6 V/cm at 14 °C for 21 h. Reference strain *S. haemolyticus* DSM 20263 was included in every run. PFGE patterns were assigned to types by visual inspection and arbitrarily assigned a letter. Closely related PFGE-types were designated using the combination of letter (the same for all closely related PFGE-types) and integer (varying between the closely related PFGE-types).

4.5.4 The virulence-related genes

The presence of the *ica*-operon and IS*256* were determined by PCR using primers targeting the *icaA* gene and IS*256* transposase, respectively, as described previously (Ziebuhr et al. 1999). The *agr*-typing was performed by multiplex-PCR using primers specific to the alleles of *agr* type I, II and III (Lina et al. 2003). Subsequently, *agr* type was assigned according to the size of the PCR product. The presence of ACME was inferred from multiplex-PCR using primers specific for the *arcA* gene and the *opp3AB* gene (Diep et al. 2008). ACME types were assigned according to the presence of the genes (Miragaia et al. 2009).

In the following *S. epidermidis* isolates the presence of the *icaA* gene, IS*256*, ACME were determined (Figure 6C, Figure 7C):

- all isolates from blood cultures,
- the first isolate of each distinct MLVA-type from gut of each neonate in Study A (in these isolates *agr* type was also determined)
- the first isolate of each distinct MLVA-type from stool samples, skin swabs and BM of each neonate/mother in Study B.

Additionally, the presence of the *icaA* gene, IS*256* and ACME was determined in *S. haemolyticus* isolates from blood cultures and the first isolate of each distinct MLVA-type from BM of each mother in Study B (Figure 7C).

4.5.5 Antibacterial susceptibility

In Study A, in all *S. epidermidis* isolates from gut and blood cultures, MICs to benzylpenicillin, oxacillin, gentamicin, vancomycin, clindamycin, and ciprofloxacin were determined by broth microdilution according to the Clinical and Laboratory Standards Institute instructions (CLSI 2012) (Figure 6C). The results were interpreted according to the clinical breakpoints by European Committee on Antimicrobial Susceptibility Testing (EUCAST 2015).

4.5.6 The *mecA* gene and SCC*mec*-typing

The presence of the *mecA* gene was determined in the following isolates as described by Kondo *et al*. (Kondo et al. 2007) (Figure 6C, Figure 7C):

- all isolates from blood cultures,
- the first *S. epidermidis* isolate of each distinct MLVA-type from gut of each neonate in Study A
- the first *S. epidermidis* isolate of each distinct MLVA-type from stool samples, skin swabs and BM of each neonate/mother in Study B,
- all staphylococcal isolates other than *S. epidermidis* from BM samples in Study B.

Additionally, in *S. epidermidis* isolates from blood cultures and the first isolate of each distinct MLVA-type from gut of each neonate in Study A (Figure 6C), SCC*mec* type was determined. For determination of the type of *ccr* gene complex M-PCR 1, as described elsewhere (Kondo et al. 2007), was used. Primers mecI-F and mecI-R were used for detection of class A *mec* gene complex (Zhang et al. 2005), IS1272-F and mecR1-R for class B *mec* gene complex (Zhang et al. 2005), IS2 and ma7 for class C2 *mec* gene complex (Kondo et al. 2007). Types were assigned according to the IWG-SCC guidelines (IWG-SCC 2009), as described in Table 4.

4.6 Data analysis

The software program R was used for statistical analyses (version 3.2.2; © 2015 The R Foundation for Statistical Computing; last accessed February 17, 2017). The categorical variables were compared by Fisher exact test and continuous variables by Mann-Whitney or Kruskal-Wallis test, as appropriate.

In Study A, analysis of neonatal and maternal characteristics associated with colonization with *S. epidermidis* (defined as the presence of *S. epidermidis* in at least one rectal swab) compared with the absence was performed by mixed effects logistic regression model, considering the unit and time period as random effects, using R package lme4. All the characteristics associated with colonization at a p-value of <0.1 in univariate models were subsequently analysed in multivariate backward elimination logistic regression and removed in the order of insignificance. Only one of highly correlated variables was used in multivariate analysis to avoid confounding. Therefore, GA rather than BW (Spearman's rank correlation rho between GA and BW 0.939) was included in the multivariate analysis.

In Study B, maternal and neonatal characteristics associated with the presence of *mecA*-positive staphylococci in at least one BM sample were assessed by Firth logistic regression (Heinze and Schemper 2002) to account for complete or quasi-complete separation of data, adjusted for GA, using R package logistf.

Genetic diversity of MLVA-types was characterized by calculating Simpson's index of diversity (SID) and 95% confidence intervals (CI) on the Comparing Partitions website (http://www.comparingpartitions.info/; last accessed March 22, 2017).

Similarity of neonatal gut and skin and mother's BM samples in two study groups (preterm vs term neonates and their mothers) in terms of MLVA-types was characterized by calculating the Bray-Curtis similarity index between pooled MLVA-types from two sample types collected in the same week. The pairwise similarity indices were calculated in the software program EstimateS 9.10 (Colwell 2013).

5 RESULTS AND DISCUSSION

5.1 Study population

A total of 221 preterm and 55 term neonates from Study A and 49 preterm and 20 term neonates and 69 mothers from Study B were included in the studies covered by this thesis. A total of 1242 rectal swabs in Study A and 296 stool samples, 266 skin swabs and 251 BM samples in Study B were collected.

5.1.1 Neonates

Of preterm neonates 69.7% (n=154) and 79.6% (n=39) in Study A and Study B, respectively, were very preterm (GA <32 weeks), half were born by Caesarean section and three quarters required central venous catheter (CVC) or invasive respiratory support (Table 7).

According to the inclusion criteria all neonates in Study A received antibacterial treatment that may decrease the overall abundance of staphylococci in gut (Greenwood et al. 2014) or enrich gut microbiota with *mecA*-positive staphylococci (Gibson et al. 2016). However, the use of antibiotics is very high, up to 99.8%, in the hospitalized neonates (Cantey et al. 2015b), exemplified also by antibacterial treatment of nearly all preterm neonates in Study B. Moreover, the majority (62–79%) of antibiotics are prescribed within the first three days of life (Cantey et al. 2015b; Metsvaht et al. 2015), as were antibiotics in Study A. Thus, despite the antibacterial treatment colonization of neonates in Study A should reflect colonization of overall population of neonates hospitalized in the NICU.

Preterm neonates in Study A and B differed in many aspects. Length of NICU stay was significantly shorter in preterm neonates in Study A compared with Study B (Table 7). Strong correlation between length of NICU stay and duration of CVC (Spearman's rank correlation rho 0.839) or antibacterial treatment (0.705) probably accounts for significantly shorter duration of CVC and antibacterial treatment in preterm neonates in Study A compared with Study B. Inclusion of only those preterm neonates in Study B in whom BM-feeding was initiated within the first week of life most likely accounts for significantly smaller proportion of preterm neonates receiving BM within the first week of life in Study A compared with Study B (29% (n=64) vs 100% (n=49); p<0.001). Of note, Study A was conducted 8 years earlier than Study B.

Differences in the inclusion criteria resulted in significant differences between term neonates in the two studies. Most importantly, all term neonates in Study A were hospitalized in the NICU and received antibacterial agents, in contrast to none of term neonates in Study B (Table 7). Less term neonates in Study A had received mother's own BM within the first week of life (16.4% (n=9)) compared with Study B where all term neonates were exclusively BM-fed (p<0.001). Still, the two groups had similar median GA and BW.

In Study B, all preterm neonates received BM by nasogastric feeding tubes and all term neonates were directly breastfed.

Table 7. Characteristics of the neonates in Study A and Study B

	Hospitalized preterm neonates		Term neonates	
	Study A (n=221)	**Study B (n=49)**	**Hospitalized, Study A (n=55)**	**Healthy, Study B (n=20)**
Neonatal characteristics at admission				
GA – weeks; median (IQR)	29 (26–32)	28 (25–30)	40 (38–40)	40 (39–40)
BW – grams; median (IQR)	1312 (875–1790)	1154 (814–1564)	3580 (3082–4105)	3651 (3324–3970)
Caesarean section – n (%)	133 (60.2)	27 (55.1)	22 (40)	0[3]
Male – n (%)	125 (56.6)	30 (61.2)	32 (58.2)	7 (45)
In Unit A – n (%)	118 (53.4)	19 (38.8)	24 (43.6)	–
Neonatal characteristics during NICU stay				
CVC – n (%)	183 (82.8)	41 (85.7)	30 (54.5)	–
Duration of CVC – days; median (IQR)	6 (2–12)	11 (7–17)[1]	1 (0–4.5)	–
Invasive respiratory support – n (%)	174 (78.7)	36 (73.5)	35 (63.6)	–
Duration of invasive respiratory support – days; median (IQR)	2 (0–6)	2 (0–8)	1 (0–3.5)	–
Use of any antibacterial agent – n (%)	221 (100)	48 (98)	55 (100)	–
Duration of antibacterial treatment – days; median (IQR)	6 (3–12)	11 (7–16)[1]	6 (3–7)	–
LOS – n (%)	54 (24.4)	15 (30.6)	1 (1.8)	–
NEC – n (%)	15 (6.8)	3 (6.1)	0 (0)	–
Length of NICU stay – days; median (IQR)	9 (5–19)	17 (9–27)[2]	4 (2.5–6)	–

–, not applicable
[1]$p<0.001$ and [2]$p=0.001$ between preterm neonates in Study B and Study A; [3]$p=0.002$ between term neonates in Study B and Study A

5.1.2 Mothers

In Study B, according to the inclusion criteria mothers of term neonates had not received antibiotics and had not been hospitalized for reasons other than delivery within three months prior to and one month after delivery (Table 8). During the same time period, all mothers of preterm neonates had been hospitalized for delivery or other reasons for median (IQR) of 7 (4–23) days and

72.9% had received antibacterial treatment for median (IQR) of 8.5 (5.25–14.75) days. The most frequently used antibiotics were cefuroxime, penicillin, and ampicillin/amoxicillin received by 38.8% (n=19), 28.6% (n=14), and 14.3% (n=7) of mothers, respectively. Thus, as antibacterial treatment may increase the prevalence of colonization with methicillin-resistant CoNS (Morgenstern et al. 2016) and during hospitalization rapid acquisition of methicillin-resistant *S. epidermidis* strains occur (Widerström et al. 2016), the majority of the mothers of preterm neonates had risk factors for colonization with resistant CoNS strains.

Table 8. Characteristics of the mothers in Study B

	Mothers of preterm neonates (n=49)	**Mothers of term neonates (n=20)**
Duration of pregnancy – weeks; median (IQR)	28 (25–30)	40 (39–40)
Delivery by Caesarean section – n (%)	27 (55.1)	0
Age of mother – years; median (IQR)[1]	31 (26.8–36)	30.5 (26–31.3)
Previous births – n; median (IQR)[1]	2 (1–2)	2 (2–2)
Hospitalizations		
Hospitalization within 3 months prior to delivery – n (%)	34 (69.4)	–
Duration of hospitalization 3 months prior to delivery[2] – days; median (IQR)	2 (0–6)	–
Hospitalization within 1 month after delivery – n (%)	49 (100)	20 (100)
Duration of hospitalization within 1 month after delivery[2] – days; median (IQR)	4 (2.5–4.5)	2 (1.75–3)
Hospitalization in the neonatology unit as a caretaker of neonate – n (%)	17 (34.7)	–
Duration of hospitalization as a caretaker of neonate – days; median (IQR)	0 (0–9)	–
Antibacterial treatment		
Antibiotics within 3 months prior to delivery – n (%)	23 (46.9)	–
Duration of treatment with antibiotics within 3 months prior to delivery[1] – days; median (IQR)	0 (0–4.5)	–
Antibiotics within 1 month after delivery[1] – n (%)	33 (68.8)	–
Duration of treatment with antibiotics within 1 month after delivery[1] – days; median (IQR)	3 (0–8)	–

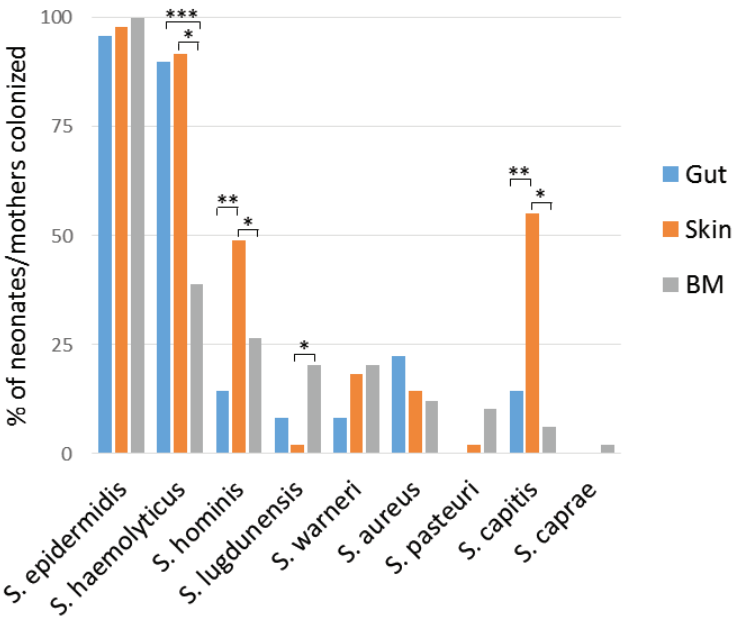
–, not applicable
Data for [1] one mother, [2] two mothers, were not available.

5.2 CoNS species colonizing neonates and BM

CoNS were present in rectal swabs of 21 of 22 (95.5%) neonates with LOS caused by CoNS in Study A. Staphylococci were present in 275 of 296 (92.9%) of stool samples, 263 of 266 (98.9%) of skin swabs and 248 of 251 (98.8%) of BM samples in Study B.

5.2.1. *S. epidermidis* colonizing neonates and BM

S. epidermidis colonized gut of 47 (95.9%) and skin of 48 (98%) preterm neonates and BM of all their mothers in Study B (Figure 8).



Figue 8. Prevalence of colonization with different species in preterm neonates and their mothers in Study B

Asterisk (*) indicates statistically significant ($p<0.05$) difference between the prevalence on skin and in BM, two asterisks (**) between skin and gut, three asterisks (***) between gut and BM.

In term neonates *S. epidermidis* was the commonest colonizer as well, isolated from skin and gut of all neonates and BM of all their mothers in Study B (Figure 9).

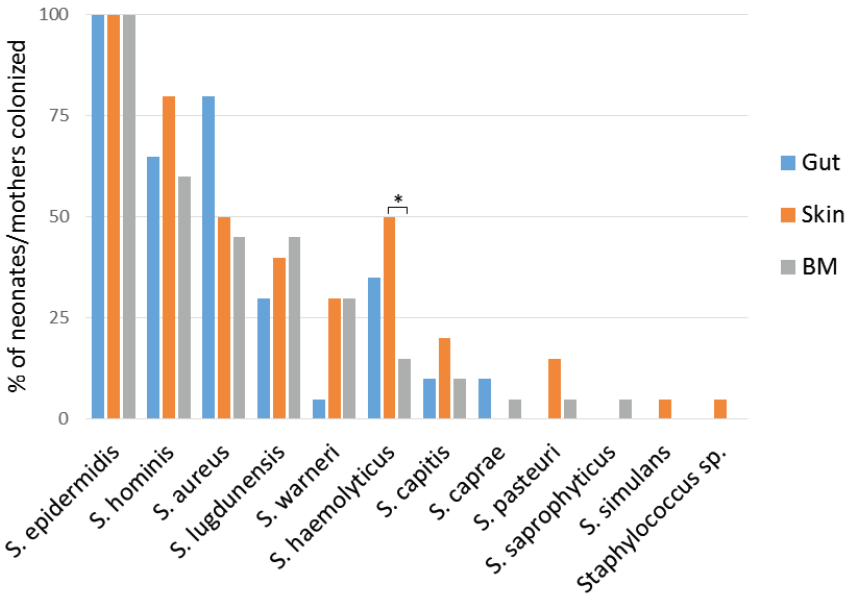


Figure 9. Prevalence of colonization with different species in term neonates and their mothers in Study B

Asterisk (*) indicates statistically significant ($p<0.05$) difference between the prevalence on skin and in BM. *Staphylococcus* sp. designates one isolate that could not be identified to the species level by MALDI-TOF mass-spectrometry.

In contrast, in Study A *S. epidermidis* was isolated from rectal swabs of only 42.1% (n=93) preterm and 23.6% (n=13) term neonates. According to a multivariate mixed effects model that included neonatal and maternal characteristics associated with the colonization with *S. epidermidis* at a p-value of <0.1 in univariate models (Paper II, Table 2), longer duration of NICU stay was the only neonatal characteristic associated with the colonization with *S. epidermidis* (odds ratio (OR) 1.04, 95% CI 1.02–1.05; $p<0.001$). Thus, the lower prevalence of colonization with *S. epidermidis* in Study A could in part be attributable to shorter duration of NICU stay (9 (5–19) in Study A vs 17 (9–27) in Study B; $p=0.001$). In addition, in Study A only one colony of each distinct morphological type was isolated. However, morphology of colony may not distinguish between CoNS species (Kleeman et al. 1993), possibly resulting in underestimation of the diversity of CoNS colonizing gut and consequently the prevalence of colonization with *S. epidermidis*.

5.2.2 Other CoNS species colonizing neonates

Preterm compared with term neonates were less commonly colonized in gut and/or on skin with *S. aureus* (prevalence of colonization in gut and/or on skin 26.5% vs 85%; $p<0.001$), *S. hominis* (51% vs 90%; $p=0.006$) and *S. lugdunensis*

(8.2% vs 60%; p<0.001), but more commonly with *S. haemolyticus* (91.8% vs 55%; p=0.001). The prevalence of colonization with *S. capitis* was higher only on skin of preterm compared with term neonates (55.1% vs 20%; p=0.017), but not in gut. These differences are also reflected in the distribution of staphylococcal species among isolates from gut and skin (Figure 10).

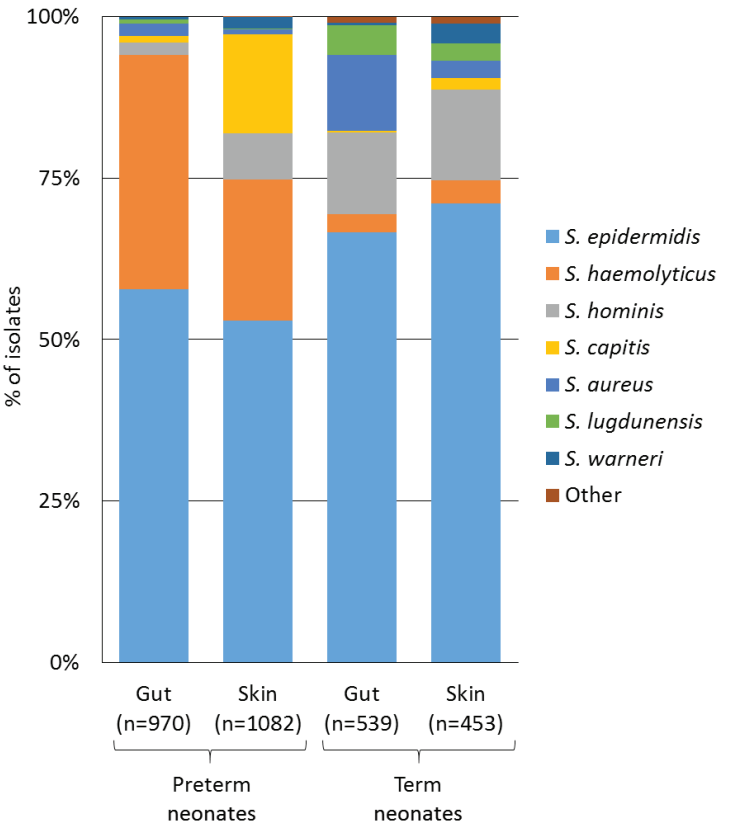


Figure 10. Distribution of staphylococcal species among isolates from gut and skin of neonates in Study B

The carriage rates of *S. haemolyticus* and *S. capitis* are in accordance with previous studies, as the prevalence of skin colonization with *S. haemolyticus* is 40–50% and with *S. capitis* is 4–5% in non-hospitalized term neonates (Carr and Kloos 1977; Marchini et al. 2005), but vary between 9–58% and 2–17%, respectively, in hospitalized neonates (de Silva et al. 2001; Jain et al. 2004). Considering that *S. haemolyticus* and *S. capitis* have emerged as important pathogens in NICU (Rasigade et al. 2012; Pereira et al. 2014; Butin et al. 2016) the high carriage rate of *S. haemolyticus* and *S. capitis* in hospitalized neonates could be in part attributable to the acquisition of strains adapted to NICU environment. Indeed, in contrast to other CoNS species, the prevalence of colonization with *S. haemolyticus* increases during hospitalization (Hira et al.

2013), resulting in colonization of gut in as many as 87% of hospitalized neonates (Aujoulat et al. 2014). The high prevalence of these species is worrisome, because *S. haemolyticus* is the most resistant CoNS species with propensity to infect the most premature neonates (Klingenberg et al. 2007) and recently identified clone of *S. capitis*, present in many units across the world (Butin et al. 2016; Ehlersson et al. 2017), is possibly associated with higher morbidity (Ben Said et al. 2016).

The carriage rates of *S. aureus* and *S. hominis* are also in accordance with literature, as of term neonates up to 60% are colonized with *S. aureus* (Lindberg et al. 2004; Benito et al. 2015) and 55% with *S. hominis* in gut (Martín et al. 2012), in contrast to 27–36% (Moles et al. 2013; Moles et al. 2015a) and 9–20% (Aujoulat et al. 2014; Said et al. 2014) of preterm neonates, respectively. The carriage of *S. lugdunensis* in healthy term (Marchini et al. 2005) and neonates hospitalized in the NICU (Center et al. 2003; Ternes et al. 2013) has been so far reported only in single cases. However, *S. lugdunensis* is common in healthy adults of whom 67% are colonized in at least one body site (Bieber and Kahlmeter 2010) that is comparable to the 60% in term neonates in the current study. Although similarly to *S. haemolyticus* and *S. capitis*, *S. aureus* may be endemic in NICU, particularly its methicillin-resistant strains (Geraci et al. 2014), none of LOS cases occurring in the 49 preterm neonates included in Study B were caused by *S. aureus* suggesting the absence of a widespread clone. In NICU, dissemination of *S. lugdunensis* virulent strains has not been reported so far and only one outbreak of *S. hominis* has been described (Chaves et al. 2005). Therefore, the low prevalence of *S. aureus*, *S. lugdunensis* and *S. hominis* in preterm neonates may be attributable in part to their less frequent clonal spread in NICU environment compared with *S. haemolyticus* and *S. capitis*.

5.2.3 Other CoNS species colonizing BM

Mothers of preterm compared with term neonates harboured less commonly *S. aureus* (12.2% vs 45%; p=0.008) and *S. hominis* (26.5% vs 60%; p=0.019) in BM. The prevalence of colonization of BM with *S. lugdunensis* was somewhat less common (20.4% vs 45%) and *S. haemolyticus* more common (38.8% vs 15%) in mothers of preterm compared with term neonates, but these differences were not statistically significant. *S. capitis* was similarly rare in mothers of preterm and term neonates (6.1% vs 10%). These differences are reflected in the distribution of staphylococcal species among isolates from BM of mothers of preterm and term neonates (Figure 11).

Median (IQR) count of staphylococci in BM of mothers of preterm neonates was significantly higher than count in BM of mothers of term neonates (4.61 (4.22–4.99) vs 3.05 (2.40–3.53); p<0.001) (Paper III, Figure S2).

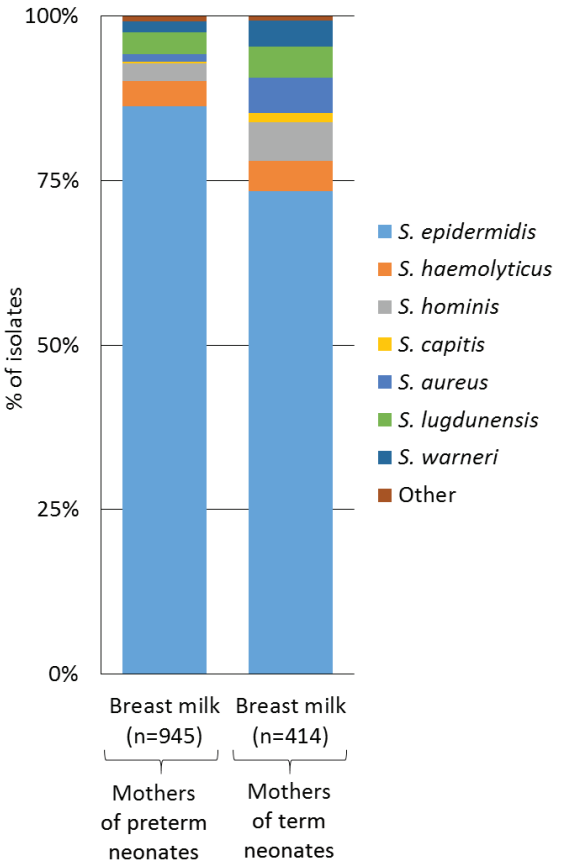


Figure 11. Distribution of staphylococcal species among isolates from BM of mothers of preterm and term neonates in Study B

The prevalence of colonization of BM with *S. aureus* in mothers of term neonates exceeds the previously reported range from 0 to 28.6% (Benito et al. 2015; Cullinane et al. 2015; Boix-Amorós et al. 2016). Although *S. aureus* in BM has been associated with the risk of development of mastitis (Cullinane et al. 2015; Jiménez et al. 2015), none of the mothers complained about any mastitis-related symptoms during the study period. The proportion of BM samples in mothers of preterm and term neonates containing *S. aureus* (5.8% and 22.8%) was lower than the prevalence of colonization (i.e. the presence of *S. aureus* in at least one BM sample from one mother), suggesting intermittent carriage rather than persistent colonization that is in accordance with high variability of the composition of BM microbiota (Jost et al. 2013; Boix-Amorós et al. 2016).

5.2.4 CoNS species common to neonates and BM

In both, preterm and term neonates CoNS species distribution in gut was similar to that in mother's BM, except for the higher prevalence of *S. haemolyticus* in gut of preterm neonates (Figure 8). *S. aureus* was more common in gut of term neonates compared with BM of their mothers, although that was not statistically significant (p=0.05) (Figure 9). Similarity of gut and BM colonization is in accordance with previous findings that, overall, microbiota composition of gut reflects that of BM, particularly in terms of staphylococci within the first weeks of life (Collado et al. 2016; Murphy et al. 2017). Therefore, the above-described differences in CoNS species between preterm and term neonates may in part result from the similar differences between CoNS species in BM of their mothers.

Skin colonization differed in preterm neonates from their mother's BM in the prevalence of *S. haemolyticus*, *S. capitis*, *S. hominis* and *S. lugdunensis* (Figure 8). In contrast, the prevalence of colonization with different CoNS species on skin of term neonates and in BM of their mothers was similar, except for the higher prevalence of *S. haemolyticus* on skin (Figure 9). Thus, while colonization of term neonates resembles largely that of BM, the results suggest that there are other sources of colonizing CoNS species for skin of preterm neonates than mother's BM.

The higher prevalence of *S. haemolyticus* on skin and gut of neonates compared with mother's BM is in accordance with the literature. While *S. haemolyticus* is a relatively rare colonizer of BM (Heikkilä and Saris 2003; Marín et al. 2009; Jost et al. 2013; Filleron et al. 2014), it is present on skin of 40–50% of healthy term neonates (Carr and Kloos 1977; Marchini et al. 2005) and may colonize gut of nearly all preterm neonates (Aujoulat et al. 2014). The high prevalence of *S. haemolyticus* in preterm neonates may result in part from the acquisition of strains spreading in NICU, as longer NICU stay increases the risk of colonization with *S. haemolyticus* (Hira et al. 2013). However, in adults the carriage rate of *S. haemolyticus* on skin or in nares is low, varying between 4–14% (Lina et al. 2003; Alvarez et al. 2014; Cavanagh et al. 2016). Thus, overall higher prevalence of *S. haemolyticus* in neonates or infants compared with adults cannot be excluded, but this warrants further studies. Such uncommonly high prevalence of gut colonization in the neonatal period and significant decrease thereafter is typical for *S. aureus* (Lindberg et al. 2011; Stokholm et al. 2016) and probably contributed to the higher prevalence of *S. aureus* in term neonates compared with BM of their mothers.

5.3 Virulence of *S. epidermidis* colonizing neonates and BM

5.3.1 The virulence-related genes in *S. epidermidis* colonizing neonates

The carriage rate of the *icaA* gene was similar, but that of IS*256* was higher in gut-colonizing *S. epidermidis* from preterm neonates in Study A compared with those in Study B (Figure 12). The overall prevalence of ACME was significantly higher in Study A compared with Study B, whereas type I and II prevailed in the first, but type III in the latter. In isolates from skin of preterm neonates in Study B, only the carriage rate of IS*256* was higher compared with isolates from gut.

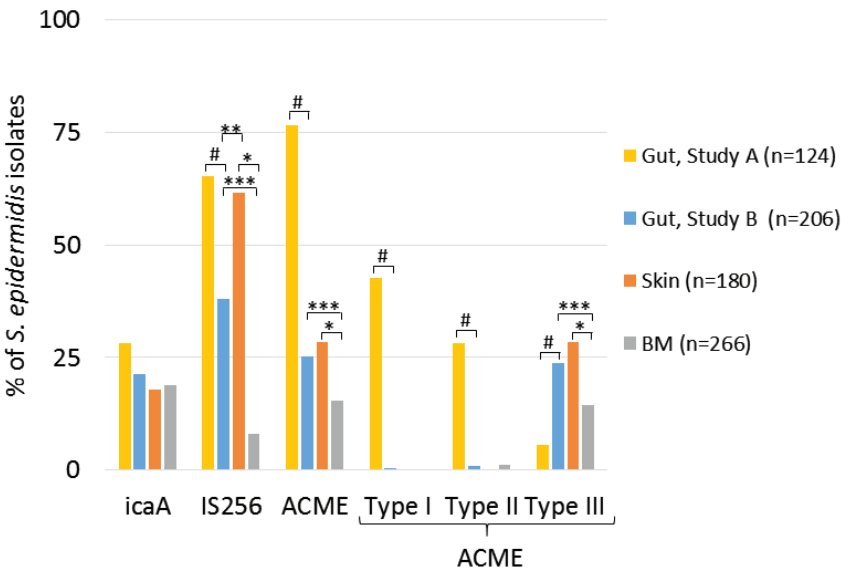


Figure 12. Carriage rate of the virulence-related genes in *S. epidermidis* from preterm neonates and their mothers

Asterisk (*) indicates statistically significant ($p<0.05$) difference between the carriage rate in isolates from skin and BM, two asterisks (**) between skin and gut, three asterisks (***) between gut and BM in Study B. Number sign (#) indicates statistically significant ($p<0.05$) difference between isolates from gut of neonates in Study A compared with Study B.

S. epidermidis from healthy term neonates carried the *icaA* gene, IS*256* and ACME significantly less commonly than those colonizing gut of hospitalized term neonates in Study A (Figure 13) or gut or skin of preterm neonates in Study B (Paper IV Table 2). In contrast, *S. epidermidis* colonizing hospitalized term compared with preterm neonates in Study A were similar in terms of the carriage rate of the *icaA* gene (26.7% vs 28.2%), IS*256* (53.3% vs 65.3%), ACME (80% vs 76.6%), *agr* type I (53.3% vs 58.9%), *agr* type II (40% vs 34.7%) and *agr* type III (6.7% vs 5.6%).

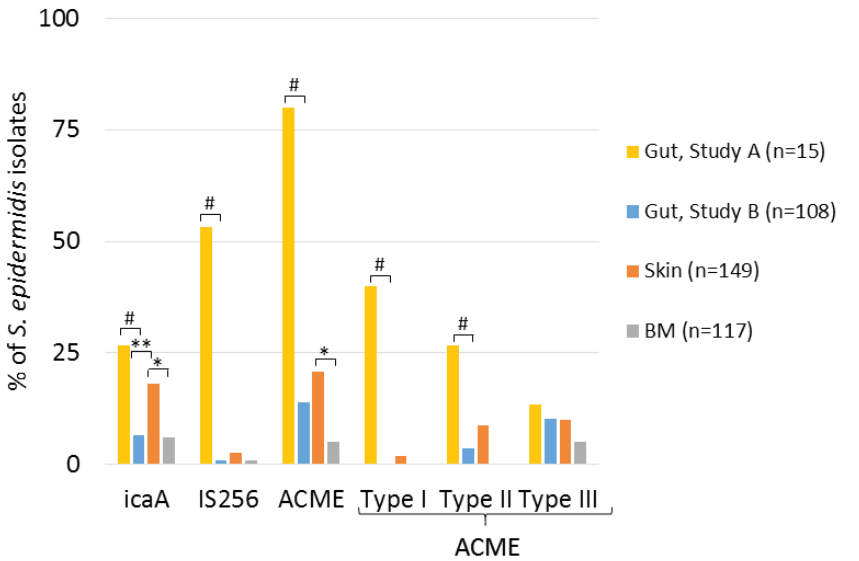


Figure 13. Carriage rate of the virulence-related genes in *S. epidermidis* from term neonates and their mothers

Asterisk (*) indicates statistically significant ($p<0.05$) difference between the carriage rate in isolates from skin and BM, two asterisks (**) between skin and gut in Study B. Number sign (#) indicates statistically significant ($p<0.05$) difference between isolates from gut of neonates in Study A compared with Study B.

Only few studies have characterized the virulence-related genes in CoNS colonizing neonates. The low carriage rate of IS*256* in *S. epidermidis* colonizing healthy term neonates is in accordance with the absence of IS*256* in isolates in a previous study (Hell et al. 2013), but the prevalence of the *icaA* gene in the current study was lower than 29–44% found by others (Jiménez et al. 2008b; Hell et al. 2013). In *S. epidermidis* colonizing preterm neonates, the prevalence of the *icaA* gene varies between 40–53% (de Silva et al. 2002; Salgueiro et al. 2017) and only 5% carry ACME (Salgueiro et al. 2017) that is higher and lower, respectively, compared with the carriage rates in our studies. Such discrepancies with the previous studies could in part result from the characteristics of the strains spreading in the geographical location at the time of the study. For example, the commonest STs of *S. epidermidis* differ from each other in terms of the virulence-related genes, consistently in different studies (Li et al. 2009; Du et al. 2013; Hellmark et al. 2013), including Study A (Table 12, Paper II Table 3) – ST2 harbours the *icaA* gene and IS*256*, ST5 carries IS*256*, but not the *icaA* gene and ST59 does not possess the *icaA* gene, and only rarely IS*256*. Geographical variation in the carriage rate of ACME, from 37.5% to 90.9% (Barbier et al. 2011), may also result from different characteristics of the most prevalent strains.

No studies have compared the characteristics of CoNS colonizing non-hospitalized and hospitalized neonates. Still, the higher carriage rate of the

virulence-related genes in the latter group is expected as the *icaA* gene and IS*256* (Tolo et al. 2016) and *agr* type I (Carmody and Otto 2004) are associated with hospital environment rather than community. Although ACME is often present in carriage isolates from healthy volunteers (Tolo et al. 2016), this element was somewhat more common in isolates colonizing preterm than term neonates in Study B. Still, in addition to colonization, ACME possibly contributes to shuffling between hospital and community (Rolo et al. 2012) and may confer some advantage for colonization and transmission (Miragaia et al. 2009) also in hospital.

5.3.2 The virulence-related genes in *S. epidermidis* colonizing BM

Similarly to their neonates, mothers of preterm compared with term neonates harboured in BM *S. epidermidis* carrying the *icaA* gene (18.8% vs 6%; p=0.002), IS*256* (7.9% vs 0.9%; p=0.013) and ACME (15.4% vs 5.1%; p=0.008) and *S. haemolyticus* carrying IS*256* (61.9% vs 10%; p=0.02) at higher rate.

Of the virulence-related genes, only the presence of the *icaA* gene has been described in CoNS colonizing BM that was harboured by 18–23% of *S. epidermidis* isolates in mothers of healthy term neonates (Jiménez et al. 2008a; Jiménez et al. 2008b; Delgado et al. 2009). This is similar to the prevalence of the *icaA* gene in mothers of preterm neonates, but higher than in mothers of term neonates in Study B. As *mecA*-positive isolates are more likely to carry the *icaA* gene (Tolo et al. 2016), the geographical variation in methicillin-resistance (Morgenstern et al. 2016) may also be reflected in the variation of the prevalence of the *icaA* gene between studies.

Despite higher carriage rate of the virulence-related genes in CoNS compared with mothers of term neonates, *S. epidermidis* colonizing BM of mothers of preterm neonates compared with gut or skin of their neonates carried IS*256* and ACME at significantly lower rate (Figure 12). Such difference is expected, because hospital environment is an important source of staphylococci colonizing preterm neonates (Krediet et al. 2001), while staphylococci in BM should largely represent commensal strains in community. Accordingly, the difference in the carriage rate of the virulence-related genes between isolates from preterm neonates compared with their mothers should be similar to the difference between isolates from preterm and term neonates.

5.3.3 Dynamics of virulence in *S. epidermidis* colonizing neonates and BM

In preterm neonates in Study B, the carriage rate of IS*256* and the *icaA* gene was similar in gut- and skin-colonizing isolates in the first week of life (Figure 14). By the fourth week of life, the proportion of IS*256*-positive isolates had decreased in gut, being significantly lower than that on skin (18.4% vs 66.7%; p<0.001).

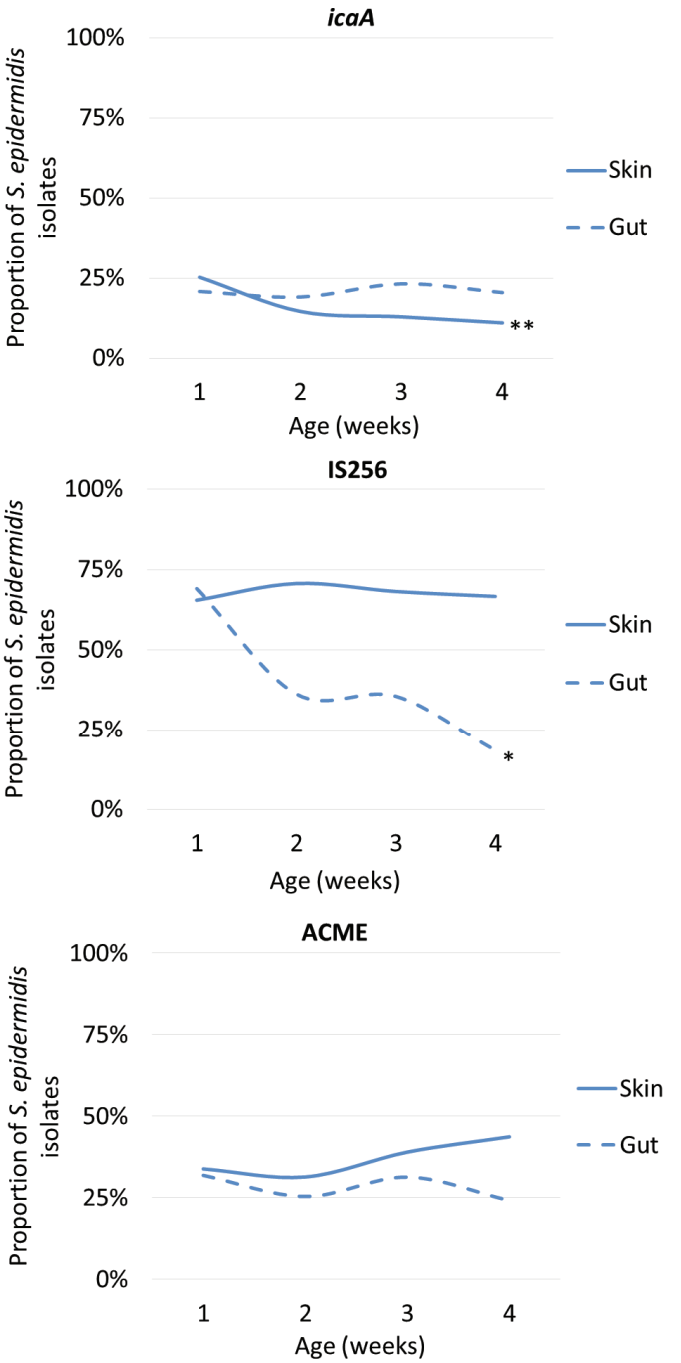


Figure 14. Proportion of *icaA*-, IS*256*- and ACME-positive isolates among *S. epidermidis* from gut and skin of preterm neonates in Study B during the first month of life

Asterisk (*) indicates statistically significant ($p<0.05$) difference between *S. epidermidis* isolated in the first compared with the fourth week of life from gut, two asterisks (**) statistically significant ($p=0.005$) difference between *S. epidermidis* isolated in the first compared with the fourth week of life from skin.

This considerable decline may account for the overall lower carriage rate of IS*256* in isolates from gut compared with skin of preterm neonates in Study B and in gut isolates from Study B compared with Study A (Figure 12). On skin, the proportion of *icaA*-positive isolates decreased, but was only somewhat lower than that in gut isolates in the fourth week of life (11.1% vs 20.6%; p=0.053). No significant changes over time in the prevalence of ACME occurred on skin or in gut, but in the fourth week of life, the prevalence of ACME was higher in isolates from skin compared with gut (43.7% vs 24.1%; p=0.001).

In BM *S. epidermidis* carrying the virulence-related genes were isolated later compared with isolates not carrying the respective gene: ACME (median (IQR) 8 (6–13) days after birth vs. 6 (4–8); p=0.023), *icaA* (13 (6–17) vs. 6 (4–8), p=0.002) and IS*256* (13.5 (8.75–20) vs. 6 (4–8), p<0.001). In line with it, the cumulative proportion of mothers of preterm neonates harboring *icaA*-, IS*256*- or ACME-positive *S. epidermidis* in BM increased during the first month after delivery (Paper III, Figure 3B). However, the proportion of isolates carrying virulence-related genes did not change during the first month of life in mothers of preterm neonates (Paper III Figure 3A) and also not in term neonates and their mothers.

5.4 Antimicrobial resistance of CoNS colonizing neonates and BM

Antimicrobial susceptibility was determined only in *S. epidermidis* in Study A. Nearly all isolates colonizing gut of neonates were resistant to penicillin and the majority to oxacillin and gentamicin, but all were susceptible to vancomycin (Table 9). Isolates from preterm compared with those from term neonates were more frequently resistant to penicillin and oxacillin, but not to other antibiotics tested.

Table 9. Resistance rate of *S. epidermidis* from gut of hospitalized neonates in Study A

	Isolates from		
	Preterm neonates (n=124)	**Term neonates (n=15)**	**p-value**
Penicillin – n (%)	124 (100)	13 (86.7)	0.003
Oxacillin – n (%)	116 (93.5)	11 (73.3)	0.032
Gentamicin – n (%)	110 (88.7)	10 (66.7)	–
Ciprofloxacin – n (%)	47 (37.9)	7 (46.7)	–
Clindamycin – n (%)	88 (29)	6 (40)	–
Vancomycin – n (%)	0	0	–

–, not significant

Oxacillin-susceptible compared with -resistant isolates were isolated earlier (median (IQR) 2 (1.75–4) vs 6 (4–11) days of life; p=0.001) and carried less frequently IS*256* (0% vs 70.1%; p<0.001), belonged more commonly to *agr* type III (25% vs 3.9%; p=0.019) and were less often resistant to gentamicin (0% vs 94.5%; p<0.001).

The high resistance rates in *S. epidermidis* colonizing hospitalized neonates to oxacillin and gentamicin are in accordance with previous studies reporting rates of 75–97% and 43–87%, respectively (Scheifele and Bjornson 1988; Bialkowska-Hobrzanska et al. 1993; de Silva et al. 2001; Jain et al. 2004). The absence of resistance to vancomycin and the resistance rate to ciprofloxacin are also within the range previously reported (0% and 5–63%, respectively) (de Silva et al. 2001; Jain et al. 2004). Still, the resistance to clindamycin was considerably lower in Study A compared with previously reported 87% (Bialkowska-Hobrzanska et al. 1993).

In light of the association of IS*256* and gentamicin-resistance with the hospital environment (Du et al. 2013) and *agr* type III with community (Carmody and Otto 2004), oxacillin-susceptible strains in Study A could have originated from a source representing community, such as mother. Indeed, mother's skin is colonized with CoNS mostly susceptible to various antibiotics (Hira et al. 2013) and skin-to-skin contact with mother promotes colonization with methicillin-susceptible staphylococci (Lamy Filho et al. 2015). As larger proportion of term compared with preterm neonates was born vaginally (40% vs 60.2%; p=0.011), exposure to staphylococci during delivery (Stokholm et al. 2014) may have resulted in acquisition of strains from mother. Term compared with preterm neonates were hospitalized in the NICU later (median (IQR) age 1 (0–4) vs 4 (2–8) hours; p<0.001). Thus, longer duration of exposure to isolates in other wards of hospital where CoNS are less resistant than in intensive care units (Lenart-Boroń et al. 2016), may have also contributed to colonization with oxacillin-susceptible *S. epidermidis*.

5.4.1 The *mecA* gene in *S. epidermidis* colonizing neonates

The carriage rate of the *mecA* gene in *S. epidermidis* colonizing preterm neonates and hospitalized term neonates was high, although in the latter group the proportion of *mecA*-positive *S. epidermidis* was lower than in preterm neonates in Study A (Table 10). In contrast, healthy term neonates were colonized with *S. epidermidis* carrying the *mecA* gene at lower rate compared with preterm neonates and hospitalized term neonates. In Study A, *mecA*-positive *S. epidermidis* mostly carried SCC*mec* type IV (43.9%), followed by type III (2.2%), type V (2.2%), type VI (1.4%), similarly in term and preterm neonates. Still, large proportion of isolates (41.7%) harboured non-typeable SCC*mec* that mostly contained multiple *ccr* and/or *mec* gene complexes (25.8%), no *ccr* and/or *mec* gene complex (10.1%) or combination of *ccr* and *mec* gene complex not assigned a type number (5.8%).

Table 10. Carriage rate of the *mecA* gene in *S. epidermidis* colonizing gut or skin of neonates

	Isolates from		p-value
	Preterm neonates	Term neonates	
Study A			
Gut – n (%)	116/124 (93.5)	11/15 (73.3)	0.032
Study B			
Gut – n (%)	150/206 (72.8)[1]	3/108 (2.8)[2]	<0.001
Skin – n (%)	163/180 (90.6)[3]	10/149 (6.7)	<0.001

[1]$p<0.001$ and [2]$p<0.001$ between gut-colonizing *S. epidermidis* in preterm and term, respectively, neonates in Study A vs Study B; [3]$p<0.001$ between skin- vs gut-colonizing isolates in preterm neonates in Study B

The high *mecA* carriage rate in preterm neonates is in accordance with 95% in CoNS colonizing skin or gut of hospitalized preterm neonates at the age of 7 days (Hira et al. 2013) or 84% in CoNS from nares at discharge (Ternes et al. 2013), whereas large proportion carry non-typeable SCC*mec* elements (Ternes et al. 2013; Salgueiro et al. 2017). The prevalence of *mecA* gene in healthy term neonates was lower compared to previously found 33% (Jiménez et al. 2008b), but the results should be interpreted in the light of significant variation in methicillin-resistance rates between different geographical regions (Morgenstern et al. 2016).

As the *mecA* gene is associated with hospital environment rather than community (Tolo et al. 2016), the difference between the *mecA* prevalence in healthy non-hospitalized and hospitalized neonates is expected. While healthy term neonates are mostly exposed to community strains that commonly exhibit low resistance rates (Cavanagh et al. 2016), hospitalized neonates become colonized with resistant strains on the hands of healthcare workers or in the air of NICU (Krediet et al. 2001).

5.4.2 The *mecA* gene in CoNS colonizing BM

Similarly to neonates, the mothers of preterm and term neonates differed in terms of the presence of the *mecA* gene in colonizing staphylococci. In BM of mothers of preterm compared with term neonates, the carriage rate of the *mecA* gene was significantly higher in *S. epidermidis* (32.7% vs 2.6%; $p<0.001$) and *S. haemolyticus* (90.5% vs 10%; $p<0.001$). Similar difference between the mothers was also evident when comparing the prevalence of colonization in at least one BM sample with *mecA*-positive *S. epidermidis* (77.6% vs 15%; $p<0.001$), *S. haemolyticus* (34.7% vs 5%; $p<0.05$) and any *mecA*-positive staphylococcus (83.7% vs 25%; $p<0.001$).

The carriage rate of the *mecA* gene in CoNS from BM of mothers of term neonates in the current study is lower than previously reported 13–44% (Jiménez et al. 2008a; Jiménez et al. 2008b; Delgado et al. 2009), possibly in

part attributable to geographical variation in antimicrobial resistance rates (Morgenstern et al. 2016). The risk of colonization with methicillin-resistant CoNS is increased by use of antimicrobial agents (Morgenstern et al. 2016) and hospitalization (Widerström et al. 2016). These may explain the higher carriage rate of the *mecA* gene in mothers of preterm compared with term neonates, as only mothers of preterm neonates had received antibiotics and were hospitalized for longer period of time than mothers of term neonates (Table 8).

Still, mothers of preterm neonates were colonized with *S. epidermidis* carrying the *mecA* gene at lower rate compared with isolates colonizing gut (32.7% vs 72.8%; $p<0.001$) and skin (32.7% vs 90.6%; $p<0.001$) of preterm neonates. Although staphylococci in BM and colonization sites of preterm neonates have not been simultaneously studied, CoNS on the hands of mothers carried the *mecA* gene at significantly lower rate compared with isolates from skin or gut of preterm neonates (7–30% vs 55–95%) (Hira et al. 2013). Such discrepancy between mothers and neonates may be explained by different sources of colonizing strains. Mothers probably carry the strains spreading in community that have low antimicrobial resistance rate, such as 4.1% to cefoxitin (Cavanagh et al. 2016). In contrast, preterm neonates in hospital become colonized with resistant strains present on the hands of healthcare workers or in the air of NICU (Krediet et al. 2001).

5.4.3 Dynamics of *mecA* in *S. epidermidis* colonizing neonates and BM

In preterm neonates in the first week of life, the *mecA* carriage rate in *S. epidermidis* from skin and gut was similarly high (89.4% vs 93.6%), decreasing thereafter (Figure 15). In the fourth week of life the carriage rate of the *mecA* gene in isolates from gut was somewhat lower than in isolates from skin (73.8% vs 84.1%; $p=0.056$). This decline may account for the overall lower prevalence of the *mecA* gene in gut compared with skin isolates from preterm neonates in Study B (Table 10).

These results are in contrast with a previous study on preterm neonates, where the proportion of *mecA*-positive CoNS had decreased on skin from 95% at the age of 7 days to about 82% by the age of 21 days, but remained as high as 95–100% in gut throughout the first three weeks of life (Hira et al. 2013). In another study, among nasal CoNS isolates the carriage rate of the *mecA* gene increased from 60% at admission to 84% at discharge (Ternes et al. 2013). The differences in dynamics of the *mecA* gene may be explained in part by comparison of isolates collected at different ages of neonates, for example there were no data on isolates colonizing in the fourth week of life available (Hira et al. 2013). In addition, differences in clinical practice influencing colonization, such as Kangaroo care (Lamy Filho et al. 2015), cannot be excluded, as no data on duration of contact with mothers were available in neither of the studies (Hira et al. 2013; Ternes et al. 2013).

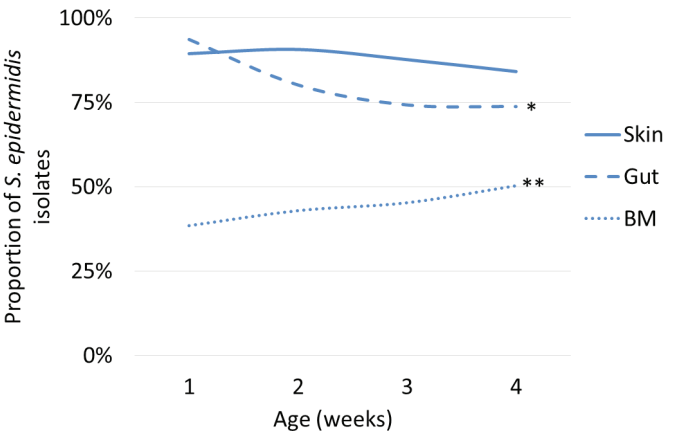


Figure 15. The proportion of *mecA*-positive among all *S. epidermidis* isolates from gut or skin of preterm neonates and BM of their mothers during the first month of life

Asterisk (*) indicates statistically significant ($p<0.001$) difference between *S. epidermidis* isolated in the first compared with the fourth week of life from gut, two asterisks (**) statistically significant ($p=0.0337$) difference between *S. epidermidis* isolated in the first compared with the fourth week of life from BM.

In term neonates and their mothers no significant changes in the carriage rate of the *mecA* gene during the first month after delivery was observed, but in BM of mothers of preterm neonates, the carriage rate increased from the first to the fourth week of life (Figure 15). Moreover, in BM *S. epidermidis* carrying the *mecA* gene were isolated later compared with isolates not carrying the *mecA* gene (8 (6–12.75) vs. 6 (4–8), $p=0.001$) and the cumulative proportion of mothers of preterm neonates harbouring *mecA*-positive *S. epidermidis* in BM increased during the first month after delivery (Paper III, Figure 3B).

Although dynamics of the carriage rate of the *mecA* gene in CoNS in BM has not been studied, for other microorganisms, such as methicillin-resistant *S. aureus* (Kayıran et al. 2014), it has been shown that BM cultures initially showing no growth of resistant bacteria may contain these later. This may be due to variability of the composition of BM and thus intermittent presence of specific bacteria, exemplified by the presence of resistant *K. pneumoniae* or *Escherichia coli* strains in only 46–62% of samples from one mother (Rettedal et al. 2012; Nakamura et al. 2016). Also acquisition of resistant bacteria into BM from NICU cannot be excluded, as has been described for *K. pneumoniae* (Rettedal et al. 2012).

5.4.4 Risk factors for colonization of BM with *mecA*-positive CoNS

Adjusted for duration of pregnancy, longer duration of hospitalization of mother within one month after delivery, hospitalization of mother in neonatology unit as a caretaker of a neonate, and use of arterial catheter or antibacterial agent in a

neonate increased the odds of colonization of mother's BM with *mecA*-positive CoNS (Table 11).

Table 11. Maternal and neonatal characteristics associated with the presence of staphylococci carrying the *mecA* gene in BM

	***mecA*-positive staphylococci in BM**		**OR (95% CI)**
	No (n=23)	**Yes (n=46)**	
Maternal characteristics			
Duration of pregnancy – w; median (IQR)	39 (29–40)	29 (26–33)	0.84 (0.76–0.93)
Duration of hospitalization within 1 month after delivery[1] – days; median (IQR)	2 (2–3)	4 (3–5)	2.34 (1.28–4.29)
Hospitalized as a caretaker of neonate – n (%)	0	17 (40)	24.9 (1.27–485)
Neonatal characteristics			
Use of arterial catheter – n (%)	5 (21.7)	39 (84.8)	17.4 (2.60–116)
Use of any antibacterial agent – n (%)	7 (30.4)	41 (89.1)	48.5 (2.60–906)

Only statistically significant characteristics are shown, full table is presented in Paper III (Table S1, Table S2).
[1]Data for two mothers were not available.

Duration of pregnancy as a risk factor may be in part attributable to the significant correlation with other risk factors, such as duration of postpartum hospitalization (Spearman's rank correlation rho –0.34; p=0.005). Furthermore, GA was significantly smaller in neonates with arterial catheter (median (IQR) 28 (25–30) vs 39 (39–40); p<0.001) or requiring antibacterial treatment (median (IQR) 28 (25–30) vs 40 (39–40); p<0.001) compared with other neonates. Although no association between duration of pregnancy and hospitalization as a caretaker was observed, only mothers of preterm neonates were hospitalized as caretakers.

Factors influencing BM colonization with resistant bacteria have been scarcely described. In one study, the *mecA* carriage rate in *S. epidermidis* was higher in women with mastitis compared with healthy women (62.5% vs 33%) (Delgado et al. 2009). Furthermore, CoNS in women with breast pain had resistance rate to oxacillin 47% (Witt et al. 2014) that was somewhat higher than in healthy mothers of term neonates (<10% to 39%) in other studies (Jiménez et al. 2008a; Jiménez et al. 2008b; Delgado et al. 2009). However, none of the mothers in the current study complained about any mastitis-related symptoms or pain.

Considering studies on other patient groups, hospitalization of mother, including hospitalization as a caretaker of neonate, as a risk factor of colonization with *mecA*-positive staphylococci is in accordance with increased carriage rate of methicillin-resistant *S. epidermidis* after admission to hospital

(Widerström et al. 2016). Although antibacterial treatment may increase the risk of colonization with methicillin-resistant staphylococci (Morgenstern et al. 2016) this has not been shown unequivocally (Lebeaux et al. 2012) and antibiotics were also not risk factors in the current study.

Antibacterial treatment increases the risk of colonization with resistant CoNS in hospitalized neonates (Ternes et al. 2013; Gibson et al. 2016), but data on the influence of clinical characteristics, such as the presence of arterial catheter, on colonization with CoNS are limited. Still, arterial catheter increases the risk of colonization with other bacteria spreading in NICU (Adamson et al. 2012). Therefore, use of arterial catheter and antibiotics in neonate as risk factors of maternal colonization may be explained by the role of neonatal microbiota in colonization of mother, similarly to the influence of maternal colonization on neonate (Lamy Filho et al. 2015).

5.5 MLVA-types of CoNS colonizing neonates and BM and causing LOS

In Study A, a total of 139 *S. epidermidis* isolates were categorised into 55 distinct MLVA-types, whereas six commonest MLVA-types accounted for 51.1% of all isolates. The prevalence of colonization with these MLVA-types was low (<10%) (Table 12), probably in part reflecting the overall low prevalence of colonization with *S. epidermidis*. All the commonest MLVA-types, except for MT43, were detected in both units, Unit A and Unit B (Paper II Figure 1A).

Table 12. Prevalence and characteristics of the commonest *S. epidermidis* MLVA-types colonizing gut of neonates in Study A

MLVA-type (corresponding ST)	Number (%) of neonates colonized		Number (%) of isolates carrying the gene			
	Preterm (n=221)	Term (n=55)	*mecA*	*icaA*	IS*256*	ACME
MT34 (ST2)	19 (8.6)	2 (3.6)	21 (100)	21 (100)	19 (90.5)	19 (90.5)
MT21 (ST5)	17 (7.7)	2 (3.6)	19 (100)	0 (0)	19 (100)	16 (84.2)
MT23 (ST5)	10 (4.5)	1 (1.8)	11 (100)	0 (0)	11 (100)	9 (81.8)
MT41 (ST81)	7 (3.2)	0 (0)	7 (100)	0 (0)	1 (14.3)	6 (85.7)
MT48 (ST59)	6 (2.7)	1 (1.8)	7 (100)	0 (0)	1 (14.3)	4 (57.1)
MT43 (ST81)	6 (2.7)	0 (0)	6 (100)	0 (0)	1 (16.7)	2 (33.3)

In Study B, of all 2933 isolates of *S. epidermidis* from gut, skin and BM 2930 were typeable by MLVA yielding total of 434 distinct MLVA-types. Total of 23 MLVA-types colonized at least five neonates in gut or on skin (Figure 16A) and accounted for 44.4% of all isolates. Nine of the 23 commonest MLVA-types colonized only preterm neonates and their mothers, hereafter designated as predominant NICU strains, and accounted for 49.5% (560 of 1132) of all isolates from preterm neonates and 6% (49 of 816) of isolates from their mothers. Of the 23 commonest MLVA-types 15 were isolated from both units, Unit A and Unit B.

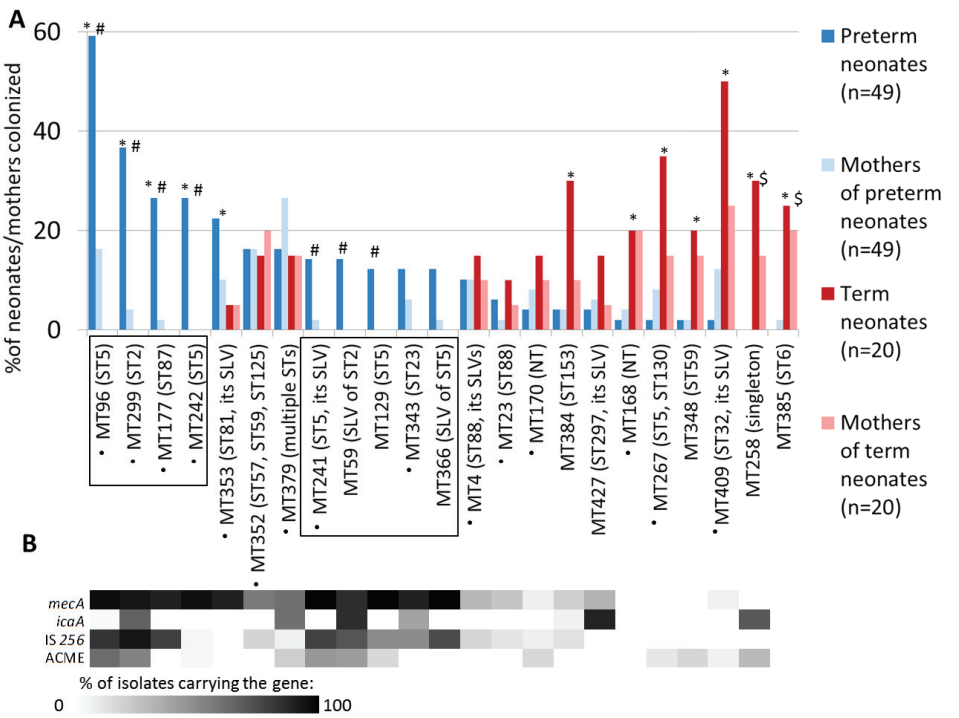


Figure 16. Prevalence and characteristics of MLVA-types colonizing at least five neonates in gut and/or on skin

(A) Prevalence of colonization among neonates and mothers. ST or its SLV that MLVA-type corresponded to is shown (MT379 corresponded to ST35, ST54, ST142, SLV of ST143, ST487 and its SLV). Black boxes indicate predominant NICU strains, i.e., 9 MLVA-types of the 23 most common MLVA-types that colonized only preterm neonates and their mothers. Dots indicate MLVA-types that were isolated from both units, Unit A and Unit B; MLVA-types without dots were isolated from Unit B and/or term neonates/their mothers only. Asterisk (*) indicates statistically significant (p-value <0.05) difference in the prevalence between preterm and term neonates, number sign (#) between preterm neonates and their mothers and dollar sign ($) between mothers of preterm and term neonates. Note that the y-axis ranges from 0% to 60%. (B) The carriage rate of the *mecA*, the *icaA* gene, IS*256*, and ACME. Each column of the heatmap corresponds to the MLVA-types shown in panel A. The darker is the color, the higher is the carriage rate, ranging from 0% (indicated in white) to 100% (black).

Median (IQR) number of distinct MLVA-types was lower in preterm compared with term neonates, both in gut (4 (3–5) vs 6 (3.75–7); p=0.055) and on skin (3 (3–5) vs 7 (5.5–9.25); p<0.001), but similar in mothers of preterm and term neonates (5 (3–7) vs 6 (3.75–8)).

Among 54 MLVA-typeable isolates of *S. haemolyticus* in BM a total of 19 distinct MLVA-types were detected. Three commonest MLVA-types of *S. haemolyticus* accounted for 35.5% (11 of 31) of isolates and colonized only mothers of preterm neonates – MT19 (n=5, 10.2%), MT14 and MT18 (both n=3, 6.1%).

Of eight bloodstream isolates from neonates with LOS caused by *S. epidermidis* in Study A five were the commonest MLVA-types – MT34 (n=4) and MT23 (n=1). Four *S. epidermidis* isolates of total of six that caused LOS in preterm neonates in Study B were available for typing and all were predominant NICU strains – MT96 (n=2), MT241 (n=1), MT242 (n=1). Of eight *S. haemolyticus* isolates causing total of 7 episodes of LOS (one episode was caused by two distinct MLVA-types) in Study B, six were the commonest MLVA-types – MT19 (n=3), MT14 (n=2), and MT18 (n=1).

The results of our studies are in accordance with the few previous studies on molecular epidemiology of CoNS colonizing hospitalized neonates. First, similarly to the current results, previous studies have found that predominant CoNS strains may constitute 52% of all isolates from skin swabs and blood cultures (de Silva et al. 2001), whereas single most common strain can colonize 25–39% of hospitalized neonates (Sloos et al. 1996; Sloos et al. 1998; Kornienko et al. 2016). Second, the presence of the commonest colonizing MLVA-types among LOS causing agents reflects previous findings that predominant strains are equally distributed between skin colonizers, blood culture contaminants and invasive isolates (de Silva et al. 2001; Klingenberg et al. 2007). Third, genetic similarity of predominant *S. epidermidis* strains colonizing neonates in NICUs located nearby have been reported previously (Sloos et al. 1998).

5.5.1 Molecular characteristics of predominant MLVA-types

In Study A, the six commonest MLVA-types carried the *mecA* gene, IS*256*, ACME type I and were resistant to gentamicin at significantly higher rate than all other MLVA-types (Table 13). All the six commonest MLVA-types corresponded to ST2, ST5 or ST59 or its single locus variant ST81 (Table 12).

In Study B, predominant NICU strains carried the *mecA* gene, IS*256* and ACME (p<0.001 for all) more frequently than the other 14 commonest MLVA-types that were also isolated from term neonates and their mothers (Figure 16B). Of the nine predominant NICU strains six corresponded to ST5 or its SLVs (including ST87) and two to ST2 or its SLV (Figure 16).

All five isolates of the commonest *S. haemolyticus* MLVA-type MT19 carried the *mecA* gene and IS*256*. Other common MLVA-types, MT14 and MT18, both carried the *mecA* gene at rate of 100 % (n=3), but IS*256* at rate of 33.3% (n=1).

Table 13. Virulence and resistance of the six commonest compared with all other MLVA-types colonizing neonates in Study A

	MLVA-types (number of isolates)		
	Six commonest (n=71)	**Other (n=68)**	**p-value**
The *icaA* gene	21 (29.6)	18 (26.5)	–
IS*256*	52 (73.2)	37 (54.4)	0.033
ACME			
Type I	40 (56.3)	19 (27.9)	0.001
Type II	10 (14.1)	29 (42.6)	<0.001
Type III	6 (8.5)	3 (4.4)	–
agr			
Type I	41 (57.7)	40 (58.8)	–
Type II	29 (40.8)	20 (29.4)	–
Type III	0 (0)	8 (11.8)	0.009
The *mecA* gene	71 (100)	56 (82.4)	<0.001
Gentamicin-resistance	70 (98.6)	50 (73.5)	<0.001

–, not significant

Considering the overall higher virulence of *S. epidermidis* isolated in hospital compared with community (Cherifi et al. 2013; Du et al. 2013), the finding that predominant NICU strains carried the genes at higher rate compared with the commonest MLVA-types colonizing also healthy term neonates and their mothers, is expected. Differences between the carriage rate of the virulence-related genes in the commonest compared with other MLVA-types in Study A corroborate the previous findings that predominant compared with sporadic strains that cause infections in NICU carry IS*256* or the *icaA* gene at higher rate (Foka et al. 2006; Klingenberg et al. 2007) and are more commonly resistant to antibiotics (Klingenberg et al. 2007). Still, predominant strains compared with sporadic ones may not carry the *icaA* gene (de Silva et al. 2002) and may be less commonly multidrug-resistant (Hira et al. 2007). As the virulence-related genes are differently distributed between and strongly associated with STs (Li et al. 2009; Du et al. 2013; Hellmark et al. 2013), observed also in Study A (Table 12, Paper II Table 3), the most prevalent STs at the time of study may considerably influence the results of the comparison of the gene content of predominant and less common strains.

5.5.2 Distribution of *S. epidermidis* MLVA-types colonizing neonates and BM

In Study A, the six commonest MLVA-types were similarly prevalent in preterm and term neonates (Table 12).

In Study B, five MLVA-types (all belonging to the predominant NICU strains) were more and seven were less common in preterm compared with term neonates (Figure 16). Still, all the 23 commonest MLVA-types were similarly distributed between skin and gut isolates from neonates. Seven MLVA-types colonized preterm neonates more commonly compared with their mothers and all these belonged to the predominant NICU strains. Only two MLVA-types were more common in mothers of term compared with preterm neonates. No differences were found between term neonates and their mothers.

Overall, the distribution of *S. epidermidis* strains among isolates colonizing hospitalized compared with healthy term neonates is in accordance with previous studies. First, similarly to the predominant NICU strains in Study B, *S. epidermidis* strains causing LOS in neonates hospitalized in the NICU have not been detected on skin of healthy term neonates (Hell et al. 2013) and also not on the hands of healthy people (Milisavljevic et al. 2005). Second, strains corresponding to ST2 and ST59 or ST81 have been detected in nares of neonates hospitalized in the NICU (Ternes et al. 2013; Salgueiro et al. 2017). In contrast, strains belonging to GC3, as MT409 (corresponding to ST32) in term neonates and their mothers, or GC4 that are associated with commensal lifestyle have been isolated from healthy term neonates and their mothers (Martín et al. 2012) and from fathers visiting their neonates in NICU (Tolo et al. 2016). Finally, in accordance with some strains that are equally likely to spread in hospital and community (Thomas et al. 2014), some MLVA-types were similarly prevalent in preterm and term neonates and their mothers in Study B.

5.5.3 Diversity and dynamics of MLVA-types colonizing neonates and BM

The genetic diversity of *S. epidermidis* in BM of mothers and gut and skin of term neonates (Figure 17) was high throughout the first month after delivery. However, in preterm neonates the genetic diversity of MLVA-types of *S. epidermidis* isolates from gut was low in the first week of life, increasing thereafter (Figure 17), but genetic diversity on skin remained low throughout the first month of life.

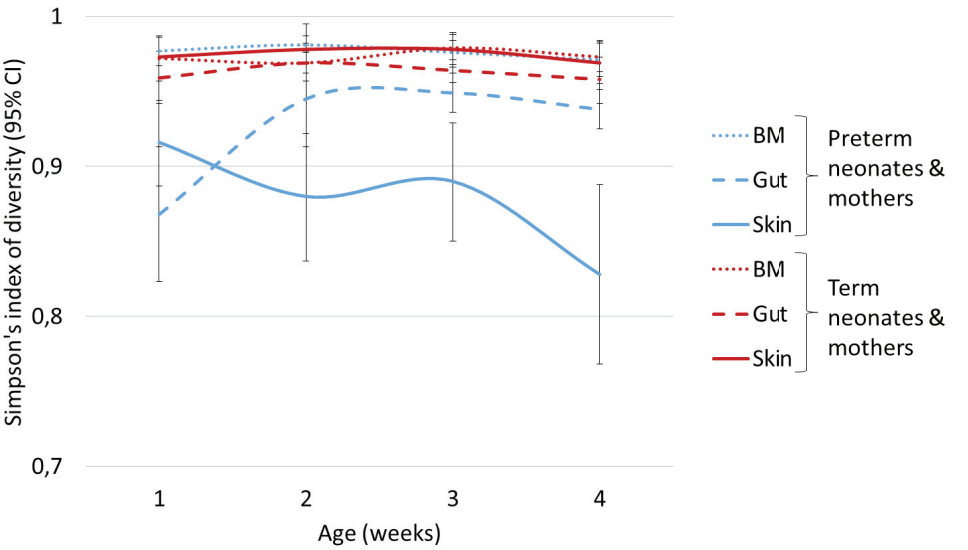


Figure 17. Simpson's index of diversity (95% CI) of MLVA-types of *S. epidermidis* isolates from neonates and their mothers in Study B during the first month after delivery
SID was calculated based on MLVA-types among all isolates collected from the indicated sample in the indicated week. Note that the scale of y-axis starts from 0.7.

The proportion of predominant NICU strains among *S. epidermidis* isolates from gut or skin of preterm neonates was similar in the first week of life, but decreased in gut and increased on skin thereafter (Figure 18). In the fourth week of life, the proportion of predominant NICU strains among all colonizing isolates was significantly larger on skin compared with gut (66.7% vs 27%; $p<0.001$). The proportion of predominant NICU strains did not change in BM of mothers of preterm neonates.

Data on genetic diversity at the strain level of CoNS colonizing preterm neonates is limited, but the overall diversity of *S. epidermidis* from skin of preterm neonates in the current study is similar to previously reported SID of 0.902–0.907 by biochemical fingerprinting (Björkqvist et al. 2010). Dynamics of diversity of microbial communities in preterm neonates has been studied mostly at the species or higher taxonomic level and these studies have reported increasing diversity of gut microbiota (Jacquot et al. 2011; Cong et al. 2016; Gregory et al. 2016), but decreasing diversity on skin within the first weeks of life (Pammi et al. 2017; Salava et al. 2017), similarly to the strain-level changes in the current study.

No studies have compared colonization with CoNS in term and preterm neonates at the strain level. The higher diversity in healthy term neonates and their mothers as well as mothers of preterm neonates compared with hospitalized preterm neonates is in line with studies on adults showing higher diversity in healthy people compared with hospitalized patients (Rolo et al. 2012; Du et al. 2013). Notably, in the current study, diversity of *S. epidermidis* MLVA-types in mothers of term neonates was higher than reported previously

for PFGE-types (median IQR 3 (2–3.5)) (Delgado et al. 2009). As the content of BM is highly variable even between samples from one mother (Sakwinska et al. 2016), such difference may be attributable to collection of multiple samples in our study in contrast to one in the other study (Delgado et al. 2009). Similar difference in the number of stool samples may account for higher diversity of *S. epidermidis* MLVA-types in gut of term neonates in Study B compared with median (IQR) of 1.5 (1–2.5) genotypes by random amplification of polymorphic DNA in a previous study (Jiménez et al. 2008b).

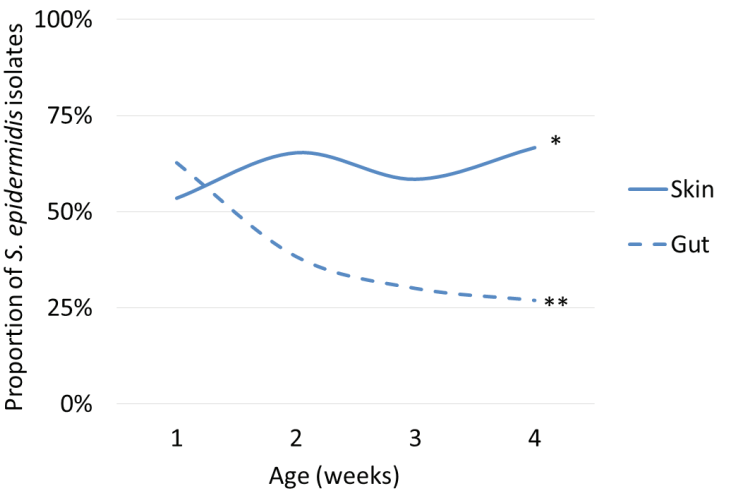


Figure 18. Proportion of predominant NICU strains among *S. epidermidis* isolates from gut and skin of preterm neonates during the first month of life

Asterisk (*) indicates statistically significant (p=0.039) difference between *S. epidermidis* isolated in the first compared with the fourth week of life from skin, two asterisks (**) statistically significant (p<0.001) difference between *S. epidermidis* isolated in the first compared with the fourth week of life from gut.

5.6 Genetic relatedness of *S. epidermidis* colonizing different body sites

Three quarters of preterm neonates and nearly all term neonates harboured indistinguishable MLVA-types on skin and in gut (Figure 19). MLVA-types indistinguishable from those in BM colonized gut of the majority of term neonates soon after birth (Figure 19), but the proportion of colonized preterm neonates increased significantly from the first to the fourth week of life (14.3% to 83.7%; p<0.001). In contrast, indistinguishable MLVA-types on skin and in BM were isolated from significantly larger proportion of term compared with preterm neonates (100% vs 34.7%; p<0.001) throughout the first month of life.

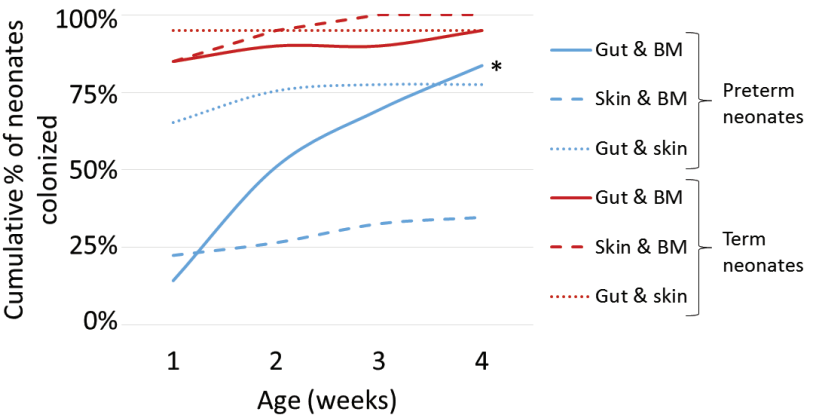


Figure 19. Cumulative proportion of neonates colonized with indistinguishable MLVA-types in gut and skin, in gut and mother's BM, on skin and mother's BM during the first month of life

Asterisk (*) indicates statistically significant ($p<0.001$) difference between the cumulative proportion of neonates colonized in gut with MLVA-type indistinguishable to that in mother's BM in the first compared with the fourth week of life.

According to Bray-Curtis similarity index *S. epidermidis* in gut of preterm neonates became more similar in terms of MLVA-types to those in BM and less similar to those on skin, but BM and skin remained dissimilar throughout the first month of life (Figure 20). In term neonates and their mothers, all pairwise comparisons of MLVA-types from different sample types revealed decreasing similarity during the first month of life (Figure 20).

The current study corroborates the clonality of colonization in neonates, i.e. the presence of indistinguishable strains in multiple body sites, as previously shown by restriction endonuclease fingerprinting of DNA and ribotyping (Bialkowska-Hobrzanska et al. 1993). Moreover, the study demonstrates once again that the majority of healthy term neonates become colonized in gut with MLVA-types indistinguishable from those in mother's BM, whereas this occurred somewhat more frequently than previously reported 55–75% of healthy term breastfed neonates (Jiménez et al. 2008b; Martín et al. 2012). In preterm neonates, our study is the first to describe colonization of neonate with *S. epidermidis* strains in mother's BM. Recently it was shown that of BM-fed preterm neonates 50% became colonized at median age of 14 days in their gut with *S. epidermidis* strain also present in mother's own BM (Gómez et al. 2016). However, in that study BM was sampled after passing it through external feeding tube system and thus BM as a source of colonizing strains could not be conclusively inferred from the data.

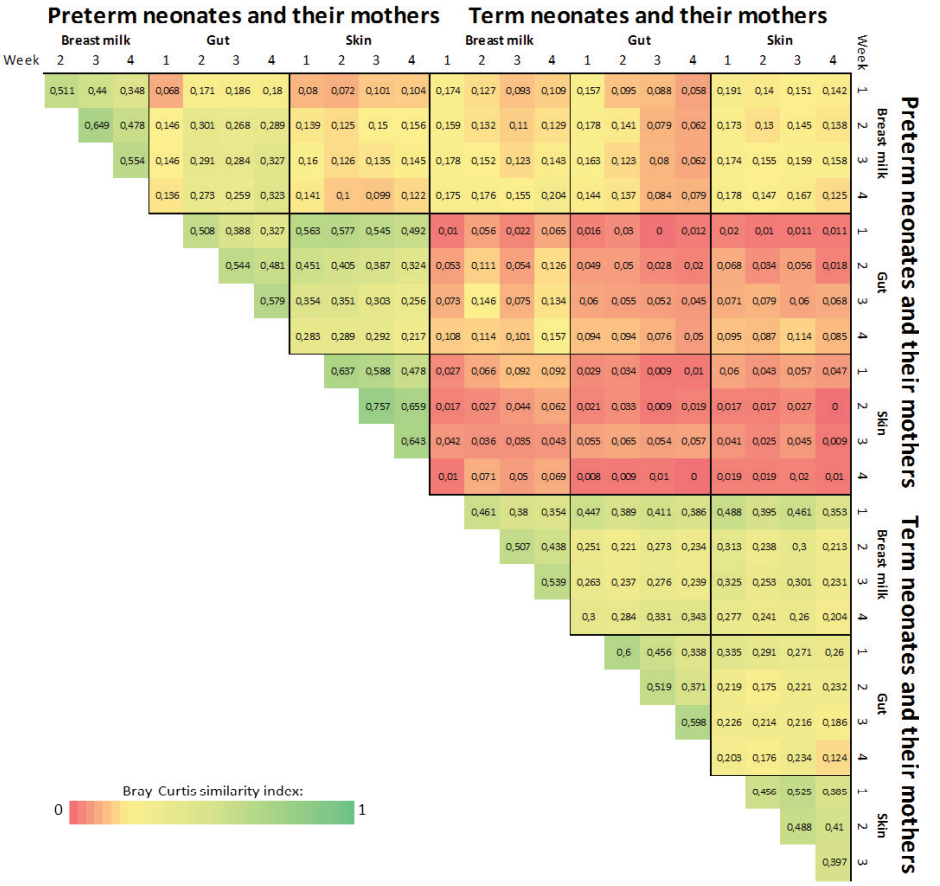


Figure 20. Similarity of neonatal skin and gut and mother's BM samples in terms of *S. epidermidis* MLVA-types during the first month of life

The heatmap is based on the Bray-Curtis similarity index of MLVA-types among all isolates collected from the indicated sample in the indicated week. Similarity of *S. epidermidis* communities increases from red (completely dissimilar communities; Bray-Curtis similarity index 0) to green (identical communities; Bray-Curtis similarity index 1).

5.6.1 Time of colonization with indistinguishable MLVA-types

In preterm neonates, indistinguishable MLVA-types on skin and in BM colonized skin earlier than BM (median (IQR) at 6 (4–14) vs 13 (8–19) days; p=0.029) (Paper IV Figure 3A); indistinguishable MLVA-types in gut and in BM colonized gut later than BM (14 (10–19) vs 9 (6–15) days; p=0.018); indistinguishable MLVA-types in gut and on skin were isolated from both sites at similar age (7.5 (4–12) vs (6 (3.25–8) days). In term neonates, indistinguishable MLVA-types were isolated from skin, gut and BM mostly within the first week of life (Paper IV Figure 3B).

5.6.2 Virulence of indistinguishable MLVA-types

In preterm neonates, indistinguishable MLVA-types in gut and in BM had lower carriage rate of the *mecA* gene, IS*256* and ACME and the proportion of predominant NICU strains was smaller compared with indistinguishable MLVA-types on skin and in BM or in gut (Table 14). There were no such differences in term neonates (Paper IV Table 3).

Table 14. Carriage rate of the virulence- and resistance-related genes in *S. epidermidis* indistinguishable MLVA-types isolated from both sample types in one mother-neonate pair

	Indistinguishable MLVA-types		
	In BM & on skin (n=44)	**In BM & in gut (n=140)**	**In gut & on skin (n=132)**
The *mecA* gene – n (%)	38 (86.4)	80 (57.1)[1]	123 (93.2)[2]
The *icaA* gene – n (%)	7 (15.9)	26 (18.6)	24 (18.2)
IS*256* – n (%)	21 (47.7)	26 (18.6)[1]	81(61.4)[2]
ACME – n (%)	20 (45.5)	30 (21.4)[1]	43 (32.6)
Predominant NICU strains – n (%)	24 (54.5)	26 (18.6)[1]	82 (62.1)[2]

[1]$p<0.003$ between indistinguishable MLVA-types in BM and in gut vs in BM and on skin; [2]$p<0.001$ between indistinguishable MLVA-types in BM and in gut vs in gut and on skin

5.7 Colonization of gut, skin and BM with CoNS subsequently causing LOS

In Study A, a total of 32 episodes of LOS caused by CoNS developed in 29 neonates. Bloodstream isolates (n=23) were available only from Unit A from 22 neonates from total of 22 episodes of LOS (caused by *S. haemolyticus* (n=12), *S. epidermidis* (n=7), *S. hominis* (n=2) and one episode was mixed (caused by *S. haemolyticus* and *S. epidermidis*)). Median age at the onset of LOS caused by CoNS was 9 days (7–13). All 22 neonates were preterm (GA <37 weeks), whereas all except one had GA <32 weeks and BW <1500 g. All except one neonate had CVC (Paper I, Table 1).

In Study B total of 14 episodes of LOS (*S. epidermidis* (n=6), *S. haemolyticus* (n=6), *S. hominis* (n=1) and one episode was mixed (caused by *S. haemolyticus* and *S. hominis*)) were caused by CoNS in 11 neonates and median (IQR) age at the onset was 8 (5–14) days. All 11 neonates were preterm (GA <37 weeks), whereas 9 of 11 were very preterm (GA <32 weeks) and VLBW. All 11 neonates had CVC. Molecular typing data for blood culture isolate and gut, skin and BM isolates of the same species as that causing LOS were available for four neonates with LOS caused by *S. epidermidis*.

Of the 26 neonates with available bloodstream isolates, 5 of 12 neonates with LOS caused by *S. epidermidis* and 10 of 13 neonates with LOS caused by

S. haemolyticus harboured strain indistinguishable by typing method from the LOS-causing strain in gut no more than 7 days prior to the onset of LOS (Figure 21). Comparison of *S. epidermidis* colonizing skin and causing LOS revealed that 2 of 4 neonates had subsequently invasive strain on skin. None of the mothers of the four preterm neonates in Study B with LOS caused by *S. epidermidis* had LOS-causing strain in BM.

Causative agent of LOS	Neonate no.		Day prior to the onset of LOS when colonizing strain was isolated													LOS strain
			20	19	18	17	16	...	7	6	5	4	3	2	1	0	
S. epidermidis (Study B)	1	gut										343, 299				**241**	**241**
		skin															241
	2	gut												**96**			**96**
		skin												**96**			**96**
	3	gut															96
		skin								**96**							**96**
	4	gut															242
		skin															242
S. epidermidis (Study A)	5								17			17					34
	6		15				15										57
	7		21			21											34
	8											**20**				**20**	**20**
	9									**34**							**34**
	10								**23**			20					**23**
	11															20	58
	12																34
S. haemolyticus (Study A)	12															**A1**	**A2**
	13								**A1**				**A1**				**A1**
	14								**A1**			**A1**					**A1**
	15														**A2**		**A2**
	16									**B**				**B**			**B**
	17											**A2**					**A2**
	18												**A2**			**A2**	**A2**
	19										**C**					**A2**	**A2**
	20															**D**	**D**
	21														**A2**		**A2**
	22											**A2**					**A2**
	23																NT
	24																NT
S. hominis (Study A)	25																NT
	26																NT

Figure 21. Time of isolation of invasive strain from gut or skin prior to the onset of LOS

Colonization of gut with in neonates in Study A in whom LOS caused by CoNS developed is shown, except for neonates no. 1 to 4 from Study B in whom *S. epidermidis* from skin was also available for typing. Number indicates MLVA-type of *S. epidermidis* and letter (and number) PFGE-type of *S. haemolyticus*. Blue indicates prior gut colonization and orange prior skin colonization with the same strain as that causing LOS. If colonization with the same species as that causing LOS was not present prior to the onset of LOS, data on colonization is left blank. NT indicates that the LOS causing isolate was not typed. In neonate no. 1, MLVA-type 96 was additionally isolated from gut at the onset of LOS (day 0). Neonate no. 12 had mixed episode of LOS, caused by *S. epidermidis* and *S. haemolyticus*.

Previous studies have demonstrated genetic similarity of invasive and gut-colonizing strains in 1 of 3 by plasmid restriction endonuclease fingerprinting (Eastick et al. 1996) and in 1 of 2 neonates with LOS caused by CoNS by restriction endonuclease fingerprinting of chromosomal DNA and ribotyping (Bialkowska-Hobrzanska et al. 1990). In more recent studies, the genetic similarity determined by PFGE was detected in 2 of 26 (Cossey et al. 2014) and similarity according to antimicrobial susceptibility pattern in 2 of 6 (Shaw et al. 2015) neonates with LOS caused by CoNS. Thus, the genetic similarity of gut-colonizing and invasive strains in 15 of 26 of neonates in our study is the highest reported so far. Invasive strains were also present on skin in 2 of 4 preterm neonates with LOS caused by *S. epidermidis*, being in the range of that previously reported (0–57%) (Valvano et al. 1988; Mueller-Premru et al. 1999; Garland et al. 2008). Although BM of mothers of neonates with LOS did not contain LOS-causing strains, it cannot be excluded that in larger study the role of BM as a source of infection-causing strains could be revealed.

6 GENERAL DISCUSSION

CoNS are the commonest causative agents of LOS in preterm neonates since 1980s (Bizzarro et al. 2005). Although IVC care bundles have significantly decreased the incidence of LOS in some centres (Schulman et al. 2011; Butler et al. 2013; Bizzarro et al. 2015), CoNS still remain responsible for the majority of cases in others (Olivier et al. 2016; Romanelli et al. 2016; Gowda et al. 2017), including NICUs participating in our studies. At the same time infection control is challenged by emerging threats, such as multidrug-resistant *S. capitis* clone highly adapted to NICU environment (Simões et al. 2016).

Increasing evidence suggests that, in addition to skin, gut may have role in the development of LOS caused by CoNS (Cossey et al. 2014; Shaw et al. 2015). Gut microbiota influences other short- and long-term illnesses, such as NEC, allergy, neurodevelopmental disorders, obesity (Groer et al. 2015; Ruiz et al. 2016). Therefore, modulation of microbiota, including by BM-feeding, has gained more interest recently (Groer et al. 2015; Ruiz et al. 2016). This thesis aimed to address the relationship between colonization of gut and skin in preterm neonates and BM of their mothers and development of LOS caused by CoNS.

6.1 Gut as a source of CoNS causing LOS

We demonstrated that invasive strains and isolates colonizing gut within a week prior to the onset of LOS were indistinguishable by MLVA in 15 of 26 episodes of LOS caused by CoNS in preterm neonates. This indicates that gut could be an entry route of CoNS into bloodstream. By now, other research groups have corroborated these findings, although in somewhat lower proportion of neonates (from 2/26 to 2/6) (Cossey et al. 2014; Shaw et al. 2015).

Although indistinguishable MLVA profiles do not prove causal relationship between prior gut colonization and subsequent infection, skin is unlikely the only source of infection-causing strains, as suggested earlier (Costa et al. 2004). Indistinguishable MLVA-types in blood cultures and on skin were found in 2 of 4 neonates from whom blood culture and skin *S. epidermidis* isolates were available for typing. We cannot exclude that strains from axillary skin are not representative to those colonizing IVCs or the surrounding skin. Still, in preterm neonates strains indistinguishable by typing method colonize various body sites (Bialkowska-Hobrzanska et al. 1993) that was corroborated also by us, as in 77.6% of preterm neonates skin and gut were colonized with indistinguishable MLVA-types. Another argument against skin as the only source of invasive strains is the absence of CVC in one neonate with LOS caused by CoNS. In other studies, considerably less, only 46–48% of neonates with LOS caused by CoNS have had CVCs (Klingenberg et al. 2007; Dimitriou et al. 2011).

Predominant NICU strains that were frequent causative agents of LOS constituted about half of all isolates from gut and skin of preterm neonates, whereas a single MLVA-type (MT96) colonized 59.2% of them. This shows that gut colonization with strains that could cause infections is not limited to those in whom LOS develops, but is widespread. Subsequent translocation of CoNS from gut into bloodstream may depend on several factors, such as the abundance of staphylococci in gut (Donnell et al. 2002; Sherman et al. 2016), overall distortion of gut microbiota (Groer et al. 2015), permeability or integrity of intestinal barrier that is influenced by duration of enteral feeding (Weaver et al. 1984), single-nucleotide polymorphisms in genes involved in intestinal mucosal structure (Srinivasan et al. 2017) and injury to intestinal wall during NEC (Hällström et al. 2004) or surgery (Donnell et al. 2002). As controlling all these factors could be difficult, reducing colonization with predominant NICU strains that have characteristics associated with development of infection, such as the *mecA* gene or advantageous genetic background, e.g. ST2, could be more feasible to prevent subsequent infection. The results of our study indicate that BM-feeding could be one approach to reduce more pathogenic strains in gut.

6.2 Influence of BM on gut colonization in preterm neonates

We demonstrated that BM is likely the source of less pathogenic strains for gut of BM-fed preterm neonates. The role of BM in gut colonization is supported by the increasing proportion of neonates colonized with MLVA-types indistinguishable from those in mother's BM, later isolation of indistinguishable MLVA-types from gut compared with BM and lower carriage rate of the virulence-related genes in BM isolates. Accordingly, initial colonization with predominant NICU strains is followed by gradual decrease in the carriage rate of IS*256* and the *mecA* gene and the proportion of predominant NICU strains among gut-colonizing *S. epidermidis* in preterm neonates

In contrast to our study, high resistance rates were sustained throughout the first three weeks of life in gut-colonizing CoNS from preterm neonates in a Dutch study (Hira et al. 2013). The discrepancy may be explained by different inclusion criteria of neonates. Study B conducted by us included preterm neonates who started to receive mother's BM within the first week of life, of whom 20.4% were late-preterm neonates with BW ranging from 1564 g to 3418 g. The inclusion criteria in the Dutch study were presumed duration of hospitalization for at least 7 days, whereas all had BW less than 1650 g. Thus, neonates in the Dutch study were probably less exposed to BM, because a proportion of VLBW neonates does not receive BM in the Netherlands (8.5–12%) (Corpeleijn et al. 2012; Corpeleijn et al. 2016) and due to smaller weight they received smaller amount of BM. Smaller exposure to BM could have contributed to lack of decrease in the resistance rates. Similarly, smaller proportion of BM-fed neonates in Study A compared with Study B (16.4% vs

100% within the first week of life) could account for the significantly higher carriage rate of the virulence-related genes in gut-colonizing *S. epidermidis* in Study A.

Colonization of gut of preterm neonates with MLVA-types indistinguishable from those in BM was delayed in preterm compared with term neonates. The lag could be attributable in part to successful establishment of initial colonization with predominant NICU strains. As the initiation of feeding with BM was delayed, occurring at the median postnatal age of 2 days, and BM accounted for median of only 27.7% of all enteral feedings within the first three days of life in our study, the lack of competition could facilitate colonization with predominant NICU strains. Antibiotics administered to nearly all neonates further promote colonization with *mecA*-positive staphylococci (Gibson et al. 2016). These may not be outcompeted by strains from mother's BM as the latter mostly do not have resistance-related factors. This is exemplified by earlier isolation of oxacillin-susceptible compared with oxacillin-resistant *S. epidermidis* from gut of neonates in Study A indicating that the first were acquired prior to NICU admission rather than during.

Second, insufficient production of BM in mothers of preterm neonates results in feeding regimens containing formula or donor milk that lack bacteria (Cossey et al. 2014) or contain them at low rate most likely due to contamination (Espinosa-Martos et al. 2013; Gómez et al. 2016). Notably, only 6.4% of preterm neonates in Study B were exclusively BM-fed during the study period despite that BM feeding was essential requirement to be included in the study. This is comparable to 3.4% in a recent study on VLBW infants in the Netherlands (Corpeleijn et al. 2016). Total daily volume of BM is considerably lower in preterm than in term neonates due to their smaller body weight, resulting in lower exposure of preterm neonates to staphylococci in BM. Although we found higher count of staphylococci in BM of mothers of preterm compared with term neonates, smaller amounts of BM may not be compensated by higher count, as such difference between mothers of preterm and term neonates is not unequivocally shown (Khodayar-Pardo et al. 2014).

Thus, as BM enriches gut with less pathogenic *S. epidermidis* strains, our results further support the recommendation of BM as the best early feeding option for preterm neonates (Eidelman et al. 2012). This is one possible approach to reduce colonization with predominant NICU strains that may ultimately result in lower risk of infection.

6.3 Contrasts between CoNS in BM of mothers of preterm and term neonates

To the best of our knowledge this is the first study characterizing CoNS in BM of mothers of preterm neonates at the strain level. We demonstrated that in contrast to mothers of term neonates, BM of mothers of preterm neonates became colonized with *mecA*-positive CoNS and virulence-gene-carrying

S. epidermidis, mostly within the first weeks after delivery. Still, in *S. epidermidis* from BM of mothers of preterm neonates the carriage rate of the virulence-related genes and the *mecA* gene was lower than in *S. epidermidis* colonizing preterm neonates. The results reflect the differences between infecting isolates from other patient groups compared with colonizing isolates from healthy people with no contact with hospital environment (Cherifi et al. 2013; Du et al. 2013). As carriage strains are less likely associated with infection development (Tolo et al. 2016), we demonstrated that BM of mothers of preterm neonates contains less pathogenic strains that has been hypothesized, but not shown.

The differences between the two groups of mothers may in part be due to the higher frequency and longer duration of antibacterial treatment and hospitalization in mothers of preterm compared with term neonates, both of which may increase the risk of colonization with methicillin-resistant CoNS (Morgenstern et al. 2016; Widerström et al. 2016). Longer duration of post-partum hospitalization as a risk factor for colonization of BM with *mecA*-positive staphylococci indicates that hospital environment, where methicillin-resistance occurs more frequently than in community (76–86.4% vs 3.3–38.4%), could be a source of such strains (Cherifi et al. 2013; Du et al. 2013).

Still, colonization likely occurred in NICU or neonatoloy unit rather than maternity ward. First, predominant NICU strains were detected among the strains acquired into BM of mothers of preterm neonates, colonizing up to 16% of them. As clones in NICU can be present elsewhere in hospital (Botelho et al. 2012), we cannot exclude the presence of predominant NICU strains in maternity ward. However, predominant strains were not detected in BM of the mothers of term neonates, who delivered in the same maternity ward as the mothers of preterm neonates. Second, *mecA*-positive staphylococci were isolated from BM mostly in the second week after delivery, when the majority of preterm neonates were in the NICU, rather than the first week, when hospitalization of mothers in maternity ward occurred. Parents of hospitalized neonates often visit NICU and their colonization with strains causing outbreaks in NICU has been reported, for example, acquisition of epidemic *S. aureus* onto nipples (Conceição et al. 2012), into nares or oropharynx (Garcia et al. 2014) or *K. pneumoniae* into BM (Rettedal et al. 2012).

Although we did not study the sources of maternal BM colonization, it may be a result of skin-to-skin contact with neonate. We demonstrated first, that indistinguishable MLVA-types on skin and BM were isolated from skin of neonate earlier than from BM and carried the virulence-related genes at high rate. Second, the association between the presence of *mecA*-positive staphylococci in BM and duration of maternal hospitalization as a caretaker of neonate suggests that mothers may acquire such strains while admitted to neonatology ward, where close contact between mother and neonate is more frequent than in NICU. Colonization of neonate with resistant and epidemic strains in NICU becomes more likely with longer duration of NICU stay (Ternes et al. 2013) and thus is highest at the discharge from NICU and at admission to neonatology

ward. The results of Study A are in accordance with this, as longer NICU stay increased the risk of colonization of neonatal gut with *S. epidermidis*, the majority of which carried the virulence-related genes, and oxacillin-resistant strains were isolated later compared with oxacillin-susceptible. Still, we cannot exclude that gradual colonization of mother may have occurred due to increasing cumulative exposure to strains in NICU environment, such as air (Krediet et al. 2001) or frequently touched surfaces (Klingenberg et al. 2001). Thus, to prevent colonization of BM the exposure of mothers to contaminated surfaces in NICU should be reduced. This includes also enhanced cleaning of environment (Butin et al. 2017a) and hand hygiene of staff (Pessoa-Silva et al. 2004). Additionally, prevention of colonization of hospitalized neonates with predominant NICU strains could reduce the risk of acquisition of these strains by mother.

Thus, we should emphasize that while BM is mostly a source of *S. epidermidis* strains with low pathogenicity for preterm neonate, it may become colonized with more pathogenic strains. As BM samples collected within the first week after delivery were mostly colonized with less pathogenic strains, beneficial effects of very early administration of BM (Corpeleijn et al. 2012; Lee et al. 2015) should be further highlighted.

6.4 Need for screening cultures of BM of mothers of preterm neonates

BM as a source of LOS-causing strains has been shown for *S. aureus* (Kayıran et al. 2014) and *E. coli* (Nakamura et al. 2016) and high count of commensal bacteria, including staphylococci has been considered as a risk factor for development of infection (Schanler et al. 2011). Thus, screening cultures (Mense et al. 2014; Bonet et al. 2015) or routine pasteurization of mother's own BM (Dicky et al. 2017) especially if given to very preterm neonates have been recommended. However, the evidence to screen or pasteurize BM is limited. To the best of our knowledge, our study was the first to determine genetic relatedness of CoNS in BM and neonatal blood cultures, although the sample size was small. In the four LOS episodes caused by *S. epidermidis* in Study B, we did not find genetic similarity between BM and blood culture isolates. Of note these results are not conclusive due to very small number of cases.

Our study showed that several aspects of colonization of BM should be considered while implementing screening cultures and interpreting the results. First, in mothers of preterm neonates the characteristics of colonizing *S. epidermidis* varied from predominant NICU strains harbouring multiple virulence-related factors to *mecA*-negative strains that belong to genetic lineage associated with commensal lifestyle. Thus, culture results at the species level are not sufficient to determine the presence of strains with higher pathogenicity. Second, high genetic diversity of isolates in BM, as is typical for community *S. epidermidis* strains (Rolo et al. 2012), would require molecular characterization of

several colonies. Notably, by isolating 5 colonies, we detected median of 2 (2–3) species and 5 (3–7) MLVA-types among *S. epidermidis* in mothers of preterm neonate. Third, high count of staphylococci could be a prerequisite for colonization of neonatal gut with less pathogenic CoNS in mother's BM. Indeed, further analysis of the data showed that higher minimum count of staphylococci in BM promoted colonization of gut with *mecA*-negative *S. epidermidis* indistinguishable from MLVA-type in BM (median (IQR) 4.39 (4.8–4.72) cfu/mL in mothers whose neonates were colonized vs 3.73 (3-11-4.15) cfu/mL if not colonized; OR (95% CI) 2.05 (1.01–4.16)) (Soeorg et al. 2017). Thus, criteria for discarding or pasteurizing BM based on number of colonies could counteract beneficial colonization. Finally, although the cumulative proportion of mothers colonized with *S. epidermidis* carrying the virulence-related genes increased, the proportion of isolates carrying the genes did not increase, suggesting intermittent or transient colonization. Thus, due to high variability of the CoNS content of BM, also shown for other bacterial species (Schanler et al. 2011; Rettedal et al. 2012; Nakamura et al. 2016), screening is probably ineffective in limiting the exposure of neonate to pathogenic strains, unless all the samples are routinely cultured (Nakamura et al. 2016).

If additionally the reduction in quality of BM samples stored in refrigerator (Slutzah et al. 2010; Takci et al. 2013) while pending culture results and the cost of culturing are taken into account, other interventions than screening should be considered to reduce potential risk of infection from BM. Our results suggested that colonization of mother with pathogenic CoNS occurs within few weeks after delivery and most likely from NICU or hospital environment. Thus, prevention of colonization of mothers should be aimed and could be achieved if exact sources and influencing factors are determined. Of note, prevention of colonization may have also effect on other pathogens possibly contaminating BM (Rettedal et al. 2012) or colonizing other body sites of mothers (Conceição et al. 2012; Garcia et al. 2014) resulting in reduced risk of infections beyond those caused by CoNS.

6.5 Limitations of the study

Some limitations of the current studies should be noted. First, the sources of invasive strains causing LOS could not be conclusively determined. Different skin sites and gut in preterm neonates are colonized with CoNS strains indistinguishable by typing method (Bialkowska-Hobrzanska et al. 1993), whereas several colonizing strains are often found, as also exemplified by us. In addition to characterizing multiple colonies of CoNS from different body sites, larger number of neonates than in our studies should be studied. Still, this was a pilot study and we achieved the aim to describe whether gut could have any role in the development of CoNS infections.

Second, neonates not receiving mother's own BM and therefore differences in colonization between BM- and formula-fed neonates were not studied. Still,

BM of mothers of preterm neonates contained staphylococci in median count of 10,000 cfu/mL, whereas pasteurized donor milk or formula should be sterile and this may affect colonization. Instead of BM, less pathogenic strains could have originated from mother's skin that is colonized with mostly susceptible CoNS (Hira et al. 2013). However, it is unlikely considering that skin-to-skin contact with mother (Lamy Filho et al. 2015), but also with healthcare workers after vacation (Hira et al. 2010), should promote less resistant strains on skin, but *S. epidermidis* on skin of preterm neonates remained largely unchanged in our study.

Third, the factors influencing maternal colonization with resistant and predominant NICU strains remain hypothetical. To more conclusively define the sources of maternal colonization, samples should be collected from mother's skin and gut, including prior to delivery, as well as NICU environment and the hands of NICU staff. Although neonate could be a source of maternal colonization, this could be more conclusively suggested if the age of neonate at the initiation of and duration of skin-to-skin contact that is probably important factor influencing transfer of strains between neonate and mother (Lamy Filho et al. 2015) was recorded.

Fourth, the current study included mostly very and extremely preterm neonates hospitalized in the NICU, receiving antibacterial agents and requiring various invasive interventions, all of which can influence colonization (Adamson et al. 2012; Gibson et al. 2016). At the other extreme, term neonates and their mothers without any perinatal health problems constituted control group to describe normal colonization. The large contrast between the two groups complicates determination of the role of neonatal and maternal characteristics influencing colonization.

6.6 Future research

Several studies have aimed to identify the sources of CoNS causing LOS (Garland et al. 2008; Cossey et al. 2014; Shaw et al. 2015), but the exact entry routes still remain unknown. Regardless of the source, the relatedness of skin and gut colonization suggests that prevention of colonization with more pathogenic strains in any body site should be aimed. Considering transmission of predominant NICU strains, prevention of colonization with potentially invasive strains could be achieved by cohorting, but this requires further studies. Although isolation of patients colonized with particular *S. epidermidis* strains has been reported only rarely, such measures have been effective in eradicating linezolid-resistant ST2 (O'Connor et al. 2015). Second, CoNS can be isolated from skin of all people (Cavanagh et al. 2016), whereas some of these strains are characterized as generalist lifestyle strains (Thomas et al. 2014), meaning that these could spread also in hospital environment. Thus, further studies are warranted to determine whether the source of strains that can spread and cause infections in NICU could be, for example, skin of parents and staff. Similarly,

recent study proposed that skin of parents could be a source of pathogenic *S. aureus* for neonates (Milstone et al. 2015).

Considering multiple reservoirs of predominant NICU strains (Krediet et al. 2001), prevention of colonization of neonates with them could be difficult. Instead, interfering with colonization could be achieved. Our study suggested that BM enriches gut of preterm neonates with less pathogenic staphylococci. While BM-feeding may not affect skin colonization, skin-to-skin contact with mother promotes neonatal colonization with methicillin-susceptible instead of methicillin-resistant strains (Lamy Filho et al. 2015). Thus, whether skin-to-skin care after delivery (Kristoffersen et al. 2016), care in single family room (Lester et al. 2014), earlier initiation and/or longer duration of Kangaroo care, including 24 hours a day implemented in some centres (Blomqvist et al. 2013), could enrich neonatal skin with less pathogenic staphylococci should be investigated.

Third, although BM promoted colonization with less pathogenic *S. epidermidis*, colonization with such strains was infrequent within the first week of life in preterm neonates. As the majority of LOS cases occur in the first weeks of life, earlier colonization with less pathogenic strains could be advantageous. Of note, neonates not hospitalized in NICU, but discharged home from maternity ward do not become colonized in gut with *S. epidermidis* strains carrying the *mecA* gene, IS*256* or belonging to STs often causing infections (Fill Malfertheiner et al. 2017). Early and frequent skin-to-skin contact with mother and early administration of BM could be one of the reasons for such colonization. Thus, as skin-to-skin contact with mother right after delivery (Kristoffersen et al. 2016) and administration of oropharyngeal colostrum (Rodriguez et al. 2010) are safe and feasible, the impact of such interventions on gut colonization of preterm neonates should be investigated.

Fourth, studies on the prevention of colonization of mothers with *mecA*-positive CoNS and predominant NICU strains are warranted. Although the current study suggested that contact with neonate could result in colonization of mother with NICU strains, Kangaroo care has multiple beneficial effects on neonate (Conde-Agudelo and Díaz-Rossello 2016) and thus limiting mother-neonate contact should not be considered as an option to prevent mother's colonization. Instead, as skin-to-skin contact with mother promotes neonatal colonization with methicillin-susceptible staphylococci (Lamy Filho et al. 2015), whether longer duration of Kangaroo care could enrich neonatal skin with less pathogenic staphylococci and thereby could reduce subsequent risk of acquisition of pathogenic strains into microbiota of mother should be studied. Furthermore, the role of other potential sources in NICU, such as air (Botelho et al. 2012), frequently touched surfaces (Klingenberg et al. 2001) or BM pumps (Engür et al. 2014), in colonization of mother should be investigated.

7 CONCLUSIONS

1. The prevalence of colonization with different staphylococcal species is similar in gut of preterm and term neonates and BM of their mothers, suggesting that feeding with BM influences gut colonization in neonates. In contrast, while the distribution of species is similar between skin and BM in term neonates and their mothers, it is different between skin and BM in preterm neonates and their mothers, indicating that skin-colonizing staphylococci are acquired from other sources than BM. The significant differences between the prevalence of different species in term and preterm neonates and mothers demonstrate that prematurity, hospitalization, antibacterial treatment or other interventions may alter the composition of staphylococcal microbiota that may influence health outcome of preterm neonates.
2. *S. epidermidis* from skin and gut of preterm neonates carry the virulence- and resistance-related genes at significantly higher rate than isolates colonizing term neonates. Overall, the high carriage rate of the virulence- and resistance-related genes and antimicrobial resistance regardless of GA of neonate indicates that this results from hospitalization to NICU rather than prematurity itself. The carriage rate of virulence-related genes in isolates from gut and skin is similarly high, showing that both body sites are important reservoirs of pathogenic strains at the time when the incidence of LOS peaks. However, in BM-fed preterm neonates gut is gradually enriched with less pathogenic *S. epidermidis* that probably originate from mother's BM
3. *S. epidermidis* in BM of mothers of preterm neonates carry the virulence- and resistance-related genes at higher rate compared with isolates from mothers of term neonates, but at lower rate compared with isolates colonizing preterm neonates. Thus, BM of mothers of preterm neonates seems to have two controversial effects. First, BM could enrich gut of preterm neonates with less pathogenic staphylococci and thereby contribute to the decreased risk of LOS. However, mother's own BM may contain more pathogenic *S. epidermidis* strains that could ultimately increase the risk of LOS in preterm neonates.
4. Of *S. epidermidis* colonizing gut and skin of preterm neonates about half belong to predominant NICU strains, that carry the virulence-related genes at higher rate compared with other strains, belong to the STs often reported to cause disease and can cause LOS. The clonal spread and the presence in hospitalized neonates, but not in non-hospitalized healthy term neonates, indicates that NICU environment is most likely the main source of predominant strains. As colonization with predominant NICU strains is widespread, colonized neonates become temporary reservoirs of them, thereby further contributing to their transmission.
5. Overall, the diversity of *S. epidermidis* in BM of mothers of preterm and term neonates is high and the commonest colonizing strains are similar.

Thus, mother's BM contains mostly CoNS representing community strains. Still, mothers of preterm neonates acquire into their BM pathogenic *S. epidermidis* and *S. haemolyticus* strains that are exclusively found to colonize or infect preterm, but not term neonates. Considering also the acquisition of CoNS carrying the virulence-related genes within the first week after delivery, the most likely source of more pathogenic strains is hospital or NICU.

6. In the first week of life, nearly all term, but only less than a quarter of preterm neonates harbour on skin and in gut *S. epidermidis* strains indistinguishable from those in mother's BM. This large deviation of colonization of preterm neonates from that of term neonates, the latter most likely representing the normal colonization, shows that the early sources of colonizing bacteria of hospitalized neonates are largely other than mother's BM. Still, by the end of the first month of life, the majority of preterm neonates acquire into gut strains colonizing mother's BM, showing that BM-feeding contributes to reversing the impact of NICU environment on gut microbiota.
7. In preterm neonates, LOS-causing strains can colonize skin, but not in all cases. In more than half of preterm neonates, CoNS strains subsequently causing LOS can be isolated from gut prior to the onset of LOS. Thus, in addition to entry from skin, the role of translocation of CoNS strains from gut into bloodstream could be equally important. In the current study, invasive strains were not present in BM of mothers of preterm neonates in whom LOS caused by CoNS developed.

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9 SUMMARY IN ESTONIAN

Koagulaas-negatiivsed stafülokokid enneaegsete vastsündinute seedetraktis ja nende emade rinnapiimas

Koagulaas-negatiivsed stafülokokid (KoNS), peamiselt *Staphylococcus epidermidis*, on sagedased inimeste nahka koloniseerivad mikroorganismid (Grice and Segre 2011). Samas on KoNS ka sagedased vereringeinfektsioonide tekitajad haiglas (ECDC 2013), peamiselt immuunkomprimeeritud ja võõrkehadega (nt veresoonekateetrid) patsientidel. Enamus infektsioone põhjustavatest tüvedest on haiglakeskkonda adapteerunud – resistentsed antibiootikumidele, kannavad resistentsuse ja virulentsusega seotud geene (näiteks metitsilliinresistentsust kodeeriv *mecA* geen, biofilmi kodeeriv *icaA* geen ja geneetilistes ümberkorraldustes osalev IS*256*) ning kuuluvad teatud geneetilistesse liinidesse, peamiselt ST2 ja ST5 (ST, ingl k *sequence type*) (Cherifi et al. 2013; Du et al. 2013; Tolo et al. 2016).

Vastsündinutel on KoNS ka sagedased seedetrakti (Adlerberth et al. 2006; Hira et al. 2013) ning nende emadel rinnapiima (Hunt et al. 2011; Boix-Amorós et al. 2016) koloniseerivad bakterid. Tervete ajaliste vastsündinute seedetrakt on koloniseeritud antibiootikumidele tundlike ja virulentsusega seotud geene mittekandvate tüvedega, mis on geneetiliselt sarnased nende emade rinnapiimas olevate tüvedega (Jiménez et al. 2008b; Martín et al. 2012). Seevastu enneaegsed vastsündinud hospitaliseeritakse esimeste elutundide jooksul intensiivravi osakonda, kus nad koloniseeruvad haiglas levivate resistentsete ja virulentsete infektsioone põhjustavate tüvedega (Krediet et al. 2001). Enneaegsete vastsündinute seedetrakti koloniseerumine ema rinnapiimas leiduvate vähevirulentsete stafülokokkidega on jäänud ebaselgeks (Cossey et al. 2014).

Enneaegsetel vastsündinutel on naha (Evans and Rutter 1986) ja seedetrakti barjäär ebaküps (Weaver et al. 1984), immuunsüsteem välja arenemata (Björkqvist et al. 2004; Granslo et al. 2013) ja nad vajavad sageli invasiivseid vahendeid, sh veresoonekateetreid, mistõttu on KoNS sagedased infektsioonide tekitajad, põhjustades ligikaudu poole hilise sepsise juhtudest (tekib pärast 72 elutundi) (Mitt et al. 2014; Gowda et al. 2017). Arvatakse, et sepsist tekitavad tüved on pärit nahalt ja satuvad vereringesse veresoonekateetrite kaudu. Kuid 39–68% KoNS poolt põhjustatud hilise sepsisega vastsündinutest ei ole veresoonekateetrit (Khashu et al. 2006; Hira et al. 2007; Dimitriou et al. 2011) ning 37–83% juhtudest on veresoonekateetrit koloniseeriv tüvi erinev verekülvis olevast (Garland et al. 2001; Garland et al. 2008; Brito et al. 2014). Seetõttu võib sarnaselt teiste mikroorganismidega olla invasiivsete KoNS tüvede sattumise põhjus vereringesse translokatsioon seedetraktist (Costa et al. 2004), kuid senini on see jäänud ebaselgeks.

Hilise sepsise tekkeriski enneaegsetel vastsündinutel vähendab ema rinnapiimaga toitmine (Corpeleijn et al. 2012), mille üheks mehhanismiks on arvatud olevat seedetrakti koloniseerumine rinnapiima vähevirulentsete KoNS tüvedega (Cossey et al. 2013). Suurt kommensaalsete bakterite, sh stafülokokkide,

kontsentratsiooni rinnapiimas on peetud infektsiooni riskifaktoriks (Schanler et al. 2011; Mense et al. 2014) ning teiste mikroorganismide puhul on näidatud, et rinnapiim võib olla infektsiooni tekitavate tüvede allikas enneaegse vastsündinu jaoks (Kayıran et al. 2014). Seetõttu mõnedes keskustes külvatakse või pastöriseeritakse rutiinselt rinnapiima (Bonet et al. 2015; Dicky et al. 2017), kuid sepsist põhjustavate ja rinnapiima koloniseerivate KoNS tüvede geneetilist sarnasust ei ole senini uuritud.

Uurimistöö eesmärgid

Töö üldine eesmärk oli kirjeldada enneaegsete vastsündinute seedetrakti ja naha ning nende emade rinnapiima kolonisatsiooni KoNS-dega ning hinnata kolonisatsiooni seost hilise sepsise tekkega. Mittehospitaliseeritud ajalised vastsündinud kaasati kontrollgrupina, et kirjeldada normaalset kolonisatsiooniprotsessi.

Töö konkreetsed eesmärgid:

1. Kirjeldada KoNS liike enneaegsete vastsündinute seedetraktis ja nahal ning nende emade rinnapiimas.
2. Kirjeldada virulentsusega ja resistentsusega seotud geenide olemasolu enneaegsete vastsündinute seedetrakti ja nahka koloniseerivatel *S. epidermidis* tüvedel.
3. Kirjeldada virulentsusega ja resistentsusega seotud geenide olemasolu enneaegsete vastsündinute ema rinnapiima koloniseerivatel *S. epidermidis* ja *S. haemolyticus* tüvedel.
4. Iseloomustada enneaegsete vastsündinute seedetrakti ja nahka koloniseerivate *S. epidermidis* tüvede molekulaarset epidemioloogiat.
5. Iseloomustada enneaegsete vastsündinute emade rinnapiima koloniseerivate *S. epidermidis* ja *S. haemolyticus* tüvede molekulaarset epidemioloogiat.
6. Teha kindlaks geneetiline sarnasus enneaegsete vastsündinute seedetrakti ja nahka ning nende emade rinnapiima koloniseerivate *S. epidermidis* tüvede vahel.
7. Teha kindlaks geneetiline sarnasus enneaegsete vastsündinute seedetrakti ja nahka ning nende emade rinnapiima koloniseerivate ja hiljem hilist sepsist põhjustavate KoNS tüvede vahel.

Materjal ja metoodika

Töö põhineb kahel uuringul, mis viidi läbi SA Tallinna Lastehaigla ja SA Tartu Ülikooli Kliinikumi lasteintensiivravi osakondadesse (LIRO) hospitaliseeritud vastsündinutel.

Prospektiivne kahekeskuseline klaster-randomiseeritud uuring (Uuring A), milles võrreldi ampitsilliini ja gentamütsiini ning penitsilliini ja gentamütsiini kliinilist efektiivsust varase sepsise riskiga vastsündinutel, viidi läbi 02.08.2006–30.11.2007 (Metsvaht et al. 2010). Uuringusse kaasati vastsündinud, kes olid

hospitaliseeritud osakonda esimese 72 elutunni jooksul, vajasid antibakteriaalset ravi varase sepsise tekke või riskifaktorite tõttu ning viibisid osakonnas vähemalt 24 tundi. Kokku 276 vastsündinult võeti osakonda saabumisel ja edasi kaks korda nädalas tampooniga (Nuova Aptaca, Canelli, Itaalia) rektaalkaabe kuni osakonnast lahkumiseni või 60. ravipäevani ja külvati veriagarile (Parm et al. 2010). Pärast inkubeerimist +37 °C juures 24–48 tundi isoleeriti igast morfoloogiliselt erinevast stafülokokkidele tüüpilisest kolooniast üks pesa ja määrati liigi tasemele API® Staph (bioMérieux S.A., Marcy l'Etoile, Prantsusmaa) ning *tuf* geeni sekveneerimise abil (Heikens et al. 2005).

Prospektiivne longitudinaalne kahe grupi võrdlusuuring (Uuring B), milles kirjeldati rinnapiima toidul olevate tervete ajaliste ja hospitaliseeritud enneaegsete vastsündinute seedetrakti ja naha ning nende emade rinnapiima kolonisatsiooni KoNS-dega, viidi läbi 16.01.2014–15.12.2015. Uuringusse kaasati 49 enneaegset vastsündinut (gestatsioonivanus <37 näd), kes hospitaliseeriti intensiivraviosakonda ja kellel alustati toitmist rinnapiimaga esimese elunädala jooksul, ja nende emad. Kontrollgrupina kaasati 20 tervet ajalist vastsündinut (gestatsioonivanus ≥37 näd, sünnikaal ≥1500 g), kes olid ainult rinnapiimatoidul, ja nende emad, kes ei vajanud haigla- ega antibiootikumravi kolm kuud enne kuni üks kuu pärast sünnitust. Vastsündinutelt koguti üks kord nädalas esimese elukuu jooksul steriilsesse konteinerisse roojaproov ning füsioloogilises lahuses niisutatud tampooniga (Copan Italia spa, Brescia, Itaalia) aksillaarselt võetud nahakaabe. Emadelt koguti üks kord nädalas rinnapiima, milleks pesi ema rinna seebi ja veega, seejärel puhastas desinfitseerimisvahendiga ning manuaalsel ekspressioonil pärast esimeste tilkade suunamist mujale kogus rinnapiima steriilsesse konteinerisse. Füsioloogilises lahuses tehtud 10-kordsed lahjendused proovidest külvati soola-mannitoolagarile. Pärast inkubeerimist +37 °C juures 48 tundi isoleeriti 5 kolooniat, sh kõik morfoloogiliselt erinevad, ning määrati liigi tasemele MALDI-TOF mass-spektromeetri (Bruker Daltonics, Bremen, Saksamaa) abil.

S. epidermidis tüpiseerimiseks kasutati modifitseeritud MLVA skeemi (Johansson et al. 2006; Cremniter et al. 2013). Uuringus A jäeti välja Se5 korduste puudumise ja Uuringus B lisaks Se4 liiga suurte PCR produktide ning Se8 madala tüpiseerimisvõime tõttu. Uuringus B kasutati *S. haemolyticus* tüpiseerimiseks eelnevalt kirjeldatud MLVA skeemi (Cavanagh et al. 2012). Uuringus A kasutati *S. haemolyticus* tüpiseerimiseks eelnevalt kirjeldatud PFGE meetodit (Villari et al. 2000). MLST abil tüpiseeriti *S. epidermidis* isolaadid, mis põhjustasid hilist sepsist, kuulusid sagedasemate Uuringus A ja Uuringus B vastsündinuid koloniseerivate MLVA-tüüpide hulka, või mis olid emal olemas vähemalt kahes rinnapiima proovis.

Virulentsuse ja resistentsusega seotud geenide (*icaA* geen, IS*256*, ACME, *mecA* geen) olemasolu määrati PCR abil (Ziebuhr et al. 1999; Kondo et al. 2007; Diep et al. 2008) kõigil hilist sepsist põhjustavatel isolaatidel ja igalt vastsündinult/emalt igast erinevast MLVA-tüübist esimesel isolaadil. Uuringu A *S. epidermidis* isolaatidel määrati ka *agr* (Lina et al. 2003) ja SCC*mec* tüüp (Zhang et al. 2005; Kondo et al. 2007) ning resistentsus bensüülpenitsilliini,

oksatsilliini, gentamütsiini, vankomütsiini, klindamütsiini ja tsiprofloksatsiini vastu mikrolahjendusmeetodil (CLSI 2012; EUCAST 2015).

Andmete analüüsimiseks kasutati tarkvaraprogrammi R (version 3.2.2; © 2015 The R Foundation for Statistical Computing). Kvalitatiivsete tunnuste võrdlemiseks kasutati Fisher'i täpset testi ja kvantitatiivsete tunnuste võrdlemiseks Mann-Whitney või Kruskal-Wallis'e testi. Vastsündinute seedetrakti *S. epidermidis*'ega kolonisatsiooni riskifaktorite analüüsimiseks kasutati logistilist regressiooni ning emade rinnapiima *mecA*-positiivsete stafülokokkidega kolonisatsiooni riskifaktorite analüüsimiseks Firth logistilist regressiooni.

Peamised tulemused

Uuringus B esinesid stafülokokid 92.9% roojaproovides, 98.9% nahakaabetes ja 98.8% rinnapiima proovides. *S. epidermidis* koloniseeris seedetrakti 95.9% ja nahka 98% enneaegsetest vastsündinutest ning kõikide emade rinnapiima ja kõikide ajaliste vastsündinute seedetrakti ja nahka. Enneaegsetel võrreldes ajaliste vastsündinutega oli seedetraktis ja/või nahal harvem *S. aureus* (26.5% vs 85%; p<0.001), *S. hominis* (51% vs 90%; p=0.006) ja *S. lugdunensis* (8.2% vs 60%; p<0.001), aga sagedamini *S. haemolyticus* (91.8% vs 55%; p=0.001). Enneaegsete võrreldes ajaliste vastsündinute emadel oli rinnapiimas harvem *S. aureus* (12.2% vs 45%; p=0.008) ja *S. hominis* (26.5% vs 60%; p=0.019). Võrreldes ema rinnapiimaga oli enneaegsete vastsündinute seedetraktis sagedamini *S. haemolyticus* (38.8% vs 89.3%; p<0.001) ja nahal sagedamini *S. hominis* (26.5% vs 49%; p=0.04), *S. haemolyticus* (38.8% vs 91.8%; p<0.001) ja *S. capitis* (6.1% vs 55.1%; p<0.001), kuid harvem *S. lugdunensis* (20.4% vs 2%; p=0.01). Võrreldes ema rinnapiimaga erinesid ajalised vastsündinud ainult naha kolonisatsiooni poolest *S. haemolyticus*'ega (15% vs 50%; p=0.04).

Uuringus A võrreldes Uuringuga B enneaegsete vastsündinute seedetrakti koloniseerivad *S. epidermidis* kandsid samasuguse sagedusega *icaA* geeni (28.2% vs 21.4%), kuid sagedamini *mecA* geeni (93.5% vs 72.8%; p<0.001), IS*256* (65.3% vs 37.9%; p<0.001) ja ACME (76.6% vs 25.2%; p<0.001). Uuringus B oli seedetrakti võrreldes nahka koloniseerivatel *S. epidermidis* isolaatidel väiksem *mecA* geeni (72.8% vs 90.6%; p<0.001) ja IS*256* (37.9% vs 61.7%; p<0.001), kuid sarnane *icaA* ja ACME kandlus. Tervete ajaliste vastsündinute seedetrakti koloniseerivad *S. epidermidis* kandsid harvem *mecA* geeni (2.8% vs 73.3%; p<0.001), *icaA* geeni (6.5% vs 26.7%; p=0.04), IS*256* (0.9% vs 53.3%; p<0.001) ja ACME (13.9% vs 80%; p<0.001) kui Uuringus A hospitaliseeritud ajaliste vastsündinute seedetrakti koloniseerivad *S. epidermidis*. Uuringus A erinesid enneaegsete võrreldes ajaliste vastsündinute seedetrakti koloniseerivad *S. epidermidis* ainult penitsilliin-resistentsuse (100% vs 86.7%; p=0.003), oksatsilliin-resistentsuse (93.5% vs 73.3%; p=0.032) ja *mecA* geeni (93.5% vs 73.3%; p=0.032) kandluse poolest.

Enneaegsete võrreldes ajaliste vastsündinute emade rinnapiimas oli *S. epidermidis* isolaatidel sagedamini *mecA* geeni (32.7% vs 2.6%; p<0.001), *icaA*

geeni (18.8% vs 6%; p=0.002), IS*256* (7.9% vs 0.9%; p=0.013) ja ACME (15.4% vs 5.1%; p=0.008) ja *S. haemolyticus* isolaatidel *mecA* geeni (90.5% vs 10%; p<0.001) ja IS*256* (61.9% vs 10%; p=0.02). Enneaegsete võrreldes ajaliste vastsündinute emade rinnapiim oli sagedamini koloniseeritud *mecA*-positiivse stafülokokiga (83.7% vs 25%; p<0.001), seejuures koloniseerumise riski suurendas, kohandatuna raseduse kestusele, ema pikem haiglas viibimine pärast sünnitust (OR (95% CI) 2.34 (1.28–4.29)), ema hospitaliseerimine neonatoloogia osakonda vastsündinu hooldajana (OR (95% CI) 24.9 (1.27–485)), vastsündinul arterikateetri olemasolu (OR (95% CI) 17.4 (2.60–116)) ja antibakteriaalsete ravimite kasutamine (OR (95% CI) 48.5 (2.60–906)).

Uuringu A 139 *S. epidermidis* isolaadist kuulus 51.1% kuude sagedasemasse MLVA-tüüpi. Uuringu B 2933 *S. epidermidis* isolaadist kuulus 44.4% 23 sagedasemasse MLVA-tüüpi, seejuures üheksa sagedast LIRO MLVA-tüüpi koloniseerisid ainult enneaegseid vastsündinuid ja nende emasid, moodustades vastavalt 49.5% ja 6% neid koloniseerivate *S. epidermidis* isolaatidest. Uuringus A kandsid kuus sagedasemat võrreldes ülejäänud MLVA-tüüpidega sagedamini *mecA* geeni (100% vs 82.4%; p<0.001), IS*256* (73.2% vs 54.4%; p=0.033) ja ACME tüüp I (56.3% vs 27.9%; p=0.001) ning olid resistentsemad gentamütsiinile (98.6% vs 73.5%; p<0.001). Uuringus B kandsid 9 sagedasemat LIRO MLVA-tüüpi võrreldes ülejäänud 14 sagedasema MLVA-tüübiga sagedamini *mecA* geeni (94.4% vs 30.1%; p<0.001), IS*256* (70.4% vs 6.4%; p<0.001) ja ACME (35.8% vs 7.8%; p<0.001). Uuringu A sagedasemate MLVA-tüüpide ja Uuringu B sagedaste LIRO MLVA-tüüpide seas oli ST2 ja ST5 hulka kuuluvaid MLVA-tüüpe. Uuringu B 54 *S. haemolyticus* isolaadist kuulus 35.5% kolme sagedasemasse MLVA-tüüpi, mis koloniseerisid ainult enneaegsete, kuid mitte ajaliste vastsündinute emade rinnapiima. Uuringus A põhjustasid sagedasemad *S. epidermidis* MLVA-tüübid 5/8, Uuringus B *S. epidermidis* sagedased LIRO MLVA-tüübid 4/4 ja *S. haemolyticus* kolm sagedasemat MLVA-tüüpi 6/8 vastava liigi poolt põhjustatud hilise sepsise juhtudest.

Enneaegsetel vastsündinutel oli Uuringus B esimesel elunädalal seedetrakti ja nahka koloniseerivatel *S. epidermidis* isolaatidel IS*256*, *icaA* ja *mecA* geeni kandlus sarnane, kuid neljandaks nädalaks oli IS*256* (18.4% vs 66.7%; p<0.001) ja *mecA* geeni (73.8% vs 84.1%; p=0.0556) kandlus väiksem seedetraktis kui nahal. Sagedaste LIRO MLVA-tüüpide osakaal enneaegsete seedetrakti ja nahka koloniseerivate *S. epidermidis* isolaatide hulgas oli esimesel elunädalal sarnane, kuid neljandal elunädal seedetraktis väiksem (27% vs 66.7%; p<0.001). Emade rinnapiimas oli virulentsusega või resistentsusega seotud geene kandvad *S. epidermidis* isoleeritavad hiljem kui vastavaid geene mittekandvad isolaadid: ACME (mediaan (IQR) 8 (6–13) päeva pärast sünnitust vs. 6 (4–8); p=0.023), *icaA* (13 (6–17) vs. 6 (4–8), p=0.002), IS*256* (13.5 (8.75–20) vs. 6 (4–8), p<0.001) ja *mecA* (8 (6–12.75) vs. 6 (4–8), p=0.001).

Esimese elukuu lõpuks olid *S. epidermidis* eristamatud MLVA-tüübid vastsündinu seedetraktis ja nahal olemas 77.6% ja 95%, eristamatud MLVA-tüübid vastsündinu seedetraktis ja ema rinnapiimas 83.7% ja 95%, eristamatud

MLVA-tüübid vastsündinu nahal ja ema rinnapiimas 34.7% ja 100% ($p<0.001$) ajalistest ja enneaegsetest vastsündinutest, vastavalt. Seejuures kumulatiivne osakaal enneaegsetest vastsündinutest, kelle seedetraktis olid ema rinnapiimas leiduvate MLVA-tüüpidest eristamatud MLVA-tüübid, oli esimesel elunädalal 14.3% ja neljandal elunädalal 83.7% ($p<0.001$). Enneaegse vastsündinu seedetrakti ja ema rinnapiima eristamatud MLVA-tüübid olid seedetraktis hiljem kui rinnapiimas (mediaan (IQR) 14 (10–19) vs 9 (6–15) päeva; $p=0.018$), vastsündinu naha ja ema rinnapiima eristamatud MLVA-tüübid olid nahal varem kui rinnapiimas (6 (4–14) vs 13 (8–19) päeva; $p=0.029$), vastsündinu seedetrakti ja naha eristamatud MLVA-tüübid olid nahal ja seedetraktis samal ajal (7.5 (4–12) vs (6 (3.25–8) päeva). Eristamatud MLVA-tüübid, mis koloniseerisid vastsündinu seedetrakti ja ema rinnapiima, võrreldes MLVA-tüüpidega, mis koloniseerisid nahka ja rinnapiima või seedetrakti ja nahka, omasid harvem *mecA* geeni (57.1% vs 86.4% vs 93.2%; $p<0.001$), IS*256* (18.6% vs 47.7% vs 61.4%; $p<0.001$) ja ACME (21.4% vs 45.5% vs 32.6%; $p=0.006$) ning sagedaste LIRO MLVA-tüüpide osakaal oli väiksem (18.6% vs 54.5% vs 62.1%; $p<0.001$).

Hilist sepsist põhjustanud KoNS olid olemas 22 episoodist Uuringus A (põhjustatuna *S. haemolyticus* (n=12), *S. epidermidis* (n=7), *S. hominis* (n=2) ja üks episood *S. haemolyticus* ja *S. epidermidis* poolt) ja 4 vastsündinul (kõik *S. epidermidis*) Uuringust B. Seedetraktis oli enne sepsise teket verekülvist isoleeritud *S. haemolyticus* 10 vastsündinul 13-st ja *S. epidermidis* 5 vastsündinul 12-st. Nahal oli enne sepsise teket verekülvist isoleeritud *S. epidermidis* 2 vastsündinul 4-st, kuid rinnapiimas mitte ühelgi.

Järeldused

1. Erinevate stafülokokkide liikide esinemissagedus enneaegsete ja ajaliste vastsündinute seedetraktis ja emade rinnapiimas on sarnane, viidates ema rinnapiima rollile vastsündinu seedetrakti koloniseerimises. Stafülokokkide liikide jaotus on erinev enneaegsete vastsündinute nahal ja nende emade rinnapiimas, mis näitab, et vastsündinu nahka koloniseerivad stafülokokid on pärit mujalt kui rinnapiimast.
2. Hospitaliseeritud enneaegsed vastsündinud koloniseeruvad *S. epidermidis* tüvedega, mis kannavad virulentsuse ja resistentsusega seotud geene sagedamini kui mittehospitaliseeritud ajalisi vastsündinuid koloniseerivad tüved. Esimestel elunädalatel, mil KoNS poolt põhjustatud hilise sepsise esinemissagedus on suurim, on enneaegsetel vastsündinutel seedetrakti ja nahka koloniseerivate *S. epidermidis* virulentsusega ja resistentsusega seotud geenide kandlus sarnane, mistõttu mõlemad kehapiirkonnad võivad olla invasiivsete tüvede allikad. Rinnapiimaga toidetud enneaegsetel vastsündinutel suureneb vähevirulentsete *S. epidermidis* tüvede osakaal seedetraktis, mis on tõenäoliselt pärit ema rinnapiimast.
3. Enneaegsete vastsündinute emade rinnapiima koloniseerivad *S. epidermidis* kannavad virulentsuse ja resistentsusega seotud geene sagedamini kui

ajaliste vastsündinute emade rinnapiima koloniseerivad, aga harvem kui enneaegseid vastsündinuid koloniseerivad isolaadid. Seega ühelt poolt võib rinnapiim suurendada vähevirulentsete *S. epidermidis* tüvede osakaalu enneaegse vastsündinu seedetraktis. Kuid teiselt poolt võib enneaegse vastsündinu ema rinnapiim suurendada infektsioonide tekke riski, sest on koloniseerunud virulentsete *S. epidermidis* tüvedega.

4. Enneaegsete vastsündinute seedetrakti ja nahka koloniseerivatest *S. epidermidis* isolaatidest ligikaudu pool kuulub sagedasemate LIRO tüvede hulka, mis kannavad virulentsuse ja resistentsusega seotud geene, kuuluvad sageli infektsioone põhjustavate STde hulka ja võivad tekitada hilist sepsist. Nende klonaalne levik ja puudumine mittehospitaliseeritud ajalistel vastsündinutel näitab, et selliste tüvede allikas on tõenäoliselt LIRO. Koloniseeritud vastsündinud on sagedaste LIRO tüvede ajutisteks reservuaarideks ja seetõttu soodustavad nende levikut osakonnas.
5. Enneaegsete vastsündinute emade rinnapiima *S. epidermidis* suur geneetiline mitmekesisus ja sagedasemate tüvede sarnasus ajaliste vastsündinute emade rinnapiimas leiduvatega, näitab, et emade rinnapiimas on peamiselt väljaspool haiglat levivad *S. epidermidis* tüved. Siiski võib enneaegsete vastsündinute emade rinnapiima sattuda virulentseid *S. epidermidis* ja *S. haemolyticus* tüvesid, mis koloniseerivad või põhjustavad infektsioone ainult enneaegsetel vastsündinutel ning satuvad rinnapiima tõenäoliselt LIRO-st.
6. Esimesel elunädalal on peaaegu kõik ajalised, kuid vähem kui veerand enneaegsetest vastsündinutest koloniseeritud ema rinnapiimas leiduvate tüvedest eristamatute *S. epidermidis* tüvedega. Seega on hospitaliseeritud vastsündinute esmased koloniseerijad pärit mujalt kui ema rinnapiimast. Esimese elukuu lõpuks on enamusel enneaegsetest vastsündinutest seedetraktist isoleeritav mõne ema rinnapiima tüvest eristamatu tüvi, mis näitab, et ema rinnapiimaga toitmine võib muuta hospitaliseerimise mõju enneaegse vastsündinu seedetrakti kolonisatsioonile.
7. Enneaegsetel vastsündinutel võivad hilist sepsist põhjustavad KoNS tüved koloniseerida nahka, kuid mitte kõigil juhtudel. Enam kui pooltel enneaegsetest vastsündinutest, kellel tekkis KoNS poolt põhjustatud hiline sepsis, oli invasiivne tüvi seedetraktis olemas enne hilise sepsise teket. Seega infektsioone põhjustavate KoNS tüvede translokatsioon seedetraktist võib olla sama oluline nagu nende pärinemine nahalt. Rinnapiimas hilist sepsist põhjustavaid tüvesid ei leidunud.

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PUBLICATIONS

CURRICULUM VITAE

Name: Hiie Soeorg
Date of birth: 02.06.1988
Citizenship: Estonian
Address: Tähe 27, Tartu 50103
Phone: +372 530 25440
E-mail: hiie.soeorg@ut.ee

Education

2013–2017	University of Tartu, Faculty of Medicine, PhD studies
2007–2013	University of Tartu, Faculty of Medicine, MD degree, diploma *cum laude*
2004–2007	Hugo Treffner Gymnasium
1995–2004	Tartu Karlova Gymnasium

Professional employment

2016–...	University of Tartu, Faculty of Medicine, Institute of Biomedicine and Translational Medicine, Laboratory Assistant
2015–2015	University of Tartu, Faculty of Medicine, Department of Microbiology, Laboratory Assistant
2014–2015	University of Tartu, Faculty of Medicine, Department of Microbiology, Specialist
2011–2014	University of Tartu, Faculty of Medicine, Department of Microbiology, Laboratory Assistant

Scientific work

Research fields: rotavirus – molecular epidemiology; coagulase-negative staphylococci – molecular epidemiology, virulence and resistance; pharmacodynamics of antibiotics – *in vitro* synergy of antibiotics, vancomycin heteroresistance.

Professional organisations

European Society for Paediatric Infectious Diseases (since 2011)
European Society of Clinical Microbiology and Infectious Diseases (since 2014)

Publications

1. Soeorg H, Tamm E, Huik K, Pauskar M, Mägi D, Pruudel K, Vainomäe L, Moosar L, Kirss K, Torm S, Närska M, Pütsepp A, Nurm H, Pruunsild K, Jänes A, Zilmer K, Lutsar I. Group A rotavirus genotypes circulating prior to implementation of a National Immunization Program in Estonia. Hum Vaccin Immunother. 2012 Apr;8(4):465–9.

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ELULOOKIRJELDUS

Nimi: Hiie Soeorg
Sünniaeg: 02.06.1988
Kodakondsus: eesti
Aadress: Tähe 27, Tartu 50103
Telefon: +372 530 25440
E-post: hiie.soeorg@ut.ee

Hariduskäik

2013–2017 Tartu Ülikool, arstiteaduskond, arstiteaduse doktoriõpe
2007–2013 Tartu Ülikool, arstiteaduskond, arstiõpe, diplom *cum laude*
2004–2007 Hugo Treffneri Gümnaasium
1995–2004 Tartu Karlova Gümnaasium

Teenistuskäik

2016–... Tartu Ülikool, meditsiiniteaduste valdkond, bio- ja siirdemeditsiini instituut, laborant
2015–2015 Tartu Ülikool, arstiteaduskond, mikrobioloogia instituut, laborant
2014–2015 Tartu Ülikool, arstiteaduskond, mikrobioloogia instituut, spetsialist
2011–2014 Tartu Ülikool, arstiteaduskond, mikrobioloogia instituut, laborant

Teadustegevus

Uurimisvaldkonnad: rotaviiruste genotüübiline jaotus; koagulaas-negatiivsete stafülokokkide molekulaarne epidemioloogia, virulentsusfaktorid, resistentsusmehhanismid; antibiootikumide farmakodünaamika – *in vitro* sünergism, vankomütsiini heteroresisentsus.

Kuuluvus erialaseltsidesse

Euroopa Laste Infektsioonhaiguste Selts (alates 2011. a)
Euroopa Kliinilise Mikrobioloogia ja Infektsioonhaiguste Selts (alates 2014. a)

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