Genetic Relatedness of Coagulase-negative Staphylococci From Gastrointestinal Tract and Blood of Preterm Neonates With Late-onset Sepsis

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Background: Coagulase-negative staphylococci (CoNS) are the first colonizers of gastrointestinal tract (GIT) and the commonest cause of late-onset sepsis (LOS) in preterm neonates. Intravascular catheters are considered a major source of CoNS bacteremia. However, several cases of LOS remain without an identified source. To elucidate whether GIT could be a potential source of invasive strains, we aimed to assess the molecular similarity between CoNS from blood and GIT in preterm neonates with LOS.

Methods: Altogether 22 blood and 53 GIT isolates collected from 22 neonates with LOS caused by CoNS (*Staphylococcus haemolyticus* in 13, *Staphylococcus epidermidis* in 7 and *Staphylococcus hominis* in 2 patients) were included. Rectal swabs were collected twice weekly from birth, but only isolates obtained before LOS were analyzed. *S. epidermidis* isolates were typed by multilocus variable number of tandem repeats analysis and multilocus sequence typing, *S. haemolyticus* by pulsed-field gel electrophoresis.

Results: Eighteen of 22 neonates had the same CoNS species in GIT and bloodstream; all these isolates from them (altogether 18 blood and 28 GIT isolates) underwent typing. The genotypic similarity between bloodstream and ≥1 antecedent GIT isolates was observed in 13 of 18 patients—3 of 7 with *S. epidermidis* and 10 of 11 with *S. haemolyticus* infection. The concordant GIT isolates were collected 0–7 days before the positive blood culture.

Conclusions: The similarity between CoNS from GIT and bloodstream indicates that preterm neonates harbour invasive strains in GIT before LOS. Whether there is a causal relationship between GIT colonization and LOS remains to be elucidated in further studies.

Key Words: coagulase-negative staphylococci, preterm neonates, colonization, late-onset sepsis, molecular typing

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Late-onset sepsis (LOS) is one of the most common causes of morbidity and mortality in preterm neonates. Approximately half of LOS episodes are caused by coagulase-negative staphylococci (CoNS).[1,2] Intravascular catheters (IVCs) are considered a major source of CoNS bacteremia. However, IVCs are present in two thirds of LOS cases,[3] of which molecular typing methods have confirmed the similarity between strains isolated from IVCs and blood in two thirds of cases.[4,5]

Furthermore, CoNS are the earliest and the most abundant colonizers of gastrointestinal tract (GIT).[6,7] In preterm neonates, intestinal permeability is increased[8] potentially allowing translocation of microorganisms from GIT to bloodstream. We and others have previously demonstrated molecular similarity between strains of *Escherichia coli*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Enterobacter cloacae*, *Citrobacter koseri*, *Serratia marcescens*, *Acinetobacter baumannii* and *Staphylococcus aureus* isolated from GIT and blood of neonates with LOS[9–11] suggesting possible endogenous origin of bloodstream infections. Similarly to the above-mentioned microorganisms, invasive CoNS are believed to arise from mucosal surfaces, including GIT, particularly in the absence of artificial devices and on occasions when LOS is not considered catheter-related despite the presence of an IVC.[12] However, to the best of our knowledge, only two studies with very small number of subjects have demonstrated genetic relatedness of CoNS from GIT and bloodstream. Bialkowska-Hobrzanska et al[13] by using restriction endonuclease fingerprinting demonstrated the similarity in one of two and Eastick et al[14] with plasmid restriction fragment length polymorphism in one of three neonates studied.

In the present study, we aimed to elucidate whether GIT could potentially be a source of invasive strains by assessing the genetic relatedness of CoNS isolated from GIT and bloodstream of preterm neonates with proven LOS.

MATERIALS AND METHODS

Patients

The study was seeded into a prospective, cluster-randomized trial comparing the efficacy of ampicillin plus gentamicin to penicillin plus gentamicin in neonates at risk of early-onset sepsis admitted into the third-level pediatric intensive care units of Tartu University Hospital and Tallinn Children's Hospital in Estonia between August 2, 2006, and November 30, 2007. The details of the study have been described elsewhere.[15] Of 283 patients included in that study, 29 developed 32 episodes of LOS caused by CoNS. Blood isolates of 22 episodes in 22 neonates from the pediatric intensive care unit of Tallinn Children's Hospital were available for further studies.

Proven LOS caused by CoNS was diagnosed if after the first 72 hours of life at least two clinical (apnoea or bradycardia spells, increased oxygen requirement, hyper- or hypothermia, lethargy and hypotonia, feeding intolerance, abdominal distension, hypotension, skin and subcutaneous lesions such as petechial rash, abscesses, sclerema) and two laboratory criteria of sepsis (white blood cell count <5 or >20 × 10^9 cells/L; I/T ratio >0.2; platelet count <100 × 10^9 cells/L; C-reative protein >10 mg/L) were present in addition

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to at least two blood cultures drawn no more than 72 hours apart, positive for the same species of CoNS or CoNS isolated from one blood culture with adequate antibacterial treatment given for >72 hours.

Isolates

Blood (at least 0.5 mL) was sampled through peripheral vein puncture in clinical deterioration and processed immediately in the microbiology laboratory of North Estonia Medical Centre by BACTEC 9240 system (Becton Dickinson Diagnostic Systems, MD), followed by identification of microbes to the species level by VITEK 2 system (bioMerieux S.A., Marcy-l'Etoile, France). Rectal swabs were collected with transport swabs (Nuova Aptaca, Canelli, Italy) on admission and twice a week thereafter until discharge or day 60, whichever occurred first. Samples were stored at –20°C until cultured within 2 weeks in batches. After thawing, rectal swabs were plated onto blood agar and incubated at 37°C for 24–48 hours. Each morphologically different colony type was identified to species level by API Staph system (bioMerieux S.A.) according to the manufacturer's instructions. Subsequently, the isolates were frozen in skimmed milk and stored at –80ºC until further analyses.

DNA was extracted from all GIT isolates collected before LOS (n = 53) and from all blood isolates (n = 22) with QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions and stored at –80ºC. Subsequently only isolates from patients colonized with the same CoNS species as bloodstream isolate were characterized and typed (n = 46; Fig. 1).

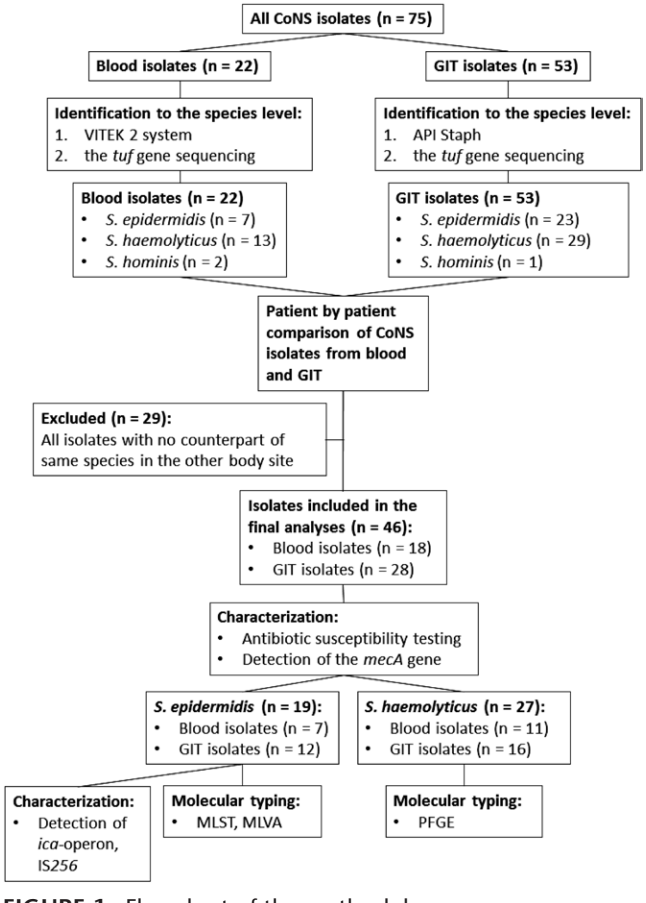

FIGURE 1. Flowchart of the methodology.

Identification to the Species Level

Final identification of isolates to the species level was performed by the *tuf* gene sequencing as described previously.[16]

Antimicrobial Susceptbility Testing

Minimum inhibitory concentrations (MIC) of benzylpenicillin, ampicillin, oxacillin, gentamicin, vancomycin, clindamycin, rifampicin and ciprofloxacin were determined by E-tests (bioMérieux S.A.) according to the European Committee on Antimicrobial Susceptibility Testing guidelines.[17] The presence of the *mecA* gene was detected by polymerase chain reaction using primers and amplification conditions as described previously.[18]

Detection of *ica*-operon and IS*256*

The presence of *ica*-operon and IS*256* in *S. epidermidis* was detected as described elsewhere.[19]

Molecular Typing

S. epidermidis isolates were typed by multilocus variable number of tandem repeats analysis (MLVA) and multilocus sequence typing (MLST) as described previously.[20,21]

As no MLVA and MLST schemes have been developed for *S. haemolyticus*, these isolates were typed by pulsed-field gel electrophoresis (PFGE) as described elsewhere[22]; reference strain DSM 20263 was included in every run. Similarity of isolates sampled from 1 patient was defined as indistinguishable PFGE pattern, that is, when no band difference occurred.[23]

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

RESULTS

All 22 neonates were preterm with median birth weight of 870 g (Table 1). Regarding the risk factors for LOS,[1] the majority (21/22) of patients had IVCs in place and were artificially ventilated. Four neonates died, none of LOS caused by CoNS. Median age at the onset of LOS was 9 days (interquartile range 7–13).

The most prevalent CoNS species responsible for LOS was *S. haemolyticus* (13/22), followed by *S. epidermidis* (7/22) and *S. hominis* (2/22). All except one neonate had prior GIT colonization with CoNS; of the remaining 21 patients, 18 had the same species in bloodstream and GIT. A total of 46 isolates were analyzed further—1 isolate per patient from bloodstream (7 *S. epidermidis*, 11 *S. haemolyticus*) and up to 2 from GIT (12 *S. epidermidis*, 16 *S. haemolyticus*; Fig. 1). The GIT isolates had been collected a median of 4 days (interquartile range 1–7) before the blood isolate.

All isolates carried the *mecA* gene and were resistant to benzylpenicillin, ampicillin and oxacillin (Table 2). All except 1 *S. haemolyticus* were resistant to gentamicin. Almost all *S. haemolyticus* and half of the *S.epidermidis* isolates were resistant to ciprofloxacin. The majority of isolates were susceptible to clindamycin and rifampicin and all to vancomycin. Generally, *S. haemolyticus* isolates exhibited higher MIC values than *S. epidermidis* to all antibiotics, except for clindamycin.

In MLST analysis, 19 *S. epidermidis* isolates were divided into 4 distinct sequence types (STs), 2 remained nontypeable (Table 3). Predominant type was ST5 (9 isolates), followed by ST20 (n = 4), ST2 and ST418 (n = 2 for both). According to the criteria by Kozitskaya et al,[24] ST418 probably arose from ST2 by a point mutation. All ST2 and ST418 isolates carried *ica*-operon and IS*256*, ST5 isolates only IS*256* and ST20 isolates only *ica*-operon. Two isolates (1 positive for *ica*-operon, the other for IS*256*) remained nontypeable by MLST due to polymerase chain reaction failure

TABLE 1. Demographic and Clinical Characteristics of the Neonates

	All Patients With CoNS LOS (n = 29)	Patients Included in the Current Study (n = 22)
GA, wk; median (IQR)	27 (25–28.5)	26.5 (25–28)
BW, wk; median (IQR)	850 (748–1185)	870 (752–1035)
Caesarean section, n (%)	15 (51.7)	10 (45.5)
Male, n (%)	18 (62)	14 (63.6)
Age at hospitalization, min; median (IQR)	60 (49–109.5)	54 (44–110)
CVC, n (%)	28 (96.6)	21 (95.5)
Duration of CVC, d; median (IQR)	19 (12–22.5)	19 (13–22)
ALV, n (%)	28 (96.6)	21 (95.5)
Duration of ALV, d; median (IQR)	11 (3.5–25)	10.5 (4–21)
GIT pathologies, n (%)		
Necrotizing enterocolitis	7 (24.1)	5 (22.7)
Gastroschisis	1 (3.4)	1 (4.5)
Small bowel atresia	1 (3.4)	0 (0)
PICU stay, d; median (IQR)	32 (20.5–47.5)	31 (19–39)
Death during PICU stay, n (%)	4 (13.8)	4 (18.2)

GA indicates gestational age; IQR, interquartile range; BW, birth weight; CVC, central venous catheter; ALV, artificial lung ventilation; PICU, pediatric intensive care unit.

at loci *arcC* or *aroE*. Overall, 9 of 19 (47.4%) isolates carried *ica*-operon and 14 of 19 (73.7%) carried IS*256*.

Nineteen *S. epidermidis* isolates were divided into 6 distinct MLVA patterns (Table 3), which were in good correlation with distribution of STs described above. Se4 locus was not amplifiable in 9 of 19 isolates, and no repeats were detected in any Se5 locus amplicons. Nine isolates were of ST5 and of MLVA-profile either 33-2-20-0 or 33-2-19-0, that is, closely related.

Five distinct PFGE patterns were identified for the 27 isolates of *S. haemolyticus* (11 blood, 16 GIT; Fig. 2 and Table 3). Two closely related PFGE-types, type A1 (7/27) and A2 (14/27), which differed by only 1 band, constituted the vast majority of them.

CoNS from GIT and blood were of similar molecular type in 3 of 7 patients infected with *S. epidermidis* and in 10 of 11 patients with *S. haemolyticus* (Table 3). Thus, 13 of 22 neonates harboured genetically similar CoNS strains in GIT and blood. All concordant GIT isolates were obtained 0–7 days before the available positive blood culture.

TABLE 2. Antibiotic Susceptibility and MIC Values of *Staphylococcus epidermidis* and *Staphylococcus haemolyticus*

	Percentage of Susceptible Organisms	MIC (mg/L)		
		MIC_{50}	MIC_{90}	Range
Staphylococcus epidermidis (n = 19)				
Ampicillin	0	1.5	7.5	0.5–96
Benzylpenicillin	0	2	8	1–48
Ciprofloxacin	52.7	0.125	3	0.094–4
Clindamycin	73.7	0.047	>256	0.023–>256
Gentamicin	0	12	24	6–48
Oxacillin	0	2	3	1–4
Rifampicin	100	0.003	0.004	0.002–0.006
Vancomycin	100	1	1.5	0.75–2
Staphylococcus haemolyticus (n = 27)				
Ampicillin	0	64	96	1.5–128
Benzylpenicillin	0	256	>256	3–>256
Ciprofloxacin	3.7	>32	>32	0.125–>32
Clindamycin	88.9	0.047	0.094	0.023–>256
Gentamicin	3.7	16	24	0.032–48
Oxacillin	0	>256	>256	0.5–>256
Rifampicin	96.7	0.006	0.008	0.003–0.12
Vancomycin	100	1.5	1.5	0.75–2

TABLE 3. Molecular Typing Patterns of Coagulase-negative Staphylococci Isolated From GIT and Blood of Neonates

Patient	Date	Isolates From GIT	Isolates From Blood
Neonates with late-onset sepsis caused by *Staphylococcus epidermidis* MLVA-profiles / STs			
1	Aug 06	31-3-11-3 / 20 (2)	39-2-12-2 / 2
2	Apr 07	31-2-11-3 / 20 (2)	31-3-11-3 / NT
3	June 07	33-2-20-0 / 5 (2)	39-2-12-2 / 2
4	**July 07**	**33-2-19-0 / 5 (2)**	**33-2-19-0 / 5**
5	**Sept 07**	**39-2-12-2 / 418 (1)**	**39-2-12-2 / 418**
6	**Oct 07**	**33-2-20-0 / 5 (1), 33-2-19-0 / 5 (1)**	**33-2-20-0 / 5**
7	Oct 07	33-2-19-0 / 5 (1)	9-2-19-1 / NT
Neonates with late-onset sepsis caused by *Staphylococcus haemolyticus* PFGE-types			
8	**Aug 06**	**A1 (2)**	**A1**
9	**Dec 06**	**A1 (2)**	**A1**
10	**Feb 07**	**A2 (1)**	**A2**
11	Feb 07	A1 (1)	A2
12	**Mar 07**	**B (2)**	**B**
13	**Apr 07**	**A2 (1)**	**A2**
14	**Apr 07**	**A2 (2)**	**A2**
15	**Apr 07**	**A2 (1), C (1)**	**A2**
16	**Aug 07**	**D (1)**	**D**
17	**Aug 07**	**A2 (1)**	**A2**
18	**Sept 07**	**A2 (1)**	**A2**

Bold rows indicate patients colonized in GIT with the same strain as blood isolate. In the brackets, the number of isolates from GIT with the respective MLVA profile or PFGE-type is indicated; from bloodstream, only one isolate was available from each patient. Only patients with microorganisms of the same species from both sites are included. MLVA profile indicates the number of repeats in the loci Se1, Se2, Se3 and Se4. Se5 was excluded from the MLVA typing scheme due to the absence of repeats in the locus in isolates typed. A capital letter was assigned to every different PFGE banding pattern; closely related PFGE-types are indicated by adding a number, for example, A1 and A2.

NT indicates nontypeable.

DISCUSSION

Our findings of molecular similarity between CoNS isolated from blood and GIT in more than half of the patients indicate that preterm neonates may harbour potentially invasive strains in GIT before LOS. Although the relatedness of GIT and blood strains of CoNS has been suggested,[12] to the best of our knowledge, this is the first study that had demonstrated it in a considerable number of neonates.

Although the genetic relatedness of GIT and invasive CoNS isolates does not prove causal relationship, the hypothesis of GIT colonization serving as a potential source of LOS is supported by several indirect findings. First, GIT has been shown to be the largest reservoir of CoNS in neonates.[14] The counts of CoNS in GIT are particularly high during the second week of life,[14] that is, the time when CoNS sepsis peaks.[2] Furthermore, the incidence of sepsis decreases when interventions affecting gut microbiota are applied, for example, earlier initiation of enteral feeding,[25] even in minimal amounts,[26] and breast-milk instead of formula.[27] In addition, pathologies of GIT, reducing the integrity of bowel wall, are risk factors for subsequent sepsis.[28] Finally, in several studies conducted with neonatal rats, translocation of enterobacteria as well as CoNS peaking in the second week of life has been detected.[29]

Of particular note are some issues regarding molecular epidemiology of CoNS. First, CoNS microbiota on skin and mucosal

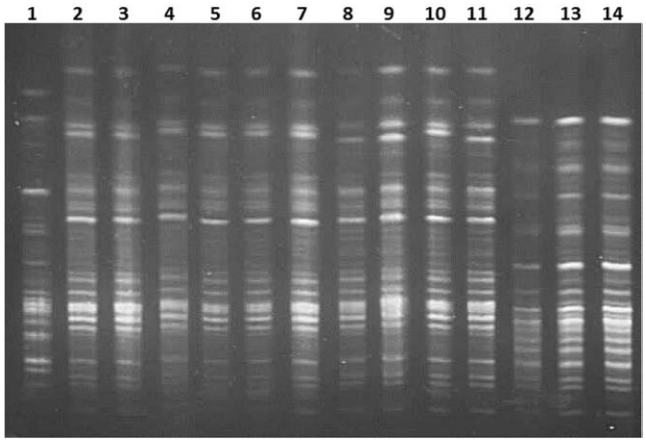

FIGURE 2. PFGE patterns of *Staphylococcus haemolyticus* isolates obtained from GIT and blood of patients 8–12. Lane 1: *S. haemolyticus* DSM 20263; lanes 2–3: GIT isolates (PFGE type A1) from patient 8; lane 4: blood isolate (A1) from patient 8; lanes 5–6: GIT isolates (A1) from patient 9; lane 7: blood isolate (A1) from patient 9; lane 8: GIT isolate (A2) from patient 10; lane 9: blood isolate (A2) from patient 10; lane 10: GIT isolate (A1) from patient 11; lane 11: blood isolate (A2) from patient 11; lanes 12–13: GIT isolates (B) from patient 12; lane 14: blood isolate (B) from patient 12.

surfaces is mainly clonal involving small number of distinct strains, some of which dominate in multiple body sites.[14,30] Thus, strains in GIT could be probably found on skin and vice versa, rendering all studies not investigating all body sites colonized with CoNS, inconclusive. This inconclusiveness is illustrated by the fact that 20%–50% of bloodstream strains remain without an identified source in studies conducted thus far and investigating skin, respiratory tract or IVCs.[4,5,13,14,31,32] Second, in some cases, colonization of IVCs with CoNS of the same molecular type as bloodstream isolate has been suggested to result from hematogenous seeding of bacteria from a distant focus,[5] which also could be GIT. Thus, evidence suggests that GIT may have a role in the pathogenesis of LOS, and it should be investigated in further studies.

In addition to demonstrating colonization of GIT by invasive strains, we found high prevalence of *ica*-operon (47.4%), IS*256* (73.7%), the *mecA* gene (100%) and resistance to gentamicin (100%) among the infecting and colonizing *S. epidermidis* isolates from hospitalized preterm neonates, which is in accordance with previous studies.[33,34] In contrast, staphylococcal strains from breast milk of mothers of healthy full-term infants carry *ica*-operon and the *mecA* gene at lower rate (20%–23% and 12%–45.1%, respectively) and are mostly sensitive to gentamicin.[35,36] In view of this, introducing less virulent strains may contribute to the decreased incidence rate of LOS in breast-fed neonates,[27] but it remains to be confirmed in further studies.

The lower MICs of ampicillin against *S. epidermidis* isolates compared with those against *S. haemolyticus* are noteworthy in relation to the comparative efficacy study.[15] About 0.1%–2.8% of administered dose of ampicillin is eliminated by biliary excretion, and the concentrations in bile exceed significantly the MICs of many pathogens.[37,38] This could explain the greater risk of colonization with *S. haemolyticus* in neonates receiving ampicillin compared with those treated with penicillin.[6,15] It should also be noted that the increased incidence rate of *S. epidermidis* LOS in penicillin treatment period[15] cannot be explained by spread of more virulent clone, as we observed different strains (ST2, ST5, ST418 and 2 nontypeable) among invasive *S. epidermidis*.

As questions may arise about the multiplicity of typing methods used in our study, an explanation should be given. Although PFGE is the most widely used method for genotyping of CoNS, it has several limitations of which the labour intensity and difficulties in interlaboratory comparison are the most notable. Thus, more rapid methods with transferability of results between laboratories, for example, MLST and MLVA, are preferred and will likely replace the methods based on banding patterns.[23] Although PFGE differentiates between *S. epidermidis* strains better than MLST, the discriminatory power of the MLVA typing scheme for *S. epidermidis* is higher than that of PFGE.[20] Even MLVA typing with only 4 loci, as in our study due to no repeats found within Se5 locus, decreases the resolution capacity only slightly.[20] Thus, using MLST and MLVA for *S. epidermidis* instead of PFGE as the most widely used typing method should not to be a concern. Furthermore, the use of different typing methods for different species will most likely not interfere with the within-species comparisons.

A few limitations should be noted. Considering the clonality of CoNS in neonates,[14,30] all possibly colonized body sites, including skin and IVCs, should be assessed to draw conclusions about the relation of the host's microbiota to CoNS infections. In addition, investigation of the colonization of hospital staff as a probable reservoir of CoNS was out of the scope of the comparative efficacy study.[15] Nevertheless, none of these devalues our findings of genetic similarity between GIT and bloodstream isolates. Second, despite identical colony morphology, several different strains can be harboured from samples.[30] In our study, only one of each morphologically distinct colony type was included. Finally, the small number of patients and isolates does not allow the interpretation of the distribution of *S. epidermidis* STs in the study setting.

In conclusion, the genetic relatedness between CoNS isolated from GIT and blood of preterm neonates with LOS indicates that newborns may be colonized with invasive strains in GIT before LOS. However, whether there is causal relationship between gut colonization and infection remains to be elucidated in further studies.

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