 10.1111/j.1469-0691.2007.01818.x

Persistent strains of coagulase-negative staphylococci in a neonatal intensive care unit: virulence factors and invasiveness

C. Klingenberg[1,2], A. Rønnestad[3], A. S. Anderson[4], T. G. Abrahamsen[3], J. Zorman[4], A. Villaruz[5], T. Flægstad[1,2], M. Otto[5] and J. Ericson Sollid[6]

[1]Department of Paediatrics, University Hospital of North Norway, [2]Department of Paediatrics, Institute of Clinical Medicine, University of Tromsø, Tromsø, [3]Department of Paediatrics, Rikshospitalet-Radiumhospitalet Medical Centre and University of Oslo, Oslo, Norway, [4]Merck & Co., Inc., Vaccine & Biologics Research, West Point, PA, [5]Laboratory of Human Bacterial Pathogenesis, Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, National Institute of Health, Hamilton, MT, USA and [6]Department of Microbiology and Virology, Institute of Medical Biology, University of Tromsø, Tromsø, Norway

ABSTRACT

Coagulase-negative staphylococci (CoNS) are the major cause of nosocomial bacteraemia in neonates. The aim of this study was to investigate whether persistent strains of CoNS possess specific bacterial characteristics as compared with sporadic non-cluster isolates. In total, 180 blood culture isolates (95 contaminants and 85 invasive isolates) obtained from a single neonatal unit over a 12-year period were studied. Pulsed-field gel electrophoresis (PFGE) identified 87 persistent CoNS strains (endemic clones). The two largest PFGE clusters belonged to a single clonal complex according to multilocus sequence typing. Patients colonised or infected with endemic clones were of lower gestational age than those infected with non-cluster strains. One *Staphylococcus haemolyticus* cluster appeared to selectively colonise and infect the most extreme pre-term infants. Endemic clones were characterised by high levels of antibiotic resistance and biofilm formation. All 51 isolates belonging to the two largest PFGE clusters were *ica* operon-positive. Genes encoding *Staphylococcus epidermidis* surface protein B and the production of phenol-soluble modulins (PSMs) were also more prevalent among endemic clones than among non-cluster strains. However, endemic clones were not more prevalent among invasive isolates than among contaminants. These findings indicate that multiple selective factors, including antibiotic resistance, biofilm formation, surface proteins with adhesive properties, and PSMs regulated by *agr*, increase the ability of CoNS to persist in a hospital environment. It may be more prudent, when searching for new therapeutic targets, to focus on ubiquitous components of CoNS instead of putative virulence factors that do not clearly contribute to increased invasive capacity.

Keywords Coagulase-negative staphylococci, endemic clones, invasive capacity, neonates, typing, virulence factors

Original Submission: 14 August 2006; **Revised Submission:** 26 April 2007; **Accepted:** 27 June 2007

Clin Microbiol Infect 2007; **13**: 1100–1111

INTRODUCTION

Coagulase-negative staphylococci (CoNS) are the major cause of nosocomial bacteraemia in neonatal intensive care units (NICUs) [1]. A matter of concern is whether these infections are caused by endemic hospital clones or by strains occurring sporadically. Persistence of endemic CoNS in single departments for many years has been documented [2–4], but a wide genetic diversity among isolates from a single unit has also been observed [5].

Persistent CoNS may possess specific characteristics that enable them to survive and persist in the NICU environment [6]. A high level of

Corresponding author and reprint requests: C. Klingenberg, Department of Paediatrics, University Hospital of North Norway, N-9038 Tromsø, Norway
E-mail: claus.klingenberg@unn.no

antibiotic resistance is considered to be a major selective force for persistence [7]. Biofilm formation is the most important virulence determinant of CoNS [8–10], but its contribution to selection and persistence in a hospital environment is unclear [3,7,9,11]. Phenol-soluble modulin (PSM) is a peptide complex produced by *Staphylococcus epidermidis*, and PSM peptides have been recognised as virulence factors with a strong pro-inflammatory capacity [12]. PSM production is strictly positively regulated by the staphylococcal quorum-sensing system *agr* (accessory gene regulator) [12], but *agr* activation is thought to inhibit biofilm formation indirectly and to increase biofilm detachment [13]. *S. epidermidis* surface (Ses) proteins are a group of putative virulence factors that facilitate bacterial adherence to host tissue, which is the first essential step in colonisation and/or infection [14,15]. The contribution of the PSM and Ses proteins to the persistence of CoNS in a hospital setting has not yet been evaluated.

In the present study, endemic clones of CoNS were identified by pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) in a single NICU over a 12-year period. The aim of the study was to evaluate the hypothesis that persistent or predominant clones in an NICU possess specific characteristics that enhance their ability to persist and cause invasive disease in neonates. In addition, the genetic diversity of isolates from a single site was assessed by means of PFGE and MLST, and these isolates were compared with *S. epidermidis* sequence types characterised previously.

MATERIALS AND METHODS

Patients and isolates

Neonates eligible for inclusion in the study were admitted to the tertiary-care NICU at Rikshospitalet Medical Centre, Oslo, Norway, between January 1989 and April 2000 and diagnosed with growth of CoNS in blood culture. In total, 180 CoNS blood isolates were obtained from 150 neonates. The diagnosis of CoNS sepsis (n = 85 isolates) required clinical signs of sepsis in a neonate aged >72 h, and a positive monomicrobial blood culture and an elevated C-reactive protein level of >10 mg/L within 2 days of blood culture. All other CoNS blood isolates, including cultures growing more than one organism, were considered to be contaminants (n = 95 isolates). At the time of blood culture, 16% of patients had an indwelling vascular line, and 25% were being treated on a ventilator. Further clinical data have been reported previously [16]. The regional committee for medical research ethics approved the collection and analysis of patient data.

Phenotypic and genotypic species identification

All isolates were identified to the species level using the ID 32 Staph system (bioMérieux, Marcy l'Etoile, France), and by amplification and direct nucleotide sequencing of PCR fragments of the *tuf* gene, using the *tuf* primers listed in Table S1 (see Supplementary material). Certain isolates yielded ambiguous results when the phenotypic and genotypic methods for species identification were compared, and also following further comparison with PFGE cluster results and PSM analyses. These isolates were additionally retested by amplification and direct nucleotide sequencing of fragments of the 16S rRNA gene, using the 16S RNA primers listed in Table S1. Definitive species identification was based on a polyphasic approach combining phenotypic and genotypic species identification, PFGE cluster data and the PSM profile.

Antibiotic resistance

MICs of oxacillin, gentamicin, erythromycin, clindamycin, fusidic acid, rifampicin and vancomycin were determined by Etest (AB Biodisk, Solna, Sweden), with susceptibilities interpreted according to CLSI guidelines [17]. PCRs for the methicillin resistance gene (*mecA*) and the most frequent aminoglycoside resistance gene (*aac(6′)-Ie-aph(2′)*-Ia) were performed using the corresponding primers listed in Table S1.

Biofilm production

Semiquantitative determination of biofilm production involved a microtitre assay as described previously [16,18]. PCR detection of *icaD*, a marker of the *ica* operon, was performed using the primers listed in Table S1.

PSMs

HPLC–mass spectrometry was used to detect and quantify PSMs in bacterial culture supernatants, as described previously [13]. Using this method, a strong correlation has been demonstrated previously among the levels of all major PSM components [12]. Consequently, exact quantification was only performed for PSMγ, and the other PSM peptides were recorded as either present or absent.

Screening for *ses* genes

PCR detection of the genes encoding SdrF, SdrG/Fbe, SesB, SesD/Bhp, SesE, SesF/Aap and SesH was performed using the corresponding primers listed in Table S1. Positive and negative controls were included in each PCR run. The PCR product obtained for *sesB* was also sequenced to determine the diversity of this surface protein among the *S. epidermidis* isolates screened (accession numbers EF424049–EF424056).

PFGE and definition of genetic relatedness

Chromosomal DNA preparation and PFGE analysis were performed as described previously [19,20]. PFGE banding patterns were analysed using GelCompar software v.2.5 (Applied Maths, Sint-Martins-Latem, Belgium). Band assignment was manually curated on all gel images after automated

band detection. Dice similarity coefficients were calculated with a band position tolerance of 1.6%. PFGE dendrograms were constructed using the unweighted pair-group method with arithmetic means (UPGMA). Isolates were first assigned to PFGE types, using either ≥75% or ≥80% band-based similarity coefficients as cut-off values. Discrepancies in cluster assignment between these two cut-off values were then resolved by visual examination of banding patterns, with interpretation of the degree of relatedness as described by Tenover *et al*. [21].

MLST

A randomly selected group of 88 *S. epidermidis* isolates was analysed using MLST. DNA isolation and nucleotide sequencing of seven *S. epidermidis* housekeeping genes (shikimate dehydrogenase, heat-shock protein, glycerol kinase, guanylate kinase, phosphate acetyltransferase, triphosphate isomerase and acetyl coenzyme A acetyltransferase) was performed as described previously by Wang *et al*. [22]. Five alleles (shikimate dehydrogenase, guanylate kinase, phosphate acetyltransferase, triphosphate isomerase and carbamate kinase (*arcC*)) were also sequenced using the PCR primers described by Wisplinghoff *et al*. [23]. The Wang and the Wisplinghoff schemes both used six of the seven genes from the *Staphylococcus aureus* MLST scheme validated previously [24]. However, the Wang scheme did not include the *arcC* allele, and the Wisplinghoff scheme did not include the glycerol kinase (*glpF*) allele. For the alleles that both schemes shared, different regions were selected for each scheme. Novel allele sequences were deposited in GenBank (EF424057–EF424061). Clonality was assessed using eBURST, a web-implemented clustering algorithm available on the MLST website (http://www.mlst.net).

Statistical analyses

The Mann–Whitney test was used for comparison of continuous non-parametric variables. The chi-square test or Fisher's exact test was used, as appropriate, for comparison of dichotomous variables. Correlations between the presence of *ses* genes and PSMs were presented as kappa values as a measure of agreement. Background results were presented as medians with interquartile range (IQR). A binary logistic regression analysis was performed to assess whether bacterial characteristics were associated independently with endemic *S. epidermidis* isolates or invasive *S. epidermidis* isolates. These results were presented as adjusted ORs with 95% CIs. All analyses were two-tailed, with $p < 0.05$ considered to be significant. Statistical analyses were performed using SPSS for Windows v.12.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Species and PFGE clusters

The 180 isolates comprised six different CoNS species: *S. epidermidis* ($n = 128$), *Staphylococcus warneri* ($n = 19$), *Staphylococcus haemolyticus* ($n = 19$), *Staphylococcus capitis* ($n = 7$), *Staphylococcus hominis* ($n = 6$) and *Staphylococcus cohnii* ($n = 1$). PFGE typing revealed a diverse population, with the 128 *S. epidermidis* isolates and the 19 *S. warneri* isolates being clustered in 45 and 12 PFGE types, respectively. *S. haemolyticus* showed the least genetic diversity, with 13 of the 19 isolates clustered in a single PFGE type. Overall, three large clusters were found to contain 14–36 isolates, and three smaller clusters contained six to nine isolates (Table 1). Two PFGE types contained four isolates, and the other 62 PFGE types contained one to three isolates. Discrepancies in cluster assignment between the 75% and 80% similarity cut-off levels were observed for 15 isolates from five clusters. After visual examination, 11 isolates were clustered according to the 75% cut-off level, and four isolates remained clustered according to the 80% cut-off level. In total, 87 clustered (endemic) isolates were isolated from 72 patients. PFGE type 27 comprised 20% ($n = 36$) of all isolates in this study, and this PFGE type was isolated every year between 1989 and

Table 1. Pulsed-field gel electrophoresis (PFGE) clusters of coagulase-negative staphylococci (CoNS) that contained at least six isolates

	Cluster					
Bacterial characteristics	**PFGE type 1 *n* = 15 (%)**	**PFGE type 19 *n* = 9 (%)**	**PFGE type 27 *n* = 36 (%)**	**PFGE type 34 *n* = 6 (%)**	**PFGE type 46 *n* = 7 (%)**	**PFGE type 51 *n* = 14 (%)**
Species	*Staphylococcus epidermidis*	*S. epidermidis*	*S. epidermidis*[a]	*S. epidermidis*	*Staphylococcus warneri*	*Staphylococcus haemolyticus*[b]
mecA-positive	15 (100)	5 (56)	35 (97)	6 (100)	4 (57)	13 (97)
icaD-positive	15 (100)	7 (78)	36 (100)	2 (33)	2 (29)	5 (36)
sesB-positive	14 (93)	5 (56)	22 (61)	5 (83)	NP	NP
PSMγ-positive	15 (100)	8 (89)	31 (86)	6 (100)	NP	NP
Invasive	9 (60)	4 (44)	21 (58)	2 (33)	2 (29)	6 (43)
Period detected	1991–1993	1991–1999	1989–1999	1996–1999	1995–2000	1991–1999

[a]One isolate was genotyped as *S. warneri*; all others were *S. epidermidis*.
[b]One isolate was genotyped as *S. warneri*; all others were *S. haemolyticus*.
NP, not present in non-*epidermidis* CoNS; PSM, phenol-soluble modulin.

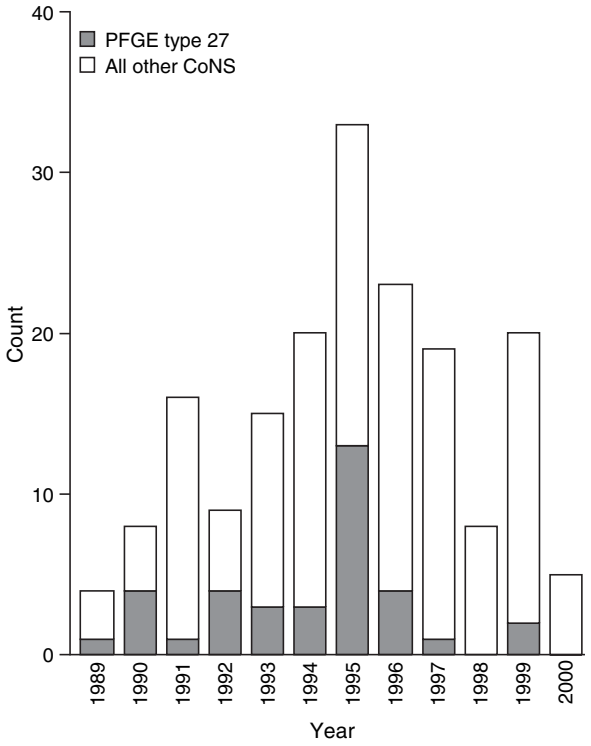

Fig.1. Annual distribution of 36 isolates of coagulase-negative staphylococci (CoNS) belonging to pulsed-field gel electrophoresis (PFGE) type 27 in comparison with the remaining 1444 CoNS isolates during the 12-year study period.

1997, and also in 1999 (Fig. 1). The highest number (n = 33) of CoNS blood culture isolates was collected in 1995; of these, 13 (39%) belonged to PFGE type 27 (Fig. 1).

MLST

In total, 88 *S. epidermidis* isolates were divided into 35 sequence types (STs). Of these, 29 STs were not detected in a previous study using the same MLST scheme [22]. Forty-three isolates were designated ST2, 26 isolates were single-locus variants of ST2, and two isolates were double-locus variants. Thus, 71 (81%) of 88 isolates analysed with MLST belonged to a large clonal complex (CC) designated CC1. Fig. S1(A) (see Supplementary material) shows an analysis of CC1 by eBURST. All PFGE type 1 isolates and 24 (88%) of the 27 PFGE type 27 isolates analysed with MLST were found to cluster in CC1 (Table 2). To further differentiate CC1 isolates, *sesB* and *arcC* sequence types were analysed. Of the 88 isolates included in this analysis, 57 were positive for *sesB*, representing six alleles. The *arcC* gene was detected in all 88 isolates, with 11 different alleles recognised, of which five were novel (Table 2). Inclusion of these alleles enabled the 71 CC1 isolates to be divided into three groups: the largest group contained isolates with *sesB*, while this gene was mostly absent from the two minor groups, as shown in Fig. S1(B). Two other CCs were identified among the remaining 17 isolates. CC2 contained six STs, with ST39 suggested as the founder, while CC3 contained two STs with no identifiable founder. Nine STs containing single isolates were detected (Table 2).

Two additional eBURST comparisons were conducted to investigate the relationship between these isolates and other global *S. epidermidis* strains. First, the STs for the 88 isolates in the present study were compared with the 50 isolates of Wang *et al*. [22]. Fig. S1(C) shows that CC1 contained 111 (80%) of 138 isolates, represented by 80 isolates from the present study and 31 isolates from the study of Wang *et al*. [22]. The remaining eight isolates from the present study resolved into two additional clusters, designated CC2 and CC3, that had not been detected previously [22]. The final eBURST analysis compared isolates from the present study with isolates deposited in the mlst.net database. This comparison used MLST analyses with the five alleles from the scheme of Wisplinghoff *et al*. [23]. As shown in Fig. S1(D), two CCs were revealed, with most (20/28) STs in one large CC. However, the dominant ST according to this typing scheme was the singleton ST19, which contained 48 isolates from the present study and two from mlst.net.

Clinical characteristics

Patients colonised or infected with cluster isolates had a significantly lower gestational age than patients with non-cluster isolates. Logically, these patients were also hospitalised for a longer period, but the blood cultures were obtained at a similar postnatal age for both groups (Table 3). Of 30 patients infected or colonised with PFGE type 27, six were born at term, while the remaining 24 were born after gestation for 23–29 weeks. Furthermore, infants colonised or infected with *S. haemolyticus* were younger (median gestation, 26 weeks; IQR 25–28 weeks) than infants

Table 2. Differentiation of 88 *Staphyloccus epidermidis* isolates by multilocus sequence typing (MLST)

ST	CC	No. of isolates	*arcC* type	*sesB* type	MLST-*arcC*-*sesB*	CC1 group	No. of isolates	PFGE type (no. of isolates)
2	1	43	1		2-1-0	b	1	26 (1)
			1	1	2-1-1	a	2	23 (1), 6 (1)
			18		2-18-0	b	1	42 (1)
			19		2-19-0	b	1	12 (1)
			20	1	2-20-1	a	1	1 (1)
			2	1	2-2-1	a	1	4 (1)
			21	1	2-21-1	a	1	27 (1)
			7		2-7-0	b	12	27 (8), 42 (1), 1 (1), 6 (1), 60 (1)
			7	1	2-7-1	a	22	27 (10), 20 (1), 1 (6); 4 (1), 5 (1), 8 (1), 30 (1), 38 (1)
			7	6	2-7-6	b	1	38 (1)
4	1	2	7		4-7-0	b	1	7 (1)
			7	1	4-7-1	a	1	19 (1)
7	1	3	1		7-1-0	c	2	31 (2)
			7	1	7-7-1	a	1	27 (1)
8	1	1	7	1	8-7-1	a	1	25 (1)
60	1	2	1		60-1-0	b	1	68 (1)
			1	1	60-1-1	a	1	31 (1)
24	1	1	7		24-7-0	b	1	27 (1)
25	S	1	1		25-1-0		1	23 (1)
26	1	1	7	1	26-7-1	a	1	1 (1)
27	S	1	1	5	27-1-5		1	19 (1)
28	1	1	7	1	28-7-1	a	1	27 (1)
29	1	1	1	1	29-1-1	a	1	33 (1)
30	1	9	7		30-7-0	b	1	20 (1)
			7	1	30-7-1	a	8	1 (4), 22 (1), 27 (3)
31	1	1	7	1	31-7-1	a	1	27 (1)
32	1	1	7		32-7-0	b	1	27 (1)
33	S	1	17	2	33-17-2		1	18 (1)
34	S	1	8		34-8-0		1	58 (1)
35	S	1	1	2	35-1-2		1	3 (1)
36	S	1	18	2	36-18-2		1	34 (1)
37	S	1	7	4	37-7-4		1	9 (1)
38	3	1	1	2	38-1-2		1	41 (1)
39	2	1	3		39-3-0		1	34 (1)
40	3	1	7		40-7-0		1	16 (1)
41	2	1	18	2	41-18-2		1	34 (1)
42	2	1	18	2	42-18-2		1	34 (1)
43	S	1	1	1	43-1-1		1	24 (1)
44	2	1	18		44-18-0		1	13 (1)
45	1	1	1		45-1-0	c	1	24 (1)
		1	7	1	45-7-1	a	1	22 (1)
46	2	1	18	2	46-18-2		1	62 (1)
47	S	1	7		47-7-0		1	63 (1)
48	2	1	7		48-7-0		1	59 (1)
49	1	1	7	1	49-7-1	a	1	1 (1)
50	1	1	7	1	50-7-1	a	1	16 (1)
51	1	1	1		51-1-0	a	1	32 (1)

ST, sequence type; CC, clonal complex; S, singleton.

Table 3. Comparison of patients with coagulase-negative staphylococci (CoNS) cluster isolates and patients with CoNS non-cluster isolates

Clinical characteristics	76 patients with CoNS cluster isolates (IQR)	74 patients with CoNS non-cluster isolates (IQR)	p value
Median birth weight, g	960 (748–1781)	2610 (843–3485)	0.007
Median gestational age, weeks	27.5 (26–33)	34 (27–40)	0.008
Median post-natal age when blood culture obtained, days	14 (5–23)	11 (2–24)	0.58
Median hospitalisation period, days	35 (11–76)	12 (7–32)	0.005
Ever with indwelling vascular line	52.0%	43.8%	0.17
Ever on ventilation	63.2%	49.3%	0.049
Invasive infection	54.0%	46.6%	0.31

IQR, inter-quartile range.

colonised or infected with other CoNS (median gestation, 29 weeks; IQR 26–40 weeks, p 0.001).

Antibiotic resistance

All isolates were susceptible to vancomycin, and there was a low rate of resistance to rifampicin. However, cluster isolates showed higher rates of resistance to oxacillin, gentamicin, fusidic acid, erythromycin and clindamycin (Tables 4 and 5). Interestingly, almost all isolates in four of the six clusters carried *mecA*, but only 57% of the non-cluster isolates were *mecA*-positive.

Table 4. *Staphylococcus epidermidis* cluster isolates compared with non-cluster isolates in terms of antibiotic resistance and virulence factors

	65 cluster isolates (PFGE type 1-19-27-34) *n* (%)	63 non-cluster isolates n (%)	p value
Antibiotic resistance[a]			
Methicillin	60 (92)	37 (59)	<0.001
Gentamicin	57 (88)	32 (51)	<0.001
Erythromycin	26 (40)	13 (21)	0.017
Clindamycin	22 (34)	8 (13)	0.005
Fusidic acid	60 (92)	44 (70)	0.001
Rifampicin	2 (3)	2 (3)	0.98
Biofilm production			
Biofilm-positive	53 (83)	25 (40)	<0.001
Median biofilm optical density	1.46	0.09	<0.001
icaD PCR-positive	59 (91)	32 (51)	<0.001
PSMs			
PSMα-positive	59 (91)	43 (68)	0.002
PSMβ1-positive	59 (91)	51 (81)	0.11
PSMβ2-positive	59 (91)	46 (73)	0.009
PSMγ-positive	60 (92)	50 (79)	0.035
PSMγ (μg/L) (median)	230	165	0.003
PSMσ-positive	46 (71)	33 (52)	0.033
PSMϵ-positive	13 (20)	17 (27)	0.35
ses genes detected by PCR			
sdrF	3 (5)	3 (5)	1.0
sdrG/fbe	27 (42)	31 (49)	0.38
sesB	46 (71)	29 (47)	0.006
sesD/bhp	0 (0)	3 (5)	0.12
sesE	35 (54)	31 (50)	0.67
sesF/aap	29 (45)	36 (57)	0.16
sesH	18 (28)	13 (21)	0.38

PFGE, pulsed-field gel electrophoresis; PSM, phenol-soluble modulin.
[a]According to CLSI breakpoint criteria [17].

Table 5. Non-*epidermidis* coagulase-negative staphylococci cluster isolates compared with non-cluster isolates

	21 cluster isolates (PFGE types 46 and 51) *n* (%)	31 non-cluster isolates *n* (%)	p value
Antibiotic resistance[a]			
Methicillin	17 (81)	16 (53)	0.052
Gentamicin	15 (71)	13 (43)	0.061
Erythromycin	13 (62)	7 (23)	0.009
Clindamycin	6 (29)	4 (13)	0.28
Fusidic acid	16 (76)	20 (67)	0.51
Rifampicin	0 (0)	0 (0)	
Biofilm production			
Biofilm-positive	3 (14)	10 (33)	0.091
Median biofilm optical density	0.06	0.07	0.091

PFGE, pulsed-field gel electrophoresis.
[a]According to CLSI breakpoint criteria [17].

Biofilm production

Biofilm formation and *icaD* were detected significantly more frequently for *S. epidermidis* cluster isolates than for non-cluster isolates (Tables 4 and 5).

PSMs

PSM peptides are only known to occur in *S. epidermidis*, and thus production of PSM peptides was investigated only in isolates of this species. As expected, there was a strong correlation (p <0.001) between the presence of PSMγ and all other PSMs: PSMβ1 (kappa 0.93); PSMβ2 (kappa 0.92); PSMα (kappa 0.91); PSMσ (kappa 0.65); and PSMϵ (kappa 0.23). Production of PSMγ was significantly greater among cluster isolates than among non-cluster isolates (Table 4). In general, all PSMs, except PSMα and PSMϵ, were detected more frequently in cluster isolates than in non-cluster isolates. Invasive *S. epidermidis* isolates were more frequently PSMγ-positive (92%) than were isolates considered to be contaminants (80%) (p 0.05). However, the mean and median PSMγ production levels did not differ significantly between invasive isolates and contaminants. No correlations were found in *S. epidermidis* between phenotypic biofilm production or the presence of *icaD* and different PSM analyses (data not shown).

S. epidermidis surface proteins

A correlation was found between the presence of *sesB* and *sesE* (kappa 0.31, p 0.001) and *sesH* (kappa 0.32, p <0.001), but there was no correlation between *sesB* and detection of other Ses-encoding genes. Table 4 shows the prevalence of *ses* genes among *S. epidermidis* cluster isolates as compared with non-cluster isolates. Invasive *S. epidermidis* isolates were more frequently *sesB*-positive (68%) than were contaminants (48%) (p 0.024). There were no differences for the other *ses* genes between invasive isolates and contaminants. There was also a correlation between the presence of *sesB* and PSMα (kappa 0.34, p <0.001) and PSMγ (kappa 0.26, p 0.003).

Median biofilm production was much greater among the 65 *sesF/aap*-positive *S. epidermidis* isolates (OD 1.63) than among the 63 *sesF/aap*-negative isolates (OD 0.25; p <0.001). This strong association between *sesF/aap* and biofilm production also remained significant (p <0.001) after adjusting for *icaD* in a linear regression model. Fourteen isolates were *ica*-negative but *sesF/aap*-positive. None of these 14 isolates produced biofilm. Only one of three *sesD/bhp*-positive isolates produced biofilm, but this isolate was also *icaD*-positive.

Factors associated independently with endemic or invasive *S. epidermidis* isolates

The variables *mecA*, *icaD*, *sesB* and PSMγ were included in a binary logistic regression model.

Compared with *S. epidermidis* non-cluster isolates, the presence of *icaD*, *mecA* and *sesB* increased the risk of an isolate belonging to the group of endemic *S. epidermidis* isolates. Adjusted ORs were: *icaD* 6.23 (95% CI 2.20–17.64; p 0.001), *mecA* 5.72 (95% CI 1.81–18.10; p 0.003) and *sesB* 2.92 (95% CI 1.25–6.84; p 0.013). Compared with *S. epidermidis* contaminants, the presence of *mecA* and *sesB* increased the risk of an isolate belonging to the group of invasive *S. epidermidis* isolates. Adjusted ORs were: *mecA* 3.08 (95% CI 1.26–7.55; p 0.011) and *sesB* 2.26 (95% CI 1.08–4.76; p 0.029).

DISCUSSION

The present study analysed bacterial factors associated with predominant and/or persistent CoNS blood culture isolates during a 12-year period in a single NICU. Two different molecular typing techniques were used to identify clusters. PFGE has been considered to be the typing method of choice for studying the clonality of bacteria within a geographically localised area, but frequently occurring mutations (high molecular clock speed) may limit its use for long-term studies [22,25]. In addition, genetic relatedness is often assessed by visual examination of banding patterns [21]. However, in longitudinal studies with a large number of isolates, a classification based on visual examination becomes difficult. Automatic classification using band-based similarity coefficients may overcome this problem [26]. For long-term studies, no general agreement exists concerning the most appropriate similarity cut-off levels for identifying related PFGE types, although arbitrarily chosen similarity levels of 70–80% are often used [7,25,27]. Unfortunately, the band position tolerance, which is critical for automatic classification of similarity [26], is not always reported. In the present study, a combination of automatic classification of similarity levels, a conservative band position tolerance (1.6%) and visual examination were used. There was a good correlation between the PFGE typing and MLST results.

Three different MLST schemes for *S. epidermidis* have been published [22,23,28]. However, the discriminatory capacity of MLST for *S. epidermidis* has been a concern [28,29]. The present study confirmed that, although MLST and PFGE results were highly concordant, MLST did not discriminate among isolates to the same extent as PFGE. This was further demonstrated by expanding the MLST scheme with two additional alleles (*arcC* and *sesB*). A similar approach has been proposed previously [29], involving sequencing of Ses protein-encoding genes (*sdrG* and *aap*), which showed promising results, either alone or in combination with MLST, as a possible new typing tool. The addition of *sesB* to the MLST scheme in the present study was also helpful for differentiation purposes; although *sesB* was only present in a subset of isolates, even the presence or absence of this gene had implications for typing.

Overall, the MLST results demonstrated the existence of one large CC of highly related isolates, including the two largest PFGE clusters. The isolates in this cluster (CC1) showed a high prevalence of the *mecA* gene and the *ica* operon. Interestingly, the dominant ST2 in the present study has also been detected in medical facilities in North America and South Africa [22]. In general, methicillin resistance and the *ica* operon seem to be virulence factors that are identified frequently in dominant STs from different continents [22,23,30]. However, comparison with isolates analysed previously with MLST also highlighted the occurrence of additional novel CCs and STs in the single NICU studied. A more comprehensive database is required to determine whether these novel strains are under-represented in the current database or are unique to the present study. Sequence-based typing systems are clearly preferred for long-term and global epidemiological studies of *S. epidermidis*, but the choice of typing system will depend on factors such as cost and the need for greater or less discrimination [29]. PFGE still compares well with MLST as a typing technique for local long-term epidemiological studies, and may even have advantages in such studies by having a higher discriminatory power.

Three large and three smaller PFGE clusters were found to persist and predominate throughout the 12-year period in the unit studied, which is a finding similar to results reported in other long-term studies [2,3,7,31]. The finding of endemic PFGE clusters indicates that cross-infection or cross-contamination has occurred within the NICU. There was a peak in the number of CoNS-positive blood cultures during 1995, and the dominant endemic PFGE type 27 also peaked at this time, indicating frequent cross-contamination, perhaps caused by an undetected lapse in the infection control policy.

All the CoNS species detected showed genetic variability. However, 13 of the 19 isolates of *S. haemolyticus* belonged to a single cluster. Previous studies have also documented outbreaks or persistence of *S. haemolyticus* strains that affected the most extreme pre-term babies in the NICU [2,32]. Whole genome sequencing and comparative genomics with other staphylococci have identified a wide range of putative virulence factors in *S. haemolyticus* [33]. However, there is still a lack of knowledge regarding the contribution of these virulence factors to the pathogenicity of *S. haemolyticus* [8].

CoNS cluster isolates in the present study were characterised by higher levels of antibiotic resistance as compared with non-cluster isolates. It has been suggested that antibiotic resistance is a major force in the selection of certain CoNS strains [7]. The most marked differences in resistance rates were observed between cluster and non-cluster isolates for β-lactam antibiotics and aminoglycosides. These two groups of antibiotics form part of the empirical antibiotic regimen in the NICU studied, which supports previous findings that in-vitro antimicrobial resistance patterns reflect the pattern of antibiotic use in a particular unit [4,5].

Biofilm formation and carriage of the *ica* operon occurred frequently among endemic clones of *S. epidermidis* in the present study. The ability of *S. epidermidis* to produce biofilm is widespread among hospital isolates [34]. However, the uniform presence of the *icaD* operon in the two large *S. epidermidis* clusters (PFGE types 1 and 27), and a much lower *icaD* prevalence in the non-cluster isolates, was a striking finding. This indicates strongly that isolates with biofilm-forming capacity have a selective advantage for survival and persistence in the NICU environment. The exact mechanism for this persistence is unclear, but it has been shown that osmo-protective factors are up-regulated in biofilms [13], so that biofilm-forming CoNS may grow well in the high-osmolarity environment of the human skin. One likely explanation of the high prevalence of endemic clones among infants with a lower gestational age is that these infants require more handling and invasive procedures, which again leads to an increased risk of being colonised by endemic clones in the NICU via the hands of healthcare workers.

The gene encoding PSMγ is located within RNAIII, the regulatory molecule of the *agr* system. PSMγ production therefore reflects *agr* activity on a translational level [35]. The *agr* operon is not involved directly in regulation of the *ica* operon [36–38]. However, there is strong evidence to support the hypothesis that low *agr* activity favours biofilm formation and the ability of *S. epidermidis* to colonise catheter material, whereas high *agr* activity increases the invasive capacity of *S. epidermidis*, but concomitantly inhibits biofilm formation [12,13,39]. It has been suggested that this influence of *agr* on biofilm production is mediated via the surfactant-like PSMs, which inhibit the later stages of biofilm formation and lead to biofilm dispersion [39]. Time-lapse studies using flow cells have shown that expression of *agr* in exposed cell clusters is followed by cell detachment from *S. aureus* biofilms [40]. In the present study, no correlation was found between the production of PSMγ and biofilm formation, which may be explained by the fact that most isolates were from patients with sepsis, and not primarily from a biofilm-associated infection resulting from implanted devices. Sepsis strains with invasive properties may differ from strains associated with implanted devices. For the latter, factors enhancing colonisation may be of greater advantage than the aggressive factors that often characterise strains causing sepsis.

In the present study, endemic clones of *S. epidermidis* showed higher levels of PSMγ, and a higher prevalence of most PSM peptides, than did non-cluster isolates. It can be speculated that production of PSM peptide, caused by an active *agr* system, is advantageous for the selection and persistence of nosocomial CoNS in this type of infection. The exact mechanisms by which an active *agr* system enhances the ability of CoNS to persist are unclear, although increased resistance to antimicrobial peptides secreted on the skin may be involved [41]. Certainly, the role of *agr* and the PSM peptides in the virulence of *S. epidermidis* in different types of infection will require further investigation.

S. epidermidis surface (Ses) proteins are cell-wall-anchored proteins that interact with targets in the host, and are important for both bacterial adherence and evasion from the host immune system [14]. The most studied Ses protein is SdrG/Fbe, a fibrinogen-binding protein. Anti-Fbe

antibodies may block bacterial adherence and decrease the severity of experimental infection in animals [42,43]. In the present study, *sdrG/fbe* was associated with neither endemic nor invasive isolates. The accumulation-associated protein (SesF/Aap) can mediate intercellular adhesion and biofilm formation, independent of the *ica* operon [44,45], and a strong and *ica*-independent association between the presence of *sesF/aap* and the level of biofilm production was found in the present study. These findings support the hypothesis that the *ica* operon and *sesF/aap* may act cooperatively in mediating *S. epidermidis* biofilm formation [45]. However, none of the *ica* operon-negative and *sesF/aap*-positive strains produced biofilm *in vitro*. SesD/Bhp may also facilitate biofilm formation [46] but, in the present study, *sesD/bhp* was detected in only three isolates, in agreement with the results of Bowden *et al*. [14].

By comparative genomics, more than ten putative Ses proteins have been detected in the *S. epidermidis* genome, but their exact function and possible role as virulence factors are still unclear [14,47,48]. In addition, there is a high genetic variability for this class of surface protein in *S. epidermidis* [48]. In the present study, only the gene encoding SesB was more prevalent among endemic *S. epidermidis* isolates than among non-cluster isolates, and this gene was also slightly more prevalent among invasive isolates than among contaminants. High titres of antibodies to different Ses proteins are found in adult patient sera, indicating that these proteins are expressed and are important during an infection [14]; this has not yet been investigated for SesB.

Ses proteins have been considered as candidates for the development of novel therapies against staphylococcal infections [14,43]. Five of the seven genes encoding Ses proteins were detected in 24–59% of all 128 *S. epidermidis* isolates investigated, while the remaining two were found only in a few isolates. This distribution differs from that reported previously [14,29], and also differs from the distribution of similar proteins in *S. aureus* [49]. Ses proteins may well constitute a group of important virulence factors, but the relatively low prevalence found in the present study suggests a limit to their potential role as general targets for new treatment or prophylactic strategies in *S. epidermidis* infections.

Endemic clones had the highest rates of antibiotic resistance and biofilm production. PFGE type 1 was positive for almost all virulence factors investigated, although only nine of 15 isolates were considered invasive. In general, no clear association was found between endemic clones and invasive capacity, which is in contrast to a previous study [2]. It is notoriously difficult to differentiate between true bloodstream infection and contamination in cases of neonatal CoNS sepsis. The definition of CoNS sepsis used was in line with that used by others [1], and is considered to be fairly robust. However, it cannot be excluded that some invasive isolates may have been contaminants and vice versa. Previous studies [16,50–52] have concluded that detection of the *ica* operon is not a suitable genetic marker for discriminating invasive from contaminating CoNS isolates. Furthermore, de Silva *et al*. [5] found that endemic clones were equally distributed among skin isolates and invasive isolates in a case-controlled study, and concluded that endemic clones with enhanced ability to cause invasive disease were not present. This is a key question in relation to the pathogenesis of neonatal CoNS disease. Efforts to reduce the frequency and severity of CoNS infections have focused on targeting either specific CoNS virulence factors [53] or quorum-sensing systems regulating these virulence factors [54]. If endemic clones do not display enhanced ability to cause invasive disease compared with sporadic strains, focusing on ubiquitous CoNS virulence factors may be more prudent when searching for novel therapeutic strategies [55].

In conclusion, a small number of clusters of CoNS persisted and predominated in the NICU studied during a 12-year period. Multiple selection forces, including antibiotic resistance, biofilm production, and other bacterial factors such as surface proteins, may increase the ability of CoNS to persist in a hospital environment. However, the endemic clones detected in the present study did not exhibit clearly increased invasive capacity, despite possessing more putative CoNS virulence factors than non-cluster isolates. Endemic clones were identified more frequently in very pre-term infants, probably as a result of frequent therapeutic handling, which enhances the risk of cross-contamination within the NICU. Clearly, multiple strategies are needed to combat nosocomial neonatal CoNS infections. Strict adherence to infection control guidelines and a meticulous hand disinfection policy is crucial for all prevention

programmes [56]. Further clinical studies are warranted to identify and delineate the clinical importance of CoNS virulence factors, and to determine how to utilise them in the design of new therapeutic strategies.

ACKNOWLEDGEMENTS

This study was supported by grants from Northern Norway Regional Health Authority and Fredriksens legat. We thank J. Mikalsen, G. Kjeldsen, L. Nordgård, M. Sletteng and E. Aarag for excellent technical assistance, and T. Wilsgård for statistical advice. We declare that none of the authors have any commercial relationship or other association that might pose a conflict of interest. This work was presented, in part, at the European Society of Paediatric Infectious Disease (ESPID) meeting in Basel, Switzerland (May 2006).

SUPPLEMENTARY MATERIAL

The following supplementary material is available for this article online at http://www.blackwell-synergy.com:

Table S1. PCR primers used in this study

Fig. S1. Analysis of multilocus sequence typing data using eBURST.

REFERENCES

1. Stoll BJ, Hansen N, Fanaroff AA *et al.* Late-onset sepsis in very low birth weight neonates: the experience of the NICHD Neonatal Research Network. *Pediatrics* 2002; **110**: 285–291.
2. Bjorkqvist M, Soderquist B, Tornqvist E *et al.* Phenotypic and genotypic characterisation of blood isolates of coagulase-negative staphylococci in the newborn. *APMIS* 2002; **110**: 332–339.
3. Huebner J, Pier GB, Maslow JN *et al.* Endemic nosocomial transmission of *Staphylococcus epidermidis* bacteremia isolates in a neonatal intensive care unit over 10 years. *J Infect Dis* 1994; **169**: 526–531.
4. Burnie JP, Naderi-Nasab M, Loudon KW, Matthews RC. An epidemiological study of blood culture isolates of coagulase-negative staphylococci demonstrating hospital-acquired infection. *J Clin Microbiol* 1997; **35**: 1746–1750.
5. de Silva GD, Justice A, Wilkinson AR *et al.* Genetic population structure of coagulase-negative staphylococci associated with carriage and disease in preterm infants. *Clin Infect Dis* 2001; **33**: 1520–1528.
6. Krediet TG, Jones ME, Janssen K, Gerards LJ, Fleer A. Prevalence of molecular types and *mecA* gene carriage of coagulase-negative staphylococci in a neonatal intensive care unit: relation to nosocomial septicemia. *J Clin Microbiol* 2001; **39**: 3376–3378.
7. Krediet TG, Mascini EM, van Rooij E *et al.* Molecular epidemiology of coagulase-negative staphylococci causing sepsis in a neonatal intensive care unit over an 11-year period. *J Clin Microbiol* 2004; **42**: 992–995.
8. Otto M. Virulence factors of the coagulase-negative staphylococci. *Front Biosci* 2004; **9**: 841–863.
9. de Silva GD, Kantzanou M, Justice A *et al.* The *ica* operon and biofilm production in coagulase-negative staphylococci associated with carriage and disease in a neonatal intensive care unit. *J Clin Microbiol* 2002; **40**: 382–388.
10. von Eiff C, Peters G, Heilmann C. Pathogenesis of infections due to coagulase-negative staphylococci. *Lancet Infect Dis* 2002; **2**: 677–685.
11. Raimundo O, Heussler H, Bruhn JB *et al.* Molecular epidemiology of coagulase-negative staphylococcal bacteraemia in a newborn intensive care unit. *J Hosp Infect* 2002; **51**: 33–42.
12. Vuong C, Durr M, Carmody AB *et al.* Regulated expression of pathogen-associated molecular pattern molecules in *Staphylococcus epidermidis*: quorum-sensing determines pro-inflammatory capacity and production of phenol-soluble modulins. *Cell Microbiol* 2004; **6**: 753–759.
13. Yao Y, Sturdevant DE, Otto M. Genomewide analysis of gene expression in *Staphylococcus epidermidis* biofilms: insights into the pathophysiology of *S. epidermidis* biofilms and the role of phenol-soluble modulins in formation of biofilms. *J Infect Dis* 2005; **191**: 289–298.
14. Bowden MG, Chen W, Singvall J *et al.* Identification and preliminary characterization of cell-wall-anchored proteins of *Staphylococcus epidermidis*. *Microbiology* 2005; **151**: 1453–1464.
15. Navarre WW, Schneewind O. Surface proteins of gram-positive bacteria and mechanisms of their targeting to the cell wall envelope. *Microbiol Mol Biol Rev* 1999; **63**: 174–229.
16. Klingenberg C, Aarag E, Ronnestad A *et al.* Coagulase-negative staphylococcal sepsis in neonates: association between antibiotic resistance, biofilm formation and the host inflammatory response. *Pediatr Infect Dis J* 2005; **24**: 817–822.
17. Clinical and Laboratory Standards Institute. *Performance standard for antimicrobial susceptibility testing,* 12th informational supplement. Wayne, PA: CLSI, 2002.
18. Christensen GD, Simpson WA, Younger JJ *et al.* Adherence of coagulase-negative staphylococci to plastic tissue culture plates: a quantitative model for the adherence of staphylococci to medical devices. *J Clin Microbiol* 1985; **22**: 996–1006.
19. Bannerman TL, Hancock GA, Tenover FC, Miller JM. Pulsed-field gel electrophoresis as a replacement for bacteriophage typing of *Staphylococcus aureus*. *J Clin Microbiol* 1995; **33**: 551–555.
20. Hanssen AM, Kjeldsen G, Sollid JU. Local variants of staphylococcal cassette chromosome *mec* in sporadic methicillin-resistant *Staphylococcus aureus* and methicillin-resistant coagulase-negative staphylococci: evidence of horizontal gene transfer? *Antimicrob Agents Chemother* 2004; **48**: 283–296.
21. Tenover FC, Arbeit RD, Goering RV *et al.* Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *J Clin Microbiol* 1995; **33**: 2233–2239.
22. Wang XM, Noble L, Kreiswirth BN *et al.* Evaluation of a multilocus sequence typing system for *Staphylococcus epidermidis*. *J Med Microbiol* 2003; **52**: 989–998.
23. Wisplinghoff H, Rosato AE, Enright MC *et al.* Related clones containing SCC*mec* type IV predominate among

clinically significant *Staphylococcus epidermidis* isolates. *Antimicrob Agents Chemother* 2003; **47**: 3574–3579.
24. Enright MC, Day NP, Davies CE, Peacock SJ, Spratt BG. Multilocus sequence typing for characterization of methicillin-resistant and methicillin-susceptible clones of *Staphylococcus aureus*. *J Clin Microbiol* 2000; **38**: 1008–1015.
25. Miragaia M, Couto I, Pereira SF *et al.* Molecular characterization of methicillin-resistant *Staphylococcus epidermidis* clones: evidence of geographic dissemination. *J Clin Microbiol* 2002; **40**: 430–438.
26. Carrico JA, Pinto FR, Simas C *et al.* Assessment of band-based similarity coefficients for automatic type and subtype classification of microbial isolates analyzed by pulsed-field gel electrophoresis. *J Clin Microbiol* 2005; **43**: 5483–5490.
27. Monsen T, Karlsson C, Wistrom J. Spread of clones of multidrug-resistant, coagulase-negative staphylococci within a university hospital. *Infect Control Hosp Epidemiol* 2005; **26**: 76–80.
28. Thomas JC, Vargas MR, Miragaia M *et al.* Improved multilocus sequence typing scheme for *Staphylococcus epidermidis*. *J Clin Microbiol* 2007; **45**: 616–619.
29. Monk AB, Archer GL. Use of outer surface protein repeat regions for improved genotyping of *Staphylococcus epidermidis*. *J Clin Microbiol* 2007; **45**: 730–735.
30. Kozitskaya S, Olson ME, Fey PD *et al.* Clonal analysis of *Staphylococcus epidermidis* isolates carrying or lacking biofilm-mediating genes by multilocus sequence typing. *J Clin Microbiol* 2005; **43**: 4751–4757.
31. Villari P, Sarnataro C, Iacuzio L. Molecular epidemiology of *Staphylococcus epidermidis* in a neonatal intensive care unit over a three-year period. *J Clin Microbiol* 2000; **38**: 1740–1746.
32. Low DE, Schmidt BK, Kirpalani HM *et al.* An endemic strain of *Staphylococcus haemolyticus* colonizing and causing bacteremia in neonatal intensive care unit patients. *Pediatrics* 1992; **89**: 696–700.
33. Takeuchi F, Watanabe S, Baba T *et al.* Whole-genome sequencing of *Staphylococcus haemolyticus* uncovers the extreme plasticity of its genome and the evolution of human-colonizing staphylococcal species. *J Bacteriol* 2005; **187**: 7292–7308.
34. Cafiso V, Bertuccio T, Santagati M *et al.* Presence of the *ica* operon in clinical isolates of *Staphylococcus epidermidis* and its role in biofilm production. *Clin Microbiol Infect* 2004; **10**: 1081–1088.
35. Otto M, Gotz F. Analysis of quorum sensing activity in staphylococci by RP-HPLC of staphylococcal delta-toxin. *Biotechniques* 2000; **28**: 1088–1096.
36. Vuong C, Gerke C, Somerville GA, Fischer ER, Otto M. Quorum-sensing control of biofilm factors in *Staphylococcus epidermidis*. *J Infect Dis* 2003; **188**: 706–718.
37. Knobloch JK, Jager S, Horstkotte MA, Rohde H, Mack D. RsbU-dependent regulation of *Staphylococcus epidermidis* biofilm formation is mediated via the alternative sigma factor sigmaB by repression of the negative regulator gene *icaR*. *Infect Immun* 2004; **72**: 3838–3848.
38. Ziebuhr W, Krimmer V, Rachid S *et al.* A novel mechanism of phase variation of virulence in *Staphylococcus epidermidis*: evidence for control of the polysaccharide intercellular adhesin synthesis by alternating insertion and excision of the insertion sequence element IS256. *Mol Microbiol* 1999; **32**: 345–356.
39. Vuong C, Kocianova S, Yao Y, Carmody AB, Otto M. Increased colonization of indwelling medical devices by quorum-sensing mutants of *Staphylococcus epidermidis* in vivo. *J Infect Dis* 2004; **190**: 1498–1505.
40. Yarwood JM, Bartels DJ, Volper EM, Greenberg EP. Quorum sensing in *Staphylococcus aureus* biofilms. *J Bacteriol* 2004; **186**: 1838–1850.
41. Yao Y, Vuong C, Kocianova S *et al.* Characterization of the *Staphylococcus epidermidis* accessory-gene regulator response: quorum-sensing regulation of resistance to human innate host defense. *J Infect Dis* 2006; **193**: 841–848.
42. Pei L, Flock JI. Functional study of antibodies against a fibrogenin-binding protein in *Staphylococcus epidermidis* adherence to polyethylene catheters. *J Infect Dis* 2001; **184**: 52–55.
43. Rennermalm A, Nilsson M, Flock JI. The fibrinogen binding protein of *Staphylococcus epidermidis* is a target for opsonic antibodies. *Infect Immun* 2004; **72**: 3081–3083.
44. Hussain M, Herrmann M, von Eiff C, Perdreau-Remington F, Peters G. A 140-kilodalton extracellular protein is essential for the accumulation of *Staphylococcus epidermidis* strains on surfaces. *Infect Immun* 1997; **65**: 519–524.
45. Rohde H, Burdelski C, Bartscht K *et al.* Induction of *Staphylococcus epidermidis* biofilm formation via proteolytic processing of the accumulation-associated protein by staphylococcal and host proteases. *Mol Microbiol* 2005; **55**: 1883–1895.
46. Tormo MA, Knecht E, Gotz F, Lasa I, Penades JR. Bap-dependent biofilm formation by pathogenic species of *Staphylococcus*: evidence of horizontal gene transfer? *Microbiology* 2005; **151**: 2465–2475.
47. Gill SR, Fouts DE, Archer GL *et al.* Insights on evolution of virulence and resistance from the complete genome analysis of an early methicillin-resistant *Staphylococcus aureus* strain and a biofilm-producing methicillin-resistant *Staphylococcus epidermidis* strain. *J Bacteriol* 2005; **187**: 2426–2438.
48. Yao Y, Sturdevant DE, Villaruz A *et al.* Factors characterizing *Staphylococcus epidermidis* invasiveness determined by comparative genomics. *Infect Immun* 2005; **73**: 1856–1860.
49. Roche FM, Massey R, Peacock SJ *et al.* Characterization of novel LPXTG-containing proteins of *Staphylococcus aureus* identified from genome sequences. *Microbiology* 2003; **149**: 643–654.
50. Vandecasteele SJ, Peetermans WE, Merckx R, Rijnders BJ, Van Eldere J. Reliability of the *ica*, *aap* and *atlE* genes in the discrimination between invasive, colonizing and contaminant *Staphylococcus epidermidis* isolates in the diagnosis of catheter-related infections. *Clin Microbiol Infect* 2003; **9**: 114–119.
51. Rohde H, Kalitzky M, Kroger N *et al.* Detection of virulence-associated genes not useful for discriminating between invasive and commensal *Staphylococcus epidermidis* strains from a bone marrow transplant unit. *J Clin Microbiol* 2004; **42**: 5614–5619.
52. Bradford R, Abdul MR, Daley AJ *et al.* Coagulase-negative staphylococci in very-low-birth-weight infants: inability of genetic markers to distinguish invasive strains from blood culture contaminants. *Eur J Clin Microbiol Infect Dis* 2006; **25**: 283–290.

53. Capparelli EV, Bloom BT, Kueser TJ *et al.* Multicenter study to determine antibody concentrations and assess the safety of administration of INH-A21, a donor-selected human staphylococcal immune globulin, in low-birth-weight infants. *Antimicrob Agents Chemother* 2005; **49**: 4121–4127.
54. Otto M. Quorum-sensing control in staphylococci—a target for antimicrobial drug therapy? *FEMS Microbiol Lett* 2004; **241**: 135–141.
55. Kocianova S, Vuong C, Yao Y *et al.* Key role of poly-gamma-DL-glutamic acid in immune evasion and virulence of *Staphylococcus epidermidis*. *J Clin Invest* 2005; **115**: 688–694.
56. Kilbride HW, Wirtschafter DD, Powers RJ, Sheehan MB. Implementation of evidence-based potentially better practices to decrease nosocomial infections. *Pediatrics* 2003; **111**: e519–e533.
57. Predari SC, Ligozzi M, Fontana R. Genotypic identification of methicillin-resistant coagulase-negative staphylococci by polymerase chain reaction. *Antimicrob Agents Chemother* 1991; **35**: 2568–2573.
58. Vakulenko SB, Donabedian SM, Voskresenskiy AM *et al.* Multiplex PCR for detection of aminoglycoside resistance genes in enterococci. *Antimicrob Agents Chemother* 2003; **47**: 1423–1426.
59. Heikens E, Fleer A, Paauw A, Florijn A, Fluit AC. Comparison of genotypic and phenotypic methods for species-level identification of clinical isolates of coagulase-negative staphylococci. *J Clin Microbiol* 2005; **43**: 2286–2290.