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ORIGINAL RESEARCH

Risk Factors for Infection with Coagulase-Negative Staphylococci in Newborns from the Neonatal Unit of a Brazilian University Hospital

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Abstract

Background: Coagulase-negative staphylococci (CoNS) are one of the most frequent causative agents of neonatal nosocomial infections, especially in premature and low-weight newborns. Risk factors for infection include extracellular polysaccharide production and consequent biofilm formation that permit adhesion to the smooth surface of catheters and other medical devices. The objective of this study was to identify CoNS strains isolated from 105 newborns admitted to the Neonatal Unit of our hospital, and to evaluate the association of biofilm production and host risk factors with the occurrence of infection.

Methods: CoNS isolates were identified and classified as significant or contaminant based on clinical and laboratory data of the newborn medical records. Perinatal risk factors for infection, neonatal clinical evolution, and antibiotic treatment were analysed. In addition, the presence of genes (*icaA*, *icaC* and *icaD*) responsible for biofilm production in CoNS was investigated.

Results: Among the 130 CoNS strains studied, 66 (50.8%) were classified as clinically significant and 64 (49.2%) as contaminant. There was no difference in the detection of biofilm-specific genes between CoNS strains isolated from newborns with (81.8%) and without infection (84.3%), although 11 (91.7%) of the 12 children whose death was related to CoNS were infected with strains that were positive for these genes. Forty-five (83.3%) of the 54 newborns infected with CoNS were premature and 33 (61.1%) had a birth weight ≤ 1,500 g. Most newborns infected with CoNS had been submitted to invasive procedures, including catheter use (85.2%), parenteral nutrition (61.1%), and mechanical ventilation (57.4%). *S. epidermidis* was the most frequently isolated species (81.5%) and was more related to infection (86.3%) than to contamination (76.5%).

Conclusion: Most newborns infected with CoNS presented factors that contributed to the colonization and development of infection with these microorganisms, including a birth weight ≤ 1,500 g, catheter complications, use of a drain, and previous antibiotic treatment. The fact that most children who died of CoNS-related infection carried strains positive for biofilm-specific genes indicates the importance of this virulence factor for the outcome of staphylococcal infections.

Keywords: biofilm, *Staphylococcus*, coagulase-negative, risk factors, infection, phenotyping methods, PCR

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Background

Coagulase-negative staphylococci (CoNS) are one of the most common causative agents of neonatal nosocomial infections,[1] especially in premature newborns or those with other types of disease hospitalized in intensive care units where they are submitted to invasive procedures and antibiotic treatment.[2,3]

Coagulase-negative staphylococci are part of the normal skin and mucosal flora and are one of the most important culture contaminants, a fact that makes the interpretation of blood culture results difficult.[4] These microorganisms are characterized by their capacity to adhere and grow on the smooth surface of catheters and other medical devices. After initial contact and attachment to a surface, bacterial cells form a biofilm that protects them against the host's defence mechanisms and antimicrobial treatment.[5] The biofilm produced by CoNS is an important virulence factor[6] and mainly consists of polysaccharide intercellular adhesin (PIA), which is encoded by the *icaADBC* operon.[7]

Studies have shown that CoNS infections are mainly caused by biofilm-producing strains and this capacity is well correlated with the outcomes of infection. It has therefore been suggested that biofilm formation might be a useful marker for the pathogenicity of a specific strain.[8] However, other studies were unable to demonstrate an association between biofilm production and infection.[6,9] These divergent results suggest that, although biofilm production is an important virulence factor, the association between biofilm formation and clinically significant infections may vary between different enviroments. In addition to biofilm production, treatment-related factors that vary from one hospital to another may contribute to the pathogenesis of CoNS infections.[2,9]

The aim of the present study was to identify CoNS strains isolated from newborns in the Neonatal Unit of the University Hospital, Botucatu School of Medicine, and to evaluate the association of biofilm production and host risk factors with the occurrence of infections caused by these microorganisms.

Materials and Methods

Strains

One hundred and thirty CoNS strains isolated from specimens obtained from 105 newborns hospitalized in the Neonatal Unit of the University Hospital, Botucatu School of Medicine (FMB), and four international reference strains (*S. epidermidis* ATCC 12228, *S. simulans* ATCC 27851, *S. warneri* ATCC 10209, and *S. xylosus* ATCC 29979) were studied. The procedures were approved by the Research Ethics Committee of the Botucatu School of Medicine.

Coagulase-negative strains isolated from internal fluids, such as blood and secretions and from foreign bodies, such as cannulas, drains, and catheters, were included. Excluded were strains isolated from newborns for whom no clinical or laboratory data comprising the week before and after isolation were available.

Identification of coagulase-negative Staphylococci

Isolates obtained from the different clinical specimens were seeded onto blood agar and stained by the Gram method for the determination of purity, morphology, and specific staining. After confirmation of these characteristics, the strains were submitted to catalase and coagulase tests.

The CoNS isolates were identified using the simplified scheme proposed by Cunha et al,[10] which is based on the utilization of sugars (xylose, sucrose, trehalose, mannitol, maltose, and fructose), production of haemolysins, urease and ornithine decarboxylase, and resistance to novobiocin. The test results were compared with those obtained for the following CoNS reference strains: *S. epidermidis* (ATCC 12228), *S. simulans* (ATCC 27851), *S. warneri* (ATCC 10209), and *S. xylosus* (ATCC 29979). After species confirmation, the strains were stored in nutrient broth with glycerol at −70 ºC.

Detection of the *icaA*, *icaC* and *icaD* genes responsible for biofilm production

Total nucleic acid was extracted from *Staphylococcus* strains cultured in blood agar, individually inoculated into brain heart infusion broth, and incubated at 37 °C for 24 h. Extraction was performed with the Illustra kit (GE Healthcare) and consisted of initial digestion of staphylococcal cells with 10 mg/ml lysozyme and 20 mg/ml proteinase K. Next, 500 µl of the extraction solution was added and the mixture was centrifuged at

10,000 × *g* for 4 min. The supernatant was transferred to a column and centrifuged at 5,000 × *g* for 1 min. The fluid collected was discarded and 500 µl of the extraction solution was again added to the column. After centrifugation, the collected fluid was discarded and 500 µl of the washing solution was added to the column. The column was centrifuged at 20,000 × *g* for 3 min and then transferred to a 1.5-ml tube. Milli-Q water (200 µl) heated to 70 °C was used for elution.

Amplification by PCR was performed in 0.5-ml microcentrifuge tubes containing 10 pmol of each oligonucleotide (Table 1), 2.0 U Taq DNA polymerase, 100 µM desoxyribonucleotide triphosphates, 10 mM Tris-HCl, pH 8.4, 0.75 mM $MgCl_2$, and 3 µl nucleic acid in a final volume of 25 µl. The reactions were incubated in an MJ Research thermocycler under the conditions described by Arciola et al:[11] denaturation at 94 °C for 5 min, followed by 50 cycles of denaturation at 94 °C for 30 s, annealing at 55.5 °C for 30 s, and extension at 72 °C for 30 s. After completion of the 50 cycles, the tubes were incubated at 72 °C for 1 min before cooling to 4 °C. *S. epidermidis* ATCC 35984 (biofilm producer) and *S. epidermidis* ATCC 12228 (non-producer) were included as positive and negative controls in all reactions.

For visualization, the amplified products were separated by electrophoresis on 2% agarose gel prepared in 0.5X TBE buffer and stained with SYBR Safe. The gels were photographed under UV transillumination.

Clinical relevance

The CoNS strains were classified as clinically significant and contaminant based on a series of clinical and laboratory data obtained from the patient records according to the criteria proposed by the CDC.[12] The clinical evolution of the newborns was evaluated in the week before and after isolation of the CoNS strains, focusing on diagnoses and clinical signs suggestive of CoNS infection. The latter are characterized by insidious and nonspecific signs and symptoms, including compromised general health, thermal instability, and the occurrence of apneas. Death of the newborn was attributed to CoNS infection if it occurred within the first 72 h after isolation of the agent, and was defined as possibly related to CoNS if it occurred 4 to 7 days after bacterial isolation. Another aspect related to clinical relevance was previous exposure to antibiotics and adequate antibiotic therapy after the bacteriological diagnosis. The definition of adequate antibiotic therapy was based on the results of the antibiogram obtained from the patient record or performed on the occasion of the study.

Strains isolated from newborns who presented the following characteristics were classified as significant: clinical presentation, haematological changes, and adequate antibiotic treatment. Strains isolated from patients who did not receive adequate antibiotic treatment and died were also classified as significant.

Strains isolated from newborns who presented only one of the above characteristics (clinical presentation, haematological changes, or adequate antibiotic therapy) were classified as contaminant. Strains isolated from newborns who presented the three criteria but whose infection was resolved without antibiotics were also classified as contaminant. Isolation of another aetiological agent from internal fluids or foreign bodies at the same time as the CoNS strains was also used as a criterion of contamination.

Statistical analysis

Data regarding the clinical relevance of the CoNS strains were analysed by the chi-squared test.[13] The Wilcoxon test was used to compare weight and age

Table 1. Oligonucleotides used for the detection of the *icaA*, *icaC*, and *icaD* genes.

Oligonucleotide	5′ to 3′ nucleotide sequence	Amplified product (bp)
icaA1	ACA GTC GCT ACG AAA AGA AA	103
icaA2	GGA AAT GCC ATA ATG AGA AC	
icaC1	TAA CTT TAG GCG CAT ATG TTT	400
icaC2	TTC CAG TTA GGC TGG TAT TG	
icaD1	ATG GTC AAG CCC AGA CAG AG	198
icaD2	CGT GTT TTC AAC ATT TAA TGC AA	

Source: Arciola et al.[11]

between groups.[14] The level of significance was set at $P < 0.05$ for all tests. Next, multivariate logistic regression analysis was performed to simultaneously evaluate the association of neonatal clinical data and biofilm production with the occurrence of CoNS infection. Results presenting a P value < 0.25 upon univariate analysis were entered into the logistic regression model.[15]

Results

Strains

One hundred and thirty CoNS strains isolated from different clinical specimens obtained from 105 newborns in the Neonatal Unit were studied. Sixty-nine strains were isolated from foreign bodies (54 from catheter tips, 5 from cannula tips, and 10 from chest drain tips), 57 from blood cultures, and 4 from secretion.

Identification of coagulase-negative Staphylococci

Table 2 shows the distribution of CoNS species classified as clinically significant and contaminant according to clinical material. The results show that *S. epidermidis* was more frequently associated with infection (86.3%) than with contamination (76.5%), but the difference was not statistically significant ($P > 0.05$). No significant differences were observed for the other species.

Clinical relevance and genes responsible for biofilm production

Among the 105 newborns studied, 54 were infected with CoNS and 51 were not (Table 3). Sixty-six (50.8%) of the 130 strains analysed for clinical significance were interpreted as clinically significant and 64 (49.2%) as contaminant. Of these, 69 were isolated from foreign bodies, with 38 (55%) being interpreted as significant, 27 (71%) were isolated from catheters, 8 (21.1%) from chest drains, and 3 (7.9%) from cannula tips.

The characteristics of the children with and without CoNS infection are shown in Table 3. Forty-five (83.3%) of the 54 children with CoNS infection were premature. Of these, 26 (48.1%) were considered to be extremely premature (gestational age < 31 weeks) versus 15 (29.4%) in the group without infection. This difference was statistically significant ($P = 0.027$). With respect to weight, 54 (61.1%) newborns with CoNS infection presented a birth weight lower than 1,500 g versus 19.6% in the group without infection ($P < 0.001$). Median birth weight also differed significantly ($P < 0.001$) between the groups with (1,238 g) and without CoNS infection (2,140 g). There was a significant difference in gender ($P = 0.022$), with 34 (63.0%) male newborns in the group with infection and 21 (41.2%) in the group without infection.

Univariate analysis of perinatal risk factors (Table 4) showed a significant difference between groups in terms of membrane rupture at >24 h ($P = 0.036$), catheter complications ($P < 0.001$), and use of a drain ($P = 0.025$).

The genes responsible for biofilm production (*icaA*, *C* and *D*) were detected in 110 of the 130 CoNS strains analysed (Fig. 1), including 54 (81.8%) in the group with infection and 56 (84.3%) in the group without infection. With respect to clinical material, the genes

Table 2. Distribution of coagulase-negative staphylococcal species classified as significant and contaminant according to clinical material.

Species	Significant			Contaminant		
	Foreign body	Blood	Secretion	Foreign body	Blood	Secretion
S. epidermidis	33	22	2	26	23	0
S. haemolyticus	1	2	0	0	2	1
S. warneri	1	1	0	2	1	0
S. xylosus	2	0	0	1	0	0
S. lugdunensis	0	1	0	0	2	0
S. capitis	1	0	0	1	0	0
S. hominis	0	0	0	0	2	0
S. simulans	0	0	0	0	1	1
S. saprophyticus	0	0	0	1	0	0
Total	38	26	2	31	31	2

Table 3. Characteristics of the newborns with and without coagulase-negative staphylococcal infection.

Characteristics	With infection		Without infection		Total		*P* value
	N	%	N	%	N	%	
GA < 31	26	48.1	15	29.4	41	39.0	0.027
GA 31–36	19	35.2	18	35.3	37	35.2	ns
GA ≥ 37	6	11.1	18	35.3	24	22.9	0.001
BW ≤ 1,500 g	33	61.1	10	19.6	43	40.1	<0.001
Median BW (g)	1,238		2,140		1,600		<0.001
Median age (days)	8.5		6		7		ns
Male gender	34	63.0	21	41.2	55	52.4	0.022
Born at HC/FMB	36	66.6	31	60.8	69	65.7	ns
Total	54	51.4	51	48.6	105	100	

Notes: BW was unknown in 3 newborns, age was unknown in 2, gender was unknown in 2, and place of birth was unknown in 3.
Abbreviations: GA, gestational age (weeks); BW, birth weight; HC/FMB, University Hospital of the Botucatu School of Medicine; ns, not significant.

responsible for biofilm production were detected in 94.4% of strains isolated from catheter tips, in 89.5% isolated from blood cultures, in 60% isolated from cannula tips, in 50% isolated from chest drains, and in 50% isolated from secretion. As can be seen in Table 5, there were no significant differences in biofilm production between the two groups or between the different clinical materials ($P > 0.05$).

Table 6 shows the results of multivariate logistic regression analysis. Calculation of the risk of infection with CoNS, reported as odds ratio, showed that newborns with a birth weight ≤1,500 g presented a 12.34 times higher chance of CoNS infection than those with higher birth weights. Newborns with catheter complications had a 12.33 times higher chance, those with a chest drain had a 6.43 times higher chance, and those undergoing previous antibiotic treatment had a 3.57 times higher chance of infection.

Eighteen (33.3%) newborns with infection died during hospitalization. Twelve (66.7%) of these deaths were related to CoNS infection. Of these, 4 newborns had been exposed to foreign bodies infected with CoNS, which were only removed on the day of death, 5 were extremely premature, 4 weighed < 1,000 g, and 11 were positive for biofilm-specific genes.

Discussion

Coagulase-negative staphylococci are pathogens that play an important role in neonatal nosocomial infections. The detection and identification of CoNS species and of risk factors for the occurrence of these infections in newborns are important for diagnosis and prevention.

In the present study, *S. epidermidis* was the most frequent species among the 130 CoNS strains isolated from newborns. Similar findings have been

Table 4. Perinatal risk factors for infection with coagulase-negative staphylococci.

Risk factor	With infection		Without infection		Total		*P* value
	N	%	N	%	N	%	
Membrane rupture >24 h	19	35.2	10	19.6	29	27.6	0.036
ICU inpatient	52	96.2	39	76.5	91	86.6	ns
Catheter	27	50.0	27	52.9	54	51.4	ns
Catheter complications	11	20.4	1	1.9	12	11.4	<0.001
Drain	8	14.8	2	2.9	10	9.5	0.025
Mechanical ventilation	31	57.4	23	45.0	54	51.4	ns
Parenteral nutrition	33	61.1	28	55.0	61	58.1	ns
Non-removal of foreign bodies	24	44.4	19	37.2	43	41.0	ns
Surgery and/or dialysis	7	13.0	10	19.6	17	16.2	ns
Total number of newborns	54	51.4	51	48.6	105	100	

Notes: Catheter use was unknown in 2 newborns, catheter complications were unknown in 2, drain use was unknown in 2, mechanical ventilation was unknown in 2, parenteral nutrition was unknown in 2, foreign body removal was unknown in 12, and surgery and/or dialysis were unknown in 6.
Abbreviations: ICU, intensive care unit; ns, not significant.

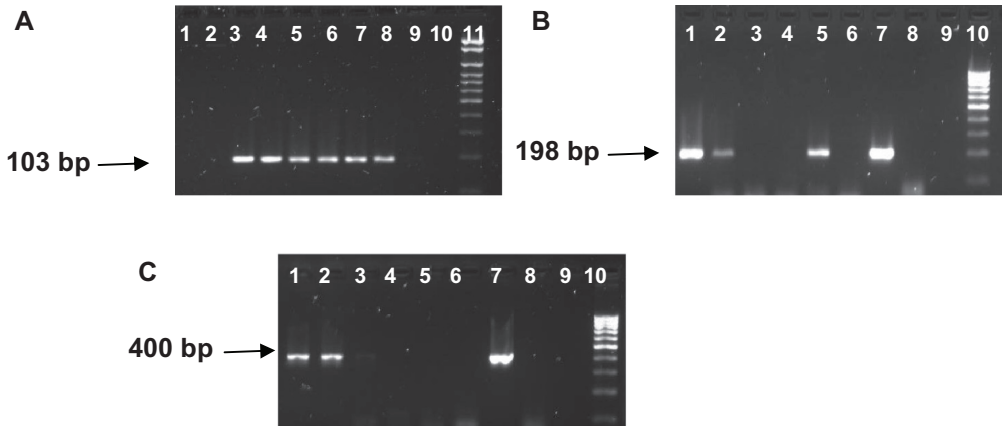

Figure 1. Electrophoresis of PCR products on agarose gel stained with SYBR Safe. (**A**) *icaA* gene (103 bp): lanes 1 and 2, negative strains; lanes 3–7, positive strains; lane 8, positive control; lane 9, negative control; lane 10, water; lane 11, molecular weight marker (100 bp). (**B**) *icaD* gene (198 bp): lanes 1, 2 and 5, positive strains; lanes 3, 4 and 6, negative strains; lane 7, positive control; lane 8, negative control; lane 9, water; lane 10, molecular weight marker (100 bp). (**C**) *icaC* gene (400 bp): lanes 1 and 2, positive strains; lanes 3–6, negative strains; lane 7, positive control; lane 8, negative control; lane 9, water; lane 10, molecular weight marker (100 bp).

reported by other investigators,[2,16] supporting the recommendation for routine identification of CoNS species since *S. epidermidis* is more strongly associated with infection than with contamination. In support of this recommendation, Pessoa-Silva et al[17] observed that 26.7% of deaths related to bloodstream infections in newborns were caused by *S. epidermidis*. In the present study, other CoNS species were also associated with infection, including 3 *S. haemolyticus* species, 2 *S. warneri* species, 2 *S. xylosus* species, 1 *S. lugdunensis* species, and 1 *S. capitis* species. Similar results have been reported by other investigators[16,18,19] who isolated these microorganisms from newborns with sepsis.

Premature newborns are more susceptible to infection, especially low-weight newborns. In the present study, most (83.3%) of the 54 newborns with CoNS infection were premature, including 48.1% extremely premature newborns (gestational age $<$ 31 weeks). These children had a median weight of 1,238 g. A birth weight $\leq$ 1,500 g was observed in 61.1% of the newborns. Similar results have been reported by other investigators.[16,20] Logistic regression analysis revealed that birth weight $\leq$ 1,500 g was a factor predisposing to CoNS infection, with a 12.3-fold increased risk. There are several factors that contribute to the susceptibility and the presence of more serious infection in these newborns, such as immaturity of the immune system characterized by phagocyte deficiency, antibody opsonisation, and complement deficiencies.[21]

No significant difference in median age was observed between newborns with and without infection (8.5 versus 6 days). Only 9.3% of the newborns developed CoNS infection within the first 24 h of life. Similarly, in the study of Cunha et al[2]

Table 5. Frequency of coagulase-negative staphylococcal strains positive for biofilm-specific genes according to clinical relevance and clinical material.

Material	*IcaA*, *C* and *D* gene-positive strains		
	With infection	Without infection	Total
Blood culture (n = 57)	25	26	51
Catheter tips (n = 54)	24	25	49
Chest drain (n = 10)	2	3	5
Cannula tips (n = 5)	2	1	3
Secretion (n = 4)	1	1	2
Total	54	56	110

Table 6. Logistic regression model.

Newborn data	*P* value	Odds ratio	95% Confidence interval	
			LL	UL
Weight $\leqslant$ 1,500 g	0.0078	12.345	2.366	64.413
Catheter complications	0.022	12.328	1.430	106.278
Drain	0.041	6.428	1.075	38.432
Previous antibiotic treatment	0.014	3.571	1.291	9.881

Abbreviations: LL, lower limit; UL, upper limit.

the infection rate was 12% in the first 48 h of life, whereas infections caused by other bacteria mainly occur during this period. Hoang et al[22] and Campeotto et al[23] reported the occurrence of colonization and infection between 6 and 7 days of life. With respect to newborn gender, 34 (63%) of the 54 children in the group with infection were boys, a statistically significant number. Similar results have been reported by Nimri et al[24] and Babazono et al,[25] who found an odds ratio of 1.86 for infection among boys compared to 1.00 among girls.

In the present study, 19 (35.2%) children of the group with infection presented prolonged (>24 h) membrane rupture ($P < 0.05$). Martius et al[26] observed a 2.9 times higher chance of infection in newborns with prolonged membrane rupture (>24 h). Patients admitted to intensive care units are more susceptible to CoNS infection because of the need for invasive procedures, immunosuppression, and use of broad-spectrum antibiotics.[27,28] Intensive care unit stay was not a risk factor in the present study. However, the children admitted to the intensive care unit were submitted to invasive procedures and antibiotic therapy, factors that indirectly render intensive care unit stay an important risk factor for these infections. Mechanical ventilation, parenteral nutrition, or catheter use was not a risk factor in the group with infection, in agreement with the results reported by Sung et al.[18] These findings can be explained by the fact that most newborns included in the study were submitted to these procedures.

Although catheter use did not differ between the groups with and without CoNS infection, catheter complications were significantly more frequent among newborns with infection (20.4%) than among those without infection (1.9%). Multivariate analysis showed a 12.32 times higher risk of infection with CoNS among newborns suffering catheter complications. This finding is in line with the results of other investigators,[22,29] who emphasize the need for care when catheters are used. Multivariate analysis showed that newborns with a chest drain had a 6.42 times higher risk of infection with CoNS. Other investigators[17,30,31] found no association between the use of chest drains and CoNS infection, although the association with other invasive procedures such as parenteral nutrition, mechanical ventilation, and catheter use was significant. Although there are no studies demonstrating a direct association between the use of drains and CoNS infection, the fact that this invasive procedure is frequently applied to newborns in intensive care units shows that the use of a drain is ultimately an important risk factor for the occurrence of these infections.

Another risk factor identified in the present study was previous antibiotic treatment in newborns with CoNS infection. Multivariate analysis showed a 3.57 times higher risk of infection in newborns previously exposed to antimicrobial agents. Similar results have been reported by other investigators.[2,32] Lopes et al[33] found an association between previous antibiotic treatment and a higher mortality rate in premature low-weight newborns. Previous exposure to antibiotics can suppress the normal flora and select resistant microorganisms.

With respect to the presence of genes responsible for biofilm production, no significant difference was observed between CoNS strains isolated from newborns with infection (81.8%) and those without infection (84.3%). There is a lack of evidence in the literature implicating biofilm formation as a risk factor for infection with CoNS.[6,9] This might be due to the fact that most CoNS species are able to produce a biofilm. In the study of Alcaraz et al[19] investigating biofilm production by CoNS in clinical and environmental samples, production was similar in both groups. The biofilm permits these microorganisms to adhere to and colonize the smooth surface of catheters and other medical devices, favouring the occurrence of infections.

Eighteen (66.7%) newborns in the group with infection died during hospitalization. Twelve (66.7%) of these deaths were related to CoNS infection, 4 newborns had been exposed to CoNS-infected foreign bodies, which were only removed at the time of death, 5 were extremely premature, 4 weighed < 1,000 g, and 11 were infected with CoNS strains positive for biofilm-specific genes. Eight (72.7%) of these 11 newborns had received adequate antimicrobial treatment but were not cured. This fact demonstrates the role of biofilm formation in treatment failure, protecting the microorganisms against the action of drugs and host defence mechanisms. In agreement with these data, Klingenberg et al[6] found an association between genotype and biofilm phenotype and resistance to antibiotics. Studies have shown

that PIA from *S. epidermidis* plays a crucial role by preventing the activation of the human innate immune system.[34,35]

Conclusion

Most newborns infected with CoNS presented factors that contributed to the colonization and development of infection with these microorganisms, including birth weight ≤1,500 g, catheter complications, use of a drain, and previous antibiotic therapy. The identification of CoNS species is an important marker of infection considering that *S. epidermidis* was the most frequently isolated aetiological agent and the species most commonly associated with infectious processes. Therefore, accurate characterization of these microorganisms is necessary when they are isolated from blood and foreign bodies of newborns, as well as careful evaluation of the clinical and laboratory data of these patients, in order to determine the clinical relevance of the isolated strains. The fact that most children who died of CoNS-related infection carried strains positive for biofilm-specific genes indicates the importance of this virulence factor for the outcome of staphylococcal infections.

Author Contributions

Adilson de Oliveira: Conceived the study, performed the microbiological tests, and wrote the article. Patrícia Sanches: Participated in the microbiological tests and clinical data analysis. João C Lyra: Contributed to the collection of material and clinical data. Maria R Bentlin: Contributed to the collection of material and clinical data. Ligia M S S Rugolo: Coordinated the material collection and clinical data analysis. Maria de Lourdes Ribeiro de Souza da Cunha: Conceived the study, coordinated the laboratory work, participated in the data analysis, and wrote the manuscript.

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Disclosures

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