Communication

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Phenotypic and Genotypic Assays to Evaluate Coagulase-Negative Staphylococci Biofilm Production in Bloodstream Infections

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Abstract: Coagulase-Negative Staphylococci (CoNS) are commensal on human body surfaces and for years, they were not considered a cause of bloodstream infection and were often regardered as contamination. However, the association of CoNS with nosocomial infection is increasingly recognized. The insertion of cannulas and intravascular catheters represents the first source of CoNS entry into the bloodstream, causing bacteremia and sepsis. They owe their pathogenic power to their ability to produce biofilms on surfaces, such as medical devices. In this study, we evaluate the adhesive capacity of CoNS isolated from blood cultures by comparing spectrophotometric phenotypic assay with genotypic analysis based on the evidence of the *ica* operon. We retrospectively reviewed the database of CoNS isolated from blood cultures, that were considered to be responsible for 361 bloodstream infections. Among them, we selected 89 ones who had full records. Our data show that *S. epidermidis* was the predominant species isolated, and with greater adhesive capacities, especially those with the complete operon. Knowing the adhesive capabilities of a microorganism responsible for sepsis can be useful in implementing appropriate corrective and preventive measures, as conventional antibiotic therapy cannot effectively eradicate biofilms.

Keywords: biofilm; bloodstream infection; catheter; coagulase-negative staphylococci (CoNS); *S. epidermidis*

1. Introduction

The coronavirus disease 2019 (COVID-19) pandemic has had a considerable impact on healthcare, especially for large number of patients requiring intensive care. The management of patients became more difficult, from diagnostic to safety measures, including delays in treatment delivery. Modifications of standards of care resulted in growing number of isolation of coagulase-negative staphylococci (CoNS) from blood culture, that remains an urgent problem to be solved. Numerous studies have shown a significant increase in incidence, especially in intensive care units during this critical period [1].

Staphylococci are commensal on human body surfaces and are also known to colonize the biomedical devices through the formation of biofilms [2]. Biofilm consists of multilayered cell clusters embedded in a matrix of extracellular polysaccharide, which facilitate the adherence of microorganism. Bacteria can attach to a wide range of surfaces, the physicochemical properties of which influence the adhesion behaviour of cells on the surface of the material and the subsequent process of biofilm formation [3]. Charge and hydrophobicity are two major components that influence bacterial-surface interaction. The chemical properties of the bacterial cell envelope may

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vary, but as a general rule, they are often negatively charged; therefore, surfaces with positive or neutral charges are more easily colonized than those with negative charges. Hydrophobic interactions can also be established between microorganisms and devices with hydrophobic surfaces [4]. The phenomenon of adhesion depends not only on the factors of the microorganism, but also on the characteristics of the material, such as roughness, surface composition, and topography; and functional chemical group modifications of the material surface. Surface modification of biomaterials is a potential strategy to prevent bacteria from attaching and forming biofilms [3]. In the clinical field, an effective modification could be coating with antibiotics [5]. The insertion of cannulas and intravascular catheters (for the administration of liquids or drugs, for hemodynamic monitoring and hemodialysis, etc.) represents the first source of CoNS entry into the bloodstream [6]. One of the major complications is represented by the possible establishment of a catheter-related sepsis, due to the biofilm formation and subsequent dissemination of microrganism. The mechanism by which CoNS attach to prosthetic material and elaborate what is now termed biofilm is being increasingly understood. One important element in this process is the *ica* operon, a gene cluster encoding the production of polysaccharide intercellular adhesin (PIA) [7]. Chromosomal *ica* gene locus comprises four intercellular adhesion genes (*icaA*, *icaB*, *icaC* and *icaD*) and one regulator gene (*icaR*), which seems to function as a repressor [8]. In staphylococci, adhesion to the surface of catheters is also mediated by covalently membrane-anchored proteins like Cell Wall-Anchored proteins (CWA) [9], noncovalently associated proteins (extracellular glycopolymers WTAs) [10], and other mucopolysaccharide factors (wall teichoic acids and lipoteichoic acids and PIA) [9]. The precise percentage of how many positive blood cultures are due to contamination varies considerably among the many studies that have attempted to estimate that number. Approximately 30% to 40% of nosocomial bloodstream infections are caused by CoNS; nevertheless, there is agreement that *S. epidermidis* is among the most frequent bacterial sources underlying bacteraemia and sepsis, with 90% of prevalence [10,11]. In this study, we evaluate the adhesive capacity of CoNS isolated from blood cultures by comparing spectrophotometric phenotypic assay with genotypic analysis based on the evidence of the *ica* operon.

2. Materials and Methods

2.1. Bacterial Isolates

The bacterial strains were stored in preservation media (BIO-RAD ©) at room temperature and revitalized for the purpose of the study. A small amount of sterile NaCl was added to each tube to resuspend the microorganism from the agarized medium, after which a drop was transferred to a COS plate with a pasteur pipette. After isolation, the plates were incubated at 37°C for 24 hours.

2.2. Phenotypic Assay

Biofilm production was investigated using Christensen's spectrophotometric method, which allows quantitative evaluation of the phenomenon [12].

The strains were resuspended in 10 ml of TSB (Tryptic Soy Broth-BD) supplemented with 0.25% w/v casamino acid and glucose. After 24h incubation at 37°C, dilutions (1:1000) were made in TSB addictioned as described above to have a bacterial suspension of about 10^5 CFU/ml. Of each dilution 200 µl were deposited in triplicate in the wells of a flat-bottom microtiter plate (Greiner BIO-ONE©) that was incubated at 37°C for 18 hours. The culture broth was removed from each well, washed twice with sterile water, and the microorganisms were fixed with absolute alcohol, after which they were washed once more and stained with crystal violet for 10 min. The dye was removed from each well, two more washes were performed and the plate was allowed to dry at room temperature.

After the BIO-RAD© (USA) instrument calibration, the spectrophotometric reading was taken at a wavelength of 570 nm, and the mathematical average of the 3 OD values relative to each strain were calculated. The observed OD value was proportional to the biofilm production of the microorganisms and allowed to classify them according to Christensen's scheme into excellent

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producers when OD>0.240, weak producers when OD was between 0.120 and 0.240, and non producers when OD < 0.120 (Christensen et al., 1982).

2.3. Genotypic Assays

2.3.1. DNA Extraction

DNA was extracted by DNeasy UltraClean Microbial Kit (Qiagen, Germany), which involved lysis of the microorganism by a combination of heat, detergents and mechanical force. DNA released from lysed cells and bound to a silica filter, was then washed and recovered in Tris buffer.

2.3.2. PCR Assays

Phenotypic identification of strains was confirmed by Polymerase Chain Reaction (PCR), amplification of the four genes of the *icaADBC* operon, responsible of PIA production, with specific primers [8] (Table 1).

Table 1. Primers and their sequences used in the study.

Gene target	Sequences	bp	Reference
icaA	F5'-TCTCTTGCAGGAGCAATCAA R5'-TCAGGCACTAACATCCAGCA	188	[13]
icaB	F5'-ATGGCTTAAAGCACACGACGC R5'-TATCGGCATCTGGTGTGACAG	526	[14]
icaC	F5'-ATCATCGTGACACACTTACTAACG R5'-CTCTCTTAACATCATTCCGACGCC	934	[8]
icaD	F5'- ATGGTCAAGCCCAGACAGAG R5'-CGTGTTTTCAACATTTAATGCAA	198	[13]

Amplification mixes contained Buffer 10X with $MgCl_2$ 15 mM, $MgCl_2$ (25 mM), Taq polymerase (5U/µl), dNTPs (10 mM), primers (10 µM) and 2 µl of genomic DNA. PCR conditions comprised an initial 5-min denaturation step at 95°C, followed by 35 cycles of 94°C for 30s, 58°C for 30s for *icaC*, while for *icaA*, *icaD* and *icaB* this phase was set to 55°C for 30s and extension for 2 min at 72°C. Amplified products were visualized by electrophoresis on 2% agarose gels stained with ethidium bromide.

3. Results

We retrospectively reviewed the database of CoNS isolated from blood cultures between January 2021 and December 2021. In our reality, Staphylococci were considered to be responsible for 490 bloodstream infectious episodes, of which 361/490 (73.7%) were sustained by CoNS. Among them, we selected 89 ones who had full records. The OD measured on microplates showed a wide distribution of values, ranging from a minimum of 0,06 to a maximum of 3,5. According to criteria established by Christensen, 49/89 (55%) were excellent producers, 20/89 (22,5%) were weak producers and 20/89 (22,5%) were non-producers (Figure 1).

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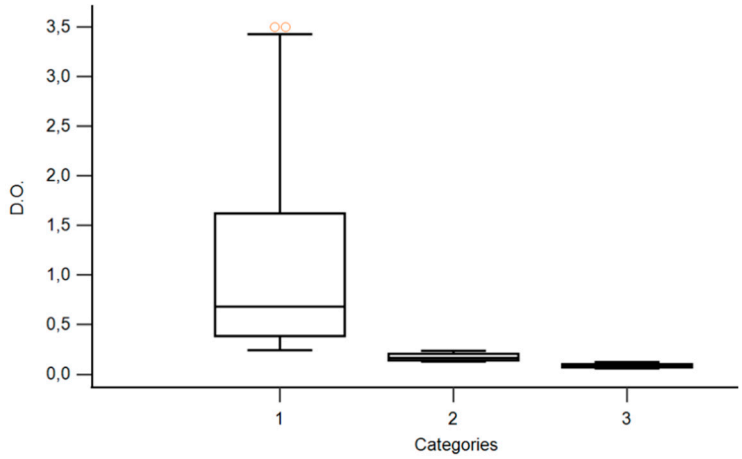

Figure 1. Box plot of distribution of the optical density (OD) of CoNS grouped by 3 categories (Excellent producers k=1, weak producers k=2, no producers k=3). The Kruskal-Wallis test showed significant differences among categories ($p<0.0001$).

Genotypic assay showed that 42/89 (47.2%) presented complete ica operon, 44/89 (49.4%) did not have the operon, 3/89 strains (3.4%), otherwise, presented 1 or 2 genes of the operon. The percentages of strains divided into the 3 categories associated with the presence of the complete operon, range, medians and CI 95%, were reported in Table 2. The Chi squared test showed significant differences among categories ($p<0.05$).

Table 2. Summary of results.

Phenotypic assay	Frequency	Range	Median (OD)	95% CI for the median	Complete operon
Excellent producer	49/89 (55.0%)	0.240-3.50	0.68	0.46-1.21	33/49 (67.3%)
Weak producer	20/89 (22.5%)	0.130-0.230	0.17	0.14-0.20	4/20 (20.0%)
No producer	20/89 (22.5%)	0.06-0.120	0.09	0.1-0.1	5/20 (25.0%)

As regards *S. epidermidis*, 42 of 57 (73,6%) strains had the complete *ica* operon, of which 33 (78,6%) were excellent biofilm producers, 4 (9,5%) were weak producers and 5 (11,9%) were non producers. The *S. epidermidis* strain that only tested positive for the *icaA* e *icaC* genes was found to be a weak producer. Comparing, instead, the 14 strains of *S. epidermidis* lacking all 4 genes, it can be said that 6 (42,85%) were excellent producers, 5 (35,71%) weak producers and 3 (21,42%) were non producers. The relation between the optical density and the presence or not of *ica* operon is represented in Table 3.

Table 3. Correlation between the biofilm production with the optical densities (phenotypic assay) and the presence of *ica* operon (genotypic assay).

Samples		Phenotypic assay		Genotypic assay			
Strain	Specie	Average o.d.	Interpretation	*icaA*	*icaD*	*icaB*	*icaC*
30678	*S.epidermidis*	3.50	EP	+	+	+	+
29789	*S.epidermidis*	3.50	EP	+	+	+	+
29216	*S.epidermidis*	3.43	EP	+	+	+	+
30667	*S.epidermidis*	3.33	EP	+	+	+	+
30203	*S.epidermidis*	3.30	EP	+	+	+	+
30383	*S.epidermidis*	2.37	EP	+	+	+	+
30428	*S.epidermidis*	2.25	EP	+	+	+	+
30371	*S.epidermidis*	2.24	EP	+	+	+	+
30344	*S.epidermidis*	1.94	EP	+	+	+	+

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30710	*S.epidermidis*	1.78	EP	+	+	+	+
29533	*S.epidermidis*	1.74	EP	+	+	+	+
30385	*S.epidermidis*	1.65	EP	+	+	+	+
30575	*S.epidermidis*	1.61	EP	+	+	+	+
29383	*S.epidermidis*	1.59	EP	+	+	+	+
30164	*S.epidermidis*	1.59	EP	-	-	-	-
29317	*S.epidermidis*	1.52	EP	+	+	+	+
30677	*S.epidermidis*	1.37	EP	+	+	+	+
30077	*S.epidermidis*	1.23	EP	+	+	+	+
29981	*S.epidermidis*	1.14	EP	+	+	+	+
30455	*S.epidermidis*	1.02	EP	-	-	-	-
30697	*S.epidermidis*	0.86	EP	+	+	+	+
29581	*S.epidermidis*	0.82	EP	+	+	+	+
30338	*S.epidermidis*	0.77	EP	+	+	+	+
29412	*S.epidermidis*	0.73	EP	+	+	+	+
30306	*S.epidermidis*	0.68	EP	+	+	+	+
30740	*S.epidermidis*	0.67	EP	+	+	+	+
29525	*S.epidermidis*	0.66	EP	-	-	-	-
30440	*S.lugdunensis*	0.64	EP	-	-	-	-
30418	*S.epidermidis*	0.63	EP	-	-	-	-
30064	*S.epidermidis*	0.62	EP	+	+	+	+
29954	*S.hominis*	0.51	EP	-	-	-	-
29638	*S.hominis*	0.46	EP	-	-	-	-
29993	*S.epidermidis*	0.43	EP	+	+	+	+
29668	*S.epidermidis*	0.43	EP	+	+	+	+
29743	*S.hominis*	0.43	EP	-	-	-	-
30789	*S.epidermidis*	0.42	EP	+	+	+	+
30607	*S.capitis*	0.39	EP	-	-	-	-
30702	*S.hominis*	0.36	EP	+	+	-	-
30478	*S.epidermidis*	0.34	EP	+	+	+	+
29769	*S.hominis*	0.32	EP	-	-	-	-
29540	*S.epidermidis*	0.29	EP	+	+	+	+
30735	*S.haemolyticus*	0.28	EP	-	-	-	-
29798	*S.epidermidis*	0.27	EP	-	-	-	-
29726	*S.epidermidis*	0.27	EP	+	+	+	+
30706	*S.epidermidis*	0.26	EP	+	+	+	+
30530	*S.haemolyticus*	0.26	EP	-	-	-	-
30242	*S.hominis*	0.25	EP	-	-	-	-
30595	*S.epidermidis*	0.25	EP	+	+	+	+
30239	*S.epidermidis*	0.24	EP	-	-	-	-
29409	*S.epidermidis*	0.23	WP	+	+	+	+
30359	*S.capitis*	0.22	WP	-	-	-	-
29846	*S.epidermidis*	0.21	WP	-	-	-	-
29655	*S.hominis*	0.21	WP	-	-	-	-
29808	*S.epidermidis*	0.21	WP	+	+	+	+
28995	*S.haemolyticus*	0.20	WP	-	-	-	-
30244	*S.epidermidis*	0.20	WP	-	-	-	-
30296	*S.haemolyticus*	0.18	WP	-	-	-	-
30288	*S.hominis*	0.18	WP	-	-	-	-
30417	*S.hominis*	0.17	WP	+	-	-	-
29618	*S.epidermidis*	0.16	WP	-	-	-	-
29972	*S.epidermidis*	0.15	WP	+	+	+	+

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30358	*S.haemolyticus*	0.15	WP	-	-	-	-
30291	*S.epidermidis*	0.15	WP	-	-	-	-
30319	*S.epidermidis*	0.14	WP	-	-	-	-
30013	*S.hominis*	0.14	WP	-	-	-	-
30773	*S.epidermidis*	0.13	WP	+	+	+	+
30387	*S.haemolyticus*	0.13	WP	-	-	-	-
29657	*S.epidermidis*	0.13	WP	+	-	-	+
29378	*S.hominis*	0.13	WP	-	-	-	-
30218	*S.epidermidis*	0.12	NP	-	-	-	-
29852	*S.haemolyticus*	0.12	NP	-	-	-	-
30539	*S.haemolyticus*	0.11	NP	-	-	-	-
29797	*S.haemolyticus*	0.11	NP	-	-	-	-
30814	*S.epidermidis*	0.10	NP	-	-	-	-
30807	*S.capitis*	0.10	NP	-	-	-	-
29834	*S.haemolyticus*	0.10	NP	-	-	-	-
29313	*S.epidermidis*	0.10	NP	+	+	+	+
29297	*S.epidermidis*	0.09	NP	+	+	+	+
29205	*S.epidermidis*	0.09	NP	-	-	-	-
29416	*S.epidermidis*	0.08	NP	+	+	+	+
29522	*S.epidermidis*	0.08	NP	+	+	+	+
29934	*S.capitis*	0.08	NP	-	-	-	-
29363	*S.epidermidis*	0.07	NP	+	+	+	+
29440	*S.hominis*	0.07	NP	-	-	-	-
29147	*S.hominis*	0.07	NP	-	-	-	-
29367	*S.haemolyticus*	0.07	NP	-	-	-	-
30761	*S.capitis*	0.06	NP	-	-	-	-
30318	*S.haemolyticus*	0.06	NP	-	-	-	-
29059	*S.haemolyticus*	0.06	NP	-	-	-	-

EP, excellent producer; WP, weak producer; NP, No producer

4. Discussion

The coagulase-negative staphylococci (CoNS) including *S. epidermidis* are ubiquitous in nature; reside on the skin of healthy individuals as normal fora. In fact, due to this phenomenon, CoNS has been emerged as common nosocomial pathogens. In addition, the ability to form biofilms on biotic as well as abiotic surfaces have made them successful human pathogens causing persistent infections leading to serious health problems. Given that biofilm is an important virulence factor that is associated with antibiotic resistance for these pathogens, early detection in clinical specimen would impose significant impact in management of staphylococcal nosocomial infections [13].

Our data show that CoNS, during the period of the pandemic Covid-19, were considered responsible for an increased number of episodes of bloodstream infections. Taking this into consideration, the study was carried out to investigate the production of biofilm by CoNS isolated from blood cultures between January 2021 and December 2021, in the different department of IRCCS Policlinico San Matteo Foundation.

Among 89 CoNS, *S. epidermidis* was the most common isolate accounting for more than half (n= 56, 62.9%) of total numbers followed by *S. hominis* (n=13, 14.6%), *S. haemolyticus* (n=13, 14.6%), *S. capitis* (n=5, 6%) and *S. lugdunensis* (n=2, 2.2%) (Table 4.)

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Table 4. CoNS strains isolated from each departments.

Departments	Total	*S. epidermidis*	*S. hominis*	*S. haemolyticus*	*S. capitis*	*S. lugdunensis*
Anesthesia and reanimation	49	36	9	4		
Surgeries	5	4			1	
Pulmonology	5	2	2	1		
Hematology	10	3		5		2
Oncologies	9	6	1	2		
Cardiology	8	5	1	1	1	
Neonatal intensive care unit	3				3	
Total	89	56	13	13	5	2

An interesting finding of this study is that only *S. epidermidis* presented the entire operon, in particular 42 of 57 strains (73,7%), of which 36 (85,1%) biofilm producers and 5 (11,9%) were non producer. Only one *S. epidermidis* strain tested positive for the *icaA* e *icaC* genes and was found to be a weak producer. The possible reasons could be the various factors such as environment, nutrition, subinhibitory concentration of certain antibiotics, and stress (temperature, osmolarity) might play a significant role in biofilm formation resulting in varied frequency of biofilm producers among clinical isolates [15,16].

Comparing, instead, the 14/57 (24,5%) strains of *S. epidermidis* negative to the genotypic test for all 4 genes, it can be said that 3 (21,42%) were non producers, but 11/14 (78,6%) were biofilm producers. This discrepancy between the phenotypic and genotypic test finds explanation in the multifactorial genesis of biofilm used by Staphylococci. In fact, this process seems not to be solely dependent on *ica* genes, and it's in line with data in the literature [8]. These findings reinforce the opinion that several mechanisms besides slime production are responsible for bacterial adhesion and hence biofilm production [17].

Antibacterial coatings depend on the covalent immobilization of antimicrobial agents on the coating surface and drug release to prevent and combat infection, while the surface modification of biomaterials affects the adhesion behavior of cells on the surfaces of implants and the subsequent biofilm formation process by altering the physical and chemical properties of the implant material surface. [3]

5. Conclusion

The results of this study showed that most CoNS strains are capable of developing biofilms, especially those with complete operon *ica*, thus increasing the pathogenic power of these strains, which are believed to be responsible for catheter colonization, bacteraemia and sepsis. This approach allowed us to underline an increase in the incidence of bloodstream infections supported by CoNS biofilm producers, especially in patients with temporary or permanent biomedical devices. However, our study confirms what has been reported previously, that, *ica* operon is largely involved in biofilm production, but it's not the only contributing factor. In fact, some strains have been shown to be capable of producing biofilm, albeit lacking this operon, because the genesis of biofilm is to be considered multifactorial.

Knowing the adhesive capabilities of a micro-organism responsible for sepsis can be useful in implementing appropriate corrective and preventive measures, such as the use of devices with antibacterial coatings, as conventional antibiotic therapy cannot effectively eradicate biofilms. The use of antibacterial coatings of medical materials not only prevents bacterial adhesion, but also directly fights biofilm infections by releasing antibacterial drugs.

In conclusion, to better solve the problem of biofilm resistance, further in-depth research is needed, such as the development of new antibacterial coatings (biodegradable and biocompatible) and the improvement of prevention by optimising medical materials and hospital infection control measures.

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