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INVESTIGATION OF BIOFILM FORMATION IN COAGULASE-NEGATIVE STAPHYLOCOCCI ISOLATED FROM PLATELET CONCENTRATE BAGS

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SUMMARY

Platelet Concentrates (PCs) are the blood components with the highest rate of bacterial contamination, and coagulase-negative staphylococci (CoNS) are the most frequently isolated contaminants. This study investigated the biofilm formation of 16 contaminated units out of 691 PCs tested by phenotypic and genotypic methods. Adhesion in Borosilicate Tube (ABT) and Congo Red Agar (CRA) tests were used to assess the presence of biofilm. The presence of *ica*ADC genes was assessed by means of the Polymerase Chain Reaction (PCR) technique. With Vitek®2, *Staphylococcus haemolyticus* was considered the most prevalent CoNS (31.25%). The CRA characterized 43.8% as probable biofilm producers, and for the ABT test, 37.5%. The *ica*ADC genes were identified in seven samples by the PCR. The ABT technique showed 85.7% sensitivity and 100% specificity when compared to the reference method (PCR), and presented strong agreement (k = 0.8). This study shows that species identified as PCs contaminants are considered inhabitants of the normal skin flora and they might become important pathogens. The results also lead to the recommendation of ABT use in laboratory routine for detecting biofilm in CoNS contaminants of PCs.

KEYWORDS: Biofilm; Staphylococci; Platelet Concentrates; Transfusion; Microbiology.

INTRODUCTION

Platelet Concentrates (PCs) are blood components widely used in patients with neoplastic diseases, who are undergoing chemotherapy, as a way to reduce the risk of bleeding[1]. However, such components are the ones with the highest rate of bacterial contamination, being considered responsible for almost all of the septic transfusion reactions [2].

According to the Brazilian Ministry of Health, approximately 3.5 million blood donations are made[3] per year in Brazil. The Brazilian Hemotherapy Service consists of a vast network that offers about 2,332 units providing services[4]. This scenario reveals the great national physical infrastructure necessary to meet the demand for blood components in the country.While Gram-positive bacteria (GP) are responsible for the highest rates of contamination in PCs (60%), the less frequent Gram-negative (GN) may present the greatest number of fatal septic reactions[5]. Among GP, coagulase-negative staphylococci (CoNS) stand out, and *Staphylococcus epidermidis* is the most frequently involved species[6,7]; and their capacity to adhere to polymer surfaces and consequent biofilm production are virulence factors of this species[8].

In recent years, CoNS have emerged as an important nosocomial pathogen, especially in immunocompromised patients, due to their ease in incorporating different resistant to antimicrobial genes, favored by the biofilm environment in the exchange of genetic material. This virulence factor has been considered one of the most significant risks to patients. The difficulty of a clinical treatment for infections associated with biofilms has direct and indirect consequences (longer hospitalization, cost of antibiotics and complementary medicines, and diagnostic procedures) on the patient's outcome and quality of life[9-11].

Biofilm is defined as a sessile microbial community surrounded by a polymeric extracellular matrix produced by the bacteria[12]. An important component in biofilm accumulation of *S. epidermidis* is the polysaccharide intercellular adhesin (PIA) and its production is mediated by the gene *ica* (intercellular gene cluster adhesion), an operon that contains the *ica*RADBC[13-16], four biosynthetic genes (*ica*ADBC), and one regulation gene (*ica*R). The PIA is a linear homopolymer composed by approximately 130 residues of N-acetylglucosamine connected by β-1, 6 links, of which 15 to 20% are deacetylated[14].

The enzymes IcaA and IcaD, when co-expressed, will show better activity for the assemblage of β(1-6)-linked glucosaminoglycans that comprise PIA. The absence of IcaD is associated with a decrease in PIA production[17], the likely IcaC functions in the extrusion of PIA from the bacterial cell[18], whereas the IcaB appears to function as a

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deacetylase[19]. Although the expression of the *ica*A gene might promote a low production of PIA, the expression of *ica*AD or *ica*ADC genes stimulates an increased production of biofilm[17,20-22]. Many factors influence the biofilm formation under physiological conditions. It is known that biofilm formation is subject to environmental conditions such as sub-inhibitory concentrations of antimicrobials, temperature rise, anaerobiosis, osmolarity, thus leading to the changing between positive and negative phenotypes of biofilm[13].

Some studies have reported that several genes are directly or indirectly involved in the molecular mechanisms of biofilm formation in *S. epidermidis*, and the most outstanding are the *atl*E gene which encodes the AtlE autolysin that is responsible for the ability to directly connect to the polymeric surface[23]; the *agr* gene, which controls biofilm formation, whose non-functionality facilitates the primary attachment of the pathogen to the surface[24]; the *luxS* gene, with a significant impact on biofilm formation, and whose deletion leads to an increase in the transcription regulation of *ica*ADBC, resulting in an increase in the PIA synthesis[25,26]; and the *ica*R gene, which is very important for biofilm formation, as it is considered a negative transcription regulator of *ica*ADBC, essential for the production of PIA[21]. Besides these genes, the proteins *Aap* (accumulation-associated protein)[10] and *Bap* (biofilm-associated protein)[27] are directly associated with biofilm formation. Therefore, it becomes increasingly clear that the potential virulence of a bacterium does not depend exclusively on a single factor.

Biofilms are highly hydrated structures containing channels that allow the internal diffusion of nutrients and oxygen, and their formation protects against the innate defense mechanisms of the host and the influx of antimicrobials, hindering drug diffusion in tissues [28], thus facilitating the development of chronic infections[29]. It is a consensus in literature that *ica*A, C and D genes are responsible for encoding biofilm-forming enzymes, evidenced in most CoNS[8,30].

This study assessed 16 bacteria isolated from 691 bags of PCs from the Blood Bank of the city of *Santa Maria*, *Rio Grande do Sul* State (HEMORGS/SM, RS), in order to detect biofilm formation in these contaminants, by means of phenotypic and genotypic methods.

MATERIALS AND METHODS

Inclusion criteria for bacterial isolates: A total of 691 PCs were analyzed, considering that 665 were obtained by centrifugation of whole blood and 26 by apheresis. All samples were collected at HEMORGS between 2009 and 2010, and the cultures were prepared from the tubular portion of the bags of platelets. After collection, the samples were sent to the Laboratory of Bacteriology, Department of Clinical and Toxicological Analysis of the Health Sciences Center of the *Universidade Federal de Santa Maria* (UFSM). The tests for detection of bacterial contamination in PCs were performed in a type II biosafety cabinet, totally exhausted. Sterilization of the tubular portion of the bags of PCs was performed with 70% alcohol for approximately one minute. Bacterial investigation of these PCs bags is described in detail by MARTINI *et al*.[31].

Since the opening of HEMORGS - *Santa Maria* in May 2008, bacteriological control has been conducted in whole blood-derived platelets (10 monthly samples) and apheresis platelets (10 monthly samples)[32]. The technique used for routine bacteriological screening at HEMORGS is described by CUNHA *et al*.[33]: approximately 300 microliters (μL) are removed from the platelet bag tubing, cultured in 2 milliliters (mL) of Mueller Hinton broth (MHB), and incubated at 35 ± 2 °C. After five days of incubation, 10 μL of the broth containing the PC sample were sub-cultured in sheep blood agar 5% (BA), and the plates were incubated in the same conditions. The samples that showed bacterial growth within 24-48 hours (h) were subcultured from initial MHB to new plates of BA, and incubated in the same conditions, in order to exclude false positives. The samples were considered "true positives" after the culture repetition and confirmation with the isolation of bacteria obtained from the first culture[31]. This quality control follows the standards recommended by the legislation, which does not propose the culture of all samples as pre-transfusion screening, but of at least 1% of the monthly production or 10 units per month[34,35].

Currently, the Ordinance No. 2.712 of November 12, 2013, redefines the technical regulation for hemotherapeutic procedures. It describes that the bacteriological control of PCs remains the same as reported by the current Resolution of the Collegiate Board of Directors (RDC) No. 57 of December 16, 2010, as well as the previous RDC No. 153 of June 14, 2004 [34,35]. Nevertheless, with the inclusion of the paragraph 8 (§ 8) of Article 116, which describes that "*Pelo alto risco de contaminação microbiológica dos concentrados de plaquetas pela sua condição de armazenamento, recomenda-se realização de avaliação de contaminação microbiológica em 100% desta produção"*, or "Due to the high risk of microbiological contamination of platelet concentrates regarding their storage conditions, it is recommended to perform the microbiological contamination assessment on 100% of this production"[36].

Assays for bacterial detection in PCs (31): The qualitative assay was performed on 612 samples, according to CUNHA *et al*.[33]. Samples that did not show any bacterial growth on BA in 48 hours were considered negative. The ones that showed development of Colony-Forming Units (CFU) were considered positive, and a new subculture was performed to exclude any possible contamination.

The quantitative assay was performed on 292 samples, as described by YOMTOVIAN *et al*.[37]. The samples that showed bacterial growth regardless of the number of colonies developed were considered positive; the multiplication factor ten was used to obtain the number of CFU/mL.

Both the qualitative[33] and the quantitative[37] techniques were used in 79 samples, with some modifications, and named *Teste do Crescimento Diário* (Daily Growth Test). At every 24 h of incubation in MHB, 10 μL of the broth containing the sample were subcultured in BA, with an automatic pipette, and plates were incubated in a bacteriological incubator (35 ± 2 °C with 5% of CO_2 for 24 – 48 h). Subsequent sowings were performed, at 24 h intervals for each analysis. Four sowings were performed altogether, and readings taken at 24, 48, 72 and 96 h after the first incubation. The samples that showed no colony growth in 48 h were considered negative, and the ones with growth were considered positive. All cultures were performed in duplicate tests, with the purpose of excluding any possible contamination during treatment.

Bacterial isolates of the 691 PCs: A total of 16 isolates of CoNS were obtained through an investigation of bacterial contamination in 691

bags of PCs, which constituted the research sample of this study. The following international reference standard strains (*American Type Culture Collection*-ATCC) were used as controls in the genotypic and phenotypic tests: *Staphylococcus epidermidis* ATCC 12228 negative control biofilm and *Staphylococcus epidermidis* ATCC 35984 positive control.

Phenotypic identification: The isolates were tested for phenotypic identification through automation, with Vitek® 2 (bioMérieux, Marcy L'Étoile, France), and the technique was performed in duplicate.

Sample preparation: In order to perform the phenotypic tests, the strains stored in Trypticase Soy Broth plus 15% of glycerol were subcultured in petri dishes containing Mueller Hinton agar, and incubated at 35 ± 2 ºC for 24 h. For the genotypic test, the process of extraction of deoxyribonucleic acid (DNA) was performed by a thermal analysis procedure[38-41]. Besides, it should be noted that the subsequent tests were performed in duplicate.

Amplification of the gene 16S of ribosomal RNA (RNAr): After extraction, each sample was submitted to electrophoresis in 1.0% agarose gel to verify the presence of DNA in the extracted material. The 16S rRNA was amplified by the Polymerase Chain Reaction (PCR).

Phenotypic methods for the research of biofilm production

Adhesion in borosilicate tube (ABT): This assay was performed as according to CHRISTENSEN *et al*[42]. Positivity was indicated by the presence of a layer of stained material adhered to the inner wall of the tubes.

Congo red agar (CRA): The test was carried out according to FREEMAN *et al.*[43]. The plates of CRA were inoculated and incubated at ambient temperature for 24 h, at 35 ± 2 ºC. It was positive (biofilm-producing) when black colonies were observed, with shiny or dry/opaque aspect. On the other hand, non-biofilm producers formed pink (P), red (R) or burgundy (B) colonies.

Genotypic method for the research of biofilm production: In order to determine the genes *ica*A, *ica*C and *ica*D, the following pairs of primers were used: *ica*A_F-ACAGTCGCTACGAAAAGAAA and *ica*A_R-GGAAATGCCATAATGAGAAC; *ica*C_F-TAACTTTAGGCGCATATGTTT and *ica*C_R-TTCCAGTTAGGCTGGTATTG; *ica*D_FATGGTCAAGCCCAGACAGAG and *ica*D_RCGTGTTTTCAACATTTAATGCAA (Ludwig Biotec®). The sequence of primers and the technique followed the instructions of ARCIOLA *et al.*[44]. A sample was considered biofilm-producing when the presence of genes *ica*AD or *ica*ADC was detected. When only one of these genes was found, the sample was considered negative for the production of biofilm[8].

Statistical analysis: The assessment of sensitivity and specificity was based on the comparison between the phenotypic methods tested with the PCR technique, which was considered a standard. The Kappa index (k) was also calculated in order to verify the agreement between the results obtained by the different methods[8,44,45].

Ethical considerations: This study was approved by the Research Ethics Committee (CEP) of UFSM, under the number 0285.0.243.000-09.

RESULTS

A total of 16 isolates of CoNS were obtained through an investigation of bacterial contamination in 691 PCs from HEMORGS: 5 *Staphylococcus haemolyticus* (31.25%), 4 *Staphylococcus epidermidis* (25%), 4 *Staphylococcus warneri* (25%) and 3 *Staphylococcus saprophyticus* (18.75%). The bacterial species isolated from contaminated PCs bags were identified by the automated system Vitek® 2, described in Figure 1.

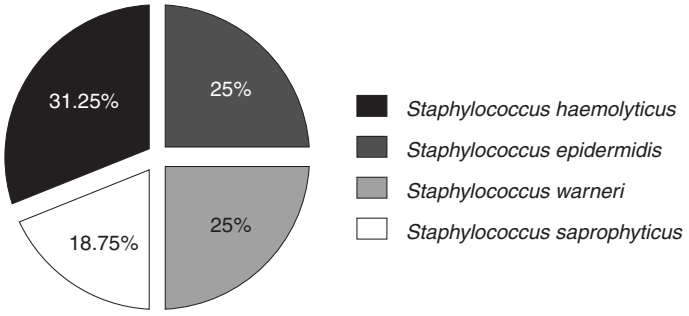

Fig. 1 - Distribution of the 16 species of coagulase-negative staphylococci isolated from 691 Platelet Concentrate bags.

Through research for the gene 16S of ribosomal RNA (rRNA-) in the samples of this study, we found that they all had great quality of extracted DNA, thus allowing the investigation of single genes. The research of the biofilm, performed with CRA, was positive in seven (43.75%) out of the 16 samples of CoNS, with the black color of the colonies evidencing probable biofilm-producing bacteria. The other strains showed other colors (B and R), indicating the absence of biofilm production. With the ABT technique, six samples (37.5%) were characterized as adherent, in other words, probable biofilm producers (Table 1).

The genotypic analysis, carried out by PCR, investigated the presence of genes *ica*A, C, and D, so we considered probable biofilm-forming the samples in which the genes *ica*AD or *ica*ADC[8] were found. The result of this analysis is detailed in Table 1. Seven out of the 16 samples (43.75%) presented the genes *ica*AD and/or *ica*ADC, concomitantly.

Comparing the results of this study, the phenotypic method of ABT and the genotypic method in biofilm production, 85.7% sensitivity and 100% specificity were obtained, showing high agreement (k = 0.87). When CRA and PCR techniques were compared, 71% of sensitivity and 78% of specificity were obtained. We also verified an agreement index k = 0.49, considering the PCR as the standard method.

DISCUSSION

In our study, all organisms isolated in the samples of contaminated PCs were also CoNS, similar to the results found by other authors[46-48]. However, no contamination was identified by GN bacteria, different from the findings of CUNHA *et al.*[33], in which 62.5% of the PCs were contaminated by GN bacteria and 37.5% by GP Regarding bacterial species, there was a predominance of *Staphylococcus haemolyticus* (31.25%), followed by *Staphylococcus epidermidis* and *Staphylococcus warneri* at a rate of 25.0% each.

Table 1

Results regarding the research of genes *ica*A, C and D in the 16 samples of coagulase-negative staphylococci isolates from Platelet Concentrates, by the Polymerase Chain Reaction technique and the phenotypic tests for biofilm production.

Samples	Species	Genes			Biofilm-positive		Slime Production	
		***ica*A**	***ica*C**	***ica*D**	***ica*AD**	***ica*ADC**	**CRA**	**ABT**
1	*S. epidermidis*	+	+	+	+	+	-	+
2	*S. epidermidis*	+	+	+	+	+	+	+
3	*S. saprophyticus*	+	+	+	+	+	+	+
4	*S. haemolyticus*	-	+	-	-	-	+	-
5	*S. saprophyticus*	-	-	-	-	-	-	-
6	*S. haemolyticus*	+	+	+	+	+	+	-
7	*S. saprophyticus*	-	-	-	-	-	-	-
8	*S. epidermidis*	-	-	-	-	-	-	-
9	*S. warneri*	-	-	-	-	-	-	-
10	*S. haemolyticus*	-	+	-	-	-	+	-
11	*S. haemolyticus*	+	+	+	+	+	+	+
12	*S. warneri*	-	-	-	-	-	-	-
13	*S. haemolyticus*	+	+	+	+	+	+	+
14	*S. epidermidis*	-	-	-	-	-	-	-
15	*S. warneri*	-	-	-	-	-	-	-
16	*S. warneri*	+	+	+	+	+	-	+

CRA = Congo red agar; ABT= Adhesion in borosilicate tube; "-" = absence; "+" = presence

A study by GRECO[49] reported that 13 contaminant CoNS strains were isolated during a routine screening of PCs by Canadian Blood Services, between January 2006 and May 2007, being *S. epidermidis* (53.8%) the predominant species, followed by *S. capitis* and *S. hominis*, 15.4% each. Moreover, ROOD *et al.*[50] described that 35.8% of contamination in PCs was caused by *S. epidermidis*, 17.9% by *S. capitis* and 14.9% by *S. saccharolyticus*. The species *S. haemolyticus* was not reported in these two recent studies, however, the only Brazilian study regarding bacterial contamination of PCs demonstrated that *S. haemolyticus* and *S. hominis* have been responsible for an individual rate of 12.5% of bacterial contamination[33].

CoNS are natural colonizers of human skin and mucosa. However, they have recently been acknowledged as important nosocomial pathogens due to their ability to form biofilm [18]. Currently, the increase of antibiotic resistance in some clinical isolates may be related to their ability to form biofilm, because the acquisition of some resistance traits is possible within biofilm, through gene transfer[51].

S. epidermidis are the most isolated[52] from blood cultures among CoNS, *S. haemolyticus* being the second most, and *S. haemolyticus* have the highest rates of resistance to antimicrobials[53]. This species can cause septicemia, peritonitis, otitis and urinary tract infections. *S. epidermidis* and *S. haemolyticus* are opportunistic human pathogens, and difficult to be eradicated because of their resistance to antibiotics[52]. In contrast to *S. epidermidis*, the molecular basis of virulence of *S. haemolyticus* is in general largely unknown; however, biofilm formation is a common clinical phenotype[54]. Furthermore, *S. haemolyticus* have the highest level of antimicrobial resistance of all CoNS and their heteroresistance to glycopeptides[53] is very common. Thus, it limits the therapeutic options and makes an *S. haemolyticus* infection a serious threat[54]. Therefore, the presence of bacterial contaminants in PCs such as *S. epidermidis* and *S. haemolyticus*, considered potentially resistant, generates a major concern due to the virulence of these strains, being directly linked to their ability to cause septic transfusion reactions and influence the clinical outcome of the receiver.

Regarding the detection values obtained in this study for the CRA method, results similar to ours have been reported by ARCIOLA *et al.*, CAFISO *et al.* and LAZZARATTO *et al.*; GRECO found 57.5%, 57%, 37.5%, 54% and 30.8%, respectively[13,44,49,55,56]. RÚZICKA *et al.* detected 43.5% of biofilm production in *S. epidermidis* isolated from blood cultures and 21.1% of isolates of skin. However, another research performed by OLIVEIRA & CUNHA, in another Brazilian state, demonstrated that 73% of the CoNS were producers of biofilm when subcultured in CRA. For the ABT test, RÚZICKA *et al.* found 53.7% and 22.4% in blood cultures and skin, respectively. OLIVEIRA & CUNHA also reported a higher rate for ABT, 82%[8,57].

The results obtained by PCR are similar to the ones found by CAFISO *et al.*, who detected the genes *ica*AD and *ica*ADC in 45% of the isolates. ARCIOLA *et al.* reported the presence of the same genes in 59% of the samples. OLIVEIRA & CUNHA found 82% of genes *ica*AD and *ica*ADC and GRECO, 23.1% of *ica*D[8,13,44,49].

In a recent study, ALI *et al.*[30], while studying *S. epidermidis* isolated from contaminated PCs bags, reported its ability to convert to a positive phenotype of biofilm when cultured under similar conditions to the PCs bags, with or without the presence of the *ica* genes. It happens because *S. epidermidis* are able to form biofilms that adhere to the plastic of platelet storage bags and platelet aggregates, as already reported by GRECO-STEWART *et al.* in strains of *Serratia marcescens*[58]. Bacteria with slower growth create a larger amount of biofilm, which makes their detection more difficult in automated culture media[30].

According to the literature, the rates of 100% sensitivity and specificity are found with ABT, and 89% sensitivity and 100% specificity with CRA, when comparing to PCR[8]. For the CRA test, GRECO reports 100% of sensitivity and 90% of specificity[49]. In our study, the ABT method was the phenotypic test with a better performance than CRA in biofilm detection, a result in agreement with OLIVEIRA & CUNHA[8].

Positive samples found in the genotypic and phenotypic tests were discussed in this study, and the presence of *ica*ADC genes was observed in seven samples: six showed biofilm formation through the ABT technique and five through CRA. One sample showed biofilm formation only via the CRA method and did not present the *ica*ADC genes. Samples were considered biofilm-producing only when *ica*AD or *ica*ADC genes were detected[8]. We believe that tested samples were negative to CRA and positive to ABT and PCR (Table 1) due to the low specificity of the CRA technique. Research tests of biofilm formation reported in the literature have shown that between the ABT and CRA techniques, the latter is considered to be less specific when compared with the standard PCR methodology. We know that the role of the icaR gene is to regulate biofilm formation and that it may influence when present. The presence of only one of the *ica*ABDC genes does not exclude the possibility of biofilm formation, perhaps to a lesser extent. Moreover, we must point out that phenotypic tests do not show 100% specificity and sensitivity.

The results have shown that the origin of the isolated bacterial species in PCs bags of this study is probably the skin. The phenotypic tests for the detection of biofilm production indicated good sensitivity and specificity when compared to the reference technique, particularly with the method of ABT that have shown high agreement. Thus, these results allow us to suggest this technique for the routine detection of CoNS strains able to produce biofilm in clinical microbiology laboratories. This is due to its easy application, low cost, high sensitivity and specificity, which ensure reliable results.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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