

Assessment of different methods for the detection of biofilm production in coagulase-negative staphylococci isolated from blood cultures of newborns

Roberta Filipini Rampelotto^{[1],[2]}, Vinicius Victor Lorenzoni^[1], Danielly da Costa Silva^{[1],[2]},
Silvana Silveira Coelho^[1], Vanessa Wust^[1], Litiérri Razia Garzon^[1],
Melise Silveira Nunes^{[1],[3]}, Bettina Meneghetti^{[1],[3]}, Patrícia Chaves Brites^[4],
Manfredo Hörner^[5] and Rosmari Hörner^{[1],[2]}

- [1]. Departamento de Análises Clínicas, Universidade Federal de Santa Maria, Santa Maria, RS, Brasil.
[2]. Programa de Pós-Graduação, Universidade Federal de Santa Maria, Santa Maria, RS, Brasil.
[3]. Laboratório de Análises Clínicas, Hospital Universitário de Santa Maria, Santa Maria, RS, Brasil.
[4]. Laboratório de Biologia Molecular, Hospital Universitário de Santa Maria, Santa Maria, RS, Brasil.
[5]. Departamento de Química, Universidade Federal de Santa Maria, Santa Maria, RS, Brasil.

Abstract

Introduction: Coagulase-negative staphylococci (CoNS) are a frequent cause of bacteremia, especially in neonates. The major virulence determinant in CoNS is the ability to produce biofilms, which is conferred by the *icaADBC* genes. This study aimed to assess different methods for the detection of biofilm formation in 176 CoNS isolates from blood cultures of newborns. **Methods:** The presence of the *icaACD* genes was assessed by polymerase chain reaction (PCR), and biofilm formation was assessed on congo red agar (CRA), by the tube method (TM), and on tissue culture plates (TCP). **Results:** Of the 176 CoNS isolates, 30.1% expressed *icaACD* and 11.4% expressed *icaAD*. The CRA assay and TM showed that 42% and 38.6% of the isolates were biofilm producing, respectively. On TCP, 40.9% of the isolates produced biofilms; 21% were weakly adherent and 19.9% were strongly adherent. When compared to the gold standard technique (PCR), the CRA assay showed 79% sensitivity and 84% specificity ($\kappa = 0.64$), TM showed 78% sensitivity and 89% specificity ($\kappa = 0.68$), and TCP showed 99% sensitivity and 100% specificity ($\kappa = 0.99$). **Conclusions:** In this study, ~42% of CoNS isolates produced biofilms, and the presence of *icaACD* was associated with a greater capacity to form biofilms. Compared to the other phenotypic methodologies, TCP is an ideal procedure for routine laboratory use.

Keywords: Biofilm. Blood culture. Coagulase-negative staphylococci. Newborn.

INTRODUCTION

Infections during the neonatal period lead to high levels of morbimortality. Sepsis is one of the most relevant complications of neonatal infections, and it increases the hospital care required for newborn babies¹⁻³. Coagulase-negative staphylococci (CoNS), which are part of the normal skin microbiota, are the most frequently isolated pathogens in blood stream infections. In particular, *Staphylococcus epidermidis* is capable of causing infections in both immunocompromised patients and newborns⁴⁻⁸.

CoNS infections are frequently associated with the use of invasive medical devices, and biofilm formation is a major

virulence determinant in this group of bacteria^{4,7-11}. The formation of biofilms allows these microorganisms to adhere to biomaterials, and biofilm formation is related to infection recurrence and therapy failure, as the organisms within biofilms are more resistant to antimicrobials and host defenses^{4,7-9,11,12}.

In recent decades, there is increasing awareness of the great difficulty in eradicating infections caused by biofilm-forming bacteria, since these microorganisms are more resistant to and can survive in the presence of various antimicrobials, requiring removal of the related medical device or surgical methods to cure the infection^{6,12-15}.

CoNS develop into biofilms via a complex, multifactorial process that can be divided into four phases: adhesion, accumulation, maturation, and detachment, each involving specific molecular factors^{8,13,16}. Studies suggest that polysaccharide intercellular adhesin (PIA) is the most important component of *Staphylococcus* spp. biofilms. Biofilm production in *Staphylococcus* spp. is mediated by the chromosomal

Corresponding author: Rosmari Hörner.

e-mail: rosmari.ufsm@gmail.com

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intracellular adhesion (*ica*) gene products, which are involved in cell adhesion and are arranged in an operon structure containing four biosynthesis genes *icaABCD*^{7,11,13}.

Considering the large number of infections caused by biofilm-forming bacteria, early detection and eradication of these microorganisms is necessary. A phenotypic method, along with detection of the genes in the *ica* operon, is generally used to verify biofilm production in *Staphylococcus* spp. infections^{7,12}. The purpose of the present study was to assess four methods for the detection of biofilm production in CoNS isolates from blood cultures of newborn patients in a tertiary hospital in southern Brazil. The four methods evaluated were tissue culture plate (TCP), the tube method (TM), congo red agar (CRA), and polymerase chain reaction (PCR). This is the first study to assess biofilm production in CoNS isolates from blood cultures of newborn patients at this hospital.

METHODS

Location and duration of study

This study was conducted at the Laboratório de Bacteriologia, Departamento de Análises Clínicas e Toxicológicas, Centro de Ciências da Saúde of Universidade Federal de Santa Maria, Santa Maria, Rio Grande do Sul, Brazil. Samples were isolated at a tertiary hospital in southern Brazil during a one-year period (2014).

Isolates

One hundred and seventy-six (176) CoNS isolates were obtained from the blood cultures of newborns. Blood cultures were required whenever a significant clinical sign was present, such as increased body temperature (>38°C), hypothermia (<36°C), leukocytosis (>10.000 leukocytes/mm³, especially with left deviation), or absolute granulocytopenia (<1000 leukocytes/mm³). For the cultures, a 3mL sample of blood was collected from each newborn and placed in a blood culture bottle. At least two blood cultures were used for each sample. Isolates were processed with the BACTEC 9240® automated blood culture system (Becton Dickson, Sparks, MD), and samples were

considered positive when two or more blood cultures developed CoNS. Species-level identification of CoNS and antimicrobial sensitivity profiles were performed with the Vitek® 2 system (bioMérieux, France). The sensitivity profiles were determined according to the Clinical and Laboratory Standard Institute (CLSI) guidelines¹⁷ at the time of the study.

Blood cultures in which CoNS growth was detected were subsequently sent to the Laboratório de Bacteriologia, Departamento de Análises Clínicas e Toxicológicas, Centro de Ciências da Saúde at UFSM, where they were inoculated on tryptone soya agar (TSA) and incubated at 35 ± 2°C for 18–24 h. The colonies were stored in tryptone soya broth (TSB) containing 15% glycerol at –80°C until biofilm detection.

PCR, TM, and CRA were performed in duplicate, and TCP was performed in quadruplicate. *S. epidermidis* American Type Culture Collection (ATCC) 12228 and *Staphylococcus aureus* ATCC 25923 (a strong biofilm producer) were used as the negative and positive controls, respectively.

Biofilm detection by the genotypic method (PCR)

DNA was extracted by the boiling method of Pérez-Pérez and Hanson¹⁸. The 16S rRNA gene sequence was determined to verify the presence of CoNS bacterial DNA, and then the *icaACD* genes were detected by PCR, which is considered the gold standard for detecting biofilm formation ability^{14,19}. The primers used, which are shown in **Table 1**, were obtained from GBT Oligos® and were designed based on the *Staphylococcus epidermidis* *icaACD* sequence (GenBank accession number U43366.1). To amplify the *icaACD* genes, a Thermo Cycler (model 2720, Biosystems) was used and programmed with the following cycling conditions: an initial step at 94°C for 5 min followed by 50 cycles of 30 sec each at 94°C, 55.5°C, and 72°C, with a final step at 72°C for 1 min. Each PCR simplex reaction contained 17.05 µL of ultrapure water, 1.75 µL of buffer (10×; Ludwig-Biotec®), 0.75 µL of MgCl₂ (50 mM; Ludwig-Biotec®), 2 µL of dNTPs (100 mM; Ludwig-Biotec®) s 0.2 µL of Taq DNA polymerase (5U/mL; Ludwig-Biotec®), 0.625 µL of forward primer, 0.625 µL of reverse primer, and 2 µL of DNA. The DNA fragments were analyzed by 1.5%

TABLE 1: Primers used for PCR.

Gene	Primer	Sequence (5'-3')	Product size (bp)
16S rRNA	16S-F	CCTATAAGACTGGGATAACTTCGGG	791
	16S-R	CTTTGAGTTTCAACCTTGCGGTCG	
<i>icaA</i>	<i>icaA</i> -F	ACAGTCGCTACGAAAAGAAA	103
	<i>icaA</i> -R	GGAAATGCCATAATGACAAC	
<i>icaC</i>	<i>icaC</i> -F	TAACTTTAGGCGCATATGTTTT	400
	<i>icaC</i> -R	TTCCAGTTAGGCTGGTATTG	
<i>icaD</i>	<i>icaD</i> -F	ATGGTCAAGCCCAGACAGAG	198
	<i>icaD</i> -R	CGTGTTTTCAACATTTAATGCAA	

agarose gel electrophoresis, and the bands were visualized with a photo documentation system (KODAK DC 290, using 1D software, version 3.6).

Biofilm detection by phenotypic methods

Congo red agar (CRA)

Isolates were analyzed according to the method described by Freeman et al.²⁰. Briefly, isolates were inoculated on sheep blood agar, incubated at $35 \pm 2^\circ\text{C}$ for 24 h, and then transferred to CRA and incubated at $35 \pm 2^\circ\text{C}$ for 24 h. Biofilm-producing isolates formed black colonies, whereas non-biofilm-producing isolates formed red colonies.

Tube method (TM)

Isolates were assessed by the TM as described by Christensen et al.²¹. They were inoculated on sheep blood agar and incubated at $35 \pm 2^\circ\text{C}$ for 24 h. Then, selected colonies were inoculated into test tubes containing 2.0 mL of TSB and incubated at $35 \pm 2^\circ\text{C}$ for 48 h. Later, the contents were removed with a pipette, and 1.0 mL of a 0.4% aqueous solution of trypan blue was added to each tube. The stain was removed with a pipette and after a 1 min incubation, the results were read by visual observation. If colored bacteria adhered to the tube wall, the isolate was considered to be a biofilm producer, whereas if no colored microorganisms adhered to the tube wall, the isolate was considered to be a non-biofilm producer.

Tissue culture plate (TCP)

Isolates were assessed by the TCP method as described by Christensen et al. (1985)²², with some modifications. The bacteria were inoculated on sheep blood agar and incubated at $35 \pm 2^\circ\text{C}$ for 24 h. Then, colonies were transferred to test tubes containing 2.0 ml of TSB and incubated at $35 \pm 2^\circ\text{C}$ for 24 h.

Using multichannel pipettes, 200 μL of TSB containing 1% glucose was added to each well of a 96-well flat bottom polystyrene tissue plate. Then, a 200 μL aliquot of the aforementioned bacterial suspension in TSB was added to each well; each isolate was inoculated into four parallel wells, and the plates were incubated at $35 \pm 2^\circ\text{C}$ for 24 h. Then, the contents of each well were aspirated, and the wells were washed four times with 200 μL of phosphate-buffered saline (PBS, pH 7.2). The wells were stained with 100 μL of 2% crystal violet for 1 min. Then, the stain was aspirated, and wells were washed with distilled water. Plates were dried for 1 h at room temperature. The optical density (OD) at 570 nm was then read with an EpochTM Multi-volume Spectrophotometer (Biotech)²².

Sterile TSB containing 1% glucose, which underwent all procedures described above, without the addition of microorganisms, was included as a medium control. A cut-off value for the detection of biofilm formation was calculated as the standard deviation multiplied by three plus the OD of the medium control (cut-off value = standard deviation \times 3 + average OD medium control)²².

Isolates were classified into three categories: non-adherent (OD less than or equal to the cut-off value, i.e., $\text{OD} \leq 0.116$), weakly adherent ($\text{OD} > 0.116$ and ≤ 0.232), and strongly adherent ($\text{OD} > 0.232$).

Statistical analysis

Sensitivity and specificity were calculated based on comparison of the phenotypic methods and PCR, which is considered a gold standard. The Kappa index (k) was also calculated to verify agreement between the results obtained from the different methods^{7,23}. Data were analyzed using the Statistical Package for Social Sciences (SPSS) 20.0 for Windows. A p value less than 0.05 was considered statistically significant, and a 95% confidence interval (CI) was used.

Ethical approval

This study was approved by the Research Ethics Committee of Universidade Federal de Santa Maria (registration number 38850614.4.0000.5346).

RESULTS

The 16S rRNA gene was amplified in all 176 CoNS isolates, and 41.5% (73/176) were positive for *icaA*, *icaC*, or *icaD* by PCR; 30.1% (53/176) carried *icaACD*, 11.4% (20/176) carried *icaAD*, and none of the isolates expressed *icaA*, *icaC*, or *icaD* alone (**Figure 1**).

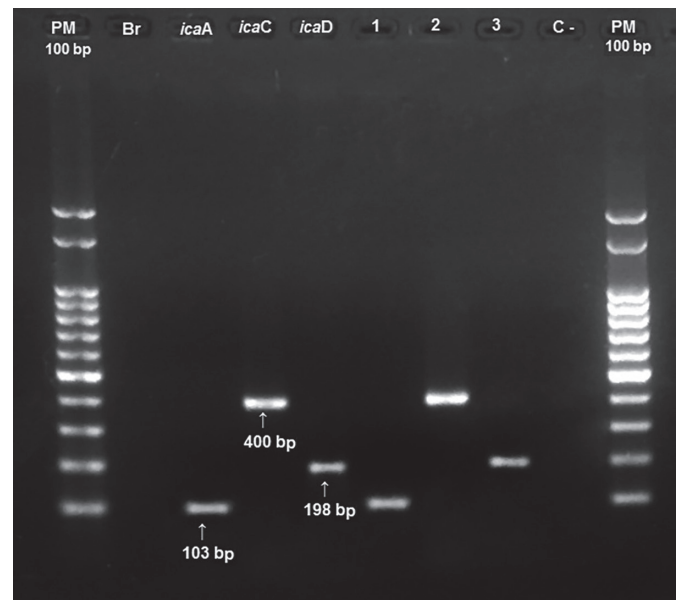


FIGURE 1: Presence of the *icaA*, *icaC*, and *icaD* genes in CoNS isolates from newborn blood cultures. Br = White; *icaA* = *icaA*-positive control; *icaC* = *icaC*-positive control; *icaD* = *icaD* positive control; 1 = *icaA*-positive isolate; 2 = *icaC*-positive isolate; 3 = *icaD*-positive isolate; C- = Negative control (*S. epidermidis* ATCC 12228); PM 100bp = DNA ladder.

On CRA, 42% (74/176) of the isolates showed black-colored colonies, indicative of biofilm production, and 58% (102/176) showed red-colored colonies, indicating no biofilm production (**Figure 2**). The TM showed that 38.6% (68/176) of isolates adhered to the tube walls and were considered positive for biofilm production, while 61.4% (108/176) of isolates did not adhere to the tube walls and were considered negative for biofilm production (**Figure 3**). In the TCP assay, 40.9% (72/176) of

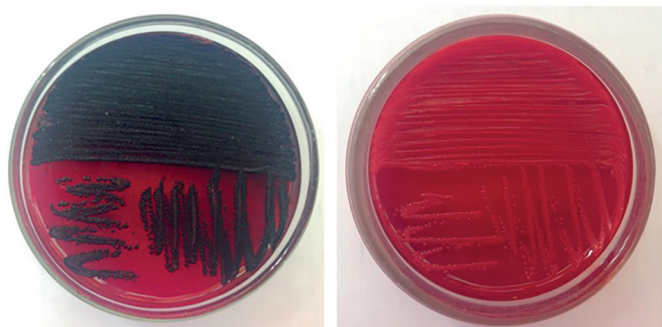


FIGURE 2: Biofilm production in CoNS isolates from newborn blood cultures as assessed by the congo red agar (CRA) assay. Left, a biofilm-producing isolate; right, a non-biofilm-producing isolate

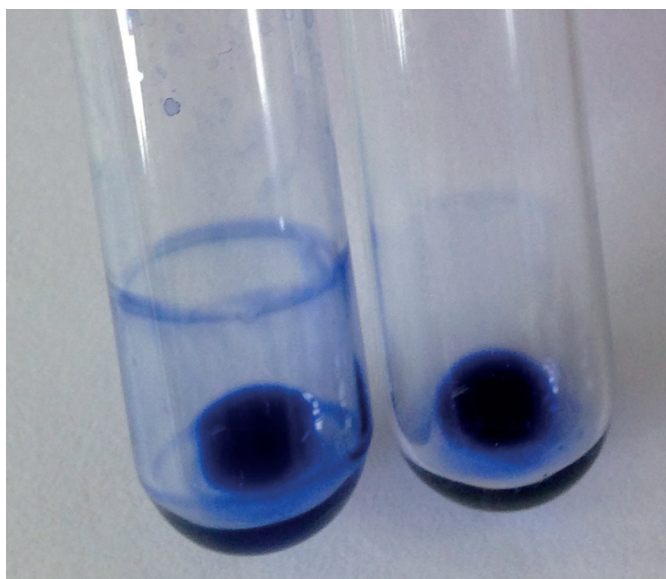


FIGURE 3: Biofilm production in CoNS isolates from newborn blood cultures as assessed by the tube method (TM). Left, a biofilm-producing isolate; right, a non-biofilm-producing isolate.

TABLE 2: Biofilm production in CoNS isolates from newborn blood cultures (n) as assessed by phenotypic techniques.

Species	PCR	CRA	TM	TCP
<i>S. epidermidis</i>	49	52	48	48
<i>S. warneri</i>	6	5	6	6
<i>S. capitis</i>	5	7	4	5
<i>S. haemolyticus</i>	4	3	3	4
<i>S. hominis</i>	3	2	2	3
CoNS	2	2	1	2
<i>S. lugdunensis</i>	1	1	1	1
<i>S. saprophyticus</i>	1	1	1	1
<i>S. xylosum</i>	1	1	1	1
<i>S. cohnii</i>	1	-	1	1
Total	73	74	68	72

PCR: polymerase chain reaction; **CRA:** congo red agar; **TM:** tube method; **TCP:** tissue culture plate; **CoNS:** coagulase-negative staphylococci.

isolates produced biofilms; 21% (37/176) were weakly adherent, and 19.9% (35/176) were strongly adherent. Of the weakly adherent isolates, 78.4% (29/37) expressed *icaAD*, and 21.6% (8/37) expressed *icaACD*. All strongly adherent isolates (35/35) expressed all three genes (*icaACD*).

In our study, CRA showed 79% sensitivity (95% CI, 70–88), 84% specificity (95% CI, 76–92), 82% accuracy, and good agreement (Kappa = 0.64); the TM assay showed 78% sensitivity (95% CI, 69–87), 89% specificity (95% CI, 82–96), and 85% accuracy, and good agreement (Kappa = 0.83); and the TCP method showed 99% sensitivity (95% CI, 97–100), 100% specificity (95% CI, 99–100), 99% accuracy, and excellent agreement (Kappa = 0.99), when compared to the gold standard, PCR.

Among the isolates that were positive for *icaACD* or *icaAD* by PCR, *S. epidermidis* was the prevalent species (67.1%, 49/73), followed by *S. warneri* (8.2%, 6/73). **Table 2** shows the number of biofilm-producing CoNS species that were positive by the four tested techniques (PCR, CRA, TM, and TCP). **Table 3** shows the percentages of biofilm-producing and non-biofilm-producing isolates that were resistant to each tested antimicrobial. All isolates in this study were sensitive to linezolid, tigecycline, and vancomycin.

DISCUSSION

In this study, we analyzed the biofilm-formation ability of 176 CoNS isolates by four different techniques. Among the 176 isolates, 41.5% of were positive for *icaA*, *icaC*, or *icaD* by PCR; 30.1% expressed *icaACD*, and 11.4% expressed *icaAD*. Oliveira and Cunha⁷ compared different methods for detecting biofilm formation in CoNS isolates from clinical specimens of newborns and the nasal cavity of healthy people in Botucatu, São Paulo, Brazil. They found that 82% of isolates were positive for *icaA*, *icaC*, or *icaD* by PCR; 40% carried *icaAD*, and 42% carried all three genes (*icaACD*). These percentages were higher than those in our study. Zalipour et al.¹² also assessed this virulence determinant in *Staphylococcus* spp. obtained from different clinical specimens collected in two school hospitals in Iran, and also found a higher rate than that in our study, as 81.9% of the *S. epidermidis* isolates expressed *icaAD*.

Regarding the phenotypic methods, 42% of the isolates showed biofilm production by the CRA assay. Similar results were reported by Öcal et al.¹¹ for CoNS isolates in samples from nasal cavities, catheters, and blood cultures of patients admitted to a hospital in Turkey; 40.3% of isolates produced biofilms by the CRA technique. In contrast, Oliveira and Cunha⁷ and Zalipour et al.¹² reported higher rates of biofilm formation on CRA, with 73% of CoNS and 70.8% of *S. epidermidis*, respectively. Hassan et al.²⁴ assessed microorganisms isolated from different clinical materials in a hospital in Pakistan, and only 10% of the tested bacteria were biofilm producing using the CRA technique.

In this study, 38.6% of isolates were considered positive for biofilm formation by the TM. Hassan et al.²⁴ and Oliveira and Cunha⁷ found somewhat higher values using TM, with ~49% and 82% biofilm-producing isolates, respectively.

TABLE 3: Percentages of biofilm-forming and non-biofilm-forming* CoNS isolates from newborn blood cultures showing resistance to tested antimicrobials.

Antimicrobials	n	Resistant biofilm producers n = 73	n	Resistant non-biofilm producers n = 103
Ciprofloxacin	51	69.9%	41	39.8%
Clindamycin	30	41.1%	10	9.7%
Erythromycin	44	60.3%	42	40.8%
Gentamicin	45	61.6%	41	39.8%
Oxacillin	71	97.3%	62	60.2%
Penicillin	73	100%	49	47.6%
Trimethoprim/ Sulfamethoxazole	41	54%	31	30.1%

*Biofilm production was assessed with the gold standard technique (PCR).

Using TCP, 40.9% of our isolates were determined to be biofilm producing; 21% were weakly adherent, and 19.9% were strongly adherent. Oliveira and Cunha⁷ observed higher indices, and 81% of the isolates were biofilm producing by TCP; 35% were weakly adherent, and 46% were strongly adherent. Rani et al.²⁵ assessed biofilm formation in *S. epidermidis* obtained from clinical specimens in a hospital in India using TCP, and all isolates produced biofilm. In contrast, Hassan et al.²⁴ reported a value closer to that in our research, with 54.5% biofilm-producing isolates.

In the present study, of the weakly adherent isolates, 78.4% carried *icaAD* and 21.6% carried *icaACD*, whereas all the strongly adherent isolates carried all three genes. None carried one *ica* gene alone. Oliveira and Cunha⁷ reported that only 56.5% of the strongly adherent isolates contained all three genes (*icaACD*). These studies demonstrate that the presence of the *icaACD* genes is an important virulence determinant in clinical isolates of CoNS, since their expression is associated with the production of PIA, a major component of biofilms in *Staphylococcus* spp.⁷. We assumed that the presence of only one of the genes in the *ica* operon is not sufficient for biofilm formation²⁶.

The expression of *icaA* alone induces low enzymatic activity, as these strains typically produce only small amounts of PIA. The presence of *icaD* favors the production of polysaccharide, and larger chains are synthesized in the presence of *icaC*. The expression of *icaAD* or *icaACD* stimulates increased biofilm production; whereas *icaB* appears to function as a deacetylase for the formation of PIA^{7,11,13,27}. In addition to this *ica* operon-related mechanism of biofilm formation, biofilms can also be formed through protein adhesion mediated by surface proteins, such as biofilm-associated protein (*Bap*), its homologs *Bhp* and accumulation associated protein (*Aap*), and extracellular matrix binding protein (*Embp*), via cell adhesion and accumulation²⁸⁻³⁰.

In our study, the CRA assay showed 79% sensitivity, 84% specificity, and 82% accuracy (kappa = 0.64); the TM assay showed 78% sensitivity, 89% specificity, and 85% accuracy (kappa = 0.68); and the TCP assay showed 99% sensitivity, 100% specificity, and 99% accuracy (kappa = 0.99), when compared to the gold standard, PCR. Oliveira and Cunha⁷

compared the three techniques used in our study (CRA, TM, and TCP) to the gold standard technique (PCR) for strains that carried the biofilm-producing genes *icaACD*. They observed 100% sensitivity and specificity for the TM, 89% sensitivity and 100% specificity for the CRA assay, and 96% sensitivity and 94% specificity for the TCP assay⁷. Thus, the authors concluded that the TM is the best method for biofilm detection due to its high sensitivity and specificity⁷. In our study, the CRA and TM assay failed to show consistent results. Thus, we concluded that TCP is the most suitable method for routine laboratory testing.

According to some researchers, the CRA assay is easier to implement than other methods; however, it is not recommended for biofilm detection in routine laboratory testing since it can yield false positive or false negative results^{7,31}. In fact, a study conducted in India showed that the TM cannot be used as a general screening test to identify biofilm-producing strains³². However, Oliveira and Cunha⁷ recommended this qualitative method (TM) for biofilm research because it is a low cost method that yields reliable results. Although the TCP method is now considered the gold standard by some researchers^{24,25,32}, as it is time-consuming, it is not widely used in routine laboratories⁷.

Among the biofilm-producing CoNS species detected by the standard technique (PCR), *S. epidermidis* was the most prevalent, at 67.1%, followed by *S. warneri* (8.2%). Oliveira and Cunha⁷ reported *S. epidermidis* as the most prevalent species, which was present at a higher rate than in our study⁷. The fact that *S. epidermidis* shows the highest rate of biofilm production capacity may be due to special mechanisms, such as their ability to colonize the surface of medical devices, resilience, and because they colonize the skin of babies as soon as they are born. Furthermore, among CoNS, *S. epidermidis* is the species most frequently isolated from neonatal infections, and it is frequently detected in infections associated with invasive devices^{7,13,15,25}.

In this study, high rates of resistance to penicillin, oxacillin, and clindamycin were detected among the biofilm-producing isolates. Research has shown that biofilm formation is associated with an increase in microbial resistance, and the acquisition of

resistance characteristics via gene transfer is possible within biofilms³³⁻³⁵.

Our results showed that approximately 42% of CoNS isolates were positive for *icaA*, *icaC*, or *icaD* by PCR, and strains containing all three genes (*icaACD*) showed a greater capacity to form biofilms. Regarding the phenotypic methodologies, the TM and CRA assay presented low of sensitivity and specificity values, and are not recommended for routine laboratory use. Thus, although the quantitative TCP method is a time-consuming technique, it would be ideal for verifying biofilm production in nosocomial CoNS isolates.

This study has some limitations. First, even in the presence of *ica* genes, a strain may not form a biofilm *in vitro* due to non-expression of these genes. Conversely, some isolates may produce biofilm, even in the absence of the *ica* genes, which could interfere with the sensitivity and specificity. In addition, it is known that biofilm production can be induced via other *ica*-independent genes, such as *Bap*, *Bhp*, *Aap*, and *Embp*, which were not examined in this study.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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