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Modification of the Congo red agar method to detect biofilm production by *Staphylococcus epidermidis*☆

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ABSTRACT

Staphylococcus epidermidis in immunocompromised patients can cause bacteremia related to the use of catheter due to biofilm production. There are different phenotypic methods to detect biofilm formation. One method is based on culture in brain heart infusion agar (BHIA) containing sucrose and red Congo dye (original Congo red agar). Our group created a new CRA formula and we have confirmed its capacity to detect biofilm production in 210 *S. epidermidis* strains, including 76 (36.2%) *icaAB* gene–positive strains. Other parameters were also evaluated. The new CRA formula that gave the best results was BHIA with sucrose (5%), Congo red (0.08%), NaCl (1.5%), glucose (2%), and vancomycin (0.5 mg/mL) (vancomycin-modified CRA—CRAmod). The CRAmod plus vancomycin may be a promising tool and can help to determine the real participation of *S. epidermidis* in the infectious process.

1. Introduction

Staphylococcus epidermidis is the most common coagulase-negative *Staphylococcus* (CNS) isolated from health care–associated infections, especially catheter-associated bacteremia and cardiovascular infections. The pathogenesis of these infections depends on the ability of the *S. epidermidis* strain to adhere onto the surface by producing an exopolymer that forms a multilayer structure known as biofilm (Aparna and Yadav, 2008; Paul and Michael, 2011).

Polysaccharide intercellular adhesin (PIA) mediates intercellular adhesion in clinical *S. epidermidis* isolates. PIA synthesis is mediated by *ica* operon, which is made up of the regulatory *icaR* gene and the *icaA*, *icaD*, *icaB*, and *icaC* genes (Fitzpatrick et al., 2005; Paul and Michael, 2011). Several studies have shown that different chemical substances or physical parameters affect the biofilm expression, such as NaCl concentration and presence or absence of oxygen (Mariana et al., 2009; Rachid et al., 2000; Stepanovic et al., 2003).

Since *S. epidermidis* is a natural inhabitant of the human microbiota, isolation of this species from clinical specimens requires differentiation of the clinical infection agent from the contaminant. This differentiation is important for the definitive diagnosis of catheter-related infections, since it can lead to a decision to remove a surgical device or change the treatment. However, such a diagnosis is hampered by the controversy over the criteria on how to determine a true bacteremia as the occurrence of false-positive results related to skin contaminants may be involved (Falagas et al., 2008; Rogers et al., 2009).

Investigation of staphylococcal biofilm can be carried out using various phenotypic methods. The Congo red agar (CRA) test developed by Freeman et al. (1989) is based on the subculture of the bacterial strains on brain heart infusion agar (BHIA), supplemented with sucrose and Congo red dye. Studies have demonstrated that this method has low accuracy, but it is cheap and easy to perform and the evaluation criteria is based on visual analysis of the color of the colonies that grow on the agar (Afreenish et al., 2011; Liberto et al., 2007). The addition or substitution of some substances or the modification of some parameters can increase the accuracy of this method as has been demonstrated for the microtitulation polystyrene test (Los et al., 2010; Mariana et al., 2009). The aim of this present study was to change the composition of the Congo red agar medium and

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incubation parameters in order to improve the accuracy of detecting biofilm produced by various *S. epidermidis* strains.

2. Materials and methods

2.1. Bacterial strains

A total of 210 *S. epidermidis* strains isolated from blood, catheter tip, and nasal secretion were evaluated. Three reference *S. epidermidis* strains were used: 1) a biofilm producer and *icaAB* genes positive (ATCC 35984), 2) a non-biofilm producer and i*caAB* genes positive (HAM 892—isogenic mutant of ATCC 35984), and 3) a non-biofilm producer and *icaAB* genes negative (ATCC 12228). The strains were stored at −20 °C in tryptic soy broth (TSB) (Difco Laboratories, Maryland, USA) with 20% glycerol.

2.2. Original CRA (CRAori) test

S. epidermidis strains were cultivated on BHIA (Difco) with 0.08% (w/v) Congo red (Sigma-Aldrich, Germany) supplemented with 5% (w/v) sucrose (Dinamica, SP, Brazil) [10]. The strains were inoculated in streaks and incubated at 35 °C under aerobic conditions for 24 and 48 h. The staphylococci biofilm producer strains formed black colonies, while the non-biofilm producer strains formed red colonies. The stains were also incubated at 35 °C under microaerophilic conditions and were evaluated after 24 and 48 h (Cotter et al., 2009).

2.3. Modified CRA (CRAmod) test

Different concentrations of NaCl (1.5%, 3%, 4%, 5%, and 7%) and glucose (1%, 2%, and 3%) were evaluated separately and together to establish the base formula of CRAmod (Rachid et al., 2000). Table 1 shows the other substances that were added to the CRAmod. The plates were incubated at 35 °C under both aerobic and microaerophilic conditions for 24 and 48 h.

2.4. Spot inoculations using the CRA method

Besides the inoculation of strains in streaks on the original or modified CRA plates, spot inoculation was also evaluated. A 4-μL aliquot of a bacterial suspension with 10^8 CFU/mL was inoculated in a spot and incubated at 35 °C under aerobic and microaerophilic conditions for 24 and 48 h. Ten strains were inoculated per plate.

The results were checked by 2 observers, and the experiments with CRA were performed at least twice.

2.5. Detection of icaAB and species-specific genes by multiplex PCR

Rapid DNA extraction was performed according to Schuenck et al. (2008). The test was performed according to Iorio et al. (2011a, 2011b) with modifications. The amplification was performed in a programmable thermal controller (Eppendorf Mastercycler Gradient, Eppendorf, Hamburg, Germany) using a 25-μL polymerase chain reaction (PCR) mixture containing: 3μL of lysed DNA, 250 μmol/L of each deoxynucleotide triphosphate (dATP, dGTP, dCTP, and dTTP) (Life Technologies, São Paulo, Brazil), 2.5 μL of 10× enzyme buffer (10 mmol/L Tris HCl, 25 mmol/L KCl), 3 mmol/L $MgCl_2$, and 1.0 U of Taq DNA polymerase (Biotools, Madrid, Spain). The following primers were used: SEpF SEpR to detect a *recN* fragment of 218 bp of *S. epidermidis* (0.4 μmol/L each) and icaAB-Fe (5′TTATCAATGCC-GAGTTGTC3′) and icaAB-Re (5′GTTTAACGCGAGYGCGCTTAT3′) (0.1 μmol/L each) to detect a 546-bp fragment of the *icaAB* genes. Cycling conditions were as follows: initial denaturation at 94 °C for 3 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 2 min, followed by a final extension step at 72 °C for 5 min. Amplified products were analyzed by electrophoresis on 2.0% agarose gel.

2.6. Statistical analysis

The Kappa test was used to analyze the level of agreement between methods. The criteria proposed by Landis and Koch (1977) were adopted for the assessment of agreement by the Kappa test. The Stata software v. 9.0 (Stata Corporation, College Station, TX, USA) was used for the calculations.

3. Results

All 210 strains were confirmed by PCR as *S. epidermidis*, and *icaAB* genes were detected in 76 (36.2%) of the strains. Table 2 shows the concordant results between the CRAori method and the presence of *icaAB* genes. Under aerobic incubation at 35 °C for 24 h, 39 (51.3%) strains that were carrying the *icaAB* genes produced biofilm, showing colonies with colors ranging from brown to black (Fig. 1). After 48 h of incubation, the number of isolates that produced biofilm in 24 h decreased to 20 (26.3%). In microaerophilic incubation, 11 (14.5%) and 4 (5.3%) strains produced biofilm at 24 and 48 h, respectively. All *icaAB*-negative strains showed colonies with colors ranging from red to dark red, being classified as non-biofilm producers (Fig. 1). Control strains ATCC 12228 and HAM 892, used as negative control strains, showed red colonies, being classified as non-biofilm producers, while the ATCC 35984 strain showed brown colonies after 24 h in aerobic and microaerophilic incubation. The same colony color was observed after 48 h for both incubations, except for the ATCC 35984 strain which showed a red colony.

Initially, inoculation onto CRAori plates was done by streaking; however, analysis of the colony colors along the streaks hampered the classification for some strains due to subtle color variations between the colonies (Fig. 2). Therefore, we evaluated spot inoculation and a more uniform color was generated making the interpretation easier (Fig. 1). Based on this result, all the other experiments using CRA plates were inoculated in spots.

Table 3 shows the results of the addition of substances to the CRAmod formula for the different incubation atmospheres. The main colors were black or brown, indicating the biofilm producers, and red or dark red for the non-biofilm producers. To analyze the effect of these substances in the CRAmod, the following clinical strains were

Table 1
Substances added to the original CRA with 1.5% NaCl and 2% glucose (CRAmod).

Substance added to CRAmod (concentration)
CaCl (0.01 mol/L) [15]
CaCl (0.02 mol/L) [15]
Mg (0.02 mol/L) [15]
Vancomycin (0.5 μg/mL)
Vancomycin (0.5 μg/mL) + Mg (0.02 mol/L)
Vancomycin (0.5μg/mL) + CaCl (0.01M)

Table 2
Relation between original Congo red agar (CRAori) method and *icaAB* genes detection in 210 *S. epidermidis* strains evaluated.

Atmosphere/time of incubation	% positive results among *icaAB* positive ($n = 76$)	% negative results among the *icaAB* negative ($n = 134$)
Aerobic/24 h	51.3% (39)	100% (134)
Aerobic/48 h	26.3% (20)	100% (134)
Microaerophilic/24 h	14.5% (11)	100% (134)
Microaerophilic/48 h	5.3% (4)	100% (134)

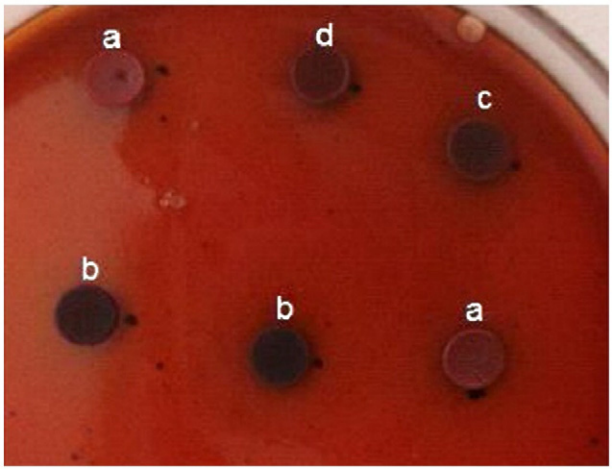

Fig. 1. Colony colors with CRA. (A) Red colonies, non-biofilm producers; (B) black colonies, biofilm producers; (C) brown colonies, biofilm producers; (D) dark red colonies, non-biofilm producers.

initially used: 143 (*icaAB*-negative/non-biofilm producer by CRAori), 252 (*icaAB*-positive/biofilm producer by CRAori), and 159 and 487 (*icaAB*-positive/non-biofilm producer by CRAori). The reference strains ATCC 35984 and HAM 892 were also evaluated. Of all the different concentrations of NaCl and glucose evaluated, only NaCl at 1.5% and glucose at 2%, tested separately, modified the colony color of ATCC 35984 from brown (in CRAori) to black. However, these substances together were able to modify the colony color of strain 487 from red (non-producer) to brown (biofilm producer), and this combination became the base of the new CRA formula (data not shown). Different concentrations of Ca^{2+} and Mg^{2+} were evaluated, but all colonies maintained the same color as by CRAmod. Finally, the addition of subinhibitory concentrations of vancomycin (0.5 μg/mL) changed both strains (487 and 159) to biofilm producers. Vancomycin plus Ca^{2+} or Mg^{2+} did not lead to any changes in the colony color. The incubation in the microaerophilic atmosphere did not cause significant changes in the colony color of these strains when compared to incubation in an aerobic atmosphere for any of the formulas.

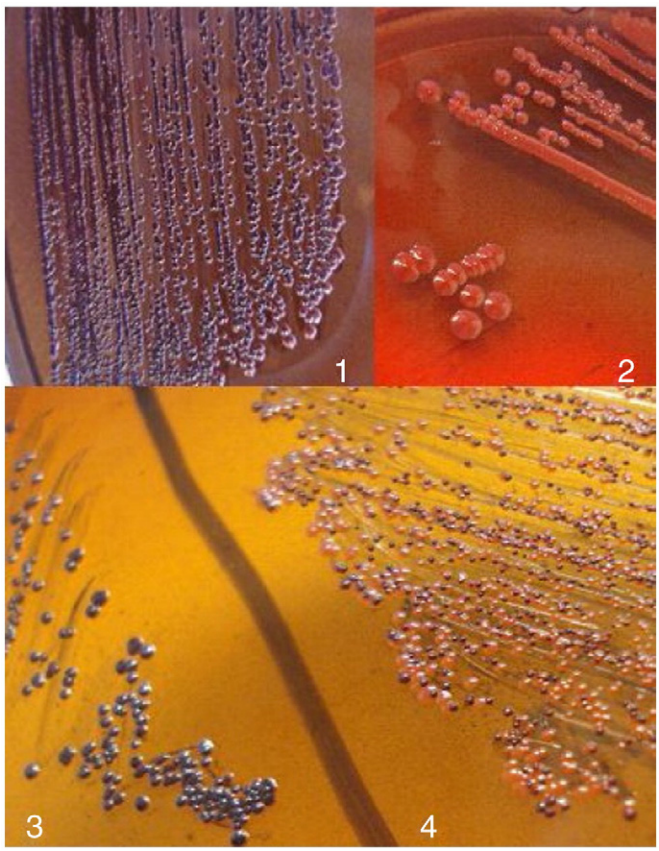

Fig. 2. Comparing the colony colors of 487 and 252 strains grooved onto CRA (1 and 2) and CRA magnesium (3 and 4). (1) Clinical strain 252 (ica positive/positive biofilm with original CRA); (2) clinical strain 487 (ica positive/negative biofilm with original CRA); (3) strain 252 with CRA magnesium; (4) strain 487 with CRA magnesium (colonies with mixed colors; red and black).

The CRAmod plus vancomycin formula was assessed further against 6 other *icaAB*-positive/non-biofilm producers according to CRAori. Five (83.4%) strains changed their phenotype to biofilm producers. Therefore, all the remaining strains were evaluated using this formula (Table 4). This formula showed a high percentage of correlation among biofilm production and the presence of the *icaAB* gene (82.9%) after 24 h of incubation; however, this percentage decreased to about 6% after 48 h. According to the Kappa index, the CRAori formula (aerobic incubation/24 h) was 0.57 when compared with the PCR results, while the CRAmod plus vancomycin (aerobic incubation/24 h) index was 0.86.

4. Discussion

The CRA method is fast, reproducible, and presents an advantage: the colonies remain viable in the medium for further analysis. Therefore the method was chosen in an attempt to improve its ability to identify biofilm production in *S. epidermidis* strains by making changes in the formula and adjusting different physical parameters. The method is easy to carry out and the results are usually based on the colony color produced, which ranges from red for non-biofilm–producing strains to black for biofilm-producing strains. Initially, the strains were inoculated in streaks onto the CRA plates to visualize the individual colonies, but this streaking can also complicate the classification due to variations between the colors of the colonies seen along the streaks. In 2002, Arciola et al. established a colorimetric scale ranging from very red to very black with 6 kinds of nuances—very red, red, bordeaux, almost black, very black, and black—for biofilm production classification (Arciola et al., 2002). However, we could not apply this scale in our study due to the closeness of the colors, which leads to different interpretations. So in order to minimize the difficulty of interpretation, we tested spot inoculation of the strains. This method gave better visualization and easier interpretations, due to the color homogeneity of the spots, especially for the biofilm-producing strains (Fig. 1). Moreover, the agglomeration of a larger number of bacterial cells could influence the biofilm expression since the regulation of associated genes is also based on *quorum sensing* (Kong et al., 2006; Novick and Geisinger, 2008). The other parameters evaluated were 2 incubation atmospheres and 2 incubation periods. The results showed that the CRA aerobic cultivation of 24-h incubation was a better indicator of biofilm production than 48 h of incubation under aerobic and, especially, microaerophilic conditions due to the occurrence of phenotypic reversion. Most of the biofilm-positive strains at 24 h were classified as biofilm-negative strains at 48 h (51.3% of strains with the *icaAB* genes were classified as biofilm producing at 24 h, and only 26.3% and 5.2% of these strains maintained this phenotype after 48 h of incubation under aerobic and microaerophilic conditions, respectively). Our results are in disagreement with Cotter et al. (2009) who showed that a lower percentage of oxygen in the atmosphere increased biofilm production by *S. epidermidis*. However, Stepanovic et al. (2003) found low biofilm production by *S. epidermidis* and *S. aureus* when submitted to a CO_2-rich atmosphere, but no differences were observed between aerobic and anaerobic incubations. Therefore, based on our best results using 24 h of incubation at 35 °C under aerobic conditions and inoculation

Table 3
Results of the colony color of *S. epidermidis* strains grown on base formula (1.5% NaCl + 2% glucose) with CRA and the addition of the different concentrations of calcium, magnesium, and vancomycin.

Atmosphere/time	Strain/*ica* operon	Ca^{2+} (0.01 mol/L)	Ca^{2+} (0.02 mol/L)	Mg^{2+} (0.02 (mol/L)	Vancomycin	Vancomycin + Mg (0.02 mol/L)	Vancomycin + Ca (0.01 mol/L)	Modified CRA	Original CRA
Aerobiosis (24 h)	HAM/+	R	R	R	R	R	R	R	R
	12228/−	R	R	R	R	R	R	R	R
	35984/+	BK	B	B	BK	B	B	BK	B
	143/−	R	R	R	R	R	R	R	R
	252/+	BK	B	BK	BK	B	B	BK	BK
	159/+	R	R	R	BK	B	R	R	R
	487/+	B	R	B	BK	DR	R	B	R
Aerobiosis (48 h)	HAM/+	R	R	R	R	R	R	R	R
	12228/−	R	R	R	R	R	R	R	R
	35984/+	B	R	B	BK	B	B	B	B
	143/−	R	R	R	R	R	R	R	R
	252/+	B	R	B	BK	B	B	BK	BK
	159/+	R	R	R	B	B	R	R	R
	487/+	R	R	R	BK	DR	R	DR	R
Microaerophilic (24 h)	HAM/+	R	R	R	R	R	R	ND	R
	12228/−	R	R	R	R	R	R	ND	R
	35984/+	B	B	R	BK	B	R	ND	B
	143/−	R	R	R	R	R	R	ND	R
	252/+	B	R	B	BK	B	B	ND	B
	159/+	R	R	R	BK	B	R	ND	R
	487/+	DR	R	R	BK	R	R	ND	R
Microaerophilic (48 h)	HAM/+	R	R	R	R	R	R	ND	R
	12228/−	R	R	R	R	R	R	ND	R
	35984/+	B	B	B	B	B	R	ND	R
	143/−	R	R	R	R	R	R	ND	R
	252/+	B	R	B	B	B	R	ND	R
	159/+	R	R	R	B	R	R	ND	B
	487/+	DR	R	R	B	R	R	ND	R

+ = Positive; − = negative; Ca = calcium; Mg = magnesium; R = red; DR = dark red; B = brown; BK = black; ND = not determined.

in spots, we decided to use these parameters to evaluate the new CRA formula.

Rachid et al. (2000) tested different NaCl and glucose concentrations in the growth medium of *S. epidermidis* strains and evaluated biofilm production using the polystyrene test. Their results showed that higher concentrations of NaCl (4% and 5%) and 1.5% and 2% of glucose were the best inducers of biofilm formation. The addition of NaCl concentrations above 2% had no effect on biofilm formation in our study, and only 1.5% NaCl promoted an increase in staining intensity of the ATCC 35984. The same phenotype alteration in strain ATCC 35984 was promoted by the addition of 2% glucose. Combinations of different concentrations of NaCl and glucose were also tested. The CRA-modified method with a combination of 1.5% NaCl with 2% glucose changed the phenotype of strain 487 (a non-biofilm producer according to the CRAori method) to a biofilm producer. This combination was chosen as the basis to evaluate the other substances added to the medium: magnesium, calcium, and vancomycin. Induction of biofilm formation with high concentrations of Ca^{2+} and Mg^{2+} was observed by Akpolat et al. (2003). They suggested that cations promoted biofilm formation by facilitating exopolysaccharide polymerization and cell adhesion by *S. epidermidis*. However, no modifications were observed in the colony color despite the addition of Ca^{2+} or Mg^{2+} and thus BHIA plus 1.5% NaCl, 2% glucose, and 0.08% Congo red was considered to be the base formula for CRAmod.

Table 4
Correlation between the modified Congo red agar (CRAmod) test with vancomycin (0.5 μg/mL) and the presence of *icaAB* genes under aerobic incubation.

Method	% positive results among *icaAB* positive ($n = 76$)	% negative results among the *icaAB* negative ($n = 134$)
CRAmod + vancomycin (24 h)	82.9% (63)	100% (134)
CRAmod + vancomycin (48 h)	76.3% (58)	100% (134)

Some studies have observed that vancomycin has no inhibitory effect on biofilm formation (Mónzon et al., 2002; Rachid et al., 2000). In contrast, other studies have shown that the presence of vancomycin at sub-MIC concentrations or even at higher concentrations (8 μg/mL) induces biofilm formation in *S. epidermidis* (Cargill and Upton, 2009; Qin et al., 2007). In our study, addition of vancomycin at sub-MIC concentration (0.5 μg/mL) to CRAmod led to phenotype change in 64.8% of strains, all of which were classified as non-biofilm producer by the CRAori method and presenting the *icaAB* genes. Combinations of vancomycin with Ca^{2+} and Mg^{2+} were also evaluated, but the results were not satisfactory. Therefore, the best results were obtained with the CRAmod formula containing BHIA + sucrose (5%) + Congo red (0.08%) + NaCl (1.5%) + glucose (2%) + vancomycin (0.5 mg/mL), and we named this media as "vancomycin CRA (CRAvc)". Using the CRAvc formula in aerobic conditions with spot inoculation and a reading at 24 h showed 82.9% positivity and was in "almost perfect agreement" with PCR (kappa = 0.86) and was superior to the other phenotypic tests such as the glass tube adhesion test (Christensen et al., 1985; Stepanovic et al., 2000) and adherence to polystyrene microplates (Christensen et al., 1982) evaluated with the same 213 strains (unpublished data). Still, the sub-MIC concentration of vancomycin in the medium did not significantly alter the growth profile of any strain that showed a visible macrocolony at 24 h of incubation similar to that observed with the original formula. Although the exact mechanism of this phenomenon is unknown, the presence of a minimum concentration of vancomycin probably acts as a stress factor against the bacterial cells, which may lead to some alterations such as cell wall thickening (Nunes et al., 2006) and may induce an increased expression of genes related to biofilm formation (Cargill and Upton, 2009; Gazzola and Cocconcelli, 2008).

CRAvc may be one more tool for the diagnosis of *S. epidermidis* infections in microbiological laboratories. However, in order to further evaluate the usefulness of this method, a larger number of *S. epidermidis* strains freshly isolated from clinical specimens of patients should be analyzed, preferably from the primary isolation

when the strains still retain their virulence characteristics expressed in “in vivo” conditions (Mathur et al., 2006).

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