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RESEARCH ARTICLE

BIOFILM DETECTION IN STAPHYLOCOCCAL STRAINS BY TWO PHENOTYPIC METHODS

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ABSTRACT

Various infections in the hospital are caused by bacterial strains of *Staphylococcus aureus*. Some strains of this species have the capacity to produce biofilm, causing infections related to the contamination of various materials or surgical devices due to their difficult eradication of the infected site. The purpose of this study was to determine the expression of slime or biofilm in strains of *Staphylococcus aureus* carriers of the *ica*A and *ica*D genes by means of two different phenotypic methods to identify the sensitivity of each of them. In the present study, 76 strains from different clinical isolates were evaluated, being found by the gold standard test of detection of biofilm producing strains used by the PCR method by the detection of the *ica*A and *ica*D genes, which was applied to all staphylococcal strains tested. Regarding the gold standard test, the results indicated that the sensitivity by the CRA method with 800 mg/L, was the lowest with 64.0%, but that by increasing the concentration of the dye to 1200 mg/L in the CRA modified, the sensitivity increased to 86%, being almost the same as the highest sensitivity obtained by the detection of biofilm in polystyrene plates by crystal violet as a developer, with a sensitivity of 89%.

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INTRODUCTION

Staphylococcus aureus is a pathogenic bacterium that produces various virulence factors such as toxins, enzymes and extracellular factors including the biofilm that allows it to adhere on host surfaces or inerts such as implants, preventing phagocytosis and its eradication from the organism (Souza, et al. 2011). The biofilm is formed by a community of bacteria within a matrix of exopolysaccharides, whose purpose is to provide protection to the staphylococcus against adverse changes in the environment, allowing them to survive even in extreme conditions, which starts when it adheres to a surface, then produce a polysaccharide called "intercellular adhesion (PIA)", which binds microorganisms together, forming similar micro-colonies fungal mushrooms (Yazdani *et al.,* 2006).

The production of biofilm is due to the expression of the "intercellular adhesion" operon (ica). The "ica" operon is composed of the *ica*R regulatory gene and four *ica* ADBC structural genes. Some studies report that the *ica* A gene codes for N-acetyl-glucosaminyltransferase, an enzyme that forms oligomers from N-acetyl-glucosamine; the *ica*D gene seems to increase the activity of the enzyme N-acetyl-glucosaminyltransferase; *ica* B expresses a deacetylase responsible for the maturation of the PIA; and finally *ica*C, it codes for an enzyme responsible for the externalization and elongation of the PIA ((Yazdani *et al.,* 2006; Götz *et al.,* 2000) .The present work aimed to determine in strains of wild *Staphylococcus aureus* carriers of *icaA* genes and *icaD* the production of biofilm, using two different phenotypic methods to identify the sensitivity of each of them.

MATERIALS AND METHODS

Strains of Staphylococcus aureus: The strains of staphylococci included in the study were donated by the Microbiology and Molecular Biology Laboratory of the Biomedical Research Center of the Autonomous University of Campeche of the City of San Francisco de Campeche. Briefly, the process is described. The strains were seeded in Tryptone Soya Broth (TSB) and incubated for 24 h at 37 °C. Subsequently, salt and mannitol agar (MSA) were striated and incubated for 24 h at 37 °C. During the identification, the strains whose gram stain, catalase test and coagulase were positive were selected to confirm their identification using the automated system Vitek II.

Detection of the slime in Congo Red Agar: The strains tested were cultured in TSB with 1% glucose, until they reached the turbidity of 0.5 of the Mac Farland Scale. Later they were sown in Congo Red Agar (CRA) with modifications to the original technique (52 g/L BHI, 36 g/L dextrose, 800 mg/L or 1200 mg/L of Congo Red dye), and incubated at 37° C for 48 h. The biofilm producing strains acquired a black and star-shaped coloration, while the non-biofilm-producing strains were red and smooth-looking colonies (Núñez *et al.,* 2018).

Detection of biofilm in 96-well plates (Crystal violet): The strains were inoculated in TSB and were expected to reach the turbidity of 0.5 of the Mac Farland scale, subsequently, 20µL of the suspension was taken and added to the sterile polystyrene 96-well microplates, after which it was added to each well. 180µL of TSB supplemented with 1% glucose. The plates were incubated for 18 h at 35 ° C. To remove the non-adherent cells, the walls were washed three times with sterile saline. Adhered bacteria were fixed with methanol for 20 min and allowed to dry at room temperature for 30 min. Adhered bacteria were stained with crystal violet (0.5%) for 15 min. The biofilm was eluted with a solution of hydrochloric acid in 5% isopropanol for 30 min without agitation. Finally, the absorbances were read at 620 nm in a plate reader for ELISA (modified of Souza *et al.,* 2011).

Genotypic detection of icaA and icaD genes: To obtain the DNA of the problem sample of interest, 4 isolated colonies of salt agar and mannitol were taken, suspended in 100 µL of sterile distilled water and heated at 100 °C for 20 min (Martín-López *et al.,* 2004). From each one of the staphylococcal strains evaluated, the DNA was isolated. Subsequently, by means of the PCR reaction and using the corresponding forward and reverse oligonucleotides, a fragment of genes *ica*A and *ica*D were amplified. The product obtained from the PCR reaction was subjected to agarose gel electrophoresis, which was then stained with ethidium bromide. Detection of fragments of genes *ica*A (560 bp) and icaD (229 bp) was done by visualization of the corresponding bands using a UV trasnsilluminator.

RESULTS

This study included 76 strains with positive tests for catalase, coagulase and gram stain. All strains were identified as *Staphylococcus aureus* by the automated system Vitek II. With respect to the production of biofilm in CRA 800 mg/L, 54% that corresponded to 41 strains presented the black coloration and starry appearance characteristic of the strains that produce silt in the culture medium; while in the CRA 1200 mg/L, 72.4% equivalent to 55 strains, were positive to the production of biofilm. The increase of the Congo Red dye to the culture medium allowed a greater detection of biofilm in CRA method by 18.4%. Regarding the method of detection of biofilm in polystyrene plates, it was observed that 75% (57 strains) produced biofilm, being 2.6% more efficient than the CRA method with higher concentration of the dye (Table 1). The gold standard test for the detection of biofilm-producing strains used as a reference was the PCR method by detecting the *icaA* and *icaD* genes, which was applied to all strains of staphylococci tested. The results indicated that 84.2% (64 strains) of the staphylococcal strains included in this study carried in their genome the genes necessary for the production of biofilm. Thus taking the detection of *icaA* and *icaD* genes as the gold standard method for the strains that are capable of producing biofilm, we have that with respect to the production of biofilm in CRA with a concentration of Congo Red dye of 800 mg/L, the sensitivity of the method was 64.0%, which increased to 86% by increasing the concentration of the dye to 1200 mg/L (Table 2). In the case of the biofilm detection method in polystyrene plates, the maximum detection of biofilm producing strains was observed with a sensitivity of 89% of true positivity as a result. However, the above showed that there were staphylococcal strains carrying the genes *ica*A and *ica*D did not express silt or biofilm by any of the two phenotypic methods used.

Table 1. Results obtained from biofilm via cra and pcr

CRA	Isolated number	PCR *icaAD*	
		Positive	Negative
PCR	64	64	00
CRA 800 mg/L	64	41	13
CRA1200 mg/L	64	55	09
PET PLAQUE	64	57	07

Table 2. Sensitivity and specificity of the tests

Test	Sensitivity (%).	Specificity (%).
PCR	100	100
CRA 0.08 %	64	100
CRA 0.12 %	86	100
PLACA PET	89	100

DISCUSSION

The pathogenesis of *Staphylococcus aureus* is attributed to the production of extracellular factors in conjunction with biofilm adhesion and formation. Regardless of whether you are colonizing a catheter or causing sepsis, this bacterium is able to attach firmly and form biofilm. The biofilm gives the bacteria the characteristic of surviving any environmental condition, making it difficult to eradicate it (Donlan and Costerton, 2002). Several studies carried out on catheters and wounds show that staphylococcal strains are generally biofilm producers, which is due to the expression of the *ica*A and *ica*D genes (Núñez *et al.,* 2018; Yazdani *et al.,* 2006); The above coincides with the results obtained with the majority of the staphylococcal strains tested in this study that were biofilm positive by the CRA method and the polystyrene plate. For the determination of slime production in CRA, modifications were made to the generally used medium (52 g/L BHI, 50 g/L dextrose and 800 mg/L Congo red) (Freeman *et al.,* 1989), using a concentration dextrose (36 g/L), as well as the same concentration and a higher concentration of the Congo Red dye (1200 mg/L), in order to evaluate the sensitivity of the

modified medium, disagreeing with results reported by another study (Yazdani *et al.,* 2006). These results demonstrate that the modified CRA (52 g/L BHI, 36 g/L dextrose and 1200 mg/L Congo red), allowed to increase the sensitivity of the medium to detect the biofilm, allowing a screening with better results in the determination of staphylococcal strains that produce this virulence factor. In this study, a high percentage of strains from different clinical isolates expressed biofilm in the phenotypic methods used, which agrees with a recent study (Núñez *et al.,* 2018); where all strains of *Staphylococcus aureus* isolated from nosocomial infections were positive to the production of biofilm. It also suggests that the biofilm for golden staphylococci is an important factor of virulence in these bacteria, which adds to its battery of virulence factors, increasing its pathogenicity, which is why rapid and early detection of the biofilm using techniques Phenotypic diseases such as those shown in this study should be included as a routine test to accompany the isolation and identification of staphylococcal strains isolated from nosocomial infections. It is also important to consider that the use of phenotypic techniques such as CRA or polystyrene plate, even when the staphylococcal strains are carriers of the *ica*A and *ica*D genes, does not imply that they express the biofilm, as with some strains found in this study or in other reports (Yazdani *et al.,* 2006; Nuryastuti *et al.,* 2008). Thus, the negative regulation of the expression of biofilm in wild staphylococcal strains to avoid expressing the silt or biofilm in the phenotypic methods used, requires further studies to elucidate this unknown.

Conclusion

A large number of strains of this study carry *ica*A and *ica*D genes in their genome, which suggests that biofilm production is an important virulence factor for this bacterium. The sensitivity of modified CRA with a concentration of Congo red dye increased the sensitivity of the method, up to almost the sensitivity observed for the polystyrene plate method with crystal violet, which was the phenotypic method with the highest sensitivity observed in our work.

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