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Research Paper

Comparison of methods for the detection of biofilm formation by *Staphylococcus aureus* isolated from bovine subclinical mastitis

Poliana de Castro Melo[1], Luciano Menezes Ferreira[1], Antônio Nader Filho[1], Luiz Francisco Zafalon[2], Hinig Isa Godoy Vicente[3], Viviane de Souza[4]

[1]Departamento de Medicina Veterinária Preventiva,
Universidade Estadual Paulista "Júlio de Mesquita Filho", Jaboticabal, SP, Brazil.
[2]Embrapa Pecuária Sudeste, São Carlos, SP, Brazil.
[3]Secretaria da Agricultura do Estado de São Paulo, Jaboticabal, SP, Brazil.
[4]Embrapa Caprinos e Ovinos, Sobral, CE, Brazil.

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Abstract

Biofilm formation is considered to be a selective advantage for *Staphylococcus aureus* mastitis isolates by facilitating bacterial persistence in the udder. It requires attachment to mammary epithelium, proliferation and accumulation of cells in multilayers. The objective of this study was to determine the sensitivity and specificity of three techniques for the detection of *S. aureus* biofilm-positive strains. Two phenotypic tests, including growth on microtitre plates and Congo red agar, were compared with a PCR technique using 94 *S. aureus* strains obtained from cows with subclinical mastitis from two farms in the state of São Paulo. These strains were characterised by *in vitro* slime production on Congo red agar, biofilm formation on microtitre plates and the presence of the *ica*A and *ica*D genes. The results revealed that 85% of the isolates tested produced slime on the Congo red agar, 98.9% of the isolates produced biofilms *in vitro* by adhering to sterile 96-well "U" bottom polystyrene tissue culture plates, and 95.7% of the isolates carried the *ica*A and *ica*D genes. The results of the phenotypic tests for biofilm formation were compared with those of the molecular analysis, and the sensitivity and specificity of the Congo red agar test were 88.9% and 100%, respectively, while those of the microtitre plate test were 100% and 25%, respectively. When the phenotypic methods for the detection of biofilm producers, namely growth on microtitre plates and Congo red agar, were compared, the sensitivity and specificity were 86% and 100%, respectively. Therefore, growth on Congo red agar and the microtitre plate test are methods that could be used to determine whether an isolate has the potential for biofilm production.

Key words: biofilms, bovine mastitis, phenotypic and molecular analysis, *Staphylococcus aureus*.

Introduction

Staphylococcus aureus is an important etiologic agent of intramammary infections in ruminants and, most *S. aureus* strains associated with mastitis are found surrounded by a layer of slime. This layer allows the pathogen to adhere to and colonise the host mammary gland epithelium (Baselga *et al.*, 1993; Bergey & Holt, 1994; Aguilar & Iturralde, 2001).

The two methods broadly used for the phenotypic identification of biofilm-producing strains are the microtitre-plate test (MtP) (Christensen *et al.*, 1985; Christensen, 1989) and the Congo red agar (CRA) test (Freeman *et al.*, 1989). The MtP was developed to replace the test tube method, which was the first method used for macroscopic estimation of bacterial biofilm on the surface of plastic tubes. The microtitre-plate technique uses a 96-well-plate spectrophotometer to measure the optical density (O.D.) of stained bacterial biofilms found on the bottom of tissue cul-

Send correspondence to P.C. Melo. Departamento de Medicina Veterinária Preventiva Universidade Estadual Paulista "Júlio de Mesquita Filho", Jaboticabal, SP, Brazil. E-mail: policame@yahoo.com.br.

ture plates and produces quantitative results (Stepanovic *et al.*, 2000; Arciola *et al.*, 2005).

The CRA plate test uses a solid medium, namely Congo red agar. This method allows for the direct analysis of the colonies and the identification of slime-forming strains (which appear as black colonies on the red agar) and non-slime-forming strains (red-coloured colonies). This is not a quantitative assay because it is based on a subjective chromatic evaluation. The strains that score positive during the test have black spikes on red colonies which remain unchanged in colour (Freeman *et al.*, 1989).

The implications of biofilm formation for infections and drug resistance have triggered an increased interest in the characterisation of the genes involved in biofilm formation. The intercellular adhesion (*ica*) locus consists of the genes *ica*ADBC, and among the *ica* genes, *ica*A and *ica*D have been reported to play a significant role in biofilm formation in *S. aureus* and *S. epidermidis* (Cramton *et al.*, 1999).

Molecular techniques have recently been used for detecting the genes responsible for the slime exopolysaccharide component of biofilms, also known as polysaccharide intercellular adhesin (PIA). PIA is made up of a linear 1,6-linked glycosaminoglycan and synthesised *in vitro* from UDP-*N*-acetylglucosamine by *N*-acetylglucosaminyltransferase, which is encoded by the intercellular adhesion (*ica*) locus, specifically by the *ica*A gene. Coexpression of *ica*A and *ica*D genes leads to the full phenotypic expression of the capsular polysaccharide (Vasudevan *et al.*, 2003).

Early detection and management of potentially pathogenic staphylococci is an essential step towards prevention and management of bovine mastitis. Therefore, there is a need to evaluate a simple and cheap method for the detection of biofilm producers. Biofilm production can be a marker of virulence and, can be detected by phenotypic assays (Baselga *et al.*, 1993; Dhanawade *et al.*, 2010).

Herein, we evaluated strains of *S. aureus* isolated from cows with subclinical mastitis by the CRA, MtP and PCR analysis. Our aim was to determine the efficacy of each of the phenotypic tests using the PCR test as the gold standard. We also compared the two phenotypic tests (MtP and CRA) each other using the MtP as the standard. The results obtained were used to review and compare the efficacy of three different detection assays for the diagnosis of biofilm staphylococci mastitis.

Materials and Methods

Bacterial strains and storage

Ninety-four *S. aureus* strains were isolated from milk samples collected from dairy cows with subclinical mastitis in two herds in the state of São Paulo. The strains were collected monthly during 12 months, from 2001 until 2002 for the first dairy herd and from 2005 until 2006 for the second dairy herd. The staphylococcal species was identified by biochemical test (Bergey & Holt, 1994; MacFaddin, 1976) and confirmed by PCR analysis (Martineau *et al.*, 1998). The biofilm-producer ATCC 25923 and the biofilm negative ATCC 12228 were used as reference strains.

Phenotypic characterization of biofilm formation by microtitre plate test

Microtitre-plate test was modified (Cucarella *et al.*, 2001). The modification was related with the dilution that according to the author was 1:40 (Cucarella *et al.*, 2001) and in this research the dilution was 1:200. Overnight cultures were diluted 1:200 with Trypticase Soy Broth (TSB, BD,NJ Franklin Lakes) containing 0.25% glucose, and 200 µL per well were seeded in sterile 96-well polystyrene tissue culture plates at 37 °C for 18 h. After three washes in phosphate buffered saline solution (pH 7.2), wells were dried for 1 h at 60 °C and the adherent biofilms were stained with 1% crystal violet for one minute. After rinsing three times with distilled water and subsequent drying at room temperature, the absorbance of the adherent biofilm was measured at 490 nm in a microplate reader (Thermoplate reader, Brasil). Uninoculated wells containing TSB with glucose served as blanks. The blank corrected absorbance values of *S. aureus* strains were used for reporting biofilm production. Strains producing a blank corrected mean absorbance value of > 0.1 were considered as weak biofilm producers, and if the value was higher than 1.0 its was considered a higher biofilm producer. (Mack *et al.*, 2000). Each strain was tested for biofilm production in duplicates and the assay was repeated three times.

Phenotypic characterization of biofilm production in CRA

The strains were cultured on CRA plates, prepared by adding 0.8 g of Congo red and 36 g of saccharose (both from Sigma, Missouri, EUA) to 1 L of BHI (Oxoid, Basingstoke, Hampshire, England). The plates were subsequently incubated for 24 h at 37 °C and overnight at room temperature. The production of rough black colonies by slime producing strains was used to differentiate them from non-slime producing *S. aureus* strains (red smooth colonies) (Freeman *et al.*, 1989).

PCR method for the identification of *ica*A and *ica*D genes responsible for PIA synthesis

Isolation of genomic DNA

S. aureus chromosomal DNA isolates were extracted with GFX kit genomic blood (Amersham Biosciences, England).

PCR

The primers for the amplification of *ica*A and *ica*D genes were designed from the published sequence of the *ica*

locus described by Cramton *et al.* (1999). For the detection of *ica*A, primers ICAAF (TCT CTT GCA GGA GCA ATC AA) and ICAAR (TCA GGC ACT AAC ATC CAG CA) were used to amplify a 188 bp fragment. Similarly for amplifying *ica*D, primers ICADF (ATG GTC AAG CCC AGA CAG AG) and ICADR (CGT GTT TTC AAC ATT TAA TGC AA) were used for a 198 bp fragment. A 20 µL reaction volume consisted of 2.5 mM MgCl2, 200 mM of each nucleotide, 1 mM of each primer, 1.25 U of Taq polymerase and 100 ng of template DNA. Thirty cycles of amplification, each consisting of denaturation at 92 °C for 45 s, annealing at 49 °C for 45 s and elongation at 72 °C for 1 min, along with a final extension at 72 °C for 7 min were performed in a thermocycler (Eppendorf, USA). The presence and size of the amplified products were confirmed by electrophoresis on 1.5% agarose gel.

Statistical analysis

Statistical analysis was performed using SAS software 2002 (SAS, 2001). The test was used for comparison of sensitivity, specificity, kappa, positive predictive value (PPV) and negative predictive value (NPV) of CRA and MtP method calculated by using Test Diag (Godoy, 1999) (analysis of 22 table). MtP method was used as standard. And for the analysis of CRA and *ica*AD genes and MtP and *ica*AD genes, the molecular analysis (presence of *ica*AD genes) was used as gold standard.

Results

Detection of biofilm production phenotype by microtitre plate test

The microtitre plate test correctly identify both the positive and the negative reference bacterial strains. Ninety-three out of 94 strains (98.9%) were found to be biofilm producers. Only 1 strain was found to be negative by both the microtitre plate test and CRA test, and this strain lacked the *ica*AD genes indicating, it was a biofilm non-producer.

Detection of the biofilm-producing phenotype by the Congo Red Agar test (CRA)

A total of 85% of the strains (n = 80) were producers of rough black colonies, and 15% strains (Freeman *et al.*, 1989) were classified as non-producers (smooth red colonies). The two reference strains, ATCC 25923 and ATCC 12228, were found to be positive and negative, as expected.

PCR detection of *ica*A and *ica*D

Ninety strains (95%) were positive by PCR for both *ica*A and *ica*D genes, as indicated by the sizes of the PCR bands observed (188 bp for *ica*A and 198 bp for *ica*D), whereas four strains were negative by PCR. All of the slime-producing strains were positive for both genes. Among non slime producers, 10 were positive for *ica* genes, which may indicate that these strains actually have the ability to produce slime. All samples that were PCR negative were also negative in the CRA test. Eighty-nine strains (94.6%) were biofilm-producers in the microtitre plate test, which were also positive by PCR. Three isolates tested positive as biofilm-producers in the microtitre plate test but were negative by PCR (Table 1).

Discussion

The ability of *S. aureus* to form biofilms helps the bacterium to survive in hostile environments within the host and is considered to be responsible for chronic or persistent infections (Christensen *et al.*, 1985; Bernardi *et al.*, 2007). Several studies have shown that the formation of slime and biofilms by *S. aureus* and *S. epidermidis* strains causing catheter-associated and nosocomial infections is associated with the presence of the *ica*A and *ica*D genes (Ziebuhr *et al.*, 1997; Arciola *et al.*, 2001, 2002). In this research, the results of a PCR test for the *ica*A and *ica*D genes and phenotypic tests were important to foment studies on mastitis and develop diagnostic tests for biofilm-producing microorganisms.

The Microtitre plate test is a convenient and economical quantitative technique for the identification of critical factors and optimal culture conditions for biofilm forma-

Table 1 - Phenotypic characterisation by CRA and microtitre plate test and genotypic identification on *ica* genes from 94 *S. aureus* strains isolated from the milk of cows with subclinical mastitis in São Paulo state.

	CRA	Microtitre Test	Presence of *ica*A gene	Presence of *ica*D gene	Isolates of *S. aureus*/%	
	Black	Strong Adherence	Positive	Positive	75 (79.7)	
	Black	Strong Adherence	Positive	Positive	5 (5.4)	
	Red	Weak Adherence	Positive	Positive	1 (1.1)	
	Red	Negative	Negative	Negative	1 (1.1)	
	Red	Strong adherence	Positive	Positive	7 (7.5)	
	Red	Strong adherence	Negative	Negative	3 (3.1)	
	Red	Strong adherence	Positive	Positive	2 (2.1)	
Total	-	-	-	-	94 /94	100

tion. This technique is used for direct detection of polysaccharide production because spectrophotometric measurements provide quantitative information on the ability of bacterial strains to rapidly grow while adhering to the substratum. However, it can be less accurate in determining their specific ability to secrete PIA because, while it is a very sensitive test, it has low specificity (Stepanovic *et al.*, 2000).

Previously, the results of CRA test and the adherence to microplates test (MtP) were compared with the presence of the *ica*A and *ica*D genes for strains of *Staphylococcus epidermidis* isolated from medical implants, and the genotypic test was used as the gold standard (Baselga *et al.*, 1993; Arciola *et al.*, 2005). The authors found that 57% of the strains were positive for the *ica*A and *ica*D genes, and three of these strains were negative by the CRA test. It was also verified that 16% of the 66% strains that produced biofilms in the MtP test were negative for *ica*A and *ica*D by PCR. In total, 10% of the 16% of biofilm-positive strains that were *ica*A and *ica*D negative were classified as weak producers of biofilms (weak adherence). The presence of the genes was best correlated with a positive CRA test (Baselga *et al.*, 1993; Arciola *et al.*, 2005; Jain & Agarwal, 2009).

In this study, the microtitre test allowed an easy and quantitative classification of the staphylococcal isolates. Matching results from both CRA and Microtitre plate test were obtained with 81 (87%) of the strains screened. Among the 94 strains tested, a low correlation was found between the results of the PCR-based analysis and the CRA test. This finding indicates that the CRA test produces a high number of false negatives.

The CRA test identified 100% of the negative biofilm producer strains, and 28.6% of the strains identified as negative by the CRA test were actually negative, based on a negative predictive value (NPV) calculation. In total, 88.9% of the strains were positive for the production of biofilm, and the probability that they were actually positive was 100%, based on a positive predictive value (PPV) calculation. Due to the number of false negative results, the negative predictive value was low which indicates reduced sensitivity. However, this test was very specific, even thought it had a low NPV (Table 2). According to the author who developed the CRA test, polysaccharides are the target of the dye. In his article, he described the use of the Congo red dye to verify the presence of polysaccharides on gram negative bacteria of aquatic origin under a light microscope. Using the dye, he was able to verify that the polysaccharides on the bacilli and the staphylococci had a similar staining pattern (Freeman *et al.*, 1989).

In this study, the colour scale for the CRA test, which has been reported previously, was not changed (Ziebuhr *et al.*, 2001). Therefore, plates that had colonies of indeterminate colour (*e.g.*, those that were dark red but were tending towards black) were repeated. Another variable that was tested was the use of sucrose. In some articles, the authors used glucose instead of sucrose, but in the present research, the results were similar for glucose and sucrose (Freeman *et al.*, 1989; Jain & Agarwal, 2009).

Table 2 - Comparison of results between *ica*A and *ica*D gene expression and CRA tests on *S. aureus* strains isolated from milk associated with bovine subclinical mastitis during the period from 2001 until 2006.

Staphylococcus aureus	PCR (*ica*A and *ica*D)			
Congo red agar (CRA)	Positive	Negative	Total	%
Positive	80	0	80	85.10
Negative	10	4	14	14.90
Total	90	4	94	100

Sensitivity - 89%.
Specificity - 100%.
Positive predictive value (PPV) - 100%.
Negative predictive value (NPV) - 28.60%.
Kappa: 0.41 and $p < 0.05$.

The results of the microtitre plate test were compared with those of the CRA test, and the results of the microtitre plate test indicate that it was better than the CRA test in the detection of biofilms *in vitro*, because of it's a higher sensitivity (100%), in detecting the positive strains. Therefore, the microtitre plate test is recommended for routine analysis of strains of *S. aureus* isolated from samples of milk. This test also had a better correlation with the presence of *ica*AD by PCR, which is correlated with the detection of the intercellular polysaccharide that is the major component of biofilm (Table 3).

In a previous study, the microtitre plate test (MtP) and CRA assay were used to identify Staphylococci biofilm-producing strains, using the MtP as the gold standard, and it was determined that the sensitivity and specificity of the Congo red agar assay were 90.63% and 90.6%, respectively, for the detection of *S. aureus* biofilm producers (Jain & Agarwal, 2009). In the present research, the samples were placed on the same microplate in duplicate, and the test was repeated three times. All of the results were very similar; therefore, there was no need to perform the test in triplicate or quadruplicate. We did not test different con-

Table 3 - Comparison of results from *ica*A and *ica*D gene expression and microtitre plate tests on *S. aureus* strains isolated from milk associated with bovine subclinical mastitis during the period from 2001 until 2006.

Staphylococcus aureus	PCR (*ica*A and *ica*D)			
Microtitre plate test (MtP)	Positive	Negative	Total	%
Positive	90	3	93	98.9
Negative	0	1	1	1.1
Total	90	4	94	100

Sensitivity - 100%.
Specificity - 25%.
Positive predictive value (PPV) - 96.70%.
Negative predictive value (NPV) - 100%.
Kappa: 0.39 and $p < 0.05$.

centrations of sugars because 98% of the samples produced biofilms in the microtitre plate test.

In this study, we also compared the results from the CRA and MtP tests using the MtP test as the gold standard, and the results revealed that the sensitivity and the specificity of the CRA test were 86% and 100%, respectively. These results suggest that the CRA test could be successfully used to detect *S. aureus* biofilm-producing strains, but when the CRA and MtP tests were compared with the molecular analysis, the results indicated that the MtP test should be the first choice because this test was more sensitive than the CRA test, identifying all of the biofilm positive strains (Table 4). The Congo red agar test has been previously compared to the microtitre plate test, which was considered to be the gold standard, in a report by Jain and colleagues (2009) published in the Journal of Microbiological Methods (Stepanovic *et al.*, 2000; Jain & Agarwal, 2009).

The presence of the *ica* locus in 95% of the mastitis *S. aureus* isolates confirms its potential role as a virulence factor in the pathogenesis of mastitis in ruminants. Several studies have reported a higher frequency of distribution of the *ica* locus in clinical isolates of *S. epidermidis* than in saprophytic strains, emphasising its utility as a virulence marker (Christensen, 1989; Costerton *et al.*, 1999; Ziebuhr *et al.*, 2001; Arciola *et al.*, 2001, 2002). The biofilm formation by strains that did not have the *ica* genes in this study could be explained by the presence of other genes, such as *bap*, which can compensate for a deficiency of *ica* genes. According to other studies, the *bap* gene in strains isolated from the bovine intramammary gland facilitated biofilm formation and the persistence of *S. aureus* (Cucarella *et al.*, 2001). In the present research, the *bap* gene was not studied.

In a previous study detecting *S. aureus* biofilm producers associated with bovine mastitis, the authors verified that the PCR technique reliably identified the biofilm-producing potential of *S. aureus* strains, which may help in the rapid detection of biofilm-producing Staphylococci. The best correlation of the PCR test with phenotypic tests occurred with the CRA assay and the microtitre plate test (MtP). In this research, the CRA and microtitre plate tests showed results that were significantly correlated with the molecular analysis (Dhanawade *et al.*, 2010).

The ability of *Staphylococcus aureus* to produce biofilm is an important factor affecting the long-term persistence of the bacteria in the mammary gland and can result in chronic mastitis and decreased efficacy of antibiotic therapy. Virulent *S. aureus* strains can be identified by the presence of genes participating in biofilm formation. However, PCR analysis only reveals the genetic predisposition for biofilm formation and expression of *ica* genes thus, the real biofilm formation must be confirmed by additional phenotypic methods.

Table 4 - Comparison of results from CRA and Microtitre plate test on *S. aureus* strains isolated from milk associated with bovine subclinical mastitis during the period from 2001 until 2006.

Staphylococcus aureus	Microtitre plate test (MtP)			
Congo red agar (CRA)	Positive	Negative	Total	%
Positive	80	0	80	85.1
Negative	13	1	14	14.9
Total	93	1	94	100

Sensitivity - 86%.
Specificity - 100%.
Positive predictive value (PPV) - 100%.
Negative predictive value (NPV) - 7.1%.
Kappa: 0.12 and $p < 0.05$.

Conclusion

The MtP method presents higher sensitivity when compared with molecular analysis to identify *S. aureus* biofilm producers. The CRA method should be used as a complementary test because of the higher specificity relative to the MtP method. All of the methods were effective at detecting *S. aureus* biofilm-producing strains, and the two classic phenotypic tests can be reliably used to detect biofilm-producing strains because they are acceptably sensitive and specific.

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