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Año
2017

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INTERNATIONAL JOURNAL OF
VETERINARY AND DAIRY SCIENCES

ORIGINAL RESEARCH ARTICLE
PUBLISHED: 03-July-2017

Biofilm formation ability and genotypic analysis of *Streptococcus uberis* isolated from bovine mastitis

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Abstract

Bovine mastitis is an important problematic in the dairy industry all over the world. *Streptococcus uberis* is a prevalent environmental pathogen implicated in bovine mastitis able to produce biofilm. The aim of this study was assay 28 *S. uberis* collected from cows with mastitis in Argentina in respect to their biofilm formation ability. Detection of biofilm related genes and genetic relationships by Pulse Field Gel Electrophoresis among the isolates were evaluated to determinate correlation with biofilm formation ability. Microtiter plate assay revealed that all of isolates produced biofilm with varied grade. Results showed that 50% of the isolates were moderate biofilm producers. Genotypic analysis of biofilm related genes demonstrated that 78.6% of isolates harbored at least one of the genes tested. Macro restriction analysis revealed 13 different patterns. Overall, the *S. uberis* isolates with the same PFGE patterns showed different biofilm related genes profiles. No correlation between the degree of biofilm produced and biofilm related genes profiles or Pulse Field Gel Electrophoresis patterns was found. Findings of the present study will contribute to improve the knowledge of this important pathogen in order to design appropriate strategies to improve the control and treatment of the disease.

Keywords: *Streptococcus uberis*, mastitis, biofilm, biofilm related genes, Pulse Field Gel Electrophoresis

INTRODUCTION

Bovine mastitis is an infectious disease difficult to eradicate and responsible for severe economic losses in dairy industry [1]. *Streptococcus uberis* is one of the most important environmental pathogen [2]. The capacity to produce biofilm is an important virulence factor for this agent. Biofilm production by intramammary pathogens provides microorganisms a higher protection against the host immune system and the action against antimicrobial agents [3]. Therefore the antibiotic therapy comes unstuck because of the inability to remove the pathogens from the mammary gland, leading to the development of chronic infections. The process of biofilm production is complex and involves several proteins and genes. Different genes have been described related to biofilm formation as *luxS, comX, comEA* and *comEC* [4]. Moreover, *hasA* gene was described as homologous to *icaA* gene of *S. aureus* [5].

A high degree of genetic variation in *S. uberis* was reported by various authors using different techniques [6-9]. Numerous genomic typing methods have been used to determine genetic relationships among bacterial isolates and pulse field gel electrophoresis (PFGE) is the current gold standard method. Molecular studies of genetic relationships based on PFGE have been performed on *S. uberis* isolates [6,10-15].

Reports concerning production of biofilm by mastitis strains have been presented [16,17]. Previously, we evaluate the influence of different factors, additives and bovine milk compounds on biofilm formation, as the presence of the *sua* gene by PCR [18] and the genetic relationships among *S. uberis* isolates by PFGE to determine whether certain PFGE patterns were associated with the most frequent virulence profiles [19]. However, there is no study determining the correlation on biofilm formation ability and genetic identity of *S. uberis*. The aim of this study was assay 28 *S. uberis* isolates collected from cows with mastitis in Argentina in respect to their biofilm formation ability. Detection of biofilm related genes and genetic relationships by PFGE among the isolates were evaluated to determinate

correlation with biofilm formation ability.

From the clinical point of view, it would be significant determine the biofilm production ability of the strains that cause mastitis as their genotypic characteristics, in order to design new and effective strategies of control and treatment.

MATERIALS AND METHODS

BACTERIAL ISOLATES

The isolates were collected from 3 herds located in the central dairy region of Argentina. The isolates were collected from cases of subclinical mastitis and cultured on Tripticase Soy agar (TSA) (Britania) with 5% of sheep blood for 24 h at 37ºC. They were presumptively identified based on colonial appearance, Gram stain reaction and catalase test. Isolates were maintained frozen at –20ºC in Tripticase Soy broth (TSB) (Britania) containing 20% glycerol.

Molecular identification by restriction fragment length polymorphism analysis of 16S rDNA (16S rDNA RFLP) using the restriction enzymes *RsaI* and *AvaII* was carried out according to Khan *et al.* 2003 [12].

A *Staphylococcus epidermidis* biofilm producer, facilitated by the Microbiology laboratory of the Department of Microbiology and Immunology of the National University of Rio Cuarto, was used as positive control for biofilm assays.

BIOFILM FORMATION

The microtiter plate assay (MTP) was carried out in order to determine the biofilm formation as described previously by Moliva et al., 2017 [18] with modifications. Two hundred of each diluted culture (1/100) in TSB was added to a sterile 96-well flat bottom polystyrene plate (Thermo Scientific Inc. Nunc Edge, USA) supplemented with 0.25% of glucose and incubated for 24 h at 37ºC. The plates were washed 3 times with phosphate buffered saline (PBS) in order to eliminate planktonic bacteria. Then, they were fixed during 45 min at 60ºC and stained with Hucker's crystal violet solution (Biopack, Argentina) for 10 min at room temperature. Cristal violet solution was removed and each well was washed 4 times with water. Subsequently, 150 µl of ethanol was added to each well and the optical density at 560 nm (OD_{560}) was measured using a ELISA reader (Labsystems Multiskan MS). Each isolate was tested four times and the assay was repeated on 2 different occasions. Positive and negative controls were included in each plate. A *Streptococcus epidermidis* was used as positive control and TSB broth without bacteria was used as negative control.

The isolates were categorized as follow:

-OD_{560} below the cutoff value, the isolate was negative biofilm producer

-OD_{560} between the cutoff value and 2 times this number, the isolate was weak biofilm producer

- OD_{560} was 2 times the cutoff value, the isolate was moderate biofilm producer

- OD_{560} greater than twice the cutoff value, the isolate was strong biofilm producer

PCR ASSAYS

Three biofilm related genes were analyzed by PCR: *comEA*(competence gene) [4], *luxS*(S-ribosylhomocysteinase) [20] and *hasA* (hyaluronic acid capsule) [21]. The oligonucleotides were synthesized by Promega Corporation, USA. A 50 µl reaction volume consisted of approximately 20 ng template DNA, 1 mM oligonucleotide primers, 0.4 mM of each of the four dNTPs, 1.5 U *Taq* polymerase and 1.5 mM $MgCl_2$. Each isolate were tested at least twice. A negative control was included in each run. PCR products were resolved on 1.2% agarose gel at 90V for 40 min. Gels were stained with GelGreen™ and photographed under UV light with MiniBisPRO gel documentation (BioAmerica, USA).

PULSE FIELD GEL ELECTROPHORESIS

Genetic relationships among *S. uberis* isolates were assessed using PFGE according to Lasagno et al., 2011 [15]. The PFGE patterns were analyzed using the Dice coefficient and the unweighted-pair group method with average linkages (UPGMA).

STATISTICAL ANALYSIS

Statistical analysis was performed using the Infostat program. X^2 test was used to determine correlation. The nominal P value for statistical significance was 0.05.

RESULTS AND DICUSSION

The bacterial isolates collected were identified pheno and genotypically as *S.uberis.* MTP revealed that all of isolates tested produced biofilm with varied grade. None isolate was determined to be negative. Among the *S. uberis,*50% of the isolates were moderate producers, 39.3% were strong biofilm producers, and 10.7% of them were weak producers. Gilchrist 2011[5] showed that *S. uberis* strains were able to form biofilm using a defined medium. In addition, biofilm production depends on grown conditions and can differ among the isolates. However, our results revealed that all the isolates had the ability to produce biofilm in TSB.

The genotypic analysis of biofilm related genes showed

that 78.6% (22/28) of isolates tested in this study harbored at least one of the genes involved in biofilm production. Seven percent of the isolates (2/28) were positive for all of the genes assayed, where as twenty one percent of the isolates (6/28) were negative for the three genes. The occurrence of *luxS, com EA* and *hasA* genes in *S. uberis* isolates were 42.8%, 21.4% and 57%, respectively.

In some pathogenic bacteria, the *luxS* gene, is involved in *quorum sensing* and it was found to be involved in biofilm formation [20]. *comEA* is a competence gene that allows the transformation of genomic DNA acting as receptor for the DNA [22]. Both genes are necessary for biofilm formation. Genes transcribing the *S. uberis* Ica homologues are forth with termed *hasA*. Gilchrist 2011 [5] reported that this gene was the most commonly detected, found in the 66.6% of the strains. The *hasA* gene product is essential for capsule production in *S. uberis* but strains isolated from cases of bovine mastitis displayed variable amounts of hyaluronic acid capsule [21]. In our study, more than the half of the isolates (57%) yielded this gene.

Biofilm related genes were present in different combinations. Eight biofilm related genes profiles were found in the *S. uberis* isolates. The most prevalent profile was *luxS-/comEA-/hasA+*. Data regarding these eight biofilm related genes profiles are summarized in **Table 1**. Compared to a study carried out by Moore 2009 [4], our results showed that a lower percentage of isolates yielded the three genes assayed.

In this study, although a low number of isolates were assayed, detection of biofilm related genes in *S. uberis* isolates was not associated with biofilm production on microtiter plate assay as demonstrated X^2 test (p=0.149, p=0.179 and p=0.109 for *luxS*, *comEA* and *hasA*, respectively). This result is in accordance with Vasudevan *et al.* 2003 [23] who reported a high prevalence of the *ica* genes among *S. aureus* mastitis isolates, but their presence was not always associated with *in vitro* biofilm formation.

Macrorestriction analysis revealed 13 different patterns named fromA to M. **Figure 1** shows the dendrogram produced by the UPGMA algorithm. Seventeen *S. uberis* isolates were grouped in 2 clusters (I and M) with 4 and 13 isolates, respectively, with identical PFGE patterns each other. Overall, *S. uberis* isolates with the same PFGE patterns showed different biofilm related genes profiles. Cluster I grouped 3 moderate biofilm producer isolates, and 2 of them had the same biofilm related gene profile. Thirty eight percent (5/13) of the isolates that grouped in cluster M were strong biofilm producers, whereas 46% (6/13) were moderate biofilm producers. It is important to note that all of the *S. Uberis* isolates negative for the three genes assayed were grouped in this cluster, although they differ in the grade of biofilm production.

Furthermore, no correlation between the degree of biofilm produced and PFGE patterns, analyzed by X^2 test, was found among the *S. uberis* isolates (p=0.352).

Results suggested that the genes assayed may be not essential for biofilm formation, as isolates lacking one or all of these genes were often biofilm producers, suggesting that the production of biofilm isolates may be regulated by others or by different genes. Moreover, the results showed that different PFGE patterns can cause mastitis, as was report previously [15,19]. Our results are consistent with Douglas et al., 2000 [11], McDougall et al., 2004 [13] and Phuetkes et al., 2001 [6]. According to Tomita et al., 2008 [9] it is likely that recombination of genomic DNA between *S. uberis* isolates may take to the genetic variability. However, this variability may be not related to the ability of the strains to form biofilm or to produce disease.

Table 1. Biofilm related genes profiles and MTP assay of *S. uberisisolates*.

Number of isolates (%)	Biofilm related genes			Biofilm gene related profile	MTP[a]		
	luxS	*comEA*	*hasA*		S	M	W
7 (25%)	-	-	+	I	2	4	1
6 (21.4%)	-	-	-	II	3	3	0
5 (17.8%)	+	-	+	III	2	2	1
4 (14.2%)	+	-	-	IV	2	1	1
2 (7%)	-	+	+	V	1	1	0
2 (7%)	+	+	+	VI	1	1	0
1(3,5%)	-	+	-	VII	0	1	0
1(3,5%)	+	+	-	VIII	0	1	0

[a]MTP= Microtiter plate assay. S= strong biofilm producer; M= moderate biofilm producer; W= weak biofilm producer

CONCLUSION

Findings of the present study demonstrated the great ability of *S. uberis* isolates to produce biofilm at different grades. In addition, the isolates displayed dissimilar biofilm related genes profiles and PFGE patterns. From the economic point of view, the routine use of genotyping is not currently feasible in veterinary practice nor in the general control of the dairy herd. Thus, the study of the biofilm forming ability as the genotypic characteristics will contribute to improve the knowledge of this important pathogen in order to design appropriate strategies to improve the control and the treatment of the disease.

ACNOWLEDGMENTS

The authors gratefully acknowledge the financial support

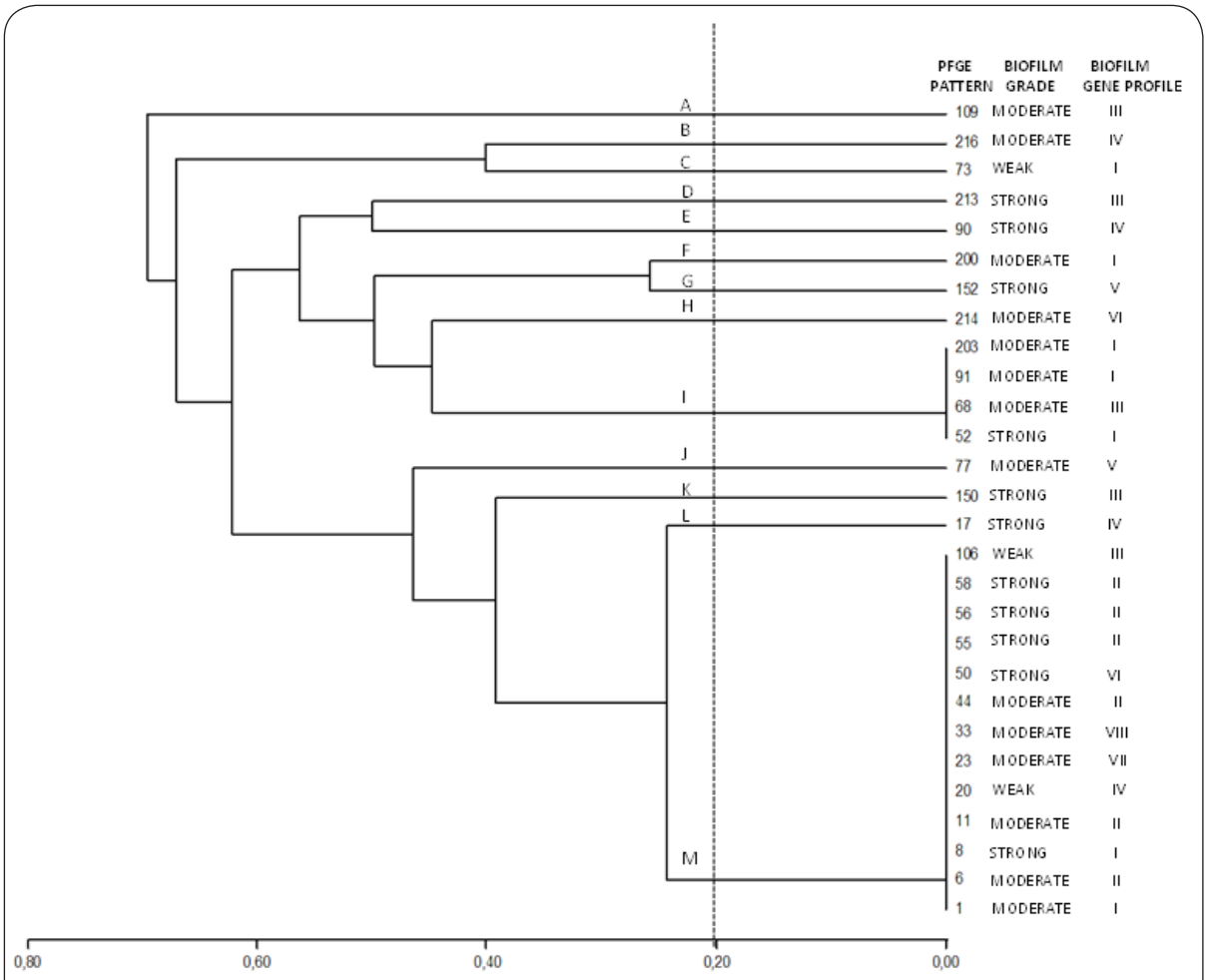

Figure 1. PFGE dendrogram of 28 *S. uberis* isolates digested with *SmaI*. Vertical dashed line indicates cut-off value (set to 80%). Clusters are indicated with letters A to M.

from Consejo Nacional de Ciencia y Tecnología (CONICET). M. Moliva is a fellow doctoral at CONICET. Dr. EB Reinoso is member of the research career of CONICET.

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