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Characterisation of *Staphylococcus aureus* isolated from cases of bovine subclinical mastitis in two Uruguayan dairy farms

Caracterización de *Staphylococcus aureus* aislados a partir de casos de mastitis subclínicas en dos tambos de Uruguay

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SUMMARY

The aim of this work was to identify and to characterise *Staphylococcus aureus* isolates associated with subclinical mastitis obtained from milk of lactating cows showing a California Mastitis Test (CMT) score result of traces, 1, 2 or 3. Coagulase, hemolysis, presence of capsule, slime formation, biofilm production, autoaggregation, hemagglutination and antibiotic susceptibility were assessed to evaluate *S. aureus* virulence factors expression potentially associated to bovine subclinical mastitis isolates. Prevalence of subclinical mastitis along the study was low and did not correlate with months or climatic variables. Most of *S. aureus* (20) were isolated from milk samples showing a CMT score result of 1. Formation of capsule, slime, biofilms and the occurrence of bacteria aggregation in all the tested isolates converged in the bacterial ability of adherence and persistence in the mammary gland and probably contribute to the further chronicity of the infection and even the colonization of dairy installations. Resistance against a set of commonly used antibiotics was low. The evaluation of virulence factors of *S. aureus* isolates in the context of subclinical mastitis in dairy farms may be useful to develop precise actions and treatments to control mastitis and to improve animal health and milk production in dairy bovine herds.

Key words: bovine subclinical mastitis, *Staphylococcus aureus*, virulence factors, Uruguay.

RESUMEN

El objetivo de este trabajo fue evaluar la prevalencia y evolución de mastitis bovina subclínica (MBS) a lo largo de un año en dos tambos comerciales localizados en un área de producción lechera (Sur) de Uruguay e identificar y caracterizar cepas de *Staphylococcus aureus* aisladas de la leche de vacas con resultados de trazas, 1, 2 o 3 correspondientes a la escala del California Mastitis Test (CMT). Producción de coagulasa, presencia de cápsula, producción de limo, formación de biofilms, autoagregación, hemaglutinación y sensibilidad a diferentes antibióticos fueron evaluadas en el caso de las cepas de *S. aureus* aisladas. La incidencia de MBS a lo largo del estudio fue baja y no se correlacionó con variables climáticas. El número de aislamientos de *S. aureus* de las muestras de leche fue bajo en comparación con estudios previos y la mayoría de ellos se obtuvo de muestras que presentaban un resultado de CMT de 1. La formación de cápsula, limo y biofilms así como la ocurrencia de autoagregación en todas las cepas confluyen en la habilidad bacteriana para adherirse y persistir en la glándula contribuyendo a la cronicidad de la infección y a la adhesión a superficies inertes. La resistencia bacteriana frente a distintos antibióticos fue baja. La evaluación de la expresión de factores de virulencia de *S. aureus* en el contexto de mastitis subclínica en tambos puede ser de utilidad para desarrollar acciones precisas y tratamientos para el control de mastitis y la promoción de la salud animal y la producción de leche.

Palabras clave: mastitis bovina subclínica, *Staphylococcus aureus*, factores de virulencia, Uruguay.

INTRODUCTION

Staphylococcus aureus is a natural inhabitant of human and animal skin and mucosal epithelia. It is extremely versatile, it causes a broad range of diseases and it is the most common etiologic agent of bovine contagious mastitis (Kerro Dego *et al* 2002), which is still the most costly disease for dairy farmers worldwide (Cressier and

Bissonnette 2011). This bacterium expresses a high number of virulence factors that vary among strains, potentially associated with the establishment and pathogenesis of intramammary inflammation (IMI).

Staphylococcal mastitis causes clinical and subclinical IMI which may persist along successive lactation periods. *S. aureus* has the ability to acquire resistance to antimicrobial drugs, making treatment difficult and often unsuccessful (Sommerhauser *et al* 2003). Prevalence of antimicrobial-resistant strains isolated from mastitic ruminants is a serious problem worldwide (Kumar *et al* 2011).

The importance of assessing the presence and combination of virulence factors associated to the mammary

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gland infection has been recently emphasised by different authors and support the idea that the development of subclinical mastitis could be related to strain-specific pathogenic attributes (Zecconi *et al* 2006).

The aim of this study was to identify and characterise *S. aureus* isolates associated with subclinical mastitis in two commercial dairy herds located in the dairy production geographic area of Uruguay (South), obtained from milk of affected cows. Expression of virulence factors by the different isolates and antimicrobial susceptibility patterns were assessed.

MATERIAL AND METHODS

COWS

The study was conducted between May 2008 to April 2009 in two dairy farms of Southern Uruguay, located close to the cities of Pando and Libertad. Somatic cells count recorded in bulk milk were lower than 200,000 cells mL⁻¹, despite a reasonably good udder health management. Cows, mostly grazed on pasture, were milked twice a day. Both herds had an annual record of about 1,500 L of milk per day.

MILK SAMPLING

All milking cows were tested monthly using the California Mastitis Test (CMT), a simple and widely used tool to detect subclinical mastitis (Persson *et al* 2011), which yields an ordinal variable related with the increase of milk somatic cell counts (SCC) as a consequence of udder inflammation (Feitosa Brito *et al* 1997). Quarters that showed CMT detectable values (traces, 1, 2 or 3, according to the intensity of the reaction) were sampled and the milk was submitted for bacterial culture. Milk samples were collected according to the National Mastitis Council guidelines (National Mastitis Council 1981).

BACTERIAL ISOLATION

Milk samples were streaked onto 5% sheep Blood Agar^{1, 2} (BA) and Baird Parker Agar² (BPA) plates and incubated aerobically at 37 °C for 24-48 h. Staphylococci were identified on the basis of Gram staining and cultural characteristics such as pigment production, haemolysis and the following biochemical reactions: catalase activity, tube coagulase test (TC), mannitol fermentation on Mannitol-Salt Agar¹, deoxyribonuclease¹ (DNase) test and Voges Proskauer (VP) test using VP broth¹ (Roberson *et al* 1992, Boerlin *et al* 2003). *S. aureus* reference

strains were ATCC 29213 (Boerlin *et al* 2003), ATCC 25923 (Kateete *et al* 2010) and ATCC 6538 (O'Reilly *et al* 2000). *Escherichia coli* (Delucchi *et al* 2008) and *Moraxella bovoculi* (Marrs *et al* 1985) were also used as controls for different tests of this study.

COAGULASE TEST

Coagulase activity was determined in tubes (tube coagulase test, TC) as described by Quinn *et al* (2004) and examined after 4 and 24 h.

DNASE TEST

This test was carried out by using commercially available DNase agar. Spot inoculation was done on the DNase agar plates and incubated at 37 °C for 1 day. After incubation, clearing around the bacterial growth was evaluated as positive.

CAPSULE TEST

The presence of bacterial capsule was determined following the microscopic method described by Türkyilmaz and Kaya (2006).

SLIME FORMATION

The Congo Red Agar (CRA) method developed by Freeman was used in this assay (Türkyilmaz and Kaya 2006). Isolates producing black colonies were regarded as slime-positive. Those showing pink colonies were considered as slime-negative isolates.

BIOFILM FORMATION

Isolates were incubated on TSA blood agar plates for 18 h at 37°C under aerobic conditions. Fresh cultures were inoculated in 5 mL of TSB glucose (0.25%) and then incubated at 37°C for 24 h under the same conditions. Biofilm formation was assessed by the microplate method (MP) proposed by Pfaller *et al* (1988) using 96 wells tissue culture plates. The plates were read using an automated plate reader (Varioscan Flash 4.00.51) at 600 nm. Sterile TSB was used as a negative control. All the experiments were performed using four replicates. Biofilm production was tested following the criteria proposed by Mathur *et al* (2006).

HEMOLYSIS

Hemolysis was evaluated on TSA plates supplemented with 10% sterile sheep blood. The plates were incubated for 24 h at 37 °C, and then overnight at 4 °C. Results were considered positive when a clear zone of complete hemolysis was detected around the colonies.

¹ Tryptic Soy Broth (TSB), Tryptic Soy Agar (TSA), Baird Parker media, Mannitol Salt Agar, DNase media, and VP Broth from HIMEDIA, India.

² Sheep Blood supplied from Biokey SRL, Uruguay.

HEMAGGLUTINATION

The hemagglutination assay was performed according to the method described by Rupp and Archer (1992) with modifications. Bacterial concentrations were adjusted using a McFarland standard of 0.5 which correlated approximately with 10^8 bacteria per mL. Hemagglutination assays were carried out in 96-well (U-shaped) microtiter plates (Costar, Cambridge, Mass.). The plates were sealed with laboratory film and shaken to ensure even mixing of the bacteria and erythrocytes, and then incubated at 4 °C for 2 h. Hemagglutination was recorded as positive when erythrocyte clumps were seen. Negative and positive controls were included in the assay.

AUTOAGGREGATION

Isolates were incubated on TSA blood agar plates for 18 h at 37°C under aerobic conditions. Fresh cultures were inoculated in 5 mL of TSB glucose (0.25%) and then incubated at 37°C for 24 h at the same conditions. Aliquots of 5 µL of the suspensions were transferred to 5 mL of TSB glucose which were incubated 48 h at the same conditions. Then, the cultures were incubated 24 h at 4° C.

200 µL were extracted from the surface of each tube of culture and transferred to a 96-well (U-shaped) microtiter plate (Costar, Cambridge, Mass.). Initial Optical Density (OD) was read at 600 nm. After this, tubes were vortexed during 30 sec and 200 µL of the cultures were transferred to a 96-well (U-shaped) microtiter plate. Final OD was read at 600 nm. Autoaggregation percentage was calculated as: $A = (1 - OD_f / OD_i) \times 100$, where A: Autoaggregation percentage; OD_f : Final Optical Density, and OD_i : Initial Optical Density.

ANTIBIOTIC SUSCEPTIBILITY PATTERNS

The Kirby-Bauer disk diffusion method (Bauer *et al* 1966) was used to determine resistance of *S. aureus* to Trimethoprim–Sulfamethoxazole, Gentamicine, Neomycin, Penicillin, Nafcillin, Clindamycin and Ciprofloxacin. Zones of inhibition were recorded at 24 and 48 h and the isolates were considered susceptible, intermediately resistant, or resistant to each antibiotic according to the National Committee for Clinical Laboratory Standards (NCCLS 1997, Gandara *et al* 2006).

STATISTICAL ANALYSIS

In order to identify *S. aureus* isolates, a Cluster analysis using de Ochiai index simultaneously to strains and to variables (coagulase, hemolysis, VP, mannitol fermentation, DNase, BPA) was performed. To compare biofilm production and autoaggregation (as percentage) an Exploratory Data Analysis was performed followed by

Kruskall-Wallis test between strains, and Mann-Whitney for strain classification. These procedures were followed by modified Bray-Curtis association index to find strains with similar behaviour for these two variables. All those procedures were performed by using PAST software (Hammer *et al* 2001); P values < 0.05 were considered significant.

RESULTS AND DISCUSSION

PREVALENCE OF SUBCLINICAL MASTITIS

Considering the total cow populations of both dairy farms, the percentage of animals that showed a detectable CMT category was 8 and 19% in the cases of Libertad and Pando, respectively. Distribution along the year did not show a defined pattern and was neither related to climatic variables like temperature, rain or humidity (data not shown). The subclinical mastitis prevalence recorded by CMT was higher in one of the farms (Pando) compared to the other located close to Libertad. This could be related to hygiene practices that were poorer in the Pando farm compared to the other one in Libertad. However, prevalence results could be considered acceptable compared to previous studies (Giannechini *et al* 2002, Plozza *et al* 2011).

Most of the *S. aureus* isolates corresponded to milk samples that showed a Grade 1 CMT result (16 out of 20) while the remaining four were isolated from milk samples that corresponded to Grade 2 (1), Grade 3 (1) and traces (2) CMT categories. The vast majority of the identified *S. aureus* corresponded to milk samples that showed a Grade 1 CMT category (16 out of 20, 80%). This indicated that *S. aureus* was predominantly isolated from animals that suffered a mild udder inflammation, result that may be taken into account for control strategies.

BACTERIAL IDENTIFICATION

A total of 508 milk samples showed a CMT detectable result and 63 isolates were characterised as Gram positive cocci and positive to catalase production. 20 of these isolates showed coagulase activity and developed a firm clot in the coagulase test tube (TC) at 4 hours and at 24 h the clot was seen in 22 isolates. Of these 22, 20 showed a consistent color change according to VP test positive reaction and were considered *S. aureus*. These 20 isolates showed hemolysis, DNase activity and grew well on MSA; 19 of them were able to ferment mannitol, which was evidenced by a color change in the MSA plates around the colonies. All strains grew on BP plates showing a characteristic morphology and dimensions but nevertheless only 12 showed a halo of lipolysis. The two remaining isolates were identified as coagulase positive *Staphylococcus* other than *S. aureus*. 3 out of the 41 isolates of Gram positive cocci without catalase activity did not evidence coagulase activity but showed a positive

oxidase test. These isolates belonged to the *Micrococcus* genus while the rest (38) were considered coagulase-negative staphylococci (CNS).

Isolates showed a positive coagulase-test result after 4 h of incubation. Isolate 20080849 was the only one that did not acidified MSA plates.

Identification of bacterial pathogens still relies mainly on phenotypic criteria due to costs and simplicity of the methods. Among coagulase-positive staphylococci, *S. aureus* is almost the only one with hemolytic activity that is regularly found in milk samples (Roberson *et al* 1992, Larsen *et al* 2002). Therefore, a combination of hemolysis and coagulase activities seems to represent an optimal criterion for the identification of *S. aureus* in cultures from milk samples (Boerlin *et al* 2003).

PRESENCE OF CAPSULE, SLIME PRODUCTION AND HEMAGGLUTINATION

All *S. aureus* isolates formed capsule and produced slime on CRA. Only 7 strains (excluding ATCC 6835 and 25923 strains used as positive controls) were able to agglutinate ovine red blood cells. Expression of capsule by *S. aureus* associated to IMI has been clearly demonstrated but this is not a constant (Buzzola *et al* 2007). In this study, we must consider that milk samples belonged to animals with subclinical mastitis. The presence of capsule enables to resist and evade successfully the first defense line of the host, even triggering different degrees of inflammation as seen in the CMT tests.

BIOFILM FORMATION

The *S. aureus* isolates were classified in high, moderate and weak biofilm producers, based on OD₅₄₀ results. One of them was a high biofilm producer (OD₅₄₀ 1,24; lower quartile value OD₅₄₀ 1,05; higher quartile value OD₅₄₀ 1,32), other 10 were moderate biofilm producers (median value OD₅₄₀ 0,42; lower quartile value OD₅₄₀ 0,33; higher quartile value OD₅₄₀ 0,53) while the remaining 9 isolates produced weak biofilms (median value OD₅₄₀ 0,21; lower quartile value OD₅₄₀ 0,17; higher quartile value OD₅₄₀ 0,25) (figure 1, panel A).

During IMI, bacterial clusters may develop within the udder and biofilm structures, may facilitate bacterial adherence and colonization of the epithelium. In general all isolates could establish biofilms in 24 h. Adhesion is a crucial early step for mammary gland infection but biofilm also may enable recurrent infections protecting bacterial cells from host defenses and the effects of antibiotics (Cucarella *et al* 2004).

AUTOAGGREGATION

The isolates showed a relative variable rate of autoaggregation values as can be seen in figure 1, Panel B. Iso-

late 20090305 showed the highest aggregation rate with a relatively small variance, similarly to strain ATCC 25923. Mann-Whitney test showed that the autoaggregation rates of the 20 isolates could be grouped in at least 6 different classes (Group1: 20090305; Group 2: 20080607, 20090109, 20080849, 20090108, 20080611; Group 3: 20090317; Group 4: 20090410; Group 5: 20080619, 20090409, 20080903, 20090307, 20080919; Group 6: 20090333, 20090323, 20080941, 20090431, 20090322, 20090436, 20090403). During the first stages of infection *S. aureus* produces hydrophobic surface proteins, particularly receptors for host components that allow the adherence to the mammary epithelium and milk fat globules and thus it disseminates in the gland also promoting intercellular aggregation (Kerro Dego *et al* 2002). Autoaggregation was seen in all isolates and also in different levels confirming bacterial adhesive capacity. However, no correlation could be established between bacterial autoaggregation and biofilm production.

ANTIBIOTIC SUSCEPTIBILITY PATTERNS

Only resistance against penicillin was observed, detected in 4 *S. aureus* isolates (20 % excluding ATCC reference strains used as controls). All isolates were susceptible to the other agents or agent combinations; none of the isolates showed resistance to more than one antimicrobial agent.

These findings were similar to those reported by Rubin *et al* (2011). Although the number of isolates could be considered as limited, our data indicate that bacterial antibiotic resistance could be low in the area, probably due to appropriate therapeutic managements.

It can be concluded that all the isolates considered in this study expressed virulence factors that potentially enhance *S. aureus* ability to colonize and persist in the mammary gland and even on inert surfaces. The increase in the knowledge of *S. aureus* involved in subclinical mastitis may be useful to design effective measures to avoid the chronicity of infection and losses of productive animals.

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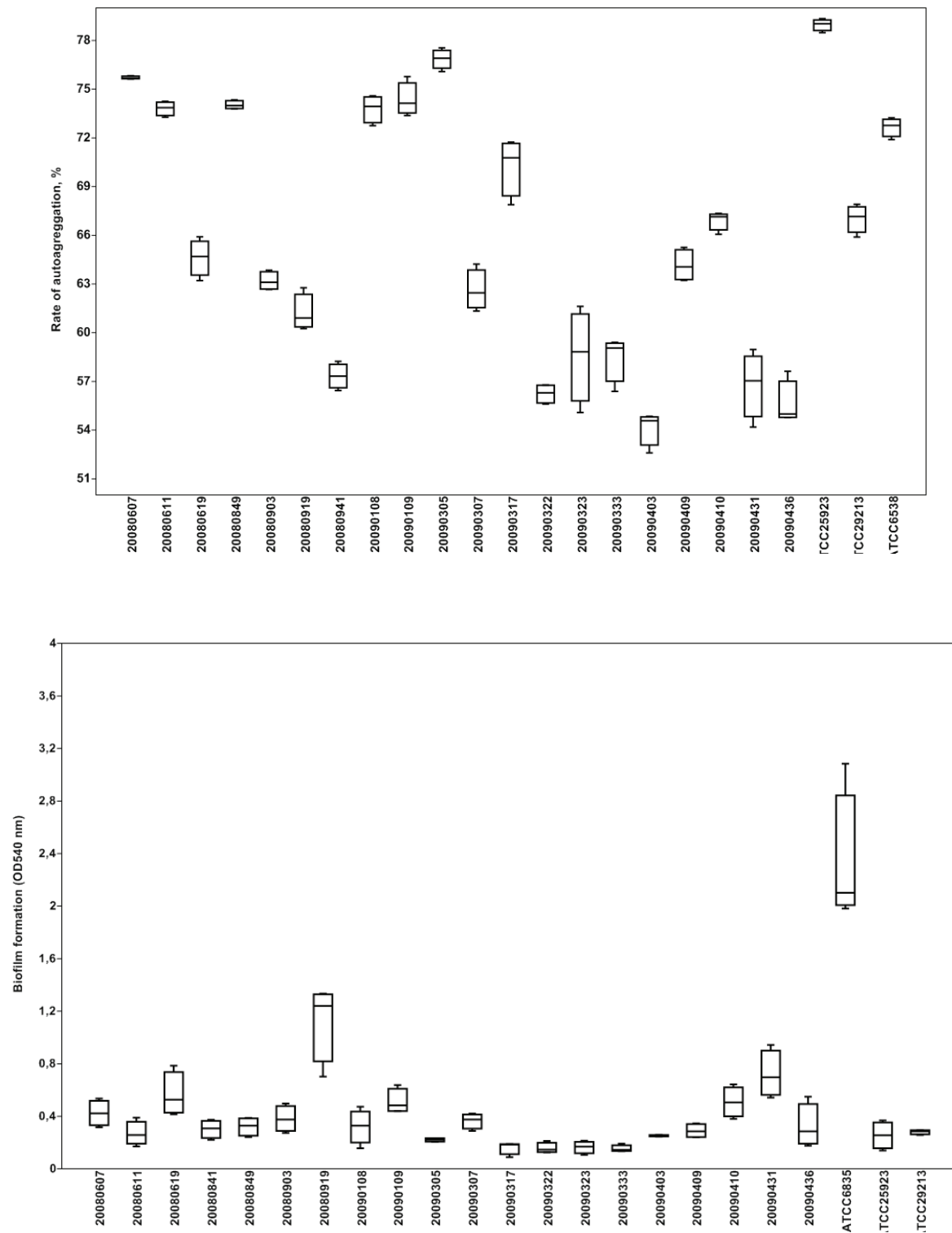


Figure 1. Panel A: Biofilm production of *S. aureus* isolates. Horizontal lines of each box correspond to the three quartiles of the replicates. Central horizontal lines correspond to the medians. Panel B: Autoaggregation rates of *S. aureus* isolates. Horizontal lines of each box correspond to the three quartiles of the replicates. Central horizontal lines correspond to the medians.

Panel A: Producción de biofilms por parte de las cepas de *S. aureus*. Las líneas horizontales de cada caja corresponden los tres cuartiles de las réplicas. Las líneas horizontales centrales corresponden a las medianas. Panel B: Autoagregación de las cepas de *S. aureus*. Las líneas horizontales de cada caja corresponden los tres cuartiles de las réplicas. Las líneas horizontales centrales corresponden a las medianas.

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