INTERPRETIVE SUMMARY

Biofilm production characterization of *mecA* and *mecC* methicillin-resistant *Staphylococcus aureus* (MRSA) isolated from bovine milk in Great Britain. By Prenafeta et al. This study aimed to determine the biofilm formation ability of *mecA* and *mecC* methicillin-resistant *Staphylococcus aureus* (MRSA) isolated from bovine milk in Great Britain. All the tested MRSA isolates were PCR positive for the *ica* genes and 50% produced biofilm in a microtiter plate assay. This is the first demonstration of biofilm production by *mecC* MRSA.

SHORT COMMUNICATION: BIOFILM PRODUCTION CHARACTERIZATION IN METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS*

Short communication: Biofilm production characterization of *mecA* and *mecC* methicillin-resistant *Staphylococcus aureus* (MRSA) isolated from bovine milk in Great Britain

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Abstract

Staphylococcus aureus is an important cause of contagious intramammary infection in dairy cattle and the ability to produce biofilm is considered to be an important virulence property in the pathogenesis of mastitis. The aim of this study was to characterize the biofilm formation capacity of methicillin-resistant *S. aureus* (**MRSA**), encoding *mecA* or *mecC*, isolated from bulk tank milk in Great Britain. For this purpose, 20 MRSA isolates were grown on microtiter plates to determine the biofilm production. Moreover, the presence of the intercellular adhesion genes *icaA* and *icaD* were analyzed by PCR. All the tested MRSA isolates were PCR positive for the *ica* genes and 10 of them (50%) produced biofilm in the microtiter plate assay. This is also the first demonstration of biofilm production by *mecC* MRSA.

Keywords: *Staphylococcus aureus*, MRSA, biofilm.

Introduction

Staphylococcus aureus is one of the most important pathogens implicated in nosocomial infections and indwelling medical devices-related infections in humans. In addition, this microorganism is a common contagious bacterium responsible for bovine mastitis.

The ability to form biofilm represents an important virulence factor in both human and bovine mammary gland *S. aureus* infections. Bacteria enclosed in the extracellular biofilm matrix and arranged in multilayers become resistant to antimicrobial agents and to the host immune system by impairing the action of phagocytic cells (Costerton et al., 1999). Such characteristics allow *S. aureus* with biofilm forming ability to adhere and colonize the mammary gland epithelium and establish persistent infections (Fox et al., 2005).

A major constituent of the staphylococcal biofilm matrix is the poly-*N*-acetyl β-1,6 glucosamine (PNAG) surface polysaccharide, involved in the intercellular adhesion and synthesized by proteins encoded by the *icaADBC* operon (Cramton et al., 1999). The *icaA* gene product is a transmembrane protein with homology to *N*-acetyl-glucosaminyltransferases, requiring the *icaD* gene product for optimal activity. *N*-acetyl-glucosamine oligomers produced by *icaAD* reach a maximal length of 20 residues and it is only when *icaAD* is coexpressed with *icaC*, which encodes a putative membrane protein, that longer oligomer chains are synthesized. IcaC is also likely to be involved in translocation of the growing polysaccharide to the cell surface. The surface-attached protein IcaB is then responsible for deacetylation of the poly-*N*-acetylglucosasmine molecule (O'Gara et al., 2007). On the other hand, *S. aureus* surface proteins such as Bap can mediate PNAG-independent biofilm development (Cucarella et al., 2001).

Recently, Bardiau et al. (2013) published that biofilm formation ability was present in all the methicillin-resistant *S. aureus* (MRSA) isolates analyzed from bovine mastitis in Belgium (n=19). Resistance to β-lactam antibiotics in MRSA is conferred by the acquisition of a mobile genetic element, the staphylococcal cassette chromosome (SCC*mec*) carrying the *mecA* gene or the recently described variant *mecC* (Garcia-Alvarez et al., 2011). *mec* genes encode an altered penicillin-binding protein, essential for cell wall biosynthesis, which has reduced affinity for β-lactam antibiotics.

The objective of the present study was to determine the capacity to form biofilm of 20 MRSA isolates from bovine bulk tank milk in Great Britain, encoding either *mecA* or *mecC* genes. Moreover, all isolates were *spa* typed. *spa* gene encodes the protein A, a multifunctional cell wall protein that binds immunoglobulins inhibiting opsonophagocytosis. Protein A is ubiquitous in *S. aureus* and is often used in strain typing on the basis of variation in the DNA sequence-encoding región Xr (Foster et al., 2014).

Materials and Methods

Isolation and identification of meticillin-resistant S. aureus from dairy cattle. Strains were collected as part of prevalence surveys of MRSA in dairy bulk tank milk in Great Britain (Paterson et al., 2012a and Paterson et al. (in press)). PCR for *mecA* and *mecC* typing was performed as previously described (Paterson et al., 2012b). Multi-locus sequence typing was performed as per Ridom GmbH (Würzburg, Germany).

Biofilm production characterization. To determine the genetic basis of biofilm formation, the intercellular adhesion genes *icaA* and *icaD* were amplified using PCR according to Zmantar et al. (2008) and sequenced to confirm the identity of the products.

A quantitative determination of the biofilm production was performed in polystyrene microtiter plates (Stepanovic et al., 2007). Overnight cultures (18 h) were diluted 1:100 in TSB with 2 % glucose, and 200 µl of this cell suspension was used to inoculate 96 flat-well microtiter plates. After 24 h incubation at 37 ºC, the content of the treated plates was decanted into a discard container. Wells were washed three times with phosphate-buffered saline (PBS; pH 7.2) and air dried. Cells were heat-fixed by exposing them to hot air at 60 ºC for 60 min. The adherent biofilm was stained with 0.1 % safranin for 1 min, removing the excess stain by rinsing with tap water. After drying, the optical density of the biofilm was measured with an ELISA microplate reader at a wavelength of 492nm. Each assay was performed four times on different days and using 8 wells in each plate for the culture of one strain. The optical density values for 32 wells were obtained for each isolate and used to determine the mean value. The ATCC 29213 human isolate (referred as SA12H in this work) was used in each assay as a weak biofilm-producer as described by Prenafeta et al. (2010). The strain SA13H was used as a positive control in each microtiter plate (strong biofilm-producer according to Prenafeta et al., 2010). Wells with uninoculated medium were used as a negative control. Strains were classified as non-biofilm producers (-) when they did not show a significant difference to the optical density mean of uninoculated wells ($P>0.05$), weak producers (+) when the optical density mean was significantly higher than than uninoculated wells ($P<0.05$) and moderate to strong biofilm-producers (++) when the optical density mean was significantly higher than the SA12H strain.

Statistical analysis. Differences of the biofilm production between isolates in the late adherence test were evaluated by one-way ANOVA using SPSS 11.5.

Results and Discussion

The *spa*-type and *mec* gene, as well as the adherence test results for the 20 MRSA isolates analyzed in this study, are summarized in Table 1. All the MRSA strains were observed to be PCR positive for both *icaA* and *icaD* genes. The culture of the 20 MRSA strains in microtiter plates revealed that 3 isolates were moderate to strong biofilm-producers, 7 isolates were weak biofilm-producers and 10 isolates did not show the ability to form biofilm.

Biofilm formation is an important virulence property of *S. aureus* and here we have investigated the biofilm production of MRSA from bulk tank milk. The *icaA* and *icaD* genes were detected in all the analyzed MRSA isolates, whereas Dhanawade et al. (2010) indicated a prevalence of these genes in 35.29 % of *S. aureus* from bovine subclinical mastitis in India.

The biofilm production in microtiter plates was confirmed in 10 of the 20 MRSA isolates. This study indicates that the ability to form biofilm is a virulence property present in 50 % of the MRSA isolated in this study from bovine milk in Great Britain, not as prevalent as Bardiau et al. (2013) observed in mastitis MRSA isolates from Belgium (100 %). This discrepancy could be due to the criteria used by Bardiau et al. (2013) to determine the biofilm formation ability in the microtiter assay, based on a cut-off defined as three standard deviations above the mean optical density of the uninoculated wells (according to Stepanovic et al., 2007), whereas in our study we used an statistical analysis to compare the mean optical density differences between the MRSA strains and the uninoculated wells or a weak biofilm-producing *S. aureus* strain.

This is the first biofilm production characterization of bovine milk MRSA isolates from Great Britain and the first demonstration of biofilm production by *mecC* MRSA which is an emerging human and animal pathogen (Paterson et al. 2014). The emergence of MRSA in cattle could imply a reduction of effective antibiotic treatments. Moreover, a

high prevalence of biofilm producing MRSA isolates could promote the chronicity of bovine mastitis, with the consequence of persistent bacterial infection and increased shedding and spread from infected animals including potential zoonotic transmission.

Acknowledgement

We thank Mireia Muntada and Anna Corominas for their excellent technical assistance in the biofilm characterization analysis.

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Table 1. *spa*-type, *mec*-gene, *icaA* and *icaD* presence and biofilm-producer phenotype among bovine methicillin-resistant *S. aureus* (MRSA) isolates. Biofilm formation is indicated by the mean OD_{492} in the microtiter plates and the standard deviation of the mean (SD). The isolates were classified as non-producers (-), weak producers (+) and moderate to strong producers (++)

Isolate	spa-type	*mec*-gene	icaA/ica D	OD_{492} (SD)	Biofilm production
6H	t6292	*mecC*	+/+	0.344 (0.170)[b]	+
6T	t6292	*mecC*	+/+	0.316 (0.187)[b]	+
13P	t843	*mecC*	+/+	0.692 (0.535)[a, b]	++
13x	t742	*mecC*	+/+	0.862 (0.486)[a, b]	++
15AL	t6300	*mecC*	+/+	0.425 (0.503)[b]	+
18-31	t843	*mecC*	+/+	0.445 (0.436)[b]	+
40-25	t011	*mecC*	+/+	0.361 (0.349)[b]	+
25-73	t1328	*mecA*	+/+	0.191 (0.143)	-
25-26	t011	*mecA*	+/+	0.129 (0.073)	-
22-79	t011	*mecA*	+/+	0.141 (0.055)	-
18-67	t843	*mecC*	+/+	0.125 (0.070)	-
30-59	t2346	*mecA*	+/+	0.146 (0.100)	-
31-7	t011	*mecA*	+/+	0.100 (0.037)	-
33-6	t6292	*mecC*	+/+	0.264 (0.261)[b]	+
34-121	t6292	*mecC*	+/+	0.235 (0.161)	-
34-122	t6292	*mecC*	+/+	0.258 (0.183)	-
37-55	t4184	*mecA*	+/+	0.391 (0.317)[b]	+
42-57	T011	*mecA*	+/+	0.649 (0.186)[a, b]	++
45-164	T015	*mecA*	+/+	0.116 (0.058)	-
27-28	t742	*mecC*	+/+	0.082 (0.037)	-
SA13H	N.d.	N.d.	+/+	0.845 (0.458)[a,b]	++
SA12H	N.d.	N.d.	N.d.	0.244 (0.07) [b]	+

[a]Denotes significant difference compared to the weak biofilm producer SA12H strain ($p<0.01$)

[b]Denotes significant difference compared to the uninoculated wells ($p<0.05$)

N.d.: not determined.