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Existence of two groups of *Staphylococcus aureus* strains isolated from bovine mastitis based on biofilm formation, intracellular survival, capsular profile and *agr*-typing

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ABSTRACT

Staphylococcus (*S.*) *aureus* is recognised worldwide as an important pathogen causing contagious acute and chronic bovine mastitis. Chronic mastitis account for a significant part of all bovine cases and represent an important economic problem for dairy producers. Several properties (biofilm formation, intracellular survival, capsular expression and group *agr*) are thought to be associated with this chronic status. In a previous study, we found the existence of two groups of strains based on the association of these features. The aim of the present work was to confirm on a large international and non-related collection of strains the existence of these clusters and to associate them with case history records. In addition, the genomes of eight strains were sequenced to study the genomic differences between strains of each cluster. The results confirmed the existence of both groups based on capsular typing, intracellular survival and *agr*-typing: strains *cap8*-positive, belonging to *agr* group II, showing a low invasion rate and strains *cap5*-positive, belonging to *agr* group I, showing a high invasion rate. None of the two clusters were associated with the chronic status of the cow. When comparing the genomes of strains belonging to both clusters, the genes specific to the group "*cap5-agr*I" would suggest that these strains are better adapted to live in hostile environment. The existence of these two groups is highly important as they may represent two clusters that are adapted differently to the host and/or the surrounding environment.
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1. Introduction

Staphylococcus aureus (*S. aureus*) is an important bacteria causing contagious bovine mastitis (Watts, 1988). *S. aureus* strains can cause acute, usually clinical, and chronic, usually subclinical, mastitis. Subclinical mastitis is characterised by a non-alteration of the milk but high somatic cell count, making the milk inappropriate for the consumers if the cell count is too high. This type of mastitis is often chronic and account for up to 30% of all bovine cases (Halasa et al., 2007), which represents an important economic problem for dairy producers with reduction in milk quantity and quality, prolonged costly antibiotic treatments and premature culling. Several properties are thought to be associated to some extent with these chronic infections: biofilm formation, intracellular survival, capsular expression and group accessory gene regulator (*agr*).

Biofilm formation is one of the most important survival mechanisms of bacteria living in the extracellular niche. It impairs

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the action of both the host immune system and antimicrobial agents (Costerton et al., 1999; Melchior et al., 2006). Second, *S. aureus* can be an intracellular pathogen of a large variety of eukaryotic cells, including epithelial cells of the mammary glands and immune cells (Almeida et al., 1996; Kerro Dego et al., 2002). Therefore, the bacteria are not only protected from the action of commonly used antibiotics in mastitis treatment (mainly β-lactams), and also able to persist in the host without causing any apparent inflammation (Boulanger et al., 2003; Garzoni and Kelley, 2009). The absence of capsular expression enhance the adherence to and the invasion of eukaryotic cells by *S. aureus* (Pohlmann-Dietze et al., 2000; Buzzola et al., 2007; Tuchscherr et al., 2005). In addition, *S. aureus* bacteria that do not express capsule induce chronic mastitis in mice, suggesting that the absence of capsule synthesis may help the bacteria to persist in the mammary glands (Tuchscherr et al., 2005). Finally, *agr* group I is associated with a persistence or with features that help the bacteria to persist in the udder: strains belonging to *agr* group I are more likely to be internalised in epithelial cells, to persist in murine mammary glands (Buzzola et al., 2007) and to be associated with penicillin resistance (Melchior et al., 2011) than the strains belonging to the other groups.

In a previous study (Bardiau et al., 2014), we correlated *agr*-typing, capsular expression, biofilm formation, and intracellular survival in a collection of Belgian *S. aureus* strains from bovine mastitis (with no case history records). We found the existence of two groups based on the association of these features: *cap5*-positive strains belonging to *agr* group I, which in vitro test negative for CP5 ELISA and show a high invasion rate in MAC-T cells, and *cap8*-positive strains belonging to *agr* group II, which express CP8 in vitro and show a low invasion rate in MAC-T cells. We hypothesised that the first group may correspond to strains adapted to the intracellular niche leading to chronic infection and that the second group may correspond to strains better adapted to the extracellular niche leading to acute infection.

The aim of this work was therefore to confirm on a large European and non-related collection of strains the existence of these two groups based on features described to be associated with long-lasting infections and to find out if these two clusters are associated with a persistence of the disease. We therefore investigated the association between *agr*-typing, capsular antigen identity and expression, biofilm formation, intracellular survival and the case history data of a collection of *S. aureus* strains isolated from cases of chronic and acute bovine mastitis in four countries (Belgium, Italy, Canada and Switzerland). Moreover, the genomes of eight strains were sequenced and compared to assess of the genomic differences between the formed clusters.

2. Materials and methods

2.1. Bacterial isolates

A total of 168 bovine *S. aureus* isolates, were collected from chronic and acute bovine intramammary infection in four countries. One hundred and two isolates were collected from chronic cases in Belgium ($n = 7$), Canada ($n = 45$), Italy ($n = 25$) and Switzerland ($n = 25$). Sixty-six isolates were collected from acute cases in Canada ($n = 46$) and Italy ($n = 20$). Strains have been isolated and identified as *S. aureus* according to the protocol described in Ote et al. (2011). Chronic mastitis was defined as recurring isolates in the same quarter of the same animal. Four *S. aureus* reference strains were included in this collection: ATCC 29740 (N305), ATCC 31885 (NL6), ATCC 49521 (Lowenstein) and ATCC 49525 (Wright).

2.2. Capsular genotyping and serotyping

Capsular genotyping was performed using PCR detection of the capsule-encoding genes *cap5* and *cap8* genes as previously described (Ote et al., 2011). Capsular serotyping was performed in in triplicates and in two independent experiments by enzyme-linked immunosorbent assay (ELISA) using specific monoclonal and polyclonal antibodies (kindly provided by GSK Biologicals, Belgium) against CAP5 and CAP8 as previously described (Bardiau et al., 2014). For the serotyping, OD values were compared to those obtained with *S. aureus* CP reference strains, namely the CP5-positive strain ATCC 49521 and CP8-positive strain ATCC 49525, and isolates that tested negative for CP5- and CP8- ELISA were defined as non-typeable (NT).

2.3. Invasion assay

Bovine mammary epithelial cells (MAC-T) were used for in vitro bacterial internalisation assays as previously described (Bardiau et al., 2014; Boulanger et al., 2007; Brouillette et al., 2003). Briefly, cell monolayers ($\sim 2.5 \times 10^5$ cells/well) were inoculated with 10^7 CFU of *S. aureus* (MOI$\sim$40) and incubated at 37 °C in 5% CO_2 for three hours. After the removal of extracellular bacteria by phosphate buffered saline (PBS) washing and lysostaphin treatment, the MAC-T cells were detached and lysed by addition of 900 μl/well of sterile distilled water containing 0.025% Triton X-100. The cell lysates were carefully suspended, serially diluted, and plated on Columbia sheep blood agar plates to quantify intracellular staphylococci. Results are expressed as a percentage of the initial inoculum and classified using the same criteria as in our previous work (Bardiau et al., 2014).

2.4. Biofilm production

Biofilm formation was evaluated by spectrophotometry in microplates using safranin staining as previously described (Bardiau et al., 2014). Briefly, overnight cultures were diluted 1:100 in tryptic soy broth (TSB) containing 0.25% glucose (TSB_{glc}), transferred into wells of sterile 96-well polystyrene tissue culture (TC) plates and incubated at 37 °C. TSB_{glc} without bacteria served as negative control. After 24 h, the plates were stained with safranin 0.1% (w/v) for 10 min. A mixture of 50% ethanol and 50% acetic acid was added to each well and plates were incubated at room temperature for 15 min. Finally, the OD of each well was measured at 490 nm using a microplate reader. The results were collected from at least two independent experiments in which the biofilm formation of each culture tested was evaluated in triplicate. The quantitative classification of biofilm production based on cut-off value (ODc) and average OD values was carried out leading to four categories of strains: no biofilm producer ($OD \leq ODc$); weak biofilm producer ($ODc < OD \leq 2 \times ODc$); moderate biofilm producer ($2 \times ODc < OD \leq 4 \times ODc$); strong biofilm producer ($4 \times ODc < OD$) (Stepanovic et al., 2007).

2.5. *agr*-typing

agr-groups were determined by multiplex PCR as previously described (Gilot et al., 2002). In brief, multiplex PCRs were performed with the following primers: Pan (5′-ATG CAC ATG GTG CAC ATG C-3′), agr1 (5′-GTC ACA AGT ACT ATA AGC TGC GAT), agr2 (5′-TAT TAC TAA TTG AAA AGT GGC CAT AGC-3′), agr3 (5′-GTA ATG TAA TAG CTT GTA TAA TAA TAC CCA G-3′), and agr4 (5′-CGA TAA TGC CGT AAT ACC CG-3′). Amplifications were performed with the following PCR program: 1 cycle at 94 °C for 1 min; 26 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min; and finally 1 cycle at

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