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Genotypic and phenotypic characterization of *Staphylococcus aureus* causing persistent and nonpersistent subclinical bovine intramammary infections during lactation or the dry period

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ABSTRACT

Staphylococcus aureus is a significant pathogen frequently causing persistent intramammary infections (IMI) in dairy cows. We compared some genotypic and phenotypic characteristics of 285 strains collected from quarter milk samples from cows with persistent and nonpersistent subclinical IMI across Canada. Variable number of tandem repeats typing was used to infer the persistence of the same *S. aureus* strain in 3 consecutive quarter milk samples collected at intervals of 3 wk during lactation or before and after dry-off. All first isolates of the series were used as the representative strains from persistent IMI and were compared with nonpersistent strains for the presence of genes *seg*, *sen*, *sec*, and *tst* as well as by *spa* typing. Biofilm production in vitro and *hld*-RNAIII expression levels were also quantified. The gene *seg* was associated with a reduction in the likelihood of the bacteria to cause a persistent IMI during lactation. Strains persisting through the dry period produced significantly more biofilm in vitro than strains that do not persist after calving. Also, we showed that strains expressing more *hld* were more likely to be nonpersistent during either lactation or through the dry period. Three *spa* types were predominant (t529, t267, and a novel type: t13401). In the strains studied, the *spa* type t529 was the most frequent, and 97.0% of the strains of this *spa* type carried both *sen* and *seg*. Strains from the *spa* type t529 were less likely to cause a persistent IMI in the dry period. Most (86.7%) of the strains of the novel *spa* type (t13401) were negative for *seg*, *sen*, or both and produced significantly more biofilm in vitro than t529 and t267. The present study expanded our current knowledge on the genotypic and phenotypic traits of *S. aureus* strains recovered from persistent and nonpersistent IMI in Canada.

Key words: dairy cow, mastitis, *Staphylococcus aureus*, subclinical, persistence

INTRODUCTION

Staphylococcus aureus is an important etiological agent of clinical and subclinical bovine mastitis. A survey on dairy farms in Canada between 2007 and 2008 by the Canadian Bovine Mastitis Research Network (**CBMRN**) showed that *S. aureus* was the most frequently recovered pathogen from mastitis cases (Reyher et al., 2011). This also supported a previous study showing that *S. aureus* was the most prevalent pathogen causing mastitis in Canadian dairy herds (Olde Riekerink et al., 2008). Of particular interest are the *S. aureus* strains responsible for subclinical and persistent IMI. The presence of such strains represents a reservoir that maintains the occurrence of *S. aureus* infections in herds.

Staphylococcus aureus produces several virulence factors allowing establishment of IMI (Sutra and Poutrel, 1994). These factors can be divided into different groups, including surface-associated factors, degradative enzymes, hemolysins, staphylococcal enterotoxins (such as SEC, SEG, SEN, and others), and the toxic shock syndrome toxin-1 (TSST-1). The enterotoxins and TSST-1 affecting humans are known as superantigen toxins because of their ability to polyclonally activate T-cells in a nonspecific manner (Dinges et al., 2000).

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In humans, it is estimated that 80% of infections are associated with biofilm-producing bacteria (Davies, 2003). Biofilms reduce opsonization by antibodies and phagocytosis, and bacteria within biofilms are less susceptible to antibiotics (Costerton et al., 1999). In bovine, biofilm formation by *S. aureus* is also considered an important virulence factor, which may influence the persistence of IMI, but the in vivo demonstration of this still remains a challenge (Melchior et al., 2006). The in vitro biofilm-forming ability of *S. aureus* strains from IMI has been evaluated in different studies using a variety of methods (Oliveira et al., 2006; Dhanawade et al., 2010).

The regulatory circuits that control *S. aureus* virulence and adaptation to its environment are complex. These regulatory systems can receive signals from the external environment to modulate biofilm formation and the production of various exoproducts in a manner that is appropriate to the infection site (Novick, 2003; Ster et al., 2005). One of the regulatory systems, Agr, encodes a 2-component quorum-sensing constituent that is activated by bacterial density through secretion of an autoinducing peptide. The activation of Agr allows production of a regulatory RNA molecule, RNAIII, which is the effector molecule of the Agr system (Novick and Geisinger, 2008). The RNAIII allows reduction of surface proteins such as adhesins required for colonization. At the same time, RNAIII permits the production of secreted proteins such as nucleases and proteases, thus helping the release of bacteria from biofilms (Novick and Geisinger, 2008; Otto, 2013). Part of the RNAIII transcript is also translated to produce delta-hemolysin (Hld), which is one of the phenol-soluble modulins involved in structuring biofilm (Periasamy et al., 2012). Measurement of *hld*-RNAIII expression levels can be used to estimate the level of activation of the Agr system in *S. aureus* (Novick, 2003).

The relative importance of the various virulence factors of *S. aureus* and the genetic background of the strains associated with bovine IMI have not been completely investigated. Studies around the world have reported a diversity of *S. aureus* genotypes associated with IMI (Akineden et al., 2001; Buzzola et al., 2001; Larsen et al., 2002; Zadoks et al., 2002; van Leeuwen et al., 2005; Haveri et al., 2008; Vautor et al., 2009; Wang et al., 2009; Hata et al., 2010; Wolf et al., 2011; Klein et al., 2012; Mitra et al., 2013). Also, *S. aureus* strains involved in bovine IMI were shown to present a variety of genotypes and phenotypes that include disease severity and IMI persistence and the capacity to produce specific toxins, biofilm, or both (Matsunaga et al., 1993; Fitzgerald et al., 2000; Cucarella et al., 2004; Zschöck et al., 2005; Zecconi et al., 2006; Haveri et al., 2007; Kalorey et al., 2007; Le Maréchal et al., 2011; Oliveira et al., 2011; Ote et al., 2011; Bardiau et al., 2014). Additional information is needed for a complete understanding of the disease and more specifically for identifying the bacterial factors responsible for the persistence of IMI. For example, it would be of interest to determine whether genotypic markers such as *seg*, *sen*, *sec*, *tst*, and the *spa* type as well as the phenotypic expression of biofilm and the level of *hld*-RNAIII expression could be of prognostic value to predict the likelihood that a *S. aureus* subclinical IMI, either during lactation or at dry-off, would become persistent. Thus, the aim of this study was to systematically compare the characteristics of *S. aureus* strains recovered from documented subclinical persistent and nonpersistent IMI.

MATERIALS AND METHODS

Bacterial Isolates Collection

A total of 285 *S. aureus* strains from subclinical IMI were used in this study. Isolates were obtained from the CBMRN Mastitis Pathogen Culture Collection (Faculté de Médecine Vétérinaire, Université de Montréal, St-Hyacinthe, QC, Canada). As described in detail in Reyher et al. (2011), all isolates were collected from 29 herds in 2007 and 2008 and were obtained from 4 different areas of Canada (Atlantic Provinces, Quebec, Ontario, and Western Canada). Systematic sampling of quarter milk samples allowed lactational isolates to be collected from subclinical IMI. Milk samples from cows with clinical mastitis were thus excluded. If the different isolates recovered from 3 successive 3-weekly quarter milk samplings (S1, S2, S3) during the lactation period shared the same variable number of tandem repeats type (**VNTR** for 5 loci, as evaluated by multiplex PCR, see subsequent description), we considered that these isolates represented the same strain and that the IMI case was persistent. In such cases, only the isolate recovered from S1 was further characterized as the representative of the persistent strain (**SUB-P**, n = 139). Those defined as nonpersistent strains (**SUB-NP**, n = 38) were present at the first sampling S1 but not at both S2 and S3. For both persistent and nonpersistent cases, at least one *S. aureus* colony from a 10-μL milk sample needed to be found for the cases to be considered real infections (Dohoo et al., 2011). Also, on average, the reported SCC for the S1 samples taken during lactation were 1,447,000 and 889,800 for persistent and nonpersistent cases, respectively. More details on sampling procedures, herd and cow characteristics, and the CBMRN isolates collection is provided in Reyher et al. (2011).