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Communications of *Staphylococcus aureus* and non-*aureus Staphylococcus* species from bovine intramammary infections and teat apex colonization

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ABSTRACT

The role of non-*aureus* staphylococci (NAS) in the risk of acquisition of intramammary infections with *Staphylococcus aureus* is vague and still under debate. The objectives of this study were to (1) investigate the distribution patterns of NAS species from milk and teat skin in dairy herds with automatic milking systems, and (2) examine if the isolated NAS influences the expression of *S. aureus* virulence factors controlled by the accessory gene regulator (*agr*) quorum sensing system. In 8 herds, 14 to 20 cows with elevated somatic cell count were randomly selected for teat skin swabbing and aseptic quarter foremilk samples from right hind and left front quarters. Teat skin swabs were collected using the modified wet-dry method and milk samples were taken aseptically for bacterial culture. Colonies from quarters with suspicion of having NAS in milk or teat skin samples (or both) were subjected to MALDI-TOF assay for species identification. To investigate the interaction between *S. aureus* and NAS, 81 isolates NAS were subjected to a qualitative β-galactosidase reporter plate assay. In total, 373 NAS isolates were identified representing 105 from milk and 268 from teat skin of 284 quarters (= 142 cows). Sixteen different NAS species were identified, 15 species from teat skin and 10 species from milk. The most prevalent NAS species identified from milk were *Staphylococcus epidermidis* (50%), *Staphylococcus haemolyticus* (15%), and *Staphylococcus chromogenes* (11%), accounting for 76%. Meanwhile, the most prevalent NAS species from teat skin were *Staphylococcus equorum* (43%), *S. haemolyticus* (16%), and *Staphylococcus cohnii* (14%), accounting for 73%. Using reporter gene fusions monitoring transcriptional activity of key virulence factors and regulators, we found that out of 81 supernatants of NAS isolates, 77% reduced expression of *hla*, encoding a-hemolysin, 70% reduced expression of RNAIII, the key effector molecule of *agr*, and 61% reduced expression of *spa* encoding protein A of *S. aureus*, respectively. Our NAS isolates showed 3 main patterns: (1) downregulation effect such as *S. chromogenes* (milk) and *Staphylococcus xylosus* (milk and teat), (2) no effect such as *Staphylococcus sciuri* (teat) and *S. vitulinus* (teat), and the third pattern (c) variable effect such as *S. epidermidis* (milk and teat) and *S. equorum* (milk and teat). The pattern of cross-talk between NAS species and *S. aureus* virulence genes varied according to the involved NAS species, habitat type, and herd factors. The knowledge of how NAS influences *S. aureus* virulence factor expression could explain the varying protective effect of NAS on *S. aureus* intramammary infections.

Key words: non-*aureus* staphylococci, *Staphylococcus aureus*, microbial interaction, bovine mastitis, protective effect

INTRODUCTION

Nowadays, non-*aureus* staphylococci (**NAS**) are the most common cause of bovine IMI in dairy herds worldwide (Braem et al., 2013; Souza et al., 2016). When studying NAS, aggregating NAS as a group without accurate species identification is no longer recommended because species-specific differences in behavior, epidemiology, ecology, and effect on udder health have been revealed (Vanderhaeghen et al., 2014). Furthermore, NAS species showed great differences in antimicrobial susceptibility and virulence factors (Sawant et al., 2009). Condas et al. (2017) concluded that considering NAS as a single group has undoubtedly contributed to apparent discrepancies among studies as to their distribution and importance in IMI.

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Previous studies have extensively investigated the epidemiological characteristics of NAS for dairy herds with conventional milking systems. However, knowledge about these characteristics or patterns is sparse for dairy cows in automatic milking systems (**AMS**; Supré et al., 2011; De Visscher et al., 2014). Management of udder health in conventional milking systems differs from AMS (Dohmen et al., 2010; Hovinen and Pyörälä, 2011). Cows in AMS can be milked up to 5 times daily without any human contact with the udder. The longer milking duration and exposure of the teat skin to disinfectants may affect the teat skin microbiota. Furthermore, risk is high for teat colonization and subsequently IMI because up to 60 cows are milked several times daily with the same robot (Rasmussen, 2006).

The epidemiological and ecological characteristics NAS isolated from milk and surrounding environment of cows differ and are associated with the identified species. Results from research studies on NAS are sometimes conflicting. Vanderhaeghen et al. (2015) reported that *S. chromogenes* is a bovine-adapted species involved in many cases of IMI, and *Staphylococcus simulans* typically causes contagious IMI, whereas *Staphylococcus xylosus* appears to be a versatile species. The NAS species originating from distinct habitats showed clear differences that may be related to their diversity in ecology and epidemiological behavior (Souza et al., 2016). These different and contradictory results about NAS characteristics may likely be due to the lack of knowledge about their ecology and epidemiology within and between species (Fry et al., 2014). Therefore, extra efforts are crucial to improve our knowledge on different traits of NAS at the species level in the different habitats for boosting our understanding of their epidemiology in dairy herd context.

The effects of NAS on the risk of acquiring *Staphylococcus aureus* IMI have yielded ongoing debate (Reyher et al., 2012; Vanderhaeghen et al., 2014). Using traditional antibiotics is the most common approach for treatment of *S. aureus* infections and bovine mastitis in general. However, this approach is associated with adverse consequences including emergence of bacterial resistance and antimicrobial residues in milk (Gomes and Henriques, 2016). Therefore, finding effective non-antibiotic antimicrobials and alternative strategies to substitute the administration of antibiotics for mastitis treatment and control is vital. Painter et al. (2014) reported that the ability of *S. aureus* to cause a wide range of infections has been ascribed to its armory of various virulence factors, many of which are under the control of the quorum-sensing accessory gene regulator (***agr***) system of *S. aureus*. Singh and Ray (2014) demonstrated that *agr* plays a central role in staphylococcal pathogenesis. The *agr* system is composed of a 2-component signal transduction system that in response to a secreted auto-inducing peptide (**AIP**) controls virulence gene expression depending on cell density. At low cell density, cell-surface-associated adhesion factors are produced, whereas at high cell density hemolysins and other secreted virulence factors are expressed (Le and Otto, 2015). Originally, the *agr* system was considered only to monitor the presence of *S. aureus* cell densities, but several studies have documented that other staphylococcal species produce AIP-like molecules, which inhibit *S. aureus agr* and toxin production (Otto et al., 2001; Canovas et al., 2016; Paharik et al., 2017). Therefore, knowledge of the microbial interactions between a variety of NAS species originating from dairy cows and dairy environment on the one hand, and *S. aureus* on the other hand, may ultimately lead to new ways of controlling infections with *S. aureus*. To the best of our knowledge, no literature is available that has investigated the cross-talk between *agr* quorum system of *S. aureus* and NAS isolated from milk as well as teat skin habitats of dairy cows at species level of NAS. The objectives of this study were to (1) investigate the distribution patterns of NAS species on quarter level from aseptic milk and teat skin samples in dairy herds with AMS, and (2) examine if the isolated NAS influences the expression of *S. aureus* virulence factors controlled by the *agr* quorum sensing system.

MATERIALS AND METHODS

Study Population

Eight dairy herds with Danish Holstein cows were selected for participation in a project on *Streptococcus agalactiae* and *Staphylococcus aureus* IMI. The herds had to have AMS with ≥3 milking robots and bulk tank milk PCR cycle threshold value ≤32 for *Streptococcus agalactiae*. About 30 to 40 lactating dairy cows were selected randomly from each herd based on the criteria of having no clinical mastitis, having SCC ≥200,000 cells/mL at the previous milk recording, and not having been treated with antimicrobials during 4 wk before sample collection. Teat skin swab and aseptic foremilk samples were collected from all quarters of selected cows. In the current study, samples from right hind and left front quarters of cows with an odd laboratory running number were included. Herd management practices and characteristics are listed in Table 1.

Sampling Procedures

Each herd was visited once to collect teat swab samples and aseptic quarter foremilk samples for bacte-