J. Dairy Sci. 98:1090–1100
http://dx.doi.org/10.3168/jds.2014-8699
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Technical note: A pilot study using a mouse mastitis model to study differences between bovine associated coagulase-negative staphylococci

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ABSTRACT

Coagulase-negative staphylococci (CNS) are a group of bacteria classified as either minor mastitis pathogens or commensal microbiota. Recent research suggests species- and even strain-related epidemiological and genetic differences within the large CNS group. The current pilot study investigated in 2 experiments whether a mouse mastitis model validated for bovine *Staphylococcus aureus* can be used to explore further differences between CNS species and strains. In a first dose titration experiment, a low inoculum dose of *S. aureus* Newbould 305 (positive control) was compared with increasing inoculum doses of a *Staphylococcus chromogenes* strain originating from a chronic bovine intramammary infection to a sham-inoculated mammary glands (negative control). In contrast to the high bacterial growth following inoculation with *S. aureus*, *S. chromogenes* was retrieved in very low levels at 24 h postinduction (p.i.). In a second experiment, the inflammation inflicted by 3 CNS strains was studied in mice. The host immune response induced by the *S. chromogenes* intramammary strain was compared with the one induced by a *Staphylococcus fleurettii* strain originating from cow bedding sawdust and by a *S. chromogenes* strain originating from a teat apex of a heifer. As expected, at 28 and 48 h p.i., low bacterial growth and local neutrophil influx in the mammary gland were induced by all CNS strains. As hypothesized, bacterial growth p.i. was the lowest for *S. fleurettii* compared with that induced by the 2 *S. chromogenes* strains, and the overall immune response established by the 3 CNS strains was less pronounced compared with the one induced by *S. aureus*. Proinflammatory cytokine profiling revealed that *S. aureus* locally induced IL-6 and IL-1β but not TNF-α, whereas, overall, CNS-inoculated glands lacked a strong cytokine host response but also induced IL-1β locally. Compared with both other CNS strains, *S. chromogenes* from the teat apex inflicted a more variable IL-1β response characterized by a more intense local reaction in several mice. This pilot study suggests that an intraductal mouse model can mimic bovine CNS mastitis and has potential as a complementary in vivo tool for future CNS mastitis research. Furthermore, it indicates that epidemiologically different bovine CNS species or strains induce a differential host innate immune response in the murine mammary gland.

Key words: coagulase-negative staphylococci strain, mouse mastitis model, bacterial growth, neutrophil influx, proinflammatory cytokine

Technical Note

Coagulase-negative staphylococci are a group of bacteria that cause mastitis in both heifers and multiparous cows (Thorberg et al., 2009; De Vliegher et al., 2012; Ajitkumar et al., 2013). Until recently, it was difficult to draw consistent conclusions on the relevance of CNS for bovine udder health. Some studies considered CNS as true mastitis pathogens, although most were retrieved from subclinical mastitis cases (Pyörälä and Taponen, 2009), whereas others considered CNS to be commensal bacteria with limited or absent negative effects on SCC, milk quality, and milk production (Schukken et al., 2009; De Vliegher et al., 2012). The development and validation of molecular identification techniques specifically for bovine-associated CNS were the basis to eliminate this confusion (Zadoks and Watts, 2009). Whereas older studies dealt with CNS as one homogenous and coherent group (Schukken et al., 2009; Piepers et al., 2010; Piepers et al., 2013), more recent studies addressed the CNS species separately, revealing heterogeneous characteristics between them (Avall-Jääskeläinen et al., 2013). In essence, 12 CNS species are frequently isolated from bovine milk. Evidence exists for diversity between and even within those species in their epidemiologic behavior and traits, such as persistence, antibiotic resistance, susceptibility to phagocytosis by mouse macrophages, and (putative) virulence (Piessens et al., 2011; Supré et al., 2011;

Received August 2, 2014.
Accepted October 20, 2014.
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Avall-Jääskeläinen et al., 2013; Vanderhaeghen et al., 2014).

To make further progress in our understanding of CNS mastitis, the interaction between CNS species and strains and the host should be studied in more detail. Ideally, such experimental infection studies are conducted using dairy cows. Unfortunately, these bovine studies are expensive and labor intensive. For those reasons, a mouse mastitis model was developed and characterized (Chandler, 1970) and has since been successfully validated for studying the specific host immune response to IMI with major mastitis pathogens, such as *Escherichia coli* and *Staphylococcus aureus* (Brouillette and Malouin, 2005; Demon et al., 2012; Demon et al., 2013), but not for CNS.

The usefulness of a mouse mastitis model as a complementary tool to investigate differences between bovine CNS species and strains was explored. Specifically, 2 experiments were performed to answer 3 clear-cut objectives: (1) whether bovine-associated CNS grow in the murine mammary gland, (2) whether they induce mastitis in mice, and (3) whether it is possible to detect differences in bacterial growth, clinical symptoms, and host immune response between CNS species or strains with a different origin using this mouse mastitis model.

The set-up of the 2 experiments is outlined in Figure 1. For both experiments, 8-wk-old female Hsd:ICR (CD1) mice that mated with 10-wk-old male Hsd:ICR (CD1) mice (Harlan Laboratories, Horst, the Netherlands) were used. Pups were weaned ±10 d after parturition to ease the intraductal accessibility. All inoculations were performed 2 h postweaning under isoflurane anesthesia combined with a long-acting analgesic buprenorphine (10 μg/kg of Vetergesic, Patheon UK Ltd., Swindon, UK) using a 32-gauge blunt needle. During both experiments, core body temperature of the mice was measured with a rectal thermistor. All experiments were approved by the committee on the ethics of animal experiments of Faculty of Veterinary Medicine, University of Ghent (permit number: EC2013/166).

In a first dose titration experiment, 4 increasing inoculum doses (i.e., 2.5×10^2, 2.5×10^3, 2.5×10^4, and 2.5×10^5 cfu/100 μL) of a bovine-associated *Staphylococcus chromogenes* strain originating from a chronic IMI [**IM** (Supré et al., 2011)] were injected in the fourth gland pair of each of the 12 mice ($n_{mice/dose} = 3$; $n_{glands/dose} = 6$) to evaluate both bacterial growth and immune cell influx in the mammary glands 24 h postinduction (**p.i.**). An additional 6 mice were inoculated with either a 100-μL sham solution (PBS + 10% glycerol; $n_{mice} = 3$) or 1×10^2 cfu/100 μL of *Staphylococcus aureus* Newbould 305 ($n_{mice} = 3$) and were included as negative and positive controls, respectively (Figure 1A). Twenty-four hours p.i., all mice were first sedated by administering a mixture of ketamine (100 mg/kg of Anesketin, Eurovet Animal Health BV, Bladel, the Netherlands) with xylazine (10 mg/kg; Xylazini Hydrochloridum, Val d'Hony-Verdifarm, Beringen, Belgium) intraperitoneally and subsequently euthanized. After euthanasia, all *S. chromogenes*-inoculated glands ($n_{glands/inoculum} = 6$) were isolated to determine their bacterial load. Likewise, the *S. chromogenes*-inoculated glands with clinical signs of inflammation after dissection (i.e., red color, hard, swollen), as well as the 2 sham- and 2 *S. aureus*-inoculated glands, were further processed for histology. To quantify bacterial growth, the mammary glands were weighed, homogenized, and spotted (20 μL) in serial logarithmic dilutions on Tryptic soy agar plates (Oxoid, Drongen, Belgium) overnight at 37°C to determine colony-forming units per 100 μL and divided by the exact weight of a mammary gland (g). For the histologic sections, mammary glands were divided in 3 transverse proportions to validate the homogeneity of the infection: near the nipple (i.e., near the inoculation site), near the lymph node, and near the back. Two mammary glands for every inoculation (sham-, *S. aureus*-, and 10^5 cfu of *S. chromogenes* IM-inoculated glands) were fixed in buffered 3.5% formaldehyde (Sigma-Aldrich, St. Louis, MO) for 24 h at room temperature (24°C). Subsequently, the samples were dehydrated and embedded in paraffin wax. Sections were deparaffinized, hydrated, and stained with hematoxylin and eosin (Sigma-Aldrich). As the inoculated mammary glands displayed clinical symptoms of inflammation following a *S. chromogenes* IM inoculum dose as high as 10^5 cfu, but not at lower doses, only these CNS-inoculated glands were collected for histological evaluation.

In the second experiment, 2 additional bovine CNS strains were included [a *Staphylococcus fleurettii* strain originating from sawdust (Supré et al., 2011) and a *S. chromogenes* strain originating from a teat apex (**TA**) of a heifer (De Vliegher et al., 2004)]. The fourth gland pair of each of the 18 mice ($n_{mice/CNS\ strain} = 6$; $n_{glands/CNS\ strain} = 12$) were injected with either *S. chromogenes* IM, *S. chromogenes* TA, or *S. fleurettii* at an inoculum dose of approx. 10^5 cfu (9.6×10^4 cfu/100 μL of *S. chromogenes* IM, 9.3×10^4 cfu/100 μL of *S. chromogenes* TA, and 8.5×10^4 cfu/100 μL of *S. fleurettii*, respectively). Three additional mice were inoculated with 2.5×10^2 cfu/100 μL of *S. aureus* Newbould 305 ($n_{mice/dose} = 3$; $n_{glands/dose} = 6$) and included as a positive control (Figure 1B). The fifth gland pair of each mouse was inoculated with 100 μL of sham solution and served as negative control. At 28 h p.i., 12 mice (3 mice per CNS strain and 3 *S. aureus* Newbould 305-inoculated mice) were sedated and euthanized as previously described. The other mice (n = 9) were se-

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