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Early host response in the mammary gland after experimental *Streptococcus uberis* challenge in heifers

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ABSTRACT

Streptococcus uberis is a highly prevalent causative agent of bovine mastitis, which leads to large economic losses in the dairy industry. The aim of this study was to examine the host response during acute inflammation after experimental challenge with capsulated *Strep. uberis*. Gene expression in response to *Strep. uberis* was compared between infected and control quarters in 3 animals. All quarters (n = 16) were sampled at 16 different locations. Microarray data showed that 239 genes were differentially expressed between infected and control quarters. No differences in gene expression were observed between the different locations. Microarray data were confirmed for several genes using quantitative PCR analysis. Genes differentially expressed due to early *Strep. uberis* mastitis represented several stages of the process of infection: (1) pathogen recognition; (2) chemoattraction of neutrophils; (3) tissue repair mechanisms; and (4) bactericidal activity. Three different pathogen recognition genes were induced: ficolins, lipopolysaccharide binding protein, and toll-like receptor 2. Calgranulins were found to be the most strongly upregulated genes during early inflammation. By histology and immunohistochemistry, we demonstrated that changes in gene expression in response to *Strep. uberis* were induced both in infiltrating somatic milk cells and in mammary epithelial cells, demonstrating that the latter cell type plays a role in milk production as well as immune responsiveness. Given the rapid development of inflammation or mastitis after infection, early diagnosis of (*Strep. uberis*) mastitis is required for prevention of disease and spread of the pathogen. Insight into host responses could help to design immunomodulatory therapies to dampen inflammation after (early) diagnosis of *Strep. uberis* mastitis. Future research should focus on development of these early diagnostics and immunomodulatory components for mastitis treatment.
Key words: mastitis, *Streptococcus uberis*, host response, microarray

INTRODUCTION

Mastitis is an inflammation of the mammary gland that is usually caused by bacterial infection. Worldwide, *Streptococcus uberis* is a common causative agent for bovine mastitis (Bradley et al., 2007; Compton et al., 2007; Olde Riekerink et al., 2008). *Streptococcus uberis* is considered an environmental pathogen; it has been recovered from soil, bedding material, and feces, as well as from several areas on the cow such as skin and udder (Smith and Hogan, 1993; Zadoks et al., 2011). In addition to clinical cases of mastitis, chronic subclinical intramammary infections with *Strep. uberis* occur frequently on dairy farms (Jayarao et al., 1999; Zadoks et al., 2003; Bradley et al., 2007). Subclinical infections often result in persistence of *Strep. uberis*, which may lead to spread of the disease (Zadoks et al., 2001, 2003). In addition, subclinical mastitis can flare up under certain conditions, causing clinical mastitis (Jayarao et al., 1999; Zadoks et al., 2003; Bradley et al., 2007). A recent study in the Netherlands showed that, despite preventive measures, prevalence of *Strep. uberis* infections has not decreased compared with 30 yr ago (Sampimon et al., 2009). Farmers' knowledge and perception play a crucial role in efforts to reduce the occurrence of mastitis based on current scientific knowledge of the disease (Jansen et al., 2010). In addition, enhanced understanding of the biology of mastitis may contribute to development of new control measures, such as immunomodulatory components to suppress (part of) the overwhelming inflammatory response. Compared with the host response to *Staphylococcus aureus* and *Escherichia coli* mastitis, the response to

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Strep. uberis mastitis is relatively poorly understood (Schukken et al., 2011; Wellnitz and Bruckmaier, 2012).

The physiological host response to infection is initiated by pathogen recognition that subsequently triggers the acute phase response, which is central to innate immunity of the host. The aim of this study was to examine the host response during acute inflammation in heifers after experimental challenge with *Strep. uberis* using microarrays and immunohistochemistry. In a previous study, the immune response to challenge with a noncapsular *Strep. uberis* wild-type strain was analyzed in heifers (Swanson et al., 2009). The strain had been isolated from a case of clinical mastitis in the field, and unencapsulated mutants of *Strep. uberis* are known to have the have the ability to cause clinical mastitis after experimental challenge (Field et al., 2003; Swanson et al., 2009). Carriage of the *has*A gene, which is required for capsule production by *Strep. uberis*, has, however, been linked to clinical mastitis, whereas absence of *has*A has been linked to subclinical infection (Pullinger et al., 2006; Tomita et al., 2008; Swanson et al., 2009). Thus, further study into the in vivo host response to encapsulated *Strep. uberis* may provide further insight into the pathogenesis of clinical *Strep. uberis* mastitis.

We used 2 encapsulated *Strep. uberis* isolates, reference strain O140J (Hill, 1988) and a Dutch field isolate (41-241), which was part of an outbreak of clinical and subclinical mastitis affecting multiple cows (Zadoks et al., 2003). The isolates belonged to sequence types 1 and 385, respectively, and are part of 2 global clonal complexes (GCC5 and GCC143, respectively) that are associated with a large number of mastitis cases in numerous countries (Zadoks et al., 2011). To evaluate the primary response to *Strep. uberis* challenge, primiparous heifers were used for infection.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

Streptococcus uberis strains O140J (Leigh et al., 1990) and 41-241 (Zadoks et al., 2001) were grown on Columbia agar blood base plates (Oxoid Ltd., London, UK) containing 6% (vol/vol) horse blood and 0.1% (vol/vol) esculin. To prepare the inoculum, cultures were grown in Todd-Hewitt broth (Oxoid) for 16 h at 37°C. Subsequently, cultures were diluted 1:10 in Todd-Hewitt broth and grown to exponential phase. Cells were washed once with PBS, and the inoculum was prepared based on the optical density of the culture. The final concentration of the inoculum was determined by plating serial dilutions on Columbia agar blood base plates containing 6% (vol/vol) horse blood and 0.1% (vol/vol) esculin.

Experimental Infection

Four Holstein-Friesian primiparous heifers in early lactation with no history of mastitis (based on clinical signs) were inoculated with *Strep. uberis*. Before inclusion in the experiment, SCC values in the quarter milk of all animals were determined to be $<1.5 \times 10^5$ cells/mL using methodology described later, and milk was checked bacteriologically by plating 100 μL on Columbia agar blood base plates containing 6% (vol/vol) horse blood and 0.1% (vol/vol) esculin for absence of *Strep. uberis* colonies under UV light. Before inoculation via the teat canal, teats were disinfected with iodine. Two diagonally opposed quarters of each animal were challenged intramammarily with *Strep. uberis*, and the remaining 2 quarters were mock challenged with PBS (0.1 *M* NaCl, 33 m*M* Na_2PO_4, 17 m*M* $NaH_2PO_4 \cdot 2H_2O$; pH 7.4) and served as within-cow control quarters. Two animals were challenged with *Strep. uberis* strain O140J (500 cfu). Because the virulence of strain 41-241 was unknown, one animal was challenged with a dose of 500 cfu and the other with 5,000 cfu. Cows were inoculated after the 1600 h milking with a short blunt-ended needle, and the udder was massaged to distribute the inoculum. After inoculation, milk and blood samples were taken twice daily at 1000 and 1600 h and kept at –20°C until use. Milk was analyzed bacteriologically as described above, and SCC of milk were determined. Rectal temperature, milking volume per quarter, hematology indices, and general health of animals were monitored throughout the experiment.

The heifers were euthanized after 2 increased SCC ($>10^6$ cfu/mL) at 2 subsequent milkings. Animals that did not display increased SCC were euthanized at the end of the experiment (4 d postchallenge). The udder was removed and tissue samples were collected from various locations of control and infected mammary quarters. Each quarter was sampled distally (teat cistern and gland cistern) and proximally (level 1 and level 2 at 4 locations: medio-cranial, medio-caudal, latero-cranial, and latero-caudal; Figure 1), immediately after the animals were euthanized. Samples from all 16 locations were snap-frozen in liquid nitrogen for RNA isolation and stored at –70°C. Samples from the same 16 locations were examined bacteriologically by swabbing samples with an inoculation loop and plating the contents on Columbia agar blood base plates containing 6% (vol/vol) horse blood and 0.1% (vol/vol) esculin and counting *Strep. uberis* colonies under UV light. Samples from the same 16 locations were fixed in formalin, embedded in paraffin, sectioned at 3 to 5 μm, and subsequently stained with hematoxylin and eosin (Merck, Darmstadt, Germany) for histological assessment or used for immunohistochemistry as described