Veterinary Microbiology 137 (2009) 326–334

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Somatic cell scores and clinical signs following experimental intramammary infection of dairy cows with a *Staphylococcus aureus* small colony variant (*S. aureus* SCV) in comparison to other bovine strains

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ARTICLE INFO

Article history:
Received 9 October 2008
Received in revised form 14 January 2009
Accepted 19 January 2009

Keywords:
Bovine mastitis
Experimental intramammary infection
Bovine *S. aureus* SCV
Persistent *S. aureus*
hemB mutant
Newbould 305

ABSTRACT

A recently isolated bovine *Staphylococcus aureus* small colony variant (SCV) was shown to persist within cultured endothelial cells. However, the clinical importance of *S. aureus* SCV in persistent infection of the mammary gland is unknown. The hypothesis tested here was a naturally occurring bovine *S. aureus* SCV, Heba3231, and a Newbould *hemB* mutant that was an artificially created SCV would establish mild intramammary infection and induce a different host response compared to their isogenic parental strains, 3231 and Newbould 305. Four groups of clinically healthy cows, 5 cows/treatment group, were infected by the intramammary route with SCV Heba3231, strain 3231, the *hemB* mutant or strain Newbould 305. Three quarters of the mammary gland of each cow were challenged with ~5000 colony-forming units of bacteria and the fourth quarter was infused with PBS. Cows were monitored and assigned clinical scores for the first 5 days post-challenge based on rectal temperature, appetite, milk yield, udder uniformity, milk appearance, bacterial culture of foremilk samples and somatic cell score (SCS). All cows were examined for chronic infection up to day 36 post-challenge. Cows challenged with SCV strains developed mastitis that was mild compared with that induced by the parent strains. Cows challenged with strain 3231 developed the most severe clinical mastitis. SCS from all treatment groups were significantly ($P < 0.05$) higher at days 2–5 and 14–36 post-infection compared to day 0 before challenge. This milder host response to SCVs may represent an adaptation of *S. aureus* to the mammary gland environment.

1. Introduction

Staphylococcus aureus mastitis is difficult to eliminate from the infected herds, despite implementation of the "five-point plan" (Neave et al., 1969) or the more recently adopted 10-point mastitis control program (National Mastitis Council, 2001). Establishment of persistent infection of the mammary gland is influenced by genetic and environmental factors that include cow effects, pathogen-related factors and the antimicrobial treatment regimen (Barkema et al., 2006). Most antibiotic treatment programs result in short-term clinical cure and relapsing infection of the cured quarters (Barkema et al., 2006). It has recently been hypothesized that persistent infection of the mammary gland may relate to the ability of specific strains of *S. aureus* to transform into a variant subpopulation known as small colony variants (SCVs) that can survive within host cells and subsequently modulate the immune response (Atalla et al., 2008).

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doi:10.1016/j.vetmic.2009.01.027

Several *in vitro* studies demonstrated the ability of *S. aureus* to invade and survive within a bovine mammary epithelial cell line (MAC-T) (Almeida et al., 1996; Bayles et al., 1998) and also resist the killing by phagocytic by macrophages (Hébert et al., 2000) and polymorphonuclear (PMN) leukocytes (Voyich et al., 2005). Recently, we isolated a bovine *S. aureus* SCV (SCV Heba3231) from a commercial herd infected with chronic *S. aureus* mastitis. The SCV Heba3231 had typical metabolic characteristics of the SCV phenotype including slow mode of growth, reduced colony size, decreased production of α-toxin, and increased resistance to gentamicin compared to its wild-type parental strain (Atalla et al., 2008). Although *in vitro* studies showed that the SCV Heba3231 had minimal deleterious effects on cultured endothelial cells (Atalla et al., 2008), the association between *S. aureus* SCV and persistent infection of the bovine mammary gland is unknown. Therefore, the objective of this study was to determine the response of the bovine mammary gland to *S. aureus* SCV and during the acute and chronic phases of experimentally induced intramammary infection compared to the response to the parental strains.

2. Materials and methods

2.1. Cows

Twenty clinically healthy Holstein dairy cows (5 cows/treatment group) at the University of Guelph dairy herd were selected for the study. Cows were in their mid- to late-lactation and in the second or third trimester of gestation. They had no previous history of clinical mastitis and were in their first to fifth lactation. Selection of each group of cows was based on low somatic cell counts (SCC) and bacterial culture. Prior to intramammary challenge, quarter foremilk samples were aseptically collected by the dairy personnel once a week for two consecutive weeks according to the International Dairy Federation standards (IDF, 1985). All teats were dipped in 1% iodine teat dip, forestripped and the first two streams of milk were discarded. All teats were dry-wiped with a clean towel and then scrubbed with cotton balls soaked in 70% isopropyl alcohol. Quarter foremilk samples were collected by hand-milking into pre-labeled, sterile snap-top plastic bottles (Fisher Scientifics, Ottawa, Canada). After sample collection, all teats were dipped in 1% iodine teat dip. Milk samples were collected at morning milking, stored at 4 °C, transported to the laboratory on ice and tested upon arrival, according to the National Mastitis Council (NMC) recommendations (Hogan et al., 1999). Briefly, a loopful (0.01 ml) of quarter milk was plated on Columbia blood agar (CBA) supplemented with 5% sheep red blood cells and on MacConkey agar plates. Plates were incubated at 37 °C and examined at 24 and 48 h. Selected cows had the following criteria: (1) quarter milk samples were negative for *S. aureus*; (2) quarter milk samples contained 500 CFU\ml or less of potential udder pathogens other than *S. aureus*, that were mostly found in mixed culture and did not influence the immune status of the mammary gland with respect to SCC and (3) quarter milk samples with SCC of less than 2×10^5 cells\ml. Cows were housed in a tie-stall barn and were fed a mixed ration formulated for the University dairy herd. They were milked using a pipeline milking system twice daily at 5:30 a.m. and 4:00 p.m. Cows used in the study were individually identified with numbered ear tags and with red band on both hind ankles. The use and care of all animals in this study has been approved by the University of Guelph Animal Care and Use Committee.

2.2. Preparation of *S. aureus* inoculum

Four *S. aureus* strains were used for the intramammary challenge: (a) the *S. aureus* SCV Heba3231 (Atalla et al., 2008) with atypical phenotypic properties including pin-point, non-hemolytic colonies on CBA, late coagulase production and inability to ferment mannitol; (b) the 3231 parent strain with typical morphological and biochemical properties including large, creamy hemolytic colonies on CBA, positive coagulase production within 18 h and sugar–alcohol fermentation (Atalla et al., 2008); (c) the Newbould *hemB*-disrupted mutant (insertional inactivation) (Brouillette et al., 2004) that has reduced colony size and a narrow zone of hemolysis on CBA compared to its parent strain; and (d) the prototypic strain *S. aureus* Newbould 305 (ATCC 29740). *S. aureus* Newbould 305 is a bovine clinical mastitis strain (Newbould, 1974) that has been routinely used for intramammary infection studies. Both the Newbould *hemB*-disrupted mutant and the prototype Newbould 305 were kindly provided by Professor F. Malouin (Department of Biological Science, University of Sherbrooke, Sherbrooke, Quebec). The term "*S. aureus* atypical phenotype" will be used to describe SCV strains that form pin-point non-hemolytic colonies on CBA after 48-h incubation with late coagulase production, while the term "*S. aureus* typical phenotype" will be used to describe wild-type strains that form large hemolytic colonies on CBA and produce coagulase within 18 h.

Each strain of *S. aureus*, stored in cryoprotection medium composed of equal volumes of skimmed milk and bovine serum albumin (BSA) at −80° C, was prepared in the early exponential growth phase as described previously (Atalla et al., 2008). Briefly, overnight bacterial culture in brain heart infusion (BHI) broth was diluted with BHI broth, placed on a rotary shaker (200 rpm) at 37 °C and adjusted to an $OD_{600\ nm}$ of 0.2 in the case of SCV Heb3231 and the Newbould *hemB* mutant or an $OD_{600\ nm}$ of 0.4 in the case of 3231 and Newbould 305. The bacterial suspension was centrifuged ($1000 \times g$) for 20 min at 4 °C. The supernatant was discarded and the pellet was suspended in sterile ice-cold, pyrogen-free, phosphate buffered saline (PBS, pH 7.4) and washed three times ($1000 \times g$) for 10 min at 4 °C. Immediately before intramammary challenge, washed cells were re-suspended in ice-cold, pyrogen-free, PBS (pH 7.4) and diluted to $\sim 1 \times 10^3$ CFU/ml based on previous direct plate counts of each strain. In addition, the CFU/ml of the prepared suspended bacterial cells was determined by direct plate count. The *hemB* mutant broth and agar cultures were supplemented with 5 μg/ml erythromycin (Sigma) (Brouillette et al., 2004).