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Pathogenesis and inflammatory response in experimental caprine mastitis due to *Staphylococcus chromogenes*

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ABSTRACT

Coagulase-negative staphylococci (CNS) are the most frequently isolated bacteria in cases of subclinical mastitis in dairy cows. CNS species may differ in their pathogenicity, but very little is known about their virulence factors or their immune response in intramammary infections. To our knowledge, no experimental studies into the mastitis pathogenesis caused by CNS have been described in lactating goats. The aim of this study was to induce an experimentally *Staphylococcus chromogenes* mastitis in lactating goats aimed at verifying if the model can be used to evaluate the inflammatory response, the dynamics of infection and the pathological findings within the first hours of intramammary inoculation. Six Saanen goats in mid-lactation were inoculated with 1×10^7 colony forming units of *S. chromogenes*. Bacterial growth peaked in milk from the challenged right halves of the mammary glands (RMG) at 4 h post inoculation (PI). Shedding of viable bacteria showed a marked decrease at 12 h PI. An increase in mean somatic cell counts was observed in the milk samples from 8 h PI onwards. Mild clinical signs were evoked by intramammary inoculation. *Staphylococcus chromogenes* could be isolated in tissue from all RMG. Histological examination of specimens of the RMG and lymph nodes of the goats showed an increased inflammatory response throughout the experiment with respect to control halves. In conclusion, the experimental inoculation of *S. chromogenes* in lactating goats is capable of eliciting an inflammatory response and capable of causing pathological changes. This research represents a preliminary study for a better knowledge of the mastitis pathogenesis caused by *S. chromogenes*.

1. Introduction

Subclinical mastitis is the predominant disease affecting dairy cows, causing great economic losses worldwide [1]. Subclinical mastitis is evidenced by a high somatic cell count (SCC) in the milk in response to an inflammatory process, but it doesn't show any visible alteration of the milk or the udder [2]. Different works report that coagulase-negative staphylococci (CNS) are the most frequently bacteria isolated from subclinical mastitis in caprine and bovine herds [3–8]. Epidemiological data suggest that not all CNS species exhibit the same degree of pathogenicity [9]. Nevertheless, very little is known about their virulence factors and the different host responses either caused by intramammary infections (IMI) with representatives of the supposed environmental or host-adapted species (or strains). Among the group of CNS commonly isolated from dairy cows mastitis, *Staphylococcus chromogenes* is one of the most prevalent [9–16].

Nowadays, the term non-aureus staphylococci (NAS) is recommended instead of CNS, because the first one includes some coagulase-positive and coagulase-variable species. Since *S. chromogenes* is coagulase-negative, the term CNS for the bacterial group to which *S. chromogenes* belongs will be used.

Development of experimental IMI models would facilitate the study of the mastitis pathogenesis caused by this pathogen. The pathogenesis of CNS in experimental bovine IMI has been studied by different authors [17,18], but the costs associated with that experimental model are the major obstacle when conducting experiments that require a

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minimal number of animals to get valid statistics. Because of that, mice and rabbits have been used as experimental animals for induced mastitis [19–21]. However, the mammary gland of these small animals differs considerably from the bovine udder. Certainly, the key characteristic for any host to qualify as a model for bacterial pathogenesis is to respond to the infectious agent in the same way as cows.

An experiment using a caprine mastitis model induced with *S. uberis* [22] has been previously carried out. The experimental inoculation of *S. uberis* in lactating goats was capable of eliciting an inflammatory response and capable of causing pathological changes, resulting in a subclinical mastitis.

Others authors have also developed experimental goat models using different mastitis pathogens, such as *Staphylococcus aureus* [23–27], *Escherichia coli* [28], *Mycoplasma agalactiae* [29,30], *Candida albicans* [31], *Chlamydia psittaci* [32], *Nocardia asteroides* [33] and *Cryptococcus neoformans* [34]. To our knowledge, no experimental studies about the mastitis pathogenesis caused by CNS have been described in lactating goats. Considering that the caprine model of mastitis is well documented and that structural genomic studies have shown that goats are closely related to bovine species [35], experimentally induced mastitis with CNS in lactating goats will try to reproduce it. Based on our previous studies [22], the aim of this research was to verify if the model can be used to evaluate the inflammatory response, the dynamics of infection and the pathological findings within the first hours of intramammary inoculation with *Staphylococcus chromogenes*, one of the most prevalent species of CNS in dairy herds.

Since CNS are frequently isolated from subclinical mastitis of goats, the dairy cows and goats benefit from this investigation.

2. Materials and methods

2.1. Animals

Six Saanen goats in mid-lactation were chosen according to the following criteria: first parity and absence of IMI and low SCC in their milk (< 250,000 cells/mL). To ensure that the dairy goats used in the experiment were free from bacterial infection at the moment of challenge, milk samples were taken from both udder halves 24, 48, 72 h prior to intramammary CNS infusions and tested for bacterial growth on trypticase soy agar (TSA) [24]. Goats used in the study were individually identified with numbered ear tags. During the experiment, the animals were housed in stalls under standardized conditions (temperature: 18–20 °C, relative humidity: 60–65%) in accordance with international guidelines for animal welfare. Goats were fed with daily ration of 1 kg of ground corn/animal and had free access to hay and water. Throughout the study, animal care met all applicable international guidelines for animal welfare.

2.2. Preparation of S. chromogenes inoculum

Staphylococcus chromogenes strain RC10-31 originally obtained from a case of subclinical bovine mastitis was used [8]. The strain was previously identified to the species level by PCR restriction fragment length polymorphism (RFLP) analysis of a partial *groEL* gene sequence [16] and subjected to the MALDI-TOF MS identification procedure (Private Hospital, Microbiology Laboratory, Universidad Nacional de Córdoba, Argentina). To corroborate the identity of the strain throughout the entire trial, streptomycin-resistant (Sm^r) isolate RC10-31 Sm^r, obtained by plating out on TSA supplemented with streptomycin (100 μg/mL) of sensitive strain *S. chromogenes* RC10-31 was used. Stock bacterial suspension was thawed and viable *S. chromogenes* RC10-31 Sm^r was quantified using serial decimal dilutions in 0.9% of saline solution before inoculation. One hundred microliters aliquots were plated onto TSA and colonies were counted after incubation at 37 °C for 24 h. The following doses were tested: 1×10^4 and 1×10^7 cfu/mL for experimental mastitis. The chosen dose was 1×10^7 cfu/mL, given that it showed changes in the RCS within a period of 24 h.

2.3. Experimental infection

Prior to intramammary challenge, both udder halves were milked by hand. Then, the teat ends were carefully cleaned with individual moistened towels and disinfected with swabs containing 70% ethanol. The halves of the mammary glands were infused through the teat canal 30 min after morning milking using a blunt cannula. A 1.0 mL inoculum containing 1.7×10^7 colony forming unit (cfu) of *S. chromogenes* was injected intracisternally into the right halves of the mammary glands of six goats. The left halves mammary glands were infused with 1.0 mL of sterile PBS, considering that each half of the udder is a semi-autonomous physiological unit and can be considered as an independent experimental unit during a relatively brief experimental bacterial challenge. After the infusion, each gland was massaged upward into the gland cistern for 30 s to distribute the inoculation dose.

Five goats were culled by barbiturate overdose, followed by exsanguination, and subjected to a complete necropsy. The goats were killed consecutively at 4–hourly intervals (8, 12, 16, 20 and 24 h) after inoculation.

Antibiotic therapy with kanamycin 100.000 U.I. and cephalexin (200 mg) was administered to the 6^{th} goat at the end of the trial (24 h PI). Two doses were administered, with an interval of 24 h between treatments.

2.4. Legislation and ethical approval

All animals involved in this investigation were cared for in accordance with CIOMS International Guiding Principles for Biomedical Research Involving Animals (1985) [36]. The study protocol was approved by the Ethics Committee for Animal Experimentation at the Universidad Nacional de Río Cuarto, Córdoba, Argentina (protocol authorization number CoEdi32-11).

2.5. Milk samples

Milk samples were collected using an aseptic technique from the right and left halves of the udder of each goat (after discarding the first few stripping of foremilk) before inoculation and 4, 8, 12, 16, 20, and 24 h post inoculation (PI). Goats were not milked during the trial. Samples were stored at 4 °C and analyzed within 24 hs.

Somatic cell counts were determined in aliquot of goat milk using a fluoro-optoelectronic counter Somacount 300 (Bentley instrument, Chaska, MN, USA) according to the manufacturer's instructions. Ethidium bromide dye was used for specific binding to the DNA in the cell nuclei.

Milk samples were serially diluted with sterile saline solution and 100 μl were plated onto TSA plates with streptomycin (100 μg/mL) to confirm the identity with the challenge strain. Number of cfu/mL was determinated after 18 hs of incubation at 37 °C. Only plates with 30-300 colonies were counted.

2.6. Blood samples

Jugular vein blood samples were collected from each animal before inoculation, and every four hours until the end of the assay. Total and differential counts of leucocytes were performed on EDTA-stabilized blood at the hour of sampling 100 cells with a Neubauer chamber were counted. Smears were stained with May Grunwald-Giemsa. The total number of leucocytes per microliter of whole blood was calculated [37].