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Correlating the immune response with the clinical–pathological course of persistent mastitis experimentally induced by *Mycoplasma agalactiae* in dairy goats

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ABSTRACT

To correlate the clinical course of mycoplasma mastitis with its immune response, right mammary glands of 15 lactating goats were inoculating with 10^{10} colony-forming units (cfu) of *Mycoplasma agalactiae* (*Ma*). Before sacrificing the animals at 5, 15 or 45 days post-inoculation (dpi), blood *Ma* antibody titres and milk mycoplasma colony and somatic cell counts were monitored. *Ma* colonised the mammary gland and milk counts increased to over 10^{12} cfu/ml within 5 dpi. During this period, an innate immune response involving neutrophils and macrophages was observed, and *Ma* antigen appeared in the degenerated acinar epithelium. From 7 dpi, a specific antibody response coincided with reduced viable mycoplasmas in milk. The humoral immune response was limited; by 37 dpi, all animals scored negative for anti-*Ma* antibodies, and around 10^{8} cfu/ml were shed. Results indicate an early immune response to *Ma* inoculation unable to control mycoplasmal invasion. An ensuing humoral response, despite reducing the mycoplasma burden, leads to chronic, persistent infection.

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1. Introduction

Contagious agalactia (CA) is one of the most serious diseases to affect small ruminants on all five continents and is considered endemic in most Mediterranean countries (Lambert, 1987; DaMassa et al., 1992; Hasso et al., 1993; Real et al., 1994; Bergonier et al., 1997). In goats, CA is a syndrome caused by several species of Mycoplasma that share tissue tropism, culture and antigenic features. These organisms include *Mycoplasma agalactiae* (*Ma*) (the main aetiological agent accounting for some 90% of outbreaks of the syndrome), *Mycoplasma mycoides* subsp. *mycoides* Large Colony (*Mmm* LC), *M. capricolum* subsp. *capricolum* (*Mcc*) and *M. putrefaciens* (*Mp*) (Lambert, 1987; Real et al., 1994).

Sick animals are the most frequent source of mycoplasmas in CA syndrome, and the mammary route is the main transmission pathway in milked adult goats. Contagious agalactia outbreaks are frequently described after parturition and the onset of milking. These situations often reflect the expression of an infection contracted after the introduction of an asymptomatic carrier in the herd during the previous lactation, and its transmission through the milk during milking (particularly machine milking) (Lambert, 1987; Kinde et al., 1994).

Clinical disease after *Ma* mammary gland infection can manifest as acute (unilateral or bilateral) mastitis, but usually progresses as a subacute or chronic disease during the course of which the pathogen is excreted in the milk during long periods of time. Symptoms observed include functional disorders of the mammary gland ranging from transient hypogalactia to abrupt and total agalactia. Disease severity usually steadily improves until clinical signs resolve, although mycoplasmas continue to be shed, and animals become asymptomatic carriers (Bergonier et al., 1997).

Despite the fact that CA has been widely described, its pathogenesis is not yet well understood. Interactions between mycoplasmas and host factors are still unclear, especially the ability of mycoplasmas to persist in host tissues despite a prominent immuno-inflammatory response. In addition, the literature lacks studies designed to correlate, during the course of mycoplasma infection in lactating goats, pathological changes, bacterial loads and clinical–pathological variables such as specific antibody titres or somatic cell counts (SCC) and bacterial counts in milk.

Here, we describe an experimental inoculation model designed to examine the kinetics of the immune reaction produced in response to bacterial persistence in the mammary gland leading to chronic *Ma* infection in dairy goats.

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2. Materials and methods

2.1. Animals, mycoplasma isolate and inoculum preparation

Nineteen adult lactating goats were selected from a dairy herd microbiologically and serologically negative for *Ma*, other mycoplasmas or bacteria or fungi (Assunção et al., 2004).

The field strain 7MAG of *Ma* was isolated in 2003 from a milk sample of a goat from an infected herd in La Palma (Canary Islands, Spain) with clinical signs of contagious agalactia including mastitis and arthritis. For inoculum preparation, *Ma* microorganisms were cultured in 200 ml of PH medium (Kirchhoff and Rosengarten, 1984). After 2 days at 37 °C, the culture was harvested by centrifugation at 10,000g for 30 min and suspended in the same volume of phosphate buffer saline, pH 7.2 (PBS). Finally, the suspension was used to fill 1.5 ml-sterile tubes and frozen at −80 °C. Viable *Ma* was quantified before inoculation by the method described by Albers and Fletcher (1982).

2.2. Experimental design

A 1.5 ml-inoculum containing 10^{10} colony-forming units (cfu) of *Ma* was injected intracisternally into the right halves of the mammary glands of 15 goats. Left mammary gland halves were inoculated in the same way with 1.5 ml of sterile PBS. Infected goats were randomly assigned to three groups of five animals each, and euthanized at 5, 15, or 45 days post infection. The remaining four goats served as uninfected controls. Animals were placed in four stalls, fed a commercial diet, and subjected to regular clinical and serological examinations. The experiment was carried out in accordance with the Code of Practice for Housing and Care of Animals used in Scientific Procedures (EU Directive 86/609/EEC).

2.3. Clinical and milk examinations

Throughout the experiment, rectal temperature and changes in the size, shape and consistency of the mammary glands and adjacent lymph nodes were recorded daily. Quantitative and qualitative changes in milk and mammary secretions were determined using the California Mastitis Test (CMT). Somatic cell counts (SCC) were also determined daily using a fluoro-optoelectronic counter Fossomatic 300 (Foss Electric, Hillerod, Denmark).

2.4. Serological examination

Blood samples were collected at 6, 12, 24, 48 and 72 h post-inoculation and then at regular 48-h intervals for serological examination. Sera were stored at −80 °C before testing at the end of the experiment. Serological examination was performed in soluble antigen coated plates using an indirect *Ma* ELISA test, according to a protocol described elsewhere (Assunção et al., 2004). Results are expressed as optical densities (OD) with an OD of 0.3 or higher taken as a positive result.

2.5. Bacteriological examination

Mammary secretions from the right teat of each goat were harvested at regular 72 h intervals and examined to quantify viable *Ma* according to previous descriptions (Albers and Fletcher, 1982). During necropsy, mammary secretions and tissue samples from the right and left halves of each mammary gland and supramammary lymph nodes were taken for microbiological analysis.

For mycoplasma isolations, solid and liquid PH media (Kirchhoff and Rosengarten, 1984) were inoculated and cultured at 37 °C. All cultures were filtered at 24 h through a 0.45-μm Millipore filter and later 200 μl of each were cultured in fresh media. Broth cultures were incubated at 37 °C and observed daily to detect turbidity. If no turbidity occurred after 15 days, the culture was considered negative. Positive cultures were streaked onto solid PH media (Kirchhoff and Rosengarten, 1984). Preliminary biochemical and serological identification were carried out on isolations from previously cloned single colonies. Biochemical profiles were recorded as sensitivity to digitonin, fermentation of glucose and mannose, arginine and urea hydrolysis, tetrazolium reduction, film and spot production and phosphatase activity (Poveda, 1998). The serological method employed was a growth inhibition test (Poveda and Nicholas, 1998) using monospecific hyperimmune antisera obtained from rabbits against the corresponding reference strains. Final identification was undertaken using specific PCR-based detection assays for *Ma* (Tola et al., 1994), *Mmm* LC and *Mcc* (Hotzel et al., 1996) and *Mp* (Peyraud et al., 2003). Amplification was performed using a Taq DNA polymerase (Bio Line) according to the manufacturer's instructions in a Mastercycler gradient (Eppendorf) DNA thermal cycler.

Other bacteria were isolated on a blood agar base (added to 5% defibrinated blood), MacConkey's agar, Baird Parker agar and finally on Saboureaud agar. The media were incubated at 37 °C under aerobic conditions for 24 h. Isolates were identified by standard biochemical tests.

2.6. Necropsy and sample collection

Once euthanized by barbiturate overdose, the goats were subjected to a complete necropsy. During the necropsy, tissue samples were collected from the right and left halves of each mammary gland and supramammary lymph nodes, fixed in 10% neutral buffered formalin, embedded in paraffin wax, and sections (4 μm) stained with haematoxylin and eosin.

2.7. Immunohistochemistry

The avidin–biotin peroxidase complex (ABC) method was used to label *Ma* in histological sections. Tissues were dewaxed and rehydrated, and endogenous peroxidase activity was blocked by incubation with 0.3% hydrogen peroxide in methanol for 30 min at room temperature. Tissue sections were then treated with pronase (Sigma Chemical Co., St. Louis, MO, USA) diluted 0.1% in phosphate-buffered saline (PBS), pH 7.2, for 5 min at room temperature. The primary polyclonal antibody 295 (VSD, Stormont, Belfast) was applied at dilution of 1:1000 in PBS for 18 h at 4 °C. To develop the immunoreaction, slides were exposed to the chromogen, 3,3′-diaminobenzidine tetrahydrochloride (Sigma Chemical Co., St. Louis, MO) for 1–2 min and counterstained with Harris' haematoxylin. Tissue sections in which the primary antibodies were replaced with PBS or normal rabbit serum were used as negative controls.

3. Results

3.1. Clinical observations

The temperature of all 15 experimentally infected goats rose transiently by 1–2 °C within 24–48 h of infection, coinciding with a reduction in appetite. Thereafter, both temperature and appetite returned to normal.

The right mammary halves of all the infected does decreased in size within the first 72 h of infection. By 4 dpi, the mammary glands were slightly swollen and showed a steady increase in size. The most noticeable inflammatory changes appeared from 6 to 14 dpi, when the infected mammary halves were severely enlarged and swollen, tender and painful (Fig. 1a). From 15 dpi onwards, the affected mammary glands showed a progressive decrease in size

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