Microbial Pathogenesis 116 (2018) 146–152

Contents lists available at ScienceDirect

Microbial Pathogenesis

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

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

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ARTICLE INFO

Keywords:
Goat mastitis model
Staphylococcus chromogenes
Somatic cell count
Bacterial growth
Histopathology

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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https://doi.org/10.1016/j.micpath.2018.01.031
Received 31 May 2017; Received in revised form 16 January 2018; Accepted 19 January 2018

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].

2.7. Clinical examination

In every sampling, the goats were also clinically examined. Clinical status consisted of changes in the size, shape and consistency of the mammary glands, adjacent lymph nodes and milk appearance. Systemic symptoms (fever), local signs (sensitive to palpation) and milk appearance were recorded during the experiment.

2.8. Bacteriological examination of tissue samples

During necropsy, tissue samples (5 g) from the right and left halves of each mammary gland (teat canal, glandular cistern, and distal, central and proximal sites) and mammary lymph nodes, were removed aseptically for bacteriological analysis and stored at −80 °C. The samples were thawed at room temperature and homogenized with a tissue disintegrator in 3.0 mL of sterile saline 0.9%. Then, viable counts (cfu/g) and identity of bacteria were determined as described for milk samples.

2.9. Tissue samples for histological analysis

After euthanasia, tissue samples were collected from the right and left halves of each mammary gland (distal, medial and proximal areas) and mammary lymph nodes. A portion of each sample was fixed in 10% neutral buffered formalin, processed through graded concentrations of alcohol and xylene, embedded in paraffin wax and sectioned in 4 mm.

2.10. Histopathology

For histological procedures, paraffin-embedded tissues were stained with hematoxylin and eosin (HE) and pathological changes were observed by light microscopy.

2.11. Statistical analysis

The analysis of SCC, numbers of cfu/mL and total and differential counts of leucocytes were modeled with PROC MIXED in SAS 9.2 (SAS Institute Inc.) using individual goat as a random effect and time as a repeated measure effect.

Somatic cell count was measured on each half gland, the natural logarithm of the somatic cell count divided by 100 was the variable analyzed. Fixed effects in the model included post inoculation time and half gland. The half gland was considered as dichotomous variables, challenged (R) versus control (L), and time post inoculation was included as a 7-level categorical variable (0, 4, 8, 12, 16, 20 and, 24 h PI). The interaction between the half gland and post inoculation time in the model was significant.

Numbers of cfu/mL were measured only on challenged the right mammary half of the mammary gland, the logarithm 10 of cfu/mL was the variable analyzed. Fixed effects in the model only included post inoculation time as a 6-level categorical variable (4, 8, 12, 16, 20 and, 24 h).

Leucocytes were measured on goats. The numbers of total and differential leucocytes were the variables analyzed. Fixed effects in the model only included post inoculation time as a 7-level categorical variable (0, 4, 8, 12, 16, 20 and, 24 h).

Compound symmetry was selected as a covariance pattern to account for the clustering of repeated samplings within half gland. Statistical significance was set at $P \leq 0.05$.

3. Results

3.1. Clinical observations

Before the experiment, all goats had normal udders and the rectal temperatures were within normal ranges. No local clinical signs or teat damage was observed after intramammary inoculation. No gross pathological findings were identified in the mammary glands corresponding to the inoculated teats. While the normal rectal temperature of adult goats is 39 °C with a range of 38.5–39.7 °C [38], only two of six goats had systemic signs (fever) reaching its maximum (40.6 °C) at 8 hs after inoculation. Contralateral mammary gland halves inoculated with sterile PBS did not exhibit clinical sings. Throughout the study, all experimentally infected goats developed subclinical mastitis; no case of clinical mastitis was observed. Mastitis pathogens were not observed in milk samples of the 6th goat not sacrificed for 3 days after the treatment.

3.2. Bacterial growth in milk

Selected goats were free of IMI with CNS and other mammary pathogens, as was indicated by the absence of bacterial growth in milk samples analyzed from the udder for 3 consecutive days just before experimental challenge. Pure cultures of *S. chromogenes* RC10-31 Sm^r were consistently recovered from milk until each of the goats were killed.

Bacterial growth peaked (2.3×10^6 cfu/mL) in milk samples obtained from challenged right halves of the mammary glands at 4 h PI (Table 1). From this time, onwards shedding of viable bacteria showed a significant decrease until the end of the experiment at 24 h PI (Table 2). Streptomycin resistance bacteria isolated from the inoculated gland allowed us to confirm that they were identical to the strain of challenge. Milk samples from the control glands were all culture-negative.

3.3. Milk analysis

The mean SCC in milk from the infected right halves of the mammary glands increased considerably from a pre-inoculation basal value at 24 h PI (Table 3). An increase in mean somatic cell counts was observed in milk samples from 8 h PI and remained constant until the end of the experience (24 h PI). The SCC of the milk of the uninfected left halves of the mammary glands of the goats remained constant over time (Table 3). Somatic cell counts of uninfected left halves of the mammary glands resulted in significantly lower cell counts compared to challenged halves from time 4 h PI to time 24 h PI (interaction halves x h PI: $P \leq 0.01$, Fig. 1).

3.4. Blood analysis

The total and differential counts of white blood cells were maintained within the reference values [39] throughout the study (Table 4). No significant differences were observed for total and differential counts of white blood cells across the study. Only the segmented neutrophils showed a significant decrease in the first 12 h PI (Table 5).

Table 1
Colony forming units (cfu) in milk of right mammary halves after inoculate with *Staphylococcus chromogenes*.

No of goats[a]	h PI[b]	*S. chromogenes* × 10^3 (cfu/mL)
		Mean (range)
6	4	2291 (200–9100)
6	8	467 (70–830)
5	12	73 (4.7–213)
4	16	9 (2.7–16.7)
3	20	2 (1.1–4.6)
2	24	2 (0.9–2.1)

[a] Number of goats evaluated.
[b] Hours post-inoculation.

Table 2
Linear mixed regression model for colony forming unit (cfu) for right mammary halves after inoculate with *Staphylococcus chromogenes*.

Effect		Estimate	SE	*P*-value	LSM[b]	SE LSM
Intercept		5,9772	0.2257	< .0001		
h PI[a]	24	−3,0080	0.2544	< .0001	2969	0.2944
	20	−2,6393	0.2181	< .0001	3338	0.2637
	16	−2,2039	0.1967	< .0001	3773	0.2463
	12	−1,5784	0.1818	< .0001	4399	0.2346
	8	−0,4665	0.1702	0.0152	5511	0.2257
	4	Reference	–	–	5977	0.2257

[a] Hours post-inoculation.
[b] Least square means.

Table 3
Somatic cell counts (SCC) in milk of left mammary halves control and right mammary halves inoculated with *Staphylococcus chromogenes*.

No of goats[a]	h PI[b]	SCC × 10^3 (cell/mL)	
		Geometric Mean (range)	
		R[c]	L[d]
6	0	95 (34–179)	82 (12–192)
6	4	7353 (2058–9879)	103 (45–199)
6	8	9780 (9657–9876)	104 (48–189)
5	12	9797 (9567–9879)	107 (54–179)
4	16	9924 (9859–9976)	110 (65–197)
3	20	9888 (9789–9999)	103 (54–184)
2	24	9487 (9431–9543)	116 (76–177)

[a] Number of goats evaluated.
[b] Hours post-inoculation.
[c] Right mammary halves.
[d] Left mammary halves.

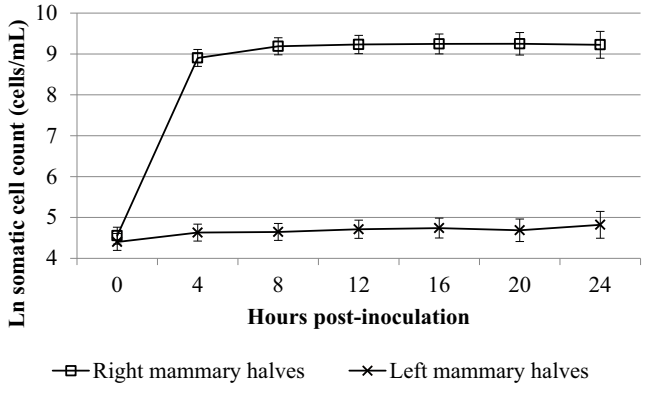

Fig. 1. Logarithmic natural of halve somatic cell count after inoculate with *Staphylococcus chromogenes*. Data are least square means ± standard error.

Table 5
Linear mixed regression model for neutrophils after inoculate with *Staphylococcus chromogenes* in peripheral blood.

Effect		Estimate	SE	*P*-value	LSM[b]	SE LSM
Intercept		6467.83	883.57	< .0001		
h PI[a]	24	−2057.61	1649.68	0.2239	4410.22	1485.54
	20	−685.74	1419.22	0.6332	5782.09	1224.56
	16	−1577.65	1287.46	0.2318	4890.19	1069.07
	12	−2743.86	1200.56	0.0310	3723.97	962.65
	8	−2431.67	1138.13	0.0426	4036.17	883.57
	4	−2487.83	1138.13	0.0384	3980.00	883.57
	0	Reference	–	–	6467.83	883.57

[a] Hours post-inoculation.
[b] Least square means.

3.5. Bacteriology of tissue samples

Staphylococcus chromogenes could be isolated in tissue samples from right mammary glands analyzed of the five goats culled. The number of bacteria in the tissues of the right mammary half of the goat killed at 12 h PI was 2.9×10^2 cfu/g. A decrease in the bacterial count from of the goat killed at 16 h PI (0.94×10^2) to that of the goat killed at 24 h PI (0.55×10^2 cfu/mL) was observed (data not shown). Similar results were observed in the proximal, medial and distal section of the inoculated mammary gland.

No other bacteria or fungi were isolated. *Staphylococcus chromogenes* was never isolated from the tissue of all unchallenged control mammary glands.

3.6. Histopathological results

Although some animals may react differently to the experimental inoculation considering the linear development of infection as time dependent, the results obtained from individual animals can be regarded as representative.

Histological examination of specimens of the right halves of the mammary glands showed an increased inflammatory response throughout the experiment and no changes were observed in the left halves of the mammary glands.

In the mammary tissue of the goat killed at 12 h PI, a vast number of cells were seen in some secretory units (Fig. 2C; white arrowhead), that were tentatively identified as neutrophils mainly based on their polymorphonuclear feature. The mammary tissue of the goat killed at 16 h PI showed more leucocytes, thus suggesting that the inflammatory process was more intense than that of the animal sacrificed at 12 h PI (Fig. 2D; white arrowhead). This finding was characterized by an increased accumulation of neutrophils in numerous secretory units.

Regarding goats killed at 20 and 24 h, the main histopathological feature was from moderate to intense neutrophil periacinar and interstitial infiltration of the mammary gland, respectively (Fig. 2E and F;

Table 4
Total and differential counting of white blood cells (cells/μL) in peripheral blood after inoculate with *Staphylococcus chromogenes*.

No of goats[a]	h PI[b]	Mean (range) of white blood cells/μL					
		Total	Neutrophils in bands	Segmented neutrophils	Eosinophils	Lymphocytes	Monocytes
6	0	11,333 (7900–15700)	79 (79–79)	3542 (3002–4345)	92 (0–237)	3779 (3160–4424)	408 (237–553)
6	4	10,067 (7900–11800)	92 (79–158)	2765 (2528–3002)	66 (0–158)	4529 (4187–4661)	448 (237–632)
6	8	9783 (7200–13400)	79 (79–79)	3239 (2686–3713)	119 (0–316)	4029 (3397–4661)	435 (237–632)
5	12	9560 (8000–13200)	95 (79–158)	3255 (2765–4187)	32 (0–79)	4155 (3081–4582)	363 (316–474)
4	16	11,075 (7100–14500)	99 (79–158)	3298 (3002–3871)	40 (0–79)	4069 (3397–4424)	395 (316–474)
3	20	11,467 (10,800–12200)	79 (79–79)	3766 (3397–4345)	79 (0–158)	3502 (2765–3950)	474 (395–553)
2	24	9700 (9500–9900)	119 (79–158)	3239 (3160–3318)	79 (0–158)	4148 (4029–4266)	316 (316–316)

[a] Number of goats evaluated.
[b] Hours post-inoculation.

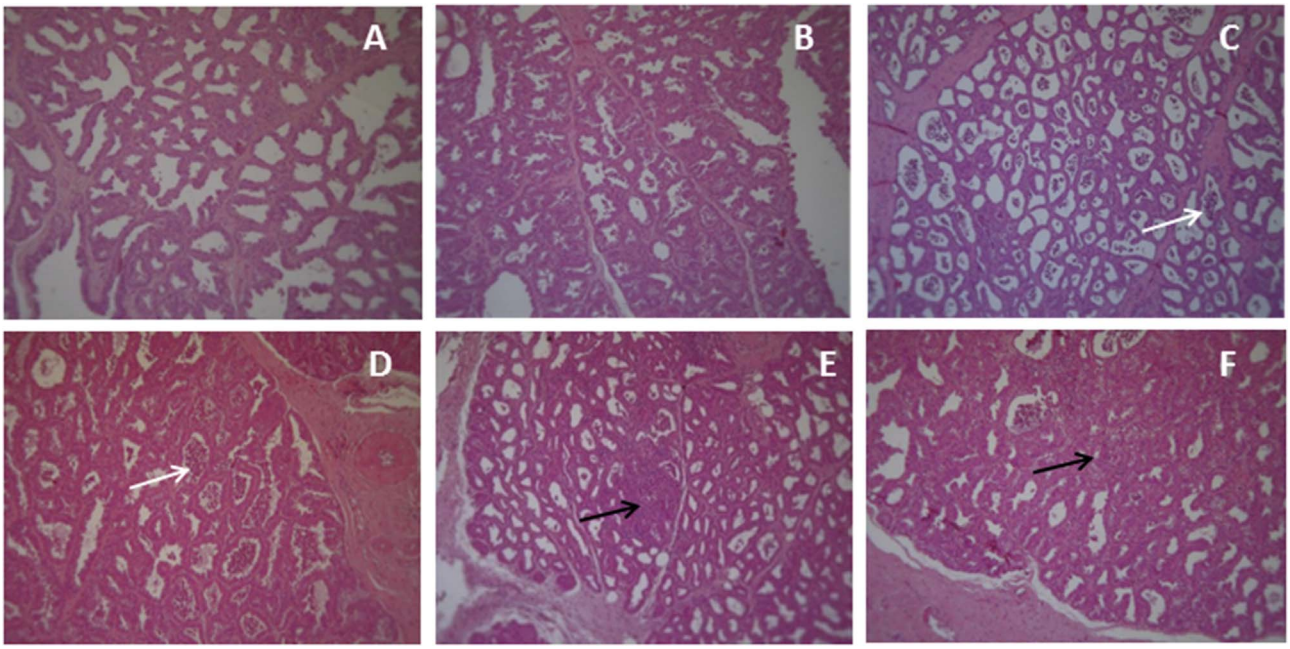

Fig. 2. Representative hematoxylin and eosin-stained sections of mammary glands isolated from goat inoculated with *Staphylococcus chromogenes*. The white arrows mark the immune cell content in the alveoli, whereas the black arrows point to the white blood cells in the interstitium. HE stain, 10× magnification, (A) 0 h PI, (B) 8 h PI, (C) 12 h PI, (D) 16 h PI (E), 20 h PI and (F) 24 h PI.

black arrowheads). Similar results were observed in the proximal, medial and distal section of the inoculated mammary gland.

The presence of segmented neutrophils (polymorphonuclear leukocytes) was observed in the interstitium of the medullary sinuses of the right lymph nodes in the goats killed at 16, 20 and 24 h PI. On the other hand, the left supramammary lymph nodes exhibited no reactive changes (data not shown). The histological findings observed in all goats could represent the response to bacterial counts reached in infected tissues.

4. Discussion

Because of the growing importance of the CNS as causative agents of bovine intramammary infections and since the pathogenicity of these bacteria is not entirely clear, animal models represent an alternative for the study of these potential pathogenic bacteria.

Although the best model for experimental mammary infection would be dairy cows, studies with bovines have costs that are difficult to pay in developing countries when a minimal number of animals is required to obtain valid statistics. Hence, the present study of pathogenesis of mastitis caused by *S. chromogenes* was carried out in a goat mastitis model.

To our knowledge, this is the first report on experimentally induced *S. chromogenes* IMI in lactating dairy goats. In the present study, no gross pathological post-mortem lesions were observed. This finding was in agreement with Mavrogianni et al. [40], who carried out an experimental mastitis with *S. chromogenes* in sheep with an identical dose as the one used in this study.

In a similar way to the results obtained in our previous work [22], all animals developed subclinical mastitis in the right halves of the mammary glands in response to intramammary bacterial infection with 1×10^7 cfu/mL of the strain *S. chromogenes* RC10-31 Sm^r. The challenge dose used in these studies was higher compared with those used by others authors to induce mastitis in goats, but with a more pathogenic bacteria such as *S. aureus* [24–26].

In the present study, the number of *S. chromogenes* in milk declined logarithmically from 4 until 16 h PI (2.3×10^6–9×10^3 cfu/mL) in the animals analyzed at each time and isolation of bacteria remained until the end of the trial (24 PI) (Table 1).

Similar results after inoculation of 1×10^8 cfu/mL of *S. uberis* in goats were obtained, where a reduction of bacteria in milk of approximately 4-log at 24 h PI was detected [22]. On the other hand, our results are similar to those obtained by Piccart et al. [41], who observed a decrease in the number of bacteria of approximately 3 log after 24 hs of the experimental inoculation of 1×10^6 cfu/mL *S. chromogenes* in cows.

Clear signs of inflammation registered after intramammary inoculation with *S. chromogenes* based on the high SCC, were similar to those obtained by Simojoki et al. [17,42,43] in cows and Pisoni et al. [24] in goats, who reported that the inflammatory response in experimentally induced intramammary infection with different CNS species, plays an important role in resolving infection.

It is known that the number of somatic cells in the milk of uninfected goats and sheep glands is typically higher than that of cows, up to 5×10^5 cells/mL [44,45]. However, in comparison to cows, much less is known about the normal composition of somatic cells in goats during lactation. Leitner et al. [45] have demonstrated that in animals free of bacteria at mid-lactation, goats had the highest number of leukocytes and segmented neutrophils with respect to that observed in cows and sheep. These authors have observed that the pattern of response to CNS IMI was basically similar, and the inter-species differences (goat, cow and sheep) in responses to intramammary infection, most likely reflected the different basal conditions of plasminogen activator-plasmin system and the type of the invading bacterial that infects each animal species.

The systemic response to intramammary infection obtained in the present study, characterized by a decrease in total blood leukocyte count, was similar to the results obtained by Cremonesi et al. [25]. The increase of SCC in milk, in accordance with the decrease of blood leukocytes, suggests the migration of the segmented neutrophil from the circulation to the mammary tissue, thus contributing to phagocytosis of bacteria as the first event in the non-specific defense mechanisms [46].

Histological examination of cistern, teat canal, proximal, central and distal mammary gland and lymph node of slaughtered animals, allowed investigating the early pathogenesis induced by *S. chromogenes* in goats, which displayed many chronological and pathological

similarities to those induced by *S. uberis* in goats [22].

The high SCC caused by experimental infection with *S chromogenes*, suggests that mastitis by this species of CNS negatively affected the quality of milk during the period studied.

Further studies should be carried out with *S. chromogenes* to cover a wide range of ecological habitats, ranging between the environment and the host. Other authors refer to differences in the elicited host response and bacterial shedding between strains belonging to the same species [41,47].

5. Conclusions

The increased SCC and histopathological changes observed in inoculated glands lead to not underestimating the importance of *S. chromogenes* as a pathogenic agent in goats. This investigation suggests that the goat model could mimic bovine *S. chromogenes* mastitis and has potential as a complementary in vivo tool for future investigations about mastitis by other CNS species.

Conflicts of interest

None.

Acknowledgements

This research was supported by grants from Secretaría de Ciencia y Técnica de la Universidad Nacional de Río Cuarto, Argentina (SECyT-UNRC). We also thank Valeria Coniglio and Matías Caverzan for collaborating with the collection of blood and milk samples. We would also like to thank Fray Carlos M. Diez, administrator of the Yucat stay, where the trials of experimental mastitis in goats were carried out.

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