

CORYNEBACTERIUM PSEUDOTUBERCULOSIS EXPERIMENTAL INFECTION OF GOATS MAMMARY GLAND

J.W. Pinheiro Junior¹, A.A.F. Oliveira¹, F.S.F. Alves², L.B.G. Silva¹, S.S.A. Rabelo¹, R.A. Mota¹

¹Universidade Federal Rural de Pernambuco, Departamento de Medicina Veterinária, Rua Dom Manuel, s/nº, CEP 52171-900, Recife, PE, Recife, Brazil. E-mail: jrwilton@bol.com.br

ABSTRACT

The objective of this study is to describe some aspects of the pathogeny of *Corynebacterium pseudotuberculosis*, isolated from a natural case of caseous lymphadenitis. The study was comprised with 2 groups of 3 animals each; inoculated with a *C. pseudotuberculosis* strain, group 1 (GI) via intra dermal and group 2 (GII) via the mammary gland. The animals were clinically examined on a daily basis and hemograms carried out over a period of time at different intervals. Only 2 animals from GII showed changes in the clinical examination characterized by hyperthermia. All the animals in both groups developed papule and edema at the inoculation site. There were no significant alterations in the erythrogram but in the leucogram there was leucocytosis indicated by the presence of neutrophilia in 2 animals from GII. Results of the cellular analysis of the gland showed that 2 animals from GII developed acute mastitis. This experiment has proven that *C. pseudotuberculosis*, isolated from a natural infection of caseous lymphadenitis, could cause clinical mastitis, losses in goat keeping and that the expression cytology test could be used as an alternative diagnosis for mastitis.

KEY WORDS: Mastitis, caseous lymphadenitis, expression cytology.

RESUMO

INFECÇÃO EXPERIMENTAL DA GLÂNDULA MAMÁRIA EM CABRAS POR *CORYNEBACTERIUM PSEUDOTUBERCULOSIS*. Objetivou-se com este trabalho descrever alguns aspectos da patogenia da infecção da glândula mamária por *Corynebacterium pseudotuberculosis* isolado de um caso natural de linfadenite caseosa. Os animais em número de seis, foram separados em dois grupos, no primeiro (GI) foi realizada inoculação da cepa de *C. pseudotuberculosis* pela via intradérmica e no segundo grupo (GII) pela via intramamária. Esses animais foram acompanhados diariamente por exame clínico e por hemogramas em diferentes tempos. Apenas dois animais do GII apresentaram alteração ao exame clínico caracterizada por hipertermia. Todos os animais (GI e GII) apresentaram pápula e edema no local da inoculação. No eritrograma, não foram observadas alterações significativas, contudo no leucograma, observou-se leucocitose por neutrofilia em dois animais do GII. A análise da celularidade das glândulas demonstrou que apenas dois animais do GII apresentaram mastite aguda. Comprovou-se com este experimento que o *C. pseudotuberculosis* isolado de infecção natural de linfadenite caseosa, pode ocasionar mastite clínica, causando prejuízos para caprinocultura, assim como a citologia de expressão pode ser utilizada como método de diagnóstico alternativo nos casos de mastite.

PALAVRAS-CHAVE: Mastite, linfadenite caseosa, citologia de expressão.

INTRODUCTION

Goat rearing is a developing activity throughout the country, although there are a greater number of goat farmers in the semi arid regions of the North East Brazil (RIBEIRO et al., 2001). Some diseases, such as mastitis and caseous lymphadenitis, reduce

production and productivity thus resulting in losses to the producers and risks to Public Health.

Caseous lymphadenitis is a chronic disease with a suppurative characteristic and necrotic inflammation characterized by internal and external abscesses and is caused by *Corynebacterium pseudotuberculosis* (NFI; NDI, 1994). It causes economic

²Embrapa, Centro Nacional de Pesquisa de Caprinos, Sobral, CE, Brazil.

losses by lowering milk and meat production, and the value of the hide, condemnation of carcasses and viscera are condemned, slaughtering of severely affected animals, veterinary expenses and labor costs in treating the abscesses and, in severe cases, death of the animal (ALVES; PINHEIRO, 1999).

ALMEIDA et al. (2005), in their study in Pernambuco State, isolated *C. pseudotuberculosis* in 60% (3/5) of lactating goats. These had mastitis and concomitant caseous lesions in the supra mammary lymph node which is characteristic of the disease.

These diseases, isolated or in conjunction with others sanitary problems of small ruminants, contribute considerably to lowering the quality of the milk, meat and hide, thus leading to financial losses what could even turn the breeding venture unviable.

Considering the importance of goat breeding in Pernambuco State and the lack of regional research that contributes in clarifying the epidemiology and the disease control, the objective of this study is to evaluate some important aspects of the *C. pseudotuberculosis* pathogeny in the mammary gland of milking goats.

MATERIAL AND METHODS

A strain of *C. pseudotuberculosis* obtained from a clinical case of caseous lymphadenitis of animals from Centro Nacional de Pesquisa em Caprinos was used in the experimental infection. The strain was identified according to the method recommended by CARTER (1988). The infective dose was $3,6 \times 10^7$ units of formed colonies (BURRELL, 1978).

Six cross breed goats in different stages of lactation, in good health condition, without any clinical sign of mastitis and with milk microbiological examination negative for *C. pseudotuberculosis* and other agents, were used in the experiment. The animals were kept in pens at the CNPC and were fed on elephant grass, corn flour ration and cotton pie, and had water *ad libitum*. The goats were separated into 2 groups of 3 animals each.

Using syringes and needles disposable, GI was infected via intra dermal and GII was inoculated with 0.2 mL of infected material directly into the parenchyma of the udder and approximately 5 cm to the right of the supra mammary lymph node. The infective dose was $3,6 \times 10^7$ units of formed colonies (BURRELL, 1978).

The clinical examination was carried out according to ROSEMBERG (1993) taking into account physiological parameters (body temperature, heart beat and respiratory rate), palpations of the superficial lymph nodes and mammary gland once a day, for one week of adaptation and the three weeks post infection (PI).

Samples of milk were collected before the inoculation to verify the state of health of the udder and after inoculation to recover the microorganism inoculated. These samples were seeded on agar base enriched with 8% sheep blood and incubated for 48 hours at 37°C. After this incubation period the morphological characteristics of the colonies were recorded and colonies smears stained by Gram technique according to QUIN et al (1994).

Hemograms carried out according to JAIN (1993) method were done for blood samples collected 72 and 8 hours before the inoculation and 48, 168, 326 and 672 hours post inoculation, comprising a total of 36 samples.

The procedure used in the expression cytology (EC) was adapted from AZÍRA method (1976) in which, after the teat cleaning with alcohol 70%, slight pressure is applied with the thumb and index finger to strip the entire canal of the teat. The material obtained was placed on an opaque histology slide, fixed with absolute methyl alcohol for ten minutes then stained with Giemsa. These slides were examined for evaluating the characteristics of stripping, composition and cell size. Cell counts were carried out on ten fields under the microscope and the mean cell count taken for the type of inflammation classification as well as cell component. The classification of the type of inflammation was carried out according to COWELL; TYLER (2002).

The animals were slaughtered on the 28th day post infection and samples of the supra mammary lymph nodes were collected for bacteriological and histopathological examination.

Statistical analysis was carried out on data of animals within the same group using the Minitab computer program. The data were tested for normality of residues and the degree of freedom in opting for the development the analysis by Nparl way analysis of non parametric variance and utilizing the Kruskal-Wallis test (SAMPAIO, 1998).

For results description the respective measurements of the central trend were used: mean and standard deviation for the variation in the erythrogram and mean, lower and superior limits for the leucogram. The level of significance used for rejecting HO (null hypothesis) was 5%.

The present study was approved by EMBRAPA Ethical Committee on March 2005.

RESULTS

Only 2 animals of GII had fever during the first 96 hours post infection. From the 5th day on, the animals had normal rectal temperature. However, one animal developed vital changes with tachycardia and tachypnea and showed physical alteration of lacteous secretion 48 hours PI. Some lumps were observed and

a reduction in milk production and turning into a purulent viscose content after 72 hours. Apathy, loss of appetite and a reduction in food consumption 72 hours post infection, remaining 48 without eating, was also observed. The other 4 animals had no fever or any other changes in their general health status.

No significant differences were observed in the variables analyzed: haematocrit (Ht), erythrocytes (He) and hemoglobin (Hb) for GI and GII animals on the different sampling times (Table 1). However, there was a Hemoglobin decrease in the GI 48 hours PI. In the leucogram there was a significant difference in the Group II animals in the rods and monocytes at different sampling times (Table 2). A significant increase of the total number of leucocytes was observed in both groups, 48 hours PI, characterizing a leucocytosis, with a decrease in number 168 hours PI. Analysis of the number of lymphocytes indicated an increase in GI forty-eight (48) hours PI with a decrease 168 hours PI.

Examination of the site of inoculation revealed papule formation 48 hours PI in all GI animals and

transformation of this into pustule on day 5 PI and developing into an ulcer fairly soon after. All the GII animals developed edema at the site of inoculation. On day 20, GI animals showed ulcer cicatrization preceded by a dry crust formation.

An increase in the supra mammary lymph nodes (lymphadenitis) was observed 48 hours post infection in all the inoculated animals except for goats number 013 and 066 (GI), which showed it 168 hours post infection.

Analysis of the gland cells of the infected animals revealed that for 2 GII animals more than 70% of the inflammatory cells were neutrophils, considered acute mastitis according to COWELL; TYLER (2002) criteria (Fig. 1). On the other hand, the neutrophils counts increased progressively over the post infection period for animals 68 and 119 and there was also an increase, predominantly in neutrophils in animal 180. The results for the EC are given in Table 3. It was not possible to carry out EC on animals 013 and 066 (GI) as these went dry during the period of adaptation.

Table 1 – Average values of the erythrogram of goats inoculated intradermically (GI) and intra-mammary (GII) with *Corynebacterium pseudotuberculosis*, CNPC, 2005.

Variables	Group1 (hours)					Group2 (hours)				
	72	8	48	336	672	72	8	48	336	672
Hematócrito (%)	23,00	24,00	22,33	21,67	21,33	23,67	25,33	25,67	25,33	22,67
HCM (pg)	9,53	8,97	8,57	12,90	12,47	8,83	9,83	9,10	7,60	8,70
VCM (fl)	28,37	28,37	24,70	26,57	26,80	27,57	30,93	26,70	26,20	24,67
CHCM (g/dl)	33,77	31,67	34,83	33,00	30,63	31,87	31,27	34,03	29,30	35,27
He (x 10 ⁶)	8,13	8,52	9,28	9,13	9,06	8,94	8,21	9,58	9,80	9,16
Hb (g/dl)	7,77	7,60	7,80	7,19	6,50	7,57	7,93	8,73	7,58	7,97

Table 2 – Leucogram median values of goats experimentally inoculated, intradermically (GI) and intramammary (GII) with *Corynebacterium pseudotuberculosis*, CNPC, 2005.

	Hours	Leucocytes	Variables				
			Segmented	Rods	Lymphocytes	Basofile	Monocytes
GI	72	9050,00	3547,50	0,00	4163,00	0,00	82,50
	8	10750,00	4212,00	0,00	4730,00	0,00	107,50
	48	24400,00	6561,00	121,50	7279,50	121,50	0,00
	168	24200,00	14762,00	0,00	9196,00	0,00	0,00
	336	11850,00	5332,50	169,00	6162,00	169,00	0,00
	672	9100,00	4185,00	0,00	3875,00	0,00	0,00
GII	72	7550,00	3895,50*	51,50	2719,50	0,00	0,00*
	8	6550,00	2947,50*	118,00	3537,00	0,00	0,00*
	48	17200,00	13167,00*	171,00	3784,00	0,00	0,00*
	168	13350,00	9078,00*	0,00	4138,50	0,00	0,00*
	336	10800,00	6696,00*	108,00	3564,00	0,00	108,00*
	672	16950,00	10000,00*	0,00	6441,00	0,00	169,50*

*Significant difference, with a confidence level of 5%.

Table 3 – Cytology Results from Expression Cytology (EC) of GI and GII animals, before and post experimental infection with *Corynebacterium pseudotuberculosis*, CNPC, 2005.

Animal	Hours			
	72 Pre-infection	48 Post-infection	432 Post-infection	600 Post-infection
013 RG (GI)	NP	NP	NP	NP
013 LG (GI)	NP	NP	NP	NP
066 RG (G)	NP	NP	NP	NP
066 LG (GI)	NP	NP	NP	NP
068 RG (GII)	40% neutrophils	40% neutrophils	50% neutrophils	NP
068 LG (GII)	10% neutrophils	< 10% neutrophils	NP	NP
117 RG (GII)	10% neutrophils	40% neutrophils	80% neutrophils	NP
117 LG (GII)	NP	NP	NP	NP
119 RG (GII)	10% neutrophils	20% neutrophils	40% neutrophils	70% neutrophils
119 LG (GII)	NP	NP	NP	NP
180 RG (GI)	NEN	NEN	30% neutrophils	NEN
180 LG (GI)	NP	NP	NP	NP

RG – right gland; LG – left gland; NP – not performed; NEN – not enough to notice.

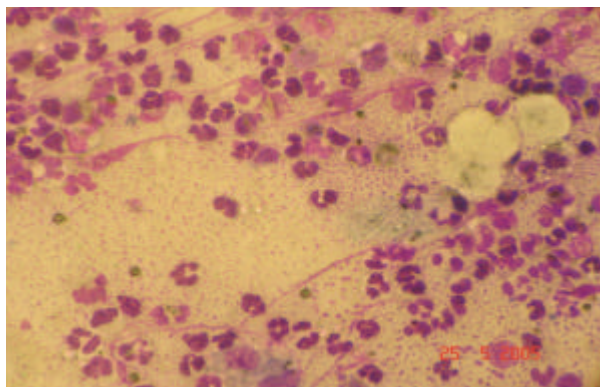


Fig. 1 – Intense inflammatory infiltrated polymorphonuclear, indicating acute infectious process. Giemsa. (x 250). Animal 117 D.

It was possible to recover and identify *C. pseudotuberculosis* from the pus of animal 66 seven (7) days after the inoculation, from the milk secretion of animal 117 forty eight (48) hours PI and from the supra mammary lymph node and mammary gland of animals 119 and 117 respectively at the time of slaughtering.

All the animals showed the supra mammary lymph nodes enlarged, but without abscesses. Visible macroscopic alterations were not seen in the organs examined, except a gelatinous edema at the sites of inoculation. Animal 117 had a large quantity of caseous secretion in the udder above the cut.

DISCUSSION

ETREBY & ABDEL-HAMID (1970) performed an experimental infection via galactofor canal in healthy

sheep with *C. ovis* and *C. pyogenes* and observed the development of chronic purulent catarrhal acute mastitis and chronic apostematous mastitis, respectively.

The microorganism in the mammary parenchyma demands chemotherapeutic action for neutrophil and macrophages that perform phagocytosis and constitute the initial focus of abscess formation that expand through the secretor tissue (ETREBY; ABDEL-HAMID, 1970).

Even though the via of inoculation in this study was different, the formation of pustules at the site of inoculation was observed (GI all animals) and 1 animal from GII had a unilateral abscess of approximately 5cm in diameter and with a purulent content in the parenchyma of the gland. Also in this animal there was a progressive increase of mammary gland cells at different times of the EC examination. There was a predominance of whole and some degenerated neutrophils, characteristic of acute inflammatory process (COWELL; TYLLER, 2002). Other GI animals had also significant increase of cells, with neutrophils predominance. Similar findings were observed by BURREL (1978) in his study with experimental inoculation of caseous lymphadenitis in sheep.

The dose of *C. pseudotuberculosis* did not cause suppurative and/or systemic caseous lesions by the used inoculation via for GI and GII animals, even though there was some alteration in the general health status of the GII animals.

According to AUGUSTINE (1982) the concentration of viable organisms in the purulent material is estimated to be from 1×10^6 to 5×10^7 per gram of pus to cause the disease in animals. Other aspect that must be considered is the period of observation that usually

is not long enough for the lesions formation in the internal lymph nodes and other organs as was described by LANGENEGGER; LANGENEGGER (1988) that kept goats under observation for 126 days. JOHNSON et al. (1993) intra dermically inoculated 4×10^6 washed bacteria and recorded a chronic disease with the formation of internal and external abscesses without any signs of the acute phase such as fever, loss of appetite and hemolysis, the period between inoculation and abscesses rupture was 3 to 6 weeks, lesser than the one observed by ASHFAQ; CAMPBELL (1979), 90 days. PEPIN et al. (1988) carried out a subcutaneous experimental infection of *C. pseudotuberculosis* on sheep at different dose rates and observed local reaction and multiple abscesses formation in the lymph nodes 21 days after inoculation.

NAGY (1976) also carried out experimental infection using different routes of infection and showed that there exists a link between the point of inoculation and the region lymph node affected. In the present study the occurrence of lesions at the site of inoculation, the formation of edema, erythema, papules, ulcers and crust in GI animals lead to the bacteria recovery from some of these lesions. However, related to the recovery of *C. pseudotuberculosis* from the lymph nodes and milk secretion, it was possible to isolate the bacteria from 50% of the animals inoculated which is in accordance with the findings of GARG; CHANDIRAMANI (1985) who also did not recover the bacteria from all the animals.

The formation of edema at the inoculation site probably occurred due to the action of the toxin produced by *C. pseudotuberculosis*, which affected the blood vessels *in vivo*. JOHNSON et al. (1993) in their study discussed the role of this toxin in the pathogenesis of naturally acquired disease.

GAMELL; TARTOUR (1974) analysed the hemograms and plasma protein of sheep experimentally infected with *C. pseudotuberculosis*, and reported a severe hemolytic anemia, described as transitory macrocytic hypochromic, as well as hypochromic normocytic anemia. This was not observed in our study.

In relation to the leucocytosis observed in the present study, JAIN (1993) reported that, among other causes, bacterial infections are responsible for leucocytosis, generally related to neutrophilia. The neutrophilia is the result of increased transference of cells from the marginal pool to the circulation, reducing the migration of cells to the tissues and increasing the production and liberation of neutrophils by the bone marrow. Also according to MEYER et al. (1995), intense acute inflammation and those similarly found in GI and GII animals, neutrophilia occurs from a stimulus and continual tissue demand, elevated production and liberation of neutrophils to suppress the tissue

demand. Even with the significant difference in the evaluation of the monocytes obtained in GII during the experimental phase, no monocytosis was observed. According to KERR (2004) monocytosis occurs just prior to chronic infections, more frequently related to parasitic or bacterial infections.

CONCLUSION

From the results of this experiment it was shown that *C. pseudotuberculosis*, isolated from a natural case of caseous lymphadenitis, could cause acute clinical mastitis. The results from the expression cytology tests were satisfactory as an auxiliary diagnostic method and could be considered for use in future studies on goats.

REFERENCES

- ALVES, F.S.F.; PINHEIRO, R.R. Linfadenite caseosa: recomendações e medidas profiláticas. *Lavoura*, n.631, p. 42-43, 1999.
- ALMEIDA, M.C.S.; SILVA, L.B.G.; MOTA, R.A.; NUNES, J.R.; SILVA, D.R. Estudo da relação entre a linfadenite caseosa e mastite em cabras criadas no Estado de Pernambuco. In: JORNADA DE INICIAÇÃO CIENTÍFICA/PIBIC/FACEPE/CNPq, 2005, Recife. *Anais. Recife*, 2005. p.81-82, 2005.
- ASHFAQ, M.K.; CAMPBELL, S.G. A survey of caseous lymphadenitis and its etiology in goats in the United States. *Veterinary Medicine of Small Animal Clinician*, v.74, p.1161-1165, 1979.
- AUGUSTINE, J.L.; RICHARDS, A.B.; RENSHAW, H.W. Concentration of *Corynebacterium pseudotuberculosis* obtained from lesions of sheep and goats with caseous lymphadenitis. In: INTERNATIONAL CONFERENCE ON GOAT PRODUCTION AND DISEASE, 3., 1982, Tucson. *Proceedings. Tucson*, 1982. p.525.
- AZEVEDO, C.F. *Criação de caprinos e ovinos no Nordeste*. Natal, Brasil, 1982. 54p.
- AZEVEDO, E.O. *Aspectos clínico epidemiológicos e diagnóstico laboratorial da Agalaxia Contagiosa dos Ovinos e Caprinos (ACOC) no Brasil*. 2005. 136p. Tese (Doutorado). Universidade Federal Rural de Pernambuco, Recife, 2005.
- AZÍRA, J. *Diagnóstico citológico en patología mamaria*. Barcelona: Espaxs, 1976. p.15-25.
- BURRELL, D.H. Experimental induction of caseous lymphadenitis in sheep by intralymphatic inoculation of *Corynebacterium ovis*. *Research in Veterinary Science*, v.24, p.269-276, 1978.
- CARTER, G.R. *Fundamentos de bacteriologia e micologia veterinária*. São Paulo: Roca, 1988. 249p.
- COWELL, R.L.; TYLER, R.D. *Diagnostic cytology and hematology of the horse*. 2nd ed. Missouri: Mosby, 2002. 260p.
- ETREBY, M.F.; ABDEL-HAMID, Y.M. Experimental Ovine Mastitis - A pathologic study. *Veterinary Pathology*, v.7, p.246-264, 1970.

- GAMEL, A.A.; TARTOUR, G. Haematological and plasma protein changes in sheeep experimentally infected with *Corynebacterium pseudotuberculosis*. *Journal Comparative Pathology*, v.84, p.477-483, 1974.
- GARG, D.N.; CHANDIRAMANI, N.K. Occurrence of *Corynebacterium ovis* infection in sheep and goats. *Indian Journal Animal Science*, v.55, n.6, p.398-402, 1985.
- JAIN, N.C. *Essentials of veterinary hematology*. 5.ed. Philadelphia: Lea & Febiger, 1993. 417p.
- JOHNSON, E.H.; VIDAL, C.E.S.; SANTA ROSA, J.; KASS, P.H. Observations on goats experimentally infected with *Corynebacterium pseudotuberculosis*. *Small Ruminant Research*, v.12, p.357-369, 1993.
- KERR, M.G. *Exames laboratoriais em medicina veterinária: bioquímica clínica e hematologia*, 2. ed. São Paulo: Roca, 2004. 436p.
- LANGENEGGER, J.; LANGENEGGER, C.H. Reprodução da linfadenite caseosa em caprinos com pequeno número de *Corynebacterium pseudotuberculosis*. *Pesquisa Veterinária Brasileira*, v.8, n.1/2, p.23-26, 1988.
- MEYER, D.J.; COLES, E.H.; RICH, L.J. *Medicina de laboratório veterinário: interpretação e diagnóstico*. São Paulo: Roca, 1995. 308p.
- NAGY, G. Caseous lymphadenitis in sheep – methods of infection. *Journal of the South African Veterinary Association*, v.47, n.3, p.197-199, 1976.
- NFI, A.N.; NDI, C.N. Caseous lymphadenitis in sheep and goats in Mankon Cameron. *Bulletin of Animal and Health Production in África*, v.42, p.163-166, 1994.
- PÉPIN, M.; PARDON P.; MARLY, J.; LANTIER, F. *Corynebacterium pseudotuberculosis* infection in adult ewes by inoculation in the external ear. *American Journal of Veterinary Research*, v.49, n.4, p.459-463, 1988.
- QUIN, P.J. CARTER, M.E.; MARKEY, B. *Clinical veterinary microbiology* London: Wolfe, 1994. 648p.
- RIBEIRO, M.G.; DIAS JÚNIOR, J.G.; PAES, A.C.; BARBOSA, P.G.; NARDI JÚNIOR, G.; LISONI, F.J.P. Punção aspirativa com agulha fina no diagnóstico do *Corynebacterium pseudotuberculosis* na linfadenite caseosa caprina. *Arquivos do Instituto Biológico*, São Paulo, v.68, n.1, p.23-28, 2001.
- ROSEMBERG, G. *Exame clínico dos bovinos*. 3. ed. Rio de Janeiro: Guanabara-Koogan, 1993. 419p.
- SAMPAIO, I.B.M. *Estatística aplicada à experimentação animal*. Belo Horizonte: Fundação de Ensino e Pesquisa em Medicina Veterinária e Zootecnia, 1998. 185p.

Received on 4/7/06

Accepted on 15/10/06