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## Research in Veterinary Science

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## High seroprevalence of caseous lymphadenitis in Brazilian goat herds revealed by *Corynebacterium pseudotuberculosis* secreted proteins-based ELISA

N. Seyffert<sup>a</sup>, A.S. Guimarães<sup>b</sup>, L.G.C. Pacheco<sup>a</sup>, R.W. Portela<sup>c</sup>, B.L. Bastos<sup>c</sup>, F.A. Dorella<sup>a</sup>, M.B. Heinemann<sup>b</sup>, A.P. Lage<sup>b</sup>, A.M.G. Gouveia<sup>b</sup>, R. Meyer<sup>c</sup>, A. Miyoshi<sup>a</sup>, V. Azevedo<sup>a,\*</sup>

<sup>a</sup>Laboratório de Genética Celular e Molecular, Departamento de Biologia Geral, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, CP 486, CEP 31270-901, Belo Horizonte, MG, Brazil

<sup>b</sup>Laboratório de Bacteriologia Aplicada, Departamento de Medicina Veterinária Preventiva, Escola de Veterinária, Universidade Federal de Minas Gerais, CEP 31270-901, Belo Horizonte, MG, Brazil

<sup>c</sup>Laboratório de Imunologia e Biologia Molecular, Departamento de Bio-Interação, Instituto de Ciências da Saúde, Universidade Federal da Bahia, CEP 40110-100, Salvador, Bahia, Brazil

## ARTICLE INFO

## Article history:

Accepted 3 July 2009  
Available online xxxx

## Keywords:

*Corynebacterium pseudotuberculosis*  
Secreted proteins  
Caseous lymphadenitis  
Goats  
ELISA  
Seroprevalence

## ABSTRACT

We conducted a seroepidemiological survey to determine the prevalence of caseous lymphadenitis (CLA) in goat herds in Minas Gerais state, Brazil. Serum samples were collected from goats ( $n = 676$ ) from 108 rural properties in 2001, covering most of the sub-regions of this ca. 586,500 square kilometer state. Antibodies against *Corynebacterium pseudotuberculosis* secreted proteins were detected by an indirect enzyme-linked immunosorbent assay (ELISA). Most of the animals (78.9%) tested positive for CLA; 98% of flocks presented at least one seropositive animal. Goats managed under an extensive production system had a significantly higher seroprevalence of CLA than those in intensive and semi-intensive operations. The age distribution of the animals in the flocks affected the prevalence of this disease; however, goat breed did not. We found seropositivity against *C. pseudotuberculosis* to be highly prevalent in these Brazilian goat herds; consequently, appropriate management practices for the control of CLA should be implemented.

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## 1. Introduction

Caseous lymphadenitis (CLA) is a chronic bacterial infection of small ruminants caused by *Corynebacterium pseudotuberculosis* (Dorella et al., 2006). Even though this Gram positive bacterium is a non spore-forming facultative intracellular pathogen, its ability to survive for long periods in the environment is a key characteristic thus contributing to the high transmission rate of CLA within a herd (Baird and Fontaine, 2007).

This disease usually presents itself with the pathognomonic abscessation of subcutaneous lymph nodes, or as a sub-clinical infection, characterized by abscesses in internal lymph nodes and in organs such as lungs, liver and kidneys (Fontaine and Baird, 2008). The epidemics of CLA transmission within a sheep flock has recently been studied by O'Reilly et al. (2008) who report that dissemination initiates with an epidemic of superficial abscesses, followed by an increase in the frequencies of abscesses in the lungs, mediastinum and bronchial lymph nodes (respiratory abscesses), which eventually become endemic.

CLA occurs worldwide, and severe problems due to this disease have been described in commercial flocks of many different countries (Al-Rawashdeh and Al-Qudah, 2000; Connor et al., 2000; Ben Said et al., 2002; Binns et al., 2002; Arsenault et al., 2003; Paton et al., 2003). Economic losses due to CLA include decreased milk production, reproductive disorders, condemnation of carcasses, and leather depreciation (Fontaine and Baird, 2008).

Caprine and ovine husbandry activities have increased considerably in various regions of Brazil during recent years, and there appears to be a tendency towards continued growth (Brazilian Agricultural Research Corporation, 2008). Although CLA has long been detected as an endemic problem in the most important goat production area of the country, the northeast region (Brown et al., 1987), a recent and comprehensive analysis of the prevalence of CLA in Brazilian goat herds is lacking, especially in emerging high-production regions, such as the state of Minas Gerais (Fig. 1). This state comprises approximately 586,500 square kilometers in the southeast region of Brazil and concentrates a considerable proportion of national agribusiness.

A large-scale collaborative project was initiated a few years ago, to characterize the ovine and caprine productions of Minas Gerais state. The information collected until present constitutes an enormous database, spanning many aspects of farming activities in

\* Corresponding author. Tel./fax: +55 31 34092610.  
E-mail address: [vasco@icb.ufmg.br](mailto:vasco@icb.ufmg.br) (V. Azevedo).

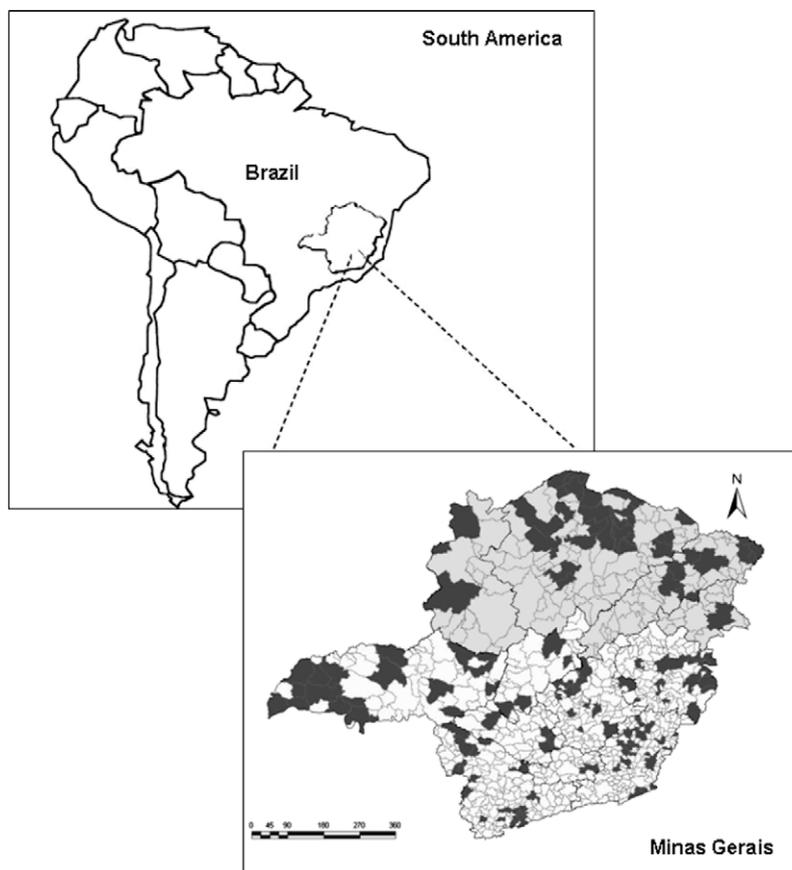


Fig. 1. Map of Minas Gerais state, Brazil, showing the regions where the samples were collected (dark patches).

different sub-regions of this state (Fig. 1); it includes the frequency of infectious diseases and of disease-related risk factors (Guzmarães, 2006). Additionally, a representative and potentially highly informative serum bank has been created, which will make possible studies of the seroprevalence of various types of infections in the goats and sheep of this region (Marques, 2006).

Our objectives were to determine the seroprevalence of CLA in goat herds of Minas Gerais state and determine whether specific aspects of caprine production in this region were correlated with these prevalence findings. An indirect enzyme-linked immunosorbent assay based on secreted proteins of *C. pseudotuberculosis* (hereafter referred to as secreted proteins-ELISA) was used to screen the goat serum bank, which is representative of the state's herds. We found a high seroprevalence (78.9%) of this disease among the goats, demonstrating that appropriate management practices for the control of CLA are urgently needed.

## 2. Materials and methods

### 2.1. Study area, animals and sera

The location of Minas Gerais state in Brazil and the municipalities that were screened in our experiment are shown in Fig. 1. The minimum number of samples needed to screen the state of Minas Gerais was calculated according to Astudillo (1979), assuming a sampling error of 20% and a confidence interval of 95% ( $z = 1.96$ ). The farms were analyzed by the Official Service of Sanitary Defense of Minas Gerais state, and information reported by owners concerning several aspects of the goat herds was collected by local veterinarians, including: age, sex and breed of the screened animals, type of production system employed, existence of other

infectious diseases, origin of the animals, and participation in expositions.

Six hundred and seventy-six serum samples were collected from 108 goat flocks in 2001. Briefly, blood was obtained by jugular puncture; following centrifugation, the sera were separated and stored at  $-20^{\circ}\text{C}$  until use. Sera from animals living in non-endemic areas were pooled and used as negative controls, and sera from animals that tested positive based on isolation of *C. pseudotuberculosis* from caseous material and on biochemical tests were also pooled and used as positive controls.

### 2.2. Secreted proteins-based ELISA

An indirect ELISA was performed to identify specific total immunoglobulins directed towards *C. pseudotuberculosis* secreted antigens, as described by Carminatti et al. (2003), with modifications. *C. pseudotuberculosis* strain 1002, isolated from a caseous granuloma found in a CLA-affected goat in Bahia state (Brazil) and identified with the API CORYNE biochemical battery (Bio-merieux, France), was used as a source for the antigens.

Briefly, total secreted antigens were obtained from 48 h cultures of *C. pseudotuberculosis* in brain heart infusion (BHI) broth (Paule et al., 2004). Polystyrene 96-well microtiter plates (Maxisorp<sup>®</sup> Nunc) were coated overnight with 100  $\mu\text{L}$  of secreted proteins diluted 100-fold into 0.05 M carbonate-bicarbonate buffer (pH 9.6). The plates were blocked with 5% skimmed milk powder diluted in 0.05 M phosphate-buffered saline (pH 7.2)–0.05% Tween 20 (PBS-T – 200  $\mu\text{L}$ /well) for 2 h at  $37^{\circ}\text{C}$ , washed once with PBS-T, and then incubated for 1 h with 50  $\mu\text{L}$  of goat sera (diluted 1:100 in PBS-T). The wells were washed three times with the same buffer and incubated for 45 min with 50  $\mu\text{L}$  of horseradish-peroxidase-

conjugated anti-goat total immunoglobulin, diluted in PBS-T to 1/10,000 (Dako Laboratories). Following five PBS-T washings, the plates were developed for 15 min with tetramethylbenzidine substrate (50  $\mu$ L/well), in the dark at room temperature. Reactions were stopped with 5% sulfuric acid (25  $\mu$ L/well), and absorbance readings were taken at 450 nm using an ELISA spectrophotometer (BIO-RAD).

Each sample was assayed in duplicate, along with appropriate positive and negative controls. All duplicates presenting differences higher than 20% were repeated, in order to confirm seropositivity status.

Since there were modifications from the original published assay, we have validated the new ELISA conditions by employing 150 serum samples from healthy animals, originated from an area which is non-endemic for CLA, as negative reference sera, and 70 serum samples from animals presenting clinical CLA, confirmed by microbiological methods, as positive reference sera. We observed a specificity of 98.5%, and sensitivity of 93.5% in the modified ELISA test, considering a cut-off point of  $OD_{450nm} = 0.350$ , calculated using the method described by Frey et al. (1998): cut-off point = mean of the OD values observed for all truly negative sera + three standard deviations.

### 2.3. Statistical analyses

Prevalence values were calculated using the software WinEpi-scope<sup>®</sup> 2.0. The influence of the different variables on the seroprevalence of CLA was evaluated using Pearson's  $\chi^2$  method, with software SPSS<sup>®</sup> 13.0.

Prevalence data were adjusted as described by Noordhuizen et al. (1997), where Real Prevalence = (apparent prevalence + specificity – 1)/(sensitivity + specificity – 1).

## 3. Results

### 3.1. General aspects of the goat herds

The frequencies of other infectious diseases in the goat herds, as well as the sanitary and zootechnical procedures employed in the farms, are presented in Tables 1 and 2. Most of the animals (76.8%) had been vaccinated against several diseases, including clostridiosis, parasitoses, anthrax, anemia, foot-and-mouth disease and hepatitis. However, only a small percentage of the goats (34.4%) were isolated from animals that presented symptoms of infection or from recently-acquired animals. Curiously, even though a great majority of the farms had purebred animals, most of them (90.1%) did not participate in animal shows (Table 1).

Ectoparasitosis, abortions, diarrhea and CLA were the most prevalent pathological disorders in the herds, based on information supplied by the owners (Table 2).

### 3.2. Seroprevalence of CLA evaluated by *C. pseudotuberculosis* secreted proteins ELISA

Sera from 676 goats were examined by ELISA for antibodies against *C. pseudotuberculosis* secreted antigens. A high proportion of the samples yielded a positive reaction for specific anti-*C. pseudotuberculosis* immunoglobulins. The apparent CLA prevalence was 72.6% and the adjusted prevalence calculated was 78.9%. About 98% of Flocks had presented at least one seropositive animal. Seropositivity rates did not differ between male and female goats (Fig. 2). The seroprevalence of CLA in animals who did not belong to a defined breed or those who belonged to the Toggenburg breed were somewhat higher however, the differences found between breeds were not significant (Fig. 3).

The prevalence of CLA was found to be significantly higher in older animals (Fig. 4). However, the high prevalence in animals less than one year old suggests that they acquire the infection quite early in life (Fig. 4). The type of animal husbandry also influenced the seroprevalence of CLA among the herd. Animals that were reared in an extensive management system had a higher prevalence of this disease than did those reared semi-extensively and intensively (Fig. 5).

## 4. Discussion

The state of Minas Gerais has a lengthy frontier with a region long recognized as endemic for CLA, the state of Bahia (Meyer, 2004). The intensive commerce of small ruminants between these two states increases the possibility that *C. pseudotuberculosis* infection may also be common in the goat herds of Minas Gerais state. Indeed, in our epidemiological survey we observed a very high seroprevalence of CLA in the goats (78.9%). Positive secreted proteins-ELISA titers against *C. pseudotuberculosis* were detected in the sera of animals from almost all of the herds (98%).

These high seroprevalence results were not expected, given that the farmers reported that only 17.5% of the goats had presented clinical signs of CLA, and this disease was considered a problem in only 31.4% of the herds (Tables 1 and 2).

Carminatti et al. (2003) found a sensitivity of 93.5% and 100% specificity of *C. pseudotuberculosis* secreted proteins-ELISA for detecting CLA in goats. The gold standard test used to classify an animal as positive for CLA is isolation of the causative bacterium from visibly-affected lymph nodes, followed by morphological and biochemical identification of the microorganism. In our modified assay, we employed different conditions, and in this way we redefined the validation criteria for this modified assay, employing a large number of truly positive and negative animals. We found a specificity of 98.5% and a sensitivity of 93.5%, when considering a cut-off point of 0.350 OD obtained by the Frey method (Frey et al., 1998), which presupposes cross-reactions and better adjusts the values within the conditions of the experiment. ELISA-based assays have been used in several previous studies to measure the prevalence of CLA (Sutherland et al., 1987; Sting et al., 1998; Dercksen et al., 2000; Binns et al., 2002), however, several factors can interfere with the performance of these diagnostic tests, including: (i) the nature of the circulating antibodies against *C. pseudotuberculosis*, which may decline during periods of pathogen dormancy or (ii) cross-reaction of the antigens derived from a *C. pseudotuberculosis* culture supernatant with antibodies produced against similar proteins from other bacteria.

Previous epidemiological studies conducted in countries initially considered CLA-free have demonstrated that the prevalence of this disease in small ruminants is usually underestimated, since its notification is not compulsory in many countries, animal owners are not aware of its economic impact and do not usually test to identify sub-clinically infected animals (Stoops et al., 1984; Paton et al., 1988; Stanford et al., 1998; Baird and Fontaine, 2007; Çetin-kaya et al., 2002; Moller et al., 2000). ELISA tests based on *C. pseudotuberculosis* antigens have already been shown to be effective in the early identification of infected sheep and goats in CLA eradication programs (Schreuder et al., 1994; Dercksen et al., 2000; Baird et al., 2004).

Most of the animals in our study originated from the southeast region of the country, and exotic purebred goat races (Saanen, Alpine, Anglo-Nubian and Toggenburg) were the most frequently encountered. Along with the proximity of Minas Gerais state to an area in which CLA is endemic, participation of animals in animal shows throughout Brazil must be also considered as a mechanism of dissemination of this disease, since no serological testing for CLA

**Table 1**

General aspects of the goat herds screened in our study, as reported by their owners in Minas Gerais state, Brazil, in 2001.

Variable	Characteristic	Goats (%)
Husbandry system	Extensive	142 (40.8)
	Semi-intensive	93 (26.5)
	Intensive	113 (32.5)
	Not informed	328
Animal origin	Southeast region	544 (80.5)
	Other regions	132 (19.5)
Participation in expositions	Yes	67 (9.9)
	No	609 (90.1)
Type of race	Exotic pure	392 (60.4)
	Exotic half-breed	12 (7.4)
	National race	48 (1.8)
	National half-breed	113 (17.5)
	Race not defined	84 (12.5)
	Not informed	27
Use of quarantine sick animals isolation?	Yes	177 (34.4)
	No	337 (65.6)
	Not informed	162
Existence of animal identification?	Yes	293 (44.1)
	No	372 (55.9)
	Not informed	11
Clinical occurrence of CLA	Yes	108 (17.5)
	No	509 (82.5)
	Not informed	59
Vaccination	CLA	1 (0.3)
	Other diseases	312 (76.8)
	No vaccination	93 (13.8)
	Not informed	270

**Table 2**

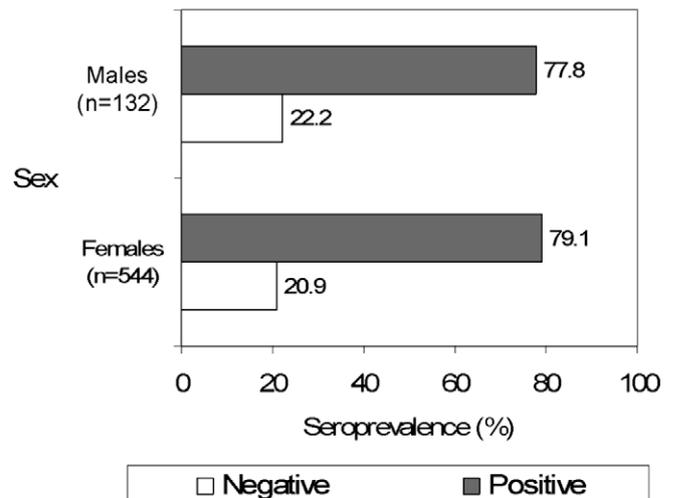
Major pathological disorders found in commercial goat herds, reported by owners in Minas Gerais state, Brazil, in 2001.

Symptoms reported by herd owners	Number of herds (n = 108) (%) <sup>a</sup>
Ectoparasitoses	58 (53.7)
Abortion	53 (49.1)
Caseous lymphadenitis	34 (31.4)
Diarrhea	32 (29.6)
Mastitis	28 (25.9)
Pneumonia	21 (19.4)
Contagious ecthyma	18 (16.6)
Keratoconjunctivitis	16 (14.8)
Pododermatitis	13 (12.1)
Arthritis	10 (9.3)
Nervous symptoms	5 (4.6)
Others	10 (9.2)

<sup>a</sup> Properties were screened by veterinarians from the Official Sanitary Vigilance Board of Minas Gerais state.

is made on the animals that are taken to such events. Only goats with external symptoms of the disease are forbidden to participate, consequently, sub-clinically infected animals from regions in which the prevalence of CLA is high can be placed in close contact with non-infected ones. Paton (2000) reported that animal transportation and commercialization can cause introduction of *C. pseudotuberculosis* into CLA-free areas.

When the serological results were stratified by age, sex and breed, some correlations with the prevalence of CLA became evident. Sex and breed of the goats did not significantly correlate with the prevalence of the disease in our study (Figs. 2 and 3), however, the seroprevalence of CLA increased with age, being 67.2% in the youngest animals and 86.9% in the oldest ones (Fig. 4). The frequency of animals who tested positive for anti-*C. pseudotuberculosis* antibodies was low in goats less than one year old. This frequency was significantly higher in goats aged one to two years



**Fig. 2.** Seroprevalence of CLA in goats according to sex, in Minas Gerais state, Brazil. No significant statistical differences between groups, by Pearson  $\chi^2$  test ( $p < 0.01$ ).

old and was even higher in those aged two to three years old. Al-Rawashdeh and Al-Qudah (2000) also reported that the prevalence of CLA increases with age. Considering that CLA is a chronic disease, with a long incubation period, it would be expected that its prevalence is higher in older animals; additionally, the longer the animal is maintained in the flock, the higher the chance that it will have already come into contact with infected animals.

The type of husbandry also significantly affected the prevalence of CLA (Fig. 5). Goats managed under an extensive production system had a higher prevalence of CLA than those managed under semi-intensive and intensive systems. This may be due to the characteristics of this disease, for abscess rupture releases huge

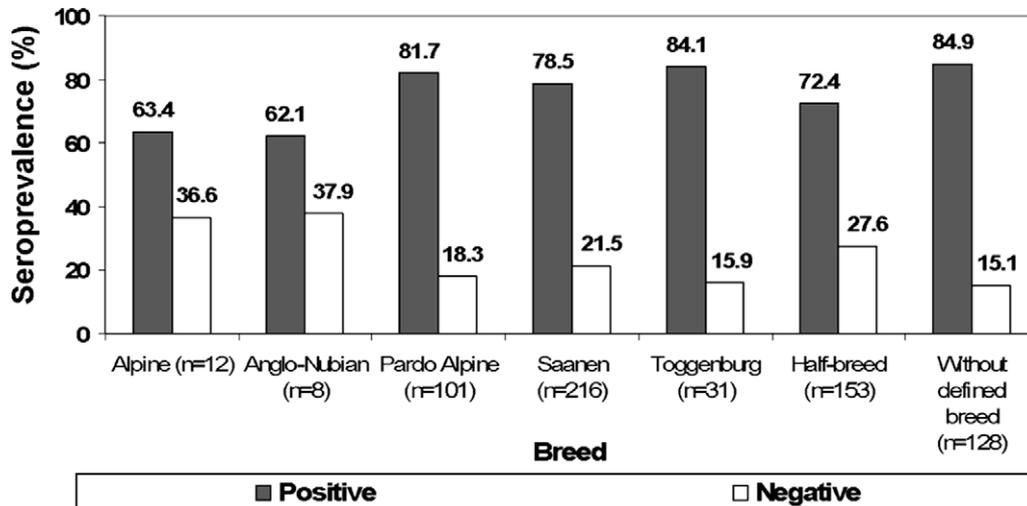


Fig. 3. Seroprevalence of CLA in goats divided by breed, in Minas Gerais State, Brazil. No significant statistical differences between groups, by the Pearson  $\chi^2$  test ( $p < 0.01$ ).

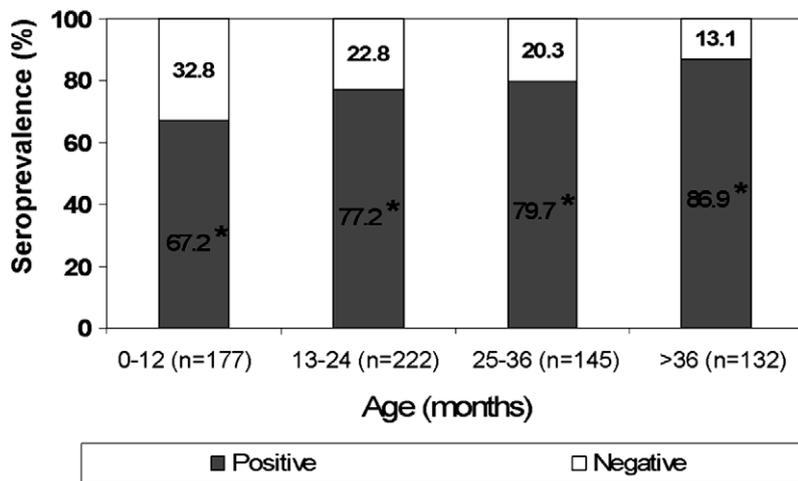


Fig. 4. Distribution of CLA seroprevalence in goats according to age, in Minas Gerais State, Brazil. Asterisks (\*) denote significant statistical differences, by Pearson  $\chi^2$  test ( $p < 0.01$ ).

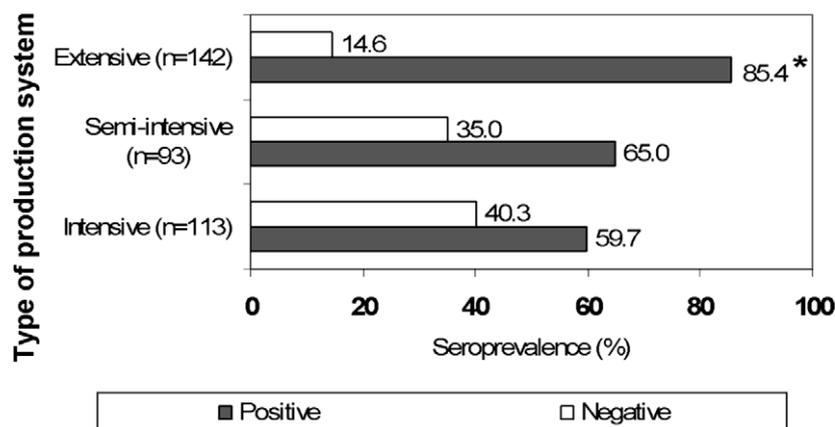


Fig. 5. Distribution of caseous lymphadenitis seroprevalence in goats according to the type of production system, in Minas Gerais state, Brazil. Asterisk (\*) denote significant statistical differences (Pearson  $\chi^2$  test,  $p < 0.05$ ).

numbers of bacteria onto the skin and fleece, resulting in contamination of the immediate environment. Other animals may then become exposed to the bacteria, either through direct physical

contact with lesions of the affected individual or indirectly via contaminated fomites (Stoops et al., 1984; Ellis et al., 1987; Paton et al., 1988; Pepin et al., 1994; Williamson, 2001).

When caprinoculture in the states of Rio de Janeiro and Minas Gerais was analyzed by Magalhães (1985), they found that 54.2% of the flocks employed an intensive production system, while 45.8% used a semi-extensive system, this is because there is a predominance of milk producing breeds in these states. An intensive production system can enhance CLA prevention, since the external lesions can be easily observed. In our study, only a small percentage of the producers indicated that CLA was a major problem in their flocks; however, the rate of infection was quite high. This could be explained by a high frequency of internal lesions that are difficult to observe.

In conclusion, when we used a highly-specific ELISA test based on secreted proteins of *C. pseudotuberculosis* to screen sera from goat flocks of Minas Gerais state in Brazil, we found a very high seroprevalence of CLA. Our finding of higher prevalence rates in older animals and in goats managed under extensive production systems could aid in planning appropriate measures for programs aimed at eradicating CLA in this emerging goat-producing region.

### Acknowledgements

We thank the ADAB (Agricultural Defense Agency of the State of Bahia) which helped us to acquire the negative control samples for this study. We are grateful to John A. McCulloch (UFMG) for reviewing the manuscript. We also thank the valuable comments made by anonymous referees. This work was supported by CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico, Brasil), CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Brasil) and FAPEMIG (Fundação de Amparo à Pesquisa do Estado de Minas Gerais, Brasil).

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