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Subclinical Diagnosis of Caseous Lymphadenitis Based on ELISA in Sheep from Brazil

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Abstract

Caseous lymphadenitis (CLA), caused by *Corynebacterium pseudotuberculosis*, is a chronic contagious disease that affects small ruminants and still remains an important problem for many lamb-producing countries. Animals are considered clinically infected when occurs abscesses in superficial lymph nodes. Visceral or internal form can coexist which no apparent clinical signs of infection are seen. The best procedure to avoid spread of the disease is elimination of infected animals. However, as the chronic and subclinical nature of the infection of CLA alternative methods are required for detection and screening. In this study, we described the performance of indirect Enzyme-Linked Immunosorbent Assay (ELISA) for diagnosis of CLA in asymptomatics sheep. Also, test culture and biochemical identification were achieved to confirm CLA infection. The serological diagnostic was performed in sheep symptomatics (n=50) and asymptomatics (n=374) from nine flocks. Analysis reported high positivity of 71% for ELISA in 85% of asymptomatic animal for CLA with a sensitivity of 88% and specificity of 31%. Results from ELISA test in asymptomatic animals against culture for caseous lymphadenitis were more specific (97%) and permitted to exclude healthy animals without symptoms. This study concluded that *C. pseudotuberculosis* infection could be widely disseminated in sheep flocks in Northwestern region of the state of São Paulo, Brazil and only one screening test is not enough. The association with indirect ELISA test and culture could better indicate the real problem of CLA in sheep flocks.

Keywords: Caseous lymphadenitis; *Corynebacterium pseudotuberculosis*; Subclinical diagnostic; ELISA

Introduction

Caseous lymphadenitis (CLA), caused by *Corynebacterium pseudotuberculosis,* is a chronic contagious disease that affects the majority of sheep-rearing areas worldwide [1-4]. The identification of infected animals, with or without clinical symptoms, is the best procedure to avoid spread and to control this disease. This identification can be realized firstly by clinical exams in which the presences of abscesses in superficial lymph nodes are observed in several animals in a group. Other bacterial pathogens are capable of producing suppurative lymphadenopathy but they tend to be sporadic in nature and are not seen as a flock problem [1].

Visceral or internal form can coexist in asymptomatic infections without any apparent clinical signs of infection, making it difficult to be diagnosed [1,4-6].

The classical or gold standard diagnosis of CLA is represented by the culture test and the identification of *C. pseudotuberculosis* from abscesses. This classical diagnosis may not always be advantageous or possible since chronic external lesions with little pus containing few viable organisms or visceral lesions cannot be sampled [1]. In such case, various diagnostic techniques have been developed. Enzyme-linked Immunosorbent Assay (ELISA) has already proven to be a versatile method to aid in CLA-control from herds and to identify sheep with visceral form without appealing to bacteriology resources [7-11].

Indirect ELISA test for the detection of anti-bodies against *C. pseudotuberculosis* secreted proteins is being performed in seroepidemiological surveys for CLA in Brazil. A high seroprevalence of this disease has been detected by using applying method either among goat (78.9%) or sheep herds (70.9%) from Minas Gerais state, despite the absence of external clinical signs of CLA in most of them (89% to 82.5%) [12,13].

Clinical examination and regular ELISA testing were able to halt the appearance of new clinical cases of CLA. Newly purchased sheep with CLA infection history should be submitted to physical examination before their introduction into naïve flocks, followed by an immediate culling of the clinically affected sheep. However, subclinical infection requests more efforts to detect and control the disease [14].

The challenge of diagnosing CLA in subclinical infections highlights the necessity for rapid, practical and available diagnostic test for CLA infection. The purpose of this study was to describe the performance of the indirect ELISA to detect CLA in asymptomatic sheep from Northwestern region of the state of São Paulo, Brazil. Additionally, bacterial culture and biochemical identification were realized in samples from asymptomatic animals and positive at indirect ELISA test to confirm CLA infection.

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Material and methods

Animals

Samples were collected from 424 sheep during the months of June and July 2007, from nine farms located in the Northwestern region of the state of São Paulo, Brazil.

The inclusion of sheep flocks in this study required the existence of apparently healthy animals with heterogeneous distribution of age, male and female, breeds and husbandry methods.

Each animal was clinically examined and classified as symptomatic or asymptomatic for CLA. Animals were considered asymptomatic when apparently healthy, without any abscess, and which could be living or not with sheep presenting clinical signs of CLA.

Bacteriological and serum samples

Bacteriological samples were collected by Fine Needle Aspiration Biopsy (FNAB). Following sterilization of the skin, FNAB was performed preferentially in the right prescapular lymph node of the animals, using a 23 G or 25 G needle and a 10 ml syringe coupled to a cytoaspirator. A single aspirate was performed, using more than 5 ml suction. Needle and syringe were transported as a unit and kept on ice until their storage at –20ºC.

Blood samples were taken using serum collection tubes and 21 G needle (BD Vacutainer Blood Collection Serum Tubes and Eclipse ™ Blood Collection Needle 21 G, Becton-Dickinson, Franklin Lakes, New Jersey). Serum samples were separated by centrifugation (800g/ 10 min) and stored at –20ºC.

Enzyme linked immunosorbent assay (ELISA)

An indirect ELISA was performed to identify total immunoglobulins directed towards *C. pseudotuberculosis* secreted antigens in sheep sera, as previously described [8,13]. Briefly, 96-well microplates (Nunc MaxiSorp, eBiosciense, San Diego, California) were coated with 100 µl per well of the supernatant of a 48-hour *C. pseudotuberculosis* culture in Brain Heart Infusion (BHI) broth, and then 1:100 diluted sheep serum samples were assayed in duplicate. Appropriate positive and negative control samples were included in all plates. Reads were taken in a microplate reader (Bio-Rad 550 Microplate Reader, Bio-Rad Laboratories, Hercules, California) at 490 nm.

The cut-off point to define seropositivity was previously described [13]. Therefore, it was calculated as the mean absorbance at $OD_{490\ nm}$ observed for all truly negative sera (n=150), plus three standard deviations (OD_{490nm}=0.32) [13,15]. Mean values for positive, negative, and blank control samples were 1.38, 0.15 and 0.05, respectively.

Bacterial culture and biochemical identification

The material in the needle and syringe of each collected sample was washed with 400 µl of sterile water and stored at -80°C with cryopreservation for microbiological examination. Twenty µl of each sample were spread on BHI agar plates and incubated at 37ºC for 48 h. *C. pseudotuberculosis*-like colonies (yellowish, dry, with irregular borders) that stained Gram-positive were tested further for biochemical properties.

The ability of *C. pseudotuberculosis* to ferment carbohydrates was evaluated in basic Cystine Tryptic Agar medium containing 1% dehydrated carbohydrates, including glucose, sucrose, maltose, mannose, mannitol, xylose, and ribose. The isolates were also evaluated for catalase and urease production, nitrate reduction and alkaline phosphatase activity, along with motility and H_2S production [15].

Synergistic haemolysis with *Rhodococcus equi* (ATCC 33701) and inhibition of *β*-haemolysis by *Staphylococcus aureus* (ATCC 25923) were also evaluated [15].

Statistical analysis

Statistical analysis was done with nonparametric statistics by entering frequencies into 2×2 tables. Thus, Chi-square Test determined the association between indirect ELISA test and clinical exam information (symptomatic and asymptomatic). Sensitivity, specificity, predictive values and accuracy were calculated with positive and negative controls. The degree of concordance among the diagnostic exams was measured by the Kappa coefficient. The alpha level for significance was 5%. The statistical analyses were made with the program OpenEpi version 2.3.

Results

This study evaluated the diagnostic performance of indirect ELISA method to detect CLA infection by secreted antigens in sheep sera from nine flocks, which were realized in association with clinical inspection. For this purpose, serum samples (n=424) were used in the test.

The distribution of the analyzed samples had mainly female (n=388), ages ranged from 1 to 12 months and originating from 3 kinds of breeds, as shown in Table 1.

CLA lesions in symptomatic sheep were most commonly observed in the prescapular lymph nodes right and left, followed by right parotid lymph node, right popliteal lymph node and right crural lymph node. The frequency of positive and negative animals from each farm, according to indirect ELISA Test, was represented in Figure 1.

A 2×2 table was used to evaluate the association between clinical inspection (symptomatic and asymptomatic) and indirect ELISA test results (Table 2), from a total of 424 sheep clinically examined, where 50 were symptomatic and 374 asymptomatic (Table 1). According to results shown in Table 2, indirect ELISA test showed 88% (95% CI: 76-94%) of sensitivity and 31% (95% CI: 27-36%) of specificity.

In order to evaluate the veracity of the results obtained from indirect ELISA test and clinical exams, regarding asymptomatic and false positive animals (n=257), bacterial culture and biochemical identification were realized in samples collected by FNAB procedure (n=113). The performance of the screening procedure (indirect ELISA test) against a gold standard (Culture test) was analyzed in a 2×2 table (Table 3).

Variable	Group	Positive(n)	Positive (%)	Total Tested (n)
Sex	Females	288	96%	388
	Males	13	4%	36
Breed	Santa Inês	172	57%	232
	Suffolk	15	5%	18
	Mixed	114	38%	174
Age (months)	1–12	41	14%	89
	13–24	209	69%	282
	25–36	51	17%	53
Clinical Symptom	Asymptomatic	257	85%	374
	Symptomatic	44	15%	50

Table 1: Distribution of results based on ELISA for *C. pseudotuberculosis* in Northwestern region of the state of São Paulo, Brazil.

		Symptomatic	Asymptomatic	Total
ELISA	+	44	257	301
	-	6	117	123
	Total	50	374	424
Chi square Test (p=0.002)				

Table 2: Association of results obtained from indirect ELISA based on clinical examination.

Parameter	Estimate	Lower - Upper 95% CIs
Sensitivity	2.469%	(0.6797, 8.563')
Specificity	96.88%	(84.26, 99.45')
Positive Predictive Value	66.67%	(20.77, 93.85')
Negative Predictive Value	28.18%	(20.62, 37.22')
Diagnostic Accuracy	29.2%	(21.61, 38.16')
Cohen's kappa	-0.0037	(-0.04164 - 0.03409)

Table 3: Performance of results from ELISA test in asymptomatic animals against culture for caseous lymphadenitis.

According to the obtained results (Table 3), by combining two diagnostic methods (Culture vs. indirect ELISA test), it was confirmed that 2 animals were truly positive and 31 were truly negative resulting in a 97% of specificity (95% CI: 84-99%).

Discussion

Corynebacterium pseudotuberculosis infection is widely disseminated in small ruminants' worldwide [2]. The present study identified the high widespread of CLA and its possible presence in all sheep herd tested in Northwestern region of the state of São Paulo, Brazil. The higher seropositivity was predominant in adults (13 to 24 months old), females and asymptomatic sheep. CLA was found ranging from 35% to 98% of seropositivity.

Curiously, even though great sanitary and zootechnical procedures were observed in two of nine farms (number 4 and 6, Figure 1), they showed the higher level of positivity. These herds were semi-intensive to intensive husbandry system, composed mainly by national type of race (Santa Inês) and the owners had enough knowledge about clinical occurrence of CLA. However, recently-acquired animals were not isolated from the others neither tested to CLA. Thus, the early identification is crucial of control and eradication programs, as described in previous epidemiological studies [9,10,13].

Some researchers have been reporting strategies to control and reduce the incidence of CLA in affected flocks. The most significant effects were seen when ELISA was regular used in association with clinical inspections in affected flocks from the United Kingdom [16]. These serological tests have been used as a research tool in others countries, as Brazil. However, such programs may be hindered by the difficulties associated with detection of subclinically infected animals [4,13,17]. Therefore, in the present study, we employed the indirect ELISA for detecting humoral responses in asymptomatics (n=374) and symptomatics (n=50) sheep. The higher sensitivity of 88% and low specificity of 31% presented 257 samples as false positive with 71% of seropositivity.

Although these results suggested that ELISA remains a good alternative to CLA-screening programs, it is questionable whether only one diagnostic test should be employed to drive CLA eradication programs [4,18]. Several factors can interfere with the interpretation of ELISAs, specifically: (i) the nature of the circulating antibodies against *C. pseudotuberculosis*, which may decline during periods of pathogen dormancy, resulting in high numbers of false-negatives; and most importantly, (ii) the inability to distinguish between previously exposed animals and those still harboring the pathogen [1,9,10].

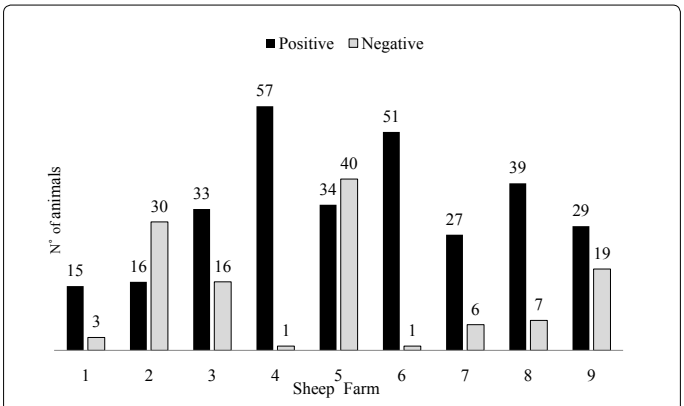

Figure 1: Distribution of positive and negative animals by ELISA from sheep farms in Northwestern region of the state of São Paulo, Brazil.

In order to confirm ELISA results and to better understand the real state of CLA infection in asymptomatic sheep, we included bacterial culture and biochemical identification in our analysis. Then, we had a new specificity of 97% with only 2% of prevalence. Based on our findings, culture and indirect ELISA test completed each other and would be a great alternative to confirm or exclude CLA infection. We believe that this association could better translate the real prevalence of properties affected and the necessity to control CLA spread in Brazil.

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