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José Romero Alexandre Alves

# Caracterização epidemiológica da linfadenite caseosa em pequenos ruminantes no Nordeste do Brasil

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Tese submetida ao Programa de Pós-Graduação em Ciência e Saúde Animal, da Universidade Federal de Campina Grande, como requisito parcial para obtenção do grau de Doutor em Ciência e Saúde Animal.

Professor Titular Dr. Clebert José Alves - Orientador

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JOSÉ ROMERO ALEXANDRE ALVES
**Doutorando**

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# RESUMO

Essa tese é composta por três artigos. No Capítulo I foi determinada a soroprevalência da infecção por *Corynebactetium pseudotuberculosis* em ovinos no Nordeste brasileiro por meio da técnica de ELISA (*enzyme linked immunosorbent assay*) indireto. Foram coletadas amostras de 2.638 ovinos provenientes de 223 propriedades de cinco estados (Ceará, Paraíba, Piauí, Rio Grande do Norte, e Sergipe). Dos 2.638 ovinos testados, 996 (37,76%; IC 95% = 35,93 – 39,62%) foram sororeativos e das 223 propriedades avaliadas, 210 (94,17%; IC 95% = 90,28 – 96,56%) resultaram positivas. O teste de ELISA-indireto apresentou sensibilidade e especificidade de 91,84% e 97,01%, respectivamente, demonstrando ser útil no diagnóstico da linfadenite caseosa (LC) em ovinos. A infecção por *C. pseudotuberculosis*, encontra-se disseminada nos rebanhos ovinos dos estados da região Nordeste do Brasil. No Capítulo II foram avaliados os fatores associados à sororeatividade para *C. pseudotuberculosis* na região Nordeste. Foram colhidas amostras de 2.312 ovinos originados de 196 propriedades estudadas no Capítulo I. Foram identificadas 93,88% (184/196) das propriedades e 37,46% (866/2.312) dos animais com resultado sorológico positivo para *C. pseudotuberculosis*. Animais de raça pura (PR = 1,189; p = 0,017), origem do reprodutor de exposição (PR = 1,192; p = 0,020), separar as crias das mães (PR = 1,132; p = 0,048), origem da água de lagoa (PR = 1,365; p = 0,002) e eliminar animais infectados como medida de controle (PR = 1,263; p = 0,027) foram identificados com fatores associados à soropositividade. Recomendam-se medidas de incrementação do controle da doença nos animais de raças puras e que participam de aglomerações animais, que visem reduzir a contaminação ambiental pelo agente, o estresse no manejo de desmama, o descarte adequado dos infectados e isolamento com inspeção em animais recém adquiridos antes de introduzi-los aos rebanhos, bem como incentivar a utilização do diagnóstico precoce e da vacinação para diminuir a ocorrência da doença. No Capítulo III foi conduzido um estudo de soroprevalência da linfadenite caseosa em caprinos comercializados em feira de animais vivos no semiárido nordestino. Foram coletadas amostras de 233 caprinos e realizado diagnóstico por meio da técnica de ELISA-indireto. Foram identificados 87 (37,34%; IC 95% = 31,38 – 43,71%) caprinos sororeativos para *C. pseudotuberculosis*. A infecção por *C. pseudotuberculosis* está presente nos rebanhos caprinos comercializados. São recomendadas políticas de incentivo ao acesso do diagnóstico precoce da doença pelos produtores e a implementação de um programa de controle da LC com o objetivo de reduzir a alta prevalência observada nas propriedades e nos animais.

**Palavras-Chave**: Ovinos, Caprinos, Soroprevalência, Doença bacteriana, Risco associado, Ensaio imunoenzimático, Programa de controle.

## ABSTRACT

This thesis consists of three articles. In Chapter I, seroprevalence of *C. pseudotuberculosis* infection in sheep in Northeastern Brazil was determined by indirect enzyme linked immunosorbent assay (i-ELISA). Samples were collected from 2,638 sheep from 223 farms of five states (Ceara, Paraiba, Piaui, Rio Grande do Norte, and Sergipe). From 2,638 sheep tested, 996 (37.76%; 95% CI = 35.93 - 39.62%) were seroreactives and from 223 properties evaluated, 210 (94.17%; 95% CI = 90.28 - 96, 56%) were positive. The i-ELISA test showed sensitivity and specificity of 91.84% e 97.01%, repectively, proving to be useful in diagnosis of caseous lymphadenitis (CLA) in sheep. In Chapter II, factors associated with seroreactivity for *C. pseudotuberculosis* in the Northeast region were evaluated. Samples were collected from 2,312 sheep from 196 farms studied in Chapter I. It was identified 93.88% (184/196) of the properties and 37.46% (866/2312) animals with positive serological result for *C. pseudotuberculosis*. Purebred animals (PR = 1.189; p = 0.017), source of exposure breeder (PR = 1.192; p = 0.020), separate lambs from ewes (PR = 1.132; p = 0.048), lakes water source ( PR = 1.65; p = 0.002) and eliminating infected animals as a control measure (PR = 1.263; p = 0.027) were identified with factors associated with seropositivity. Measures to increase disease control are recommended in purebred animals that participate in agglomerations of animals, to reduce environmental contamination by the agent, stress in handling weaning, proper disposal of the infected and isolation with inspection on recently acquired animals before introducing them to herds, as well as to encourage the use of early diagnosis and vaccination to reduce the occurrence of the disease. In Chapter III a seroprevalence study of caseous lymphadenitis was conducted in goats sold in animals fair on northeastern semiarid. Samples were collected from 233 goats and the diagnostic was performed by the i-ELISA. Eighty-seven (37.34%; 95% CI = 31.38 - 43.71%) seroreacitive goats were identified. *C. pseudotuberculosis* infection is present in marketed goat herds. Policies to encourage early access to disease diagnosis by producers and the implementation of a CLA control program are recommended to reduce the high prevalence observed in farms and animals.

**Keywords**: Sheep, Goats, Seroprevalence, Caseous lymphadenitis, Risk associated, Enzyme linked immunosorbent assay, Control program.

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## INTRODUÇÃO GERAL

A produção de pequenos ruminantes é uma atividade largamente explorada nos países tropicais. Nesse contexto, o Brasil possui um efetivo estimado em mais de 13,7 milhões de ovinos e 8,26 milhões de caprinos. Na região Nordeste a ovinocaprinocultura representa uma importante atividade, onde se concentram mais de 9 milhões de ovinos e 7,6 milhões de caprinos, representando 65,59% e 92,81% do efetivo nacional, respectivamente. A atividade está presente em mais de 385 mil criatórios ovinos e 296 mil criatórios caprinos da região, o que constitui 75,38% e 88,79% das propriedades criadoras do país, respectivamente (IBGE, 2019). A maior parte dos sistemas de criação é de subsistência, ocorrendo diversos problemas como falta de assistência técnica, deficiências de manejo e baixo nível organizacional dos produtores, que inviabilizam economicamente a atividade devido à baixa produtividade do rebanho (Sousa, 2007; Coelho et al, 2011; Holanda Filho et al, 2019).

Em algumas regiões, como o sul e sudeste brasileiro, a pecuária de pequenos ruminantes mostra-se mais organizada, viabilizando o sistema produtivo (SEBRAE, 2009). Entretanto o potencial produtivo desta atividade ainda não é satisfatório para o agronegócio nacional, onde a inexistência de fatores que impulsionem o gerenciamento e articulação do setor primário da cadeia produtiva tornam a atividade mais competitiva e viável economicamente (Aro et al, 2007).

Nesse cenário, as feiras de animais vivos na região Nordeste possuem grande expressividade como via de escoamento da produção. São locais onde se comercializa grande parte dos pequenos ruminantes pelos próprios criadores ou comerciantes intermediários, que adquirem os animais nas propriedades e os revendem nesses recintos (Nogueira Filho et al., 2010). Comumente encontrados em diversas cidades interioranas, esses eventos contribuem para o desenvolvimento e crescimento econômico regional (Maia, 2007), porém representam um risco de transmissão e disseminação de doenças infecciosas, na ausência de um controle sanitário satisfatório, onde a aglomeração de animais pode facilitar o contato entre susceptíveis e fontes de infecção.

Altas frequências de doenças, principalmente infecciosas, que ocorrem na atividade representam importante parcela das perdas produtivas do sistema de criação (Alves et al, 2007; Guilherme et al, 2017). Entre essas, a Linfadenite Caseosa (LC) causada por *Corynebacterium pseudotuberculosis* é uma enfermidade crônica e debilitante responsável por prejuízos econômicos na criação de caprinos e ovinos, principalmente devido a redução da produtividade, condenação de carcaças e

desvalorização da pele (Faccioli-Martins et al, 2014). Essa enfermidade possui potencial zoonótico, ocorrendo principalmente de forma esporádica e ocupacional (Motta et al, 2010).

A prevalência e incidência da doença nos pequenos ruminantes estão relacionadas a condições ambientais inadequadas, redução das defesas orgânicas dos animais e a ausência de programas sanitários de prevenção e controle (Alves et al, 2007). No Brasil, estima-se que a maioria dos rebanhos de ovinos e caprinos estejam infectados e que a prevalência clínica exceda 30% dos animais (Langenegger, 1991; Guimarães et al, 2011), sendo que em algumas regiões do Nordeste esse valor supera 50% (Alves et al, 1997).

Existem diversos testes de diagnóstico para a LC, sendo o cultivo microbiológico e isolamento do agente considerado o teste padrão ouro (Motta et al, 2010). Também existem as técnicas sorológicas, como o ELISA (*Enzyme Linked Immuno Sorbent Assay*), que apresenta como principal vantagem a identificação de animais acometidos com a forma subclínica da doença, a qual não é possível de ser diagnosticada pelo cultivo microbiológico. Além disso, esse teste se mostra muito útil nos estudos de prevalência dessa doença nos rebanhos e se apresenta como opção a ser utilizado em programas de controle (Faccioli-Martins et al, 2014; Oreiby, 2015).

Poucos levantamentos sorológicos têm sido realizados no Brasil, provavelmente devido às dificuldades na obtenção de insumos e a falta de uma infraestrutura necessária para realização das técnicas (Martins et al, 2010). Assim, são necessárias informações acerca da enfermidade nos pequenos ruminantes do Nordeste brasileiro para que seja possível o estabelecimento de um programa de controle eficaz, principalmente por meio da aplicação de métodos de diagnóstico que permitam avanços no conhecimento da epidemiologia da doença na região. A condução de estudos em locais com expressividade na comercialização de animais se faz necessária para avaliar possíveis rotas de transmissão, por meio do estrito contato entre animais e possíveis fontes de infecção nessas aglomerações. Considerando a importância e a necessidade de informações acerca da situação sanitária nas espécies ovina e caprina, o objetivo do estudo foi determinar os indicadores que caracterizam a situação epidemiológica da LC em pequenos ruminantes nos estados do Nordeste brasileiro e fatores associados à positividade da doença em estabelecimentos criadores e em locais de aglomeração dessas espécies na região.

A tese é composta por três Capítulos constituídos por artigos originais. O Capítulo I teve como objetivos determinar a frequência de animais e propriedades positivas para LC em ovinos em cinco estados da região Nordeste do Brasil e foi aceito para publicação pelo Periódico Tropical Animal Health and Production – Qualis B1. O segundo Capítulo

foi composto pelo artigo intitulado “Fatores associados com a soroprevalência da linfadenite caseosa em ovinos no nordeste brasileiro”, e será submetido ao periódico Transboundary and Emerging Diseases – Qualis B1. E o terceiro Capítulo foi constituído pelo artigo “Soroprevalência da linfadenite caseosa em caprinos comercializados em feira de animais no Semiárido nordestino”, publicado na Revista Semina: Ciências Agrárias – Qualis B1.

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## 1. CAPÍTULO I. Seroepidemiological study of caseous lymphadenitis in sheep from the Northeast region of Brazil using an indirect ELISA

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**Seroepidemiological study of Caseous Lymphadenitis in sheep from Northeast region of Brazil using an indirectly ELISA**

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**Abstract**

The objective of this study was to determine the frequency of seropositivity *Corynebacterium pseudotuberculosis* in sheep in five states of North-eastern Brazil, using an indirect enzyme-linked immunosorbent assay (i-ELISA). Young and adult sheep of both sexes were used. Blood samples were collected from 2,638 sheep from 223 herds across all states. For the i-ELISA, antigens produced from the strain of *C. pseudotuberculosis* BRM 029971, a bacterial isolate from the Northeast region of Brazil, were used. Sensitivity and specificity indexes were calculated for the validation of the test, using as reference 49 and 134 serum samples from sheep known to be positive and negative, respectively. The i-ELISA presented four false-negative and four false-positive results, showing a specificity of 97.01%, a sensitivity of 91.84%, and an accuracy of 95.63%. These results were calculated based on an Optical Density (OD) cut-off point = 0.138. Of the 2,638 sheep tested, 996 (37.76%, 95% CI = 35.93 - 39.62%) were seropositive, and of the 223 evaluated herds, 210 (94.17%, 95% CI = 90.28 - 96.56%) seropositive. The i-ELISA showed adequate sensitivity and specificity, proving to be a useful tool in the diagnosis of caseous lymphadenitis (CLA) in sheep. Infection by *C. pseudotuberculosis*, determined by serology, is disseminated in the sheep herds in the

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states of Northeast Brazil, with greater frequency in adults. Thus, there is a need to implement effective control measures that prevent the spread of infectious agents.

**Keywords**: Northeast Region. Caseous lymphadenitis. Enzyme-linked immunosorbent assay. Sheep. Serology.

## Introduction

Sheep production stands out as an important activity for the Northeast region of Brazil, where more than 9 million sheep are concentrated, accounting for 65.59% of the national population, present in more than 385,000 livestock establishments, which constitutes 75.38% of the breeding properties of the country (IBGE, 2019). However, high rates of morbidity and mortality, caused mostly by infectious diseases, affect sheep herds in the region and result in serious economic damage to the production chain, which constitutes a barrier to breeding activity (Alencar et al., 2010; Santos et al., 2011; Guilherme et al., 2017).

Among infectious diseases, we highlight CLA (Seyffert et al., 2010). The damage caused by this disease is related to the impairment of organic functions, such as decreased productivity, reproductive problems, mastitis, depreciation of the skin, condemnation of carcasses, and even death of the animals (Alves et al., 2007). In sheep, the occurrence of the visceral or subclinical form, with internal abscesses, has been more frequent when compared with goats, which most commonly present the clinical or superficial form of the disease (Guimarães *et al.* 2011a).

The importance of this disease is related to the economic impact caused in small ruminant rearing and is reinforced in Brazil by the increased representation of these species in the total national livestock herd (Seyffert *et al.* 2010; Carmo *et al.* 2012). In Brazil, it is estimated that many goats and sheep are infected, with prevalence levels of the clinical form exceeding 30% (Guimarães *et al.* 2011a). Thus, a comprehensive analysis of the disease in ovine breeding in the Brazilian regions is necessary.

Among the methods of diagnosing CLA, serological techniques are commonly used, highlighting ELISA, which provides adequate sensitivity and specificity, besides being very useful for studies of the prevalence of this disease in herds, and is presented as an option in control programs (Faccioli-Martins et al., 2014; Oreiby, 2015). However, few serological surveys have been performed in Brazil, probably due to the difficulties in

obtaining inputs (Martins *et al.* 2010) as well as there are no accredited laboratories to perform the serological diagnosis of the disease.

Thus, information about CLA in sheep herds of the Brazilian Northeast is necessary, through the application of effective diagnostic methods that allow knowledge of the epidemiological aspects of the disease in the region to establish an adequate control program. Therefore, the objective of this study were to determine the seropositive sheep in Brazilian states.

## Materials and Methods

### Study area

The study was conducted in the Northeast region of Brazil, which occupies the north-eastern position of the country between 1°00’ and 18°30' south latitude, and 34°20' and 48°30' west longitude from Greenwich. The region has an area of 1,561,177.8 km², equivalent to 18.3% of the Brazilian territory, covering a total of 1,793 municipalities, distributed by nine States (Bahia, Ceara, Pernambuco, Paraiba, Rio Grande do Norte, Piaui, Maranhao, Alagoas, and Sergipe) (SUDENE, 2018).

### Sampling

Samples of young (older than six months) and adult sheep of both sexes from rural properties in mesoregions with a significant population density of ovine herds were used from five Northeast states-Ceara, Rio Grande do Norte, Paraiba, Piaui, and Sergipe. The participation of the herd owners was voluntary. The inclusion of the herds was by probabilistic sampling, by randomly drawing previously listed herds, based on lists provided by breeder associations, state agricultural and cattle breeding agencies, agricultural secretaries, and the Brazilian Service to Support Micro and Small Enterprises.

The minimum number of herds to be visited was calculated using the formula for simple random sampling (Thrusfield, 2007), considering the following parameters: prevalence of herds with seropositive animals of 95.9% (Guimarães et al., 2009), a sampling error of 6%, and a confidence level of 95%. For these parameters, a sample of 42 properties per state would be necessary; however, the final sampling consisted of 50 properties in the state of Sergipe, 47 in Rio Grande do Norte, 46 in Ceara, and 57 in Piaui. In Paraiba, due to operational losses, 23 properties were sampled. Sampling was stratified according to the approximate composition of the herds, defined as 60% matrices, 35%

young (six- to twelve-months) and all rams. At each property, blood samples from 12 sheep were collected, or from all the animals on the property, if that number was less than or equal to 12.

Blood samples were collected from 2,638 sheep from 223 herds from 51 municipalities in the five Northeast states (Figure 1) from 2010 to 2012. After the samples were collected, they were submitted to the Laboratory of Bacteriology from the Brazilian Agricultural Research Corporation, Embrapa Goats and Sheep/Sobral/CE, where they were centrifuged, desorbed, separated, identified and maintained at -20° C in the institutional serum bank until serology was performed.

**Diagnostic method**

The CLA diagnostic method was an i-ELISA. The sensitivity and specificity indexes of the test were determined using as reference, 49 serum samples from animals known to be positive were used, in which the microbiological culture and the isolation and identification of the infectious agent; and 134 serum samples from sheep confirmed as negative for the disease originated from a disease-free property, located in the municipality of Bage, RS, Brazil. For the preparation of the positive and negative controls used in testing, two pools of positive and negative samples were prepared, respectively. The incubation buffer was used "blank sample". The cut-off point for the i-ELISA test was calculated according to Frey et al. (1998) with a confidence interval of 99.8%.

The i-ELISA for the detection of anti-*C. pseudotuberculosis* antibodies was performed according to the methodology described by Carminati (2005), using antigen produced from *C. pseudotuberculosis* strain 04/2014 (BRM 029971) isolated from the Northeast region of Brazil, belonging to the Collection of Pathogenic Microorganisms for Goats and Sheep from the Brazilian Agricultural Research Corporation (Embrapa), cultivated in brain-heart broth with 0.1% Tween 80 and purified by three-phase fractionation (TPP), according to Paule *et al.* (2004). The test was performed with some modifications developed by Embrapa Goats and Sheep.

Flat-bottomed polystyrene 96-well plates (Nunc Marxisorp® Thermo Fisher Scientific Waltham, Massachusetts, USA) were sensitized with 100 µL per well of antigen, diluted in 0.05M carbonate-bicarbonate buffer pH 9.6 to a final concentration of 0.5 µg/well and incubated at 4°C overnight. The plate was washed twice with buffer (PBS 0.05% Tween 20). The blockade was then performed with 100 µL/well of 2% casein solution in PBS, with subsequent incubation for 1 hour at 37°C. After washing, 100 µL

of the 1:50 diluted serum in PBS-T20 + 0.05% casein was added, and the plate was incubated for 1 hour at 37°C. Each serum and each control were tested in duplicate. After the washing sequence, 100 µL/well of rabbit total immunoglobulin anti-sheep immunoglobulin conjugate was added (Sigma-Aldrich®, A5420, Missouri, USA), labelled with peroxidase, diluted in 1:5,000 proportion and incubated for 45 minutes at 37°C. After washing, 100 µL/well of the developing solution (15 ml of citric acid solution, pH 5.0, 10 mg of orthophenylene diamine, 25 µL of 20% $H_2O_2$) was added. The plate was incubated for 15 minutes at room temperature and sheltered from light. The reaction was interrupted by adding 30 µl of $H_2SO_4$ 1:20. The reading was performed (Multiskan™ FC Microplate Photometer) using a 492 nm wavelength filter.

**Statistical analyses**

An association analysis was performed between animal categories (rams, ewes and lambs) and seropositivity by using the Chi-square test, as well as the odds ratio (OR) and respective confidence interval of 95% (CI 95%) were calculated (Thrusfield, 2007). The software SPSS 20.0 for Windows was used for analyses.

**Results**

The data obtained in the validation of the i-ELISA for the detection of anti-*C. pseudotuberculosis* antibodies are shown in Table 1. The test presented four false-negative results, demonstrating the specificity of 97.01%. Four false-positive results were observed, with a sensitivity of 91.84%. Positive predictive value and negative predictive value were 97.01% and 91.84%, respectively. The accuracy of the test was 95.63%. These results were calculated based on a cut-off point of OD = 0.138.

A total of 223 herds were evaluated, of which 210 (94.17%; 95% CI = 90.28 – 96.56%) had at least one animal with positive serology. The frequency of positive herds per state varied from 91.30% (21/23) in Paraiba to 95.65% (44/46) in Ceara Table 2.

From the 2,638 sheep evaluated, 996 (37.76%; CI 95% = 35.93 – 39.62%) were seropositive, and the highest frequence was observed in the state of Ceara (42.05%; 230/547), and the lowest in the state of Paraiba (30.96%; 87/281). From a total of 1,463 ewes tested, 715 (48.87%) presented seropositive results, highlighting the States of Ceara (52.30%; 159/304), Piaui (50.96%; 185/363), and Rio Grande do Norte (50.63%; 159/314), with the highest frequencies of seropositivity. A total of 303 rams were tested;

138 of these (45.54%) had positive results, with higher frequencies observed in the states of Ceara (57.97%; 40/69), Rio Grande do Norte (55.22%; 37/67), and Piaui (44.56%; 41/92). A total of 872 lambs were tested, and the frequency of observed seropositives was 143 (16.40%), where the lowest frequency of seropositivity was observed in the state of Sergipe (12.73%; 28/220).

There was association between the animal categories and the seropositivity ($P < 0.05$) in all states Table 3, with rams and ewes presenting significant odds of seropositivity compared to lambs, except for the Paraíba state, in which only ewes presented significant odds.

**Discussion**

The findings of the present study demonstrated the usefulness and efficacy of i-ELISA for the epidemiological diagnosis of CLA in an important area for small ruminant breeding. The method employed an antigen produced from a bacterial strain isolated of caseous abscesses collected from a sheep originated the geographical area studied. Diagnosis of *C. pseudotuberculosis* infection through the isolation and identification of the bacteria is considered the "gold standard" (Baird and Fontaine, 2007). However, diagnosis using polymerase chain reaction and serological techniques (Oreiby, 2015), especially ELISA, offers advantages in identifying infections in situations where animals do not present overt clinical symptomatology (Binns et al., 2007). Among other advantages are the testing time requirements, as well as the ease of antigen preparation and reduced resource requirements, which allow epidemiological studies and commercial applications (Nassar et al., 2014). Therefore, in addition to facilitating the diagnosis of CLA in herds, ELISA contributes to the control of subclinical infection situations, thus becoming a tool in the implementation of disease control programs.

Studies using the ELISA technique for the diagnosis of CLA in the Netherlands (Dercksen et al., 2000), Germany (Kaba et al., 2001; Sting et al., 2012), United Kingdom (Binns et al., 2007), and Canada (Menzies et al., 2004) obtained sensitivity variations between 71% and 97% and specificity between 96% and 100%, with different standardizations and antigens produced from bacterial proteins, exotoxins, interferon-γ, and recombinant phospholipase D. In Brazil, studies with ELISA tests which used proteins of *C. pseudotuberculosis* as antigens showed sensitivity variations ranging from 89% to 100% and specificity ranging from 98% to 100% (Carminati et al., 2003; Zerbinati

et al., 2007; Seyffert et al., 2010; Rebouças et al., 2013; Nassar et al., 2014). This variation was possibly due to the differences between the inputs used in the techniques and the standardization protocols. In this study, the ELISA test showed a sensitivity of 91.84%, a specificity of 97.01%, which are within the range of sensitivity and specificity results demonstrated by other studies, with indexes above 90%, allowing reliable use of this test for CLA diagnosis.

The cut-off point established for the i-ELISA (OD = 0.138) was calculated based on previously described methodology (Frey et al., 1998). The ROC curve was not applied because the applied methodology proves its employability in ELISA tests. Four animals with compatible clinical signs, proven positive through microbiological culture, and positive isolation of *C. pseudotuberculosis* presented a negative test result. This finding may represent false-negative animals due to problems in the sensitivity of the test, as well as the hypothesis that some animals do not produce antibodies to the specific antigen used (Binns et al., 2007) or have low production of anti-*C. pseudotuberculosis* antibodies, especially when the animal expresses cellular immune response against this agent (Rebouças et al., 2013). Four animals from a geographical area free of the disease presented positive results in the test. This finding may be related to cross-reactions due to infections by other bacteria such as other species of *Corynebacterium* sp., *Listeria monocytogenes*, or *Mycobacterium avium* subsp. *paratuberculosis* (Dercksen et al., 2000).

This research is noteworthy as a serological study for CLA diagnosis, with planned sampling in a specific geographic area of great importance to sheep farming in Brazil. The five states studied account for approximately 51.5% of the sheep herd in this region and 47.5% of sheep breeding operations (IBGE, 2019). Few serological surveys for *C. pseudotuberculosis* in small ruminants have been carried out in Brazil using the i-ELISA diagnostic method, which underscores the importance of such a study.

The large number of herds that had at least one positive animal (94.17%) indicates that the disease is widespread among the herds studied, corroborating with the high frequency of positive herds observed in other studies in the Northeast region. In goat breeding facilities, Farias *et al.* (2018) identified 88.5% positivity in five Northeast states and (Carmo et al., 2009) observed 82.7% in Ceara. In sheep farms from Bahia (Martins *et al.* 2010), a 56% positive frequency was identified. In Minas Gerais, the frequency of positive sheep farms ranged from 95.9% to 100% (Guimarães et al., 2011b) and Seyffert *et al.* (2010) observed 98% of goat herds were positive. It is possible that there is no

application of disease control measures in these herds or were neglected due to disinformation or difficulties in controlling CLA on the farms.

The variation of the frequency of positive properties between states was low considering that all states presented frequencies between 91.3% and 94.74% and the observed regional average was 94.17%. These data confirm the endemic character of the disease in Northeastern Brazil, with a high positivity rate for *C. pseudotuberculosis* in properties of the region. Studies suggest that variation in disease occurrence in different geographical areas may be related to factors such as breeding techniques, breed susceptibility (Aslan et al., 2016) and lack of knowledge about disease control (Duno et al., 2016 ), which may justify the results obtained in this study.

Regarding the animals tested, a serological study was performed using the i-ELISA for the diagnosis of CLA in goats in the same states that this research was conducted, with 30.4% of seropositivity being observed (Farias *et al.* 2018). In the present study, the significant difference in the frequency of seropositive sheep (37.76%) may be related to the characteristics of the disease in this species. Furthermore, the management and breeding system, combined with the gregarious characteristic of the sheep, can influence a higher occurrence of this disease in these animals..

Other studies performed in Ceara with goats (Carmo et al., 2009), and in Bahia and Pernambuco with sheep and goats (Martins et al., 2010, 2011; Alves et al., 2018) presented 26.2%, 27.54%, 54.98%, and 37.34% of seropositive animals, respectively. In Minas Gerais, the frequency of seropositivity in sheep was 70.9% (Guimarães et al., 2009) and 43.7% (Guimarães *et al.* 2011b), and 78.9% in goats (Seyffert et al., 2010). In the Distrito Federal, a prevalence of 44.0% was observed in sheep (Carmo *et al.* 2012). The difference between the results found in this and the other studies can be explained by the presence of environmental conditions favourable to the survival of *C. pseudotuberculosis*, as well as different characteristics in sheep breeding systems, which allow the spread of the disease in the animals. However, it is possible to affirm that the disease is widespread in small ruminants in several regions of the country, with a high frequency of seropositive animals.

The frequency of seropositive sheep varied between the states of Paraiba and Sergipe when compared with those observed in the states of Piaui, Ceara, and Rio Grande do Norte, with higher results from seropositive animals registered in the last three states, proving that the prevalence of this disease differs in different geographical areas. A study conducted in these states with goats showed a lower frequency of seropositive animals in

Sergipe, followed in ascending order by Ceara, Paraiba, Rio Grande do Norte, and Piaui (Farias *et al.* 2018), disagreeing with the results obtained in the present study with sheep. It is possible to suggest that differences between the breeding systems of goats and sheep, as well as the particularities of the disease, combined with the habits of each species could interfere in the chain of transmission of the disease for these species.

There was association between animal categories and the seropositivity, with adult animals (rams and ewes) presenting significant odds of seropositivity when compared to lambs, which is in agreement with other studies conducted in goats and sheep (Guimarães et al., 2009; Ribeiro et al., 2013; Duno et al., 2016; Farias et al., 2018). The chronic nature of the disease with a long incubation period may influence a higher frequency of positive adult animals (Seyffert et al., 2010). Because they remain in the herds longer, adult animals have greater contact with infection sources and thus have a greater chance of acquiring the disease (Machado et al., 2011; Souza et al., 2011). Thus, young lambs are at risk of infection due to contact with purulent material eliminated by infected animals in the breeding environment.

Ewes presented significant odds of seropositivity, which disagrees with some studies that observed no significant difference in the occurrence of LC between males and females (Guimarães *et al.* 2009; Seyffert *et al.* 2010; Farias *et al.* 2018). However, previous studies performed using agent isolation (Souza et al., 2011) and i-ELISA (Alves et al., 2018) showed a higher frequency of positive females than males. The longer residence time of the ewes in the herd provides a greater chance of these animals acquiring an infection, besides contributing to the maintenance and increased risk of transmission of the disease in the herd.

As found for ewes, rams also presented significant odds of seropositivity when comparted with lambs. This corroborates the results by Aslan et al. (2016), that found higher occurrence of the disease in rams than ewes, as well as seropositive rams have been described as a risk factor for CLA (Kaba et al., 2011). This fact may contribute to the spread of the disease among herds since the loan of these animals between owners from different farms is a common practice in the Northeast region. The importance of animals with the subclinical form of the disease, which eliminates the agent mainly by air, is highlighted, and because they do not present superficial abscesses; animals appear to be in a good state of health but may act as the source of infection for the herds. It is possible that CLA and other diseases are spread with this movement of rams between different properties without adequate sanitary control of these animals.

In this study, high frequencies of CLA-positive herds and animals were observed, suggesting the absence of effective control measures and prophylaxis to control the disease. The application of these measures varies according to the level of disease prevalence, and in the areas with high levels of infection, the implementation of strict sanitary control associated with vaccination is recommended (Guimarães *et al.* 2011a). Management practices to prevent the spread of the bacteria in the environment and herds are necessary for the efficiency of a control program (Faccioli-Martins et al., 2014).

In the future, the identification of positive animals through periodic clinical inspection and focusing on the detection of subclinical infections using serological tests such as ELISA other diagnostic tests such as intradermal (Alves and Olander, 1999), periodic herd vaccination, combined with an animal disposal program and drainage or treatment of abscesses with adequate disposal of the infectious material are possible tools for controlling the disease at regional breeding facilities. However, limitations such as the lack of adequate information from breeders and the absence of a specific control program for CLA have negatively influenced the implementation of preventive measures for avoiding the dissemination of disease (Farias *et al.* 2019; Guimarães *et al.* 2011b).

Currently, most of the breeders of small ruminants in the Brazilian Northeast identify CLA based on the presence of characteristic abscesses in the animals, being limited to the suggestive diagnosis of the disease. This observation underscores the need for the use of a confirmatory i-ELISA, which is quick, practical, and accessible to producers. In Brazil, there seems to be insufficient immunogen supply for routine vaccination for CLA in herds (Guimarães *et al.* 2011a), a limiting factor for the use of this practice. Thus, there is a need for public policies to encourage producers to obtain animals free from the disease, combined with a laboratory structure that allows access to disease diagnostics, as well as the production of vaccines and supplies with enough quantity and quality and at an affordable cost.

The i-ELISA used in this study showed adequate sensitivity and specificity, proving to be used in diagnosis of CLA in sheep, and it can be used to monitor the disease in sheep with high reliability.

Infection with *C. pseudotuberculosis*, determined by serology, is widespread in sheep herds of the states of Northeast Brazil. Attention is drawn to the need to implement effective control measures, such as the detection of animals testing positive for *C. pseudotuberculosis* using serological tests in herds and management practices that avoid the spread of the agent in the environment and the contamination of animals.

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**Statement of Animal Rights**

This project was submitted to the Comitê de Ética em Pesquisa from Centro de Saúde e Tecnologia Rural da Universidade Federal de Campina Grande and approved according to protocol number 012/2017.

**Conflict of Interest Statement**

The authors declare that they have no conflict of interest.

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**Table 1. Validation parameters found in the i-ELISA for antibody detection of anti-*Corynebacterium pseudotuberculosis* in sheep from Northeastern region of Brazil, 2018.**

| Parameter | Result |
|---|---|
| Number of sera tested | 183.00 |
| Number of true-positive sera | 45.00 |
| Number of true-negative sera | 130.00 |
| Number of false positive sera | 4.00 |
| Number of false negative sera | 4.00 |
| Cutoff Point (OD[a]) | 0.138 |
| Sensitivity (%) | 91.84 |
| Specificity (%) | 97.01 |
| Accuracy (%) | 95.63 |
| Positive predictive value | 91.84 |
| Negative predictive Value | 97.01 |

[a]OD = Optical Density

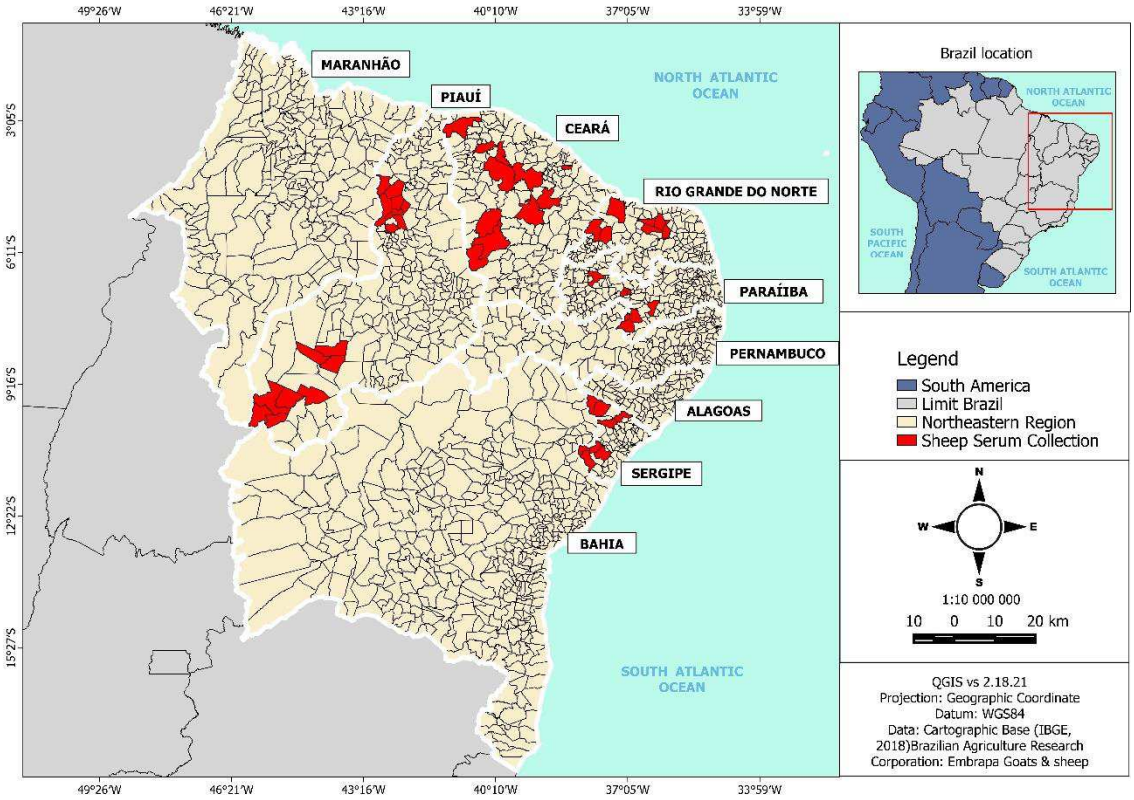
**Table 2. Frequency of ovine breeding herds tested for *Corynebacterium pseudotuberculosis* from the states of Paraiba, Rio Grande do Norte, Ceara, Piaui, and Sergipe, 2018**

| States | Total Nº | Positive (%) | CI 95% (%) |
|---|---|---|---|
| Paraiba | 23 | 21 (91.30) | 73.20 - 97.58 |
| Sergipe | 50 | 47 (94.00) | 83.78 - 97.94 |
| Rio Grande do Norte | 47 | 44 (93.62) | 82.84 - 97.81 |
| Ceara | 46 | 44 (95.65) | 85.47 - 98.80 |
| Piaui | 57 | 54 (94.74) | 85.63 - 98.19 |
| Total | 223 | 210 (94.17) | 90.28 - 96.56 |

**Table 3. Frequency of Sheep tested for *Corynebacterium pseudotuberculosis* by category, reared in the ovine breeding units of the states of Paraiba, Rio Grande do Norte, Ceara, Piaui, and Sergipe, 2018**

| State | Categories | Total number of animals | Number of positive animals (%) | Odds ratio (OR) | 95% CI for OR | P value |
|---|---|---|---|---|---|---|
| Paraíba | Rams | 22 | 6 (27.27) | 1.9 | 0.6 – 5.6 | 0.383 |
| | Ewes | 162 | 65 (40.12) | 3.4 | 1.8 – 6.3 | 0.0001 |
| | Lambs | 97 | 16 (16.49) | 1 | - | - |
| Sergipe | Rams | 53 | 14 (26.41) | 2.4 | 1.2 – 5.1 | 0.0234 |
| | Ewes | 320 | 147 (45.93) | 5.8 | 3.7 – 9.1 | 0.0001 |
| | Lambs | 220 | 28 (12.72) | 1 | - | - |
| Rio Grande do Norte | Rams | 67 | 37 (55.22) | 5.6 | 3 – 10.3 | 0.0001 |
| | Ewes | 314 | 159 (50.63) | 4.7 | 3 – 7.2 | 0.0001 |
| | Lambs | 183 | 33 (18.03) | 1 | - | - |
| Ceará | Rams | 69 | 40 (57.97) | 6.4 | 3.4 – 11.8 | 0.0001 |
| | Ewes | 304 | 159 (52.30) | 5 | 3.2 – 7.9 | 0.0001 |
| | Lambs | 174 | 31 (17.81) | 1 | - | - |
| Piauí | Rams | 92 | 41 (44.56) | 3.7 | 2.2 – 6.5 | 0.0001 |
| | Ewes | 363 | 185 (50.96) | 4.8 | 3.2 – 7.4 | 0.0001 |
| | Lambs | 198 | 35 (17.67) | 1 | - | - |
| Northeast | Rams | 303 | 138 (45.54) | 4.3 | 3.2 – 5.7 | 0.0001 |
| | Ewes | 1463 | 715 (48.87) | 4.9 | 4 - 6 | 0.0001 |
| | Lambs | 872 | 143 (16.40) | 1 | | |
| **Total** | **Animals** | **2,638** | **996 (37.76)** | **-** | **-** | **-** |

**Figure 1. Geographic distribution of the municipalities with ovine breeding herds tested for *Corynebacterium pseudotuberculosis* from the states of Paraiba, Rio Grande do Norte, Ceara, Piaui, and Sergipe, 2018**



## 2. CAPITULO II: Factors associated with the seroprevalence of caseous lymphadenitis among sheep in Northeast Brazil

Artigo submetido ao Periódico Transboundary and Emerging Diseases: Qualis B1.

# Factors associated with the seroprevalence of caseous lymphadenitis in sheep from Northeastern Brazil

**RUNNING HEAD:** Caseous lymphadenitis in Northeastern Brazil

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**ABSTRACT**

Caseous lymphadenitis (CL) is one of the main infectious diseases in sheep, responsible for great economic losses, mainly in Northeast region of Brazil, which has the largest sheep flock in the country. In addition, human cases occur mainly in people who have direct contact with infected animals. Thus, the aim of this survey was to determine the factors associated with the seroprevalence of this disease in sheep from Northeastern Brazil using a planned sample of flocks and animals. Samples were collected from 2,312 adult and young sheep of both sex from 196 farms, located in 51 municipalities in five Northeastern Brazilian states (Ceará, Paraíba, Piauí, Rio Grande do Norte, and Sergipe). Serological diagnosis was performed using the indirect ELISA technique. Factors associated with seropositivity were identified by variables extracted from epidemiological questionnaires administered to breeders, using univariate and multivariate analyses. Flock-level and animal-level prevalences were 93.88% (95% CI = 89.60 – 96.46%) 37.46% (95% CI = 35.51 – 39.45%), respectively. Factors associated with prevalence were purebred sheep (Prevalence ratio - PR = 1.189; $P$ = 0.017), ram acquisition from animal expositions (PR = 1.192; $P$ = 0.020), offspring is separated from ewes (PR = 1.132; $P$ = 0.048), water supplied to sheep from ponds (PR = 1.365; $P$ = 0.002), and elimination of infected animals (PR = 1.263; $P$ = 0.027). In view of the high prevalence found here and based on the associated factors, the implementation of an efficient CL control programme in the Northeastern Brazil is recommended.

**KEYWORDS**
*Corynebacterium pseudotuberculosis*, bacterial pathogens, indirect ELISA, herd-level and animal-level prevalences, Northeastern Brazil, epidemiology

## 1 INTRODUCTION

Sheep farming has grown in the Northeast region of Brazil, even amidst a scenario of drought and economic crisis in recent years. Contrary to the other regions of the country, there was an increase of 16.9%, from 7.7 million animals in 2006 (IBGE, 2009) to about 9.0 million in 2017, concentrating 75.38% of the raising farms and 65.59% of the national sheep flock (IBGE, 2019). In the Brazilian semiarid region, the farms raise sheep for meat production, mainly mixed or specialised breeds, such as Santa Inês, Morada Nova, and Somalis (Gonçalves Junior, 2012).

Despite being important, this activity presents low levels of performance, mainly due to the occurrence of infectious diseases (Alencar et al., 2010; Santos et al., 2011; Guilherme et al., 2017). In this context, caseous lymphadenitis (CL) due to *Corynebacterium pseudotuberculosis* is one of the major diseases affecting sheep flocks in this region (Alves et al., 2007; Faccioli-Martins et al., 2014). Factors that may contribute to high rates of disease occurrence are associated with the thorny vegetation typical of semiarid regions (Unanian et al., 1985; Guimarães et al., 2011a) and the predominantly extensive and semi-extensive farming systems, often without adequate sanitary control (Farias et al., 2019a, 2019b).

In countries where the disease occurs in sheep and goats, the implementation of control programmes aimed at reducing its prevalence and negative impacts related to economic losses have been suggested (Baird and Malone, 2010; Paton, 2010; Alloui et al., 2011; Oreiby et al., 2014; Aslan et al., 2016; Kichou et al., 2016; Windsor and Bush, 2016; Farias et al., 2018, 2019a). However, structuring the control and prevention programme requires knowledge of the disease and the possible factors associated with infection in order to implement measures to intercept the animal and environmental transmission cycle of the agent in the breeding systems.

Thus, a study to assess the factors that contribute to the occurrence of this sheep disease in the region with the highest concentration of this species in the country is necessary. Therefore, the aim of this study was to determine the factors associated with the seroprevalence of ovine caseous lymphadenitis in Northeastern Brazil.

## 2 MATERIAL AND METHODS

### 2.1 Ethics Statement

The project was reviewed and approved by the Research Ethics Committee (REC) of the Rural Health and Technology Centre of the Federal University of Campina Grande (CSTR/UFCG) under protocol number 012/2017.

### 2.2 Study area and sampling

The study was conducted in the Northeast region of Brazil, which is located between 1°00' and 18°30' south latitude and 34°20' and 48°30' west longitude and occupies 18.3% of the territory with an area of 1,561,177.8 km². It is composed by 1,793 municipalities distributed into nine states (Bahia, Ceará, Pernambuco, Paraíba, Rio Grande do Norte, Piauí, Maranhão, Alagoas, and Sergipe) (SUDENE, 2018).

The study included samples collected from adult and young sheep of both sex from farms located in mesoregions with significant sheep population densities in five Northeastern states: Ceará, Rio Grande do Norte, Paraíba, Piauí, and Sergipe. The owners' participation was voluntary. The farms were included by probabilistic sampling through a random drawing of previously listed farms ceded by the breeders' associations, State Agricultural Defence Agencies, Agriculture Departments, and the Brazilian Micro and Small Business Support Service (SEBRAE).

The minimum number of farms to be visited was calculated using the simple random sampling formula (Thrusfield, 2007), considering the following parameters: 95.9% prevalence of seropositive animals (Guimarães et al., 2009), sampling error of 6%, and 95% confidence level. These parameters required a sample of 42 farms per state; however, the final sample consisted of 43 farms in Piauí, 46 in Rio Grande do Norte, 50 in Sergipe, 34 in Ceará, and 23 in Paraíba. Although the number of visits in the latter two states was predicted, losses due to operational questions, incomplete questionnaires with less than 50% of the questions answered by the owners or specific unanswered questions made it impossible to include the total number of farms visited in the study.

The minimum number of animals to be examined in each flock was estimated to allow the classification as a positive farm, using the concept of aggregate sensitivity and specificity (Dohoo et al., 1997). The following values were used for the calculation: 89% and 99% (Rebouças et al., 2013) for sensitivity and specificity using indirect ELISA, respectively; and 70.9% (Guimarães et al., 2009) for the estimated prevalence per animal (intra-flock). The Herdacc software version 3 (Jordan, 1995) was used during this process, and a sample size was selected per flock based on a value of aggregate sensitivity and specificity ≥ 95%. Therefore, all animals were sampled in flocks with up to four sheep, and in flocks with five or more animals, at least four sheep were selected. The selection of animals within the flocks was systematic using sampling units at equal intervals, the first animal being randomly selected (Thrusfield, 2007).

Blood samples were collected from 2,312 animals from 196 farms located in 51 municipalities in five Northeastern states (Figure 1). After collection, the samples were sent to the Bacteriology Laboratory of Embrapa Goats and Sheep/CNPC-Sobral/CE, sera were obtained and stored at -20° C in the serum bank of this institution for subsequent serological testing.

**2.3 Serological diagnosis**

The i-ELISA for anti-*C. pseudotuberculosis* antibody detection was used as described by Carminati (2005), using an antigen produced from the *C. pseudotuberculosis* BRM 029971 sheep strain, belonging to the Collection of Pathogenic Microorganisms of Goats and Sheep of the Brazilian Agricultural Research Corporation (Embrapa), cultivated in brain-heart infusion with 0.1% Tween 80 and purified by three-phase fractionation (TPP), according to Paule et al. (2004). The ELISA was performed with modifications developed by Embrapa Goats and Sheep.

The 96-well flat-bottom polystyrene plate (NUNC®) was sensitised with 100 µL of antigen per well, diluted in 0.05 M carbonate bicarbonate buffer pH 9.6 to a final concentration of 0.5 µg/well and incubated overnight at 4ºC. After this step, the plate was washed twice with wash buffer (PBS 0.05% Tween 20). Then, it was blocked with 100 µL/well of 2% casein solution in PBS with subsequent incubation for one hour at 37°C. After the washing sequence, 100 µL of diluted serum in 1:50 PBS-T20 + casein 0.05% was added and the plate incubated for one hour at 37ºC. Each serum and control were tested in duplicate. After the washing sequence, 100 µL/well of the total donkey anti-sheep immunoglobulin conjugate (SIGMA® A3415) was added, marked with peroxidase, diluted in a proportion of 1:5,000 and incubated for 45 minutes at 37ºC. After the washing sequence, 100 µL/well of developing solution was added (15 ml of citric acid solution pH 5.0 + 10 mg ortho-phenylenediamine + 25 µL of $H_2O_2$ at 20%). The plate was incubated for 15 minutes at room temperature, protected from light. The reaction was interrupted by adding 30 µL of $H_2SO_4$ 1:20. Then, an ELISA reader (Multiskan™ FC Microplate Photometer) was used with a 492 nm wavelength filter.

**2.4 Epidemiological questionnaires**

A structured questionnaire with 46 closed-ended questions was designed to obtain information on (a) management practices; (b) technological profile of the farm; (c) health aspects; and (d) flock structure and composition. The questionnaires were administered to the owner or person in charge of the flock, by one of the researchers at the time of animal blood collection.

The following variables and their categories were evaluated: farm size (< 100 ha/ > 100 ha); presence of qualified labor (yes/no); presence of sheep pens (yes/no); presence of manure pits (yes/no); flock size (< 100 animals/ > 100 animals); consorted rearing of sheep/goats (yes/no); ewes are separated in maternity pens (yes/no); animals are

separated by sex (yes/no); animals are separated by age (yes/no); offspring is separated from ewes (yes/no); mortality at birth (low < 5%; high > 5%); mortality at weaning (low < 5%; high > 5%); semen acquisition for artificial insemination (yes/no); ram acquisition from animal expositions (yes/no); ram acquisition from neighbour's farms (yes/no); ram acquisition from animal fairs (yes/no); ram replacement from their own flock (yes/no); ewe replacement by acquiring semen for artificial insemination (yes/no); ewe acquisition from animal expositions (yes/no); ewe acquisition from neighbour's farms (yes/no); ewe acquisition from animal fairs (yes/no); ewe replacement from their own flock (yes/no); predominant breed (purebred/crossbred); occurrence of clinical CL in the flock (yes/no); occurrence of worm infection in the flock (yes/no); occurrence of contagious ecthyma in the flock (yes/no); occurrence of pododermatitis in the flock (yes/no); occurrence of gas gangrene in the flock (yes/no); occurrence of bronchopneumonia in the flock (yes/no); occurrence of diarrhoea in the flock (yes/no); flock vaccination (yes/no); worm control (yes/no); care for lambs at birth (yes/no); cuts umbilical cord and disinfects navel (yes/no); lamb suckle as soon as born (yes/no); lumps are scarified to control CL (yes/no); lumps are treated after bursting to control CL (yes/no); elimination of infected animals to control CL (yes/no); use of lime at the corral entrances (yes/no); cleaning of facilities (yes/no); use of quarantine or other procedure before introducing animals into the flock (yes/no); allows direct access to water sources (yes/no); water supplied to sheep from reservoirs (yes/no); water supplied to sheep from rivers (yes/no); water supplied to sheep from ponds (yes/no); water supplied to sheep from wells (yes/no).

**2.5 Analysis of factors associated with seroprevalence**

The analysis of factors associated with seroprevalence was conducted in two steps: univariable analysis and multivariable analysis. The variables were organised for presentation in ascending or descending order in relation to the seropositivity scale. When necessary, these variables were re-categorised. The lowest seropositivity category was considered the basis of comparison for the other categories. In the univariable analysis each independent variable was crossed with the dependent variable (animal serological profile – seropositive and seronegative). Later, the variables that presented a $P$-value $\leq$ 0.2 by the Chi-square test (Zar, 1999) were selected for the multivariable analysis using the robust Poisson regression model. Collinearity between independent variables was verified by the correlation analysis. In case of strong collinearity (correlation coefficient > 0.9), one of the two variables was excluded based on the biological plausibility (Dohoo

et al., 1997). To assess how well the model fits the Pearson Chi-square test was used, and the significance of the model was verified with Omnibus test. All analyses were performed with a significance level of 5% and using the SPSS 20.0 software for Windows®.

## 3 RESULTS

Of the 196 farms studied, 184 (93.88%; 95% CI = 89.60 – 96.46%) were positive for CL, and of a total of 2,312 animals sampled 866 (37.46%; 95% CI = 35.51 – 39.45%) were seropositive (Table 1). Flock-level prevalence per state range from 91.30% (Paraíba) to 93.35% (Piauí), however, there was no statistical difference ($P = 0.983$), and animal-level prevalence ranged from 30.86% (Paraíba) to 42.22% (Piauí) with statistical difference ($P < 0.001$).

In the univariable analysis the factors selected ($P \leq 0.2$) for the robust Poisson regression are presented in Table 2. The final robust Poisson regression model presented five factors associated with seropositivity: purebred sheep (Prevalence ratio - PR = 1.189; $P = 0.017$), ram acquisition from animal expositions (PR = 1.192; $P = 0.020$), offspring is separated from ewes (PR = 1.132; $P = 0.048$), water supplied to sheep from ponds (PR = 1.365; $P = 0.002$), and elimination of infected animals (PR = 1.263; $P = 0.027$) (Table 3). The model presented good fit (Pearson Chi-square: value = 1,441.964; degrees of freedom - df = 2,295; value/df = 0.628) and statistical significance (Omnibus test: likelihood ratio Chi-square = 34.17; df = 9; $P < 0.001$).

## 4 DISCUSSION

The methodology based on a planned sampled used in the present survey allowed the identification of a high flock-level prevalence (93.88%), showing that the disease is widespread in Northeastern Brazil, where high rates of seropositivity in sheep breeding farms have been found, ranging from 50.0% to 100.0% (Guimarães et al., 2011b; Carmo et al., 2012). These data suggest that CL control measures may have been neglected by producers.

The animal-level prevalence (37.46%) demonstrates the impact of the disease on sheep production in Northeastern Brazil. Serological surveys in sheep showed seropositivity ranging from 43.7% to 70.9% (Guimarães et al., 2009; Guimarães et al.,

2011b; Carmo et al., 2012) in Brazil. Worldwide, the occurrence of CL in small ruminants presents varied frequencies, 1.28% in the Paranaguá region, Venezuela (Duno et al., 2016); 6.7% in the Gharbia and Kafrelsheikh region, Egypt (Oreiby et al., 2006), 2014); 8.1% in Batna, Algeria (Alloui et al., 2011); 16.9% in Grenada (Hariharan et al., 2015); 21% in Canada (Arsenault et al., 2003); 1% to 29% in different regions and flocks in Australia (Paton et al., 2003); and 25% to 55.55% in different provinces in Turkey (Aslan et al., 2016).

Frequencies od CL found in surveys conducted in Brazilian slaughterhouses were 21.38% for goats (Barnabé et al., 2019) and 15.9% for sheep (Souza et al., 2011) in Paraíba, 43.7% for sheep in Minas Gerais (Guimarães et al., 2011b), and Rio Grande do Sul presented an estimated loss of 0.09% in condemned sheep carcasses, representing a loss equivalent to USD 24,004.24 (Machado et al., 2011). The disease was detected in sheep slaughterhouses with a prevalence of 32.65% in Egypt (Al-Gaabary et al., 2010), 16% in Morocco (Kichou et al., 2016), 13.24% in the Falkland Islands (Piotr et al., 2016), and between 7% and 11% in Argentina (Belchior et al., 2006). The disease was responsible for up to 11.9% of lung condemnations in sheep slaughterhouses in Spain (Vilallonga and Valcarcel, 2016). These differences are justified by the interference of factors such as management, breeding system, breed susceptibility and environmental conditions favourable to *C. pseudotuberculosis*. The global occurrence of the disease is evident, showing its importance in sheep farming and alerting to the economic impact it may be causing on flocks. More studies are recommended to quantify the damage caused by CL, showing the cost-benefit ratio of controlling the disease. These data may encourage farmers to use methods such as vaccination, diagnosis and other management practices that reduce disease prevalence.

No farms investigated used CL vaccination. In Brazil, there are immunogens available for disease prevention, however, they are not quite used in flocks (Faccioli-Martins et al., 2014). Recent studies identified some vaccines with proven efficacy (Syame et al., 2018), showing a tendency to use recombinant proteins as immunogens (Santana-Jorge et al., 2016; Leal et al., 2018; Silva et al., 2018; Barral et al., 2019) and reinforcing the possibility of producing more effective vaccines against the disease, which may be available to farmers in the near future. The sporadic use of CL vaccination seems to be a problem in countries where the disease exists. The use of this tool, combined with biosecurity practices, should be implemented by farmers to control the disease (Windsor

and Bush, 2016), however, further research on the cost-effectiveness of vaccination should be conducted to encourage farmers to use this control measure.

Purebred sheep had 0.189 times more likely to be seropositive, i.e., there was an 18.9% increase in prevalence compared to crossbred animals. A higher frequency of seropositivity (57%) was found in Santa Inês animals compared to crossbred animals (38%) (Ribeiro et al., 2013), and exotic purebred (61.1%) and national purebred (66.7%) compared to crossbred (48.5%) (Guimarães et al., 2009). These findings may be related to a greater use of intensive management system and greater participation of animals with superior breed standards in livestock events. Disease control measures should be intensified in purebred sheep, such as health certification proving the absence of CL in sheep with superior breed standards required during acquisition and prior to introducing the animal into the flock. The rusticity of locally adapted crossbred animals was suggested as a possible resistance factor for infectious diseases such as leptospirosis (Alves et al., 2017a; Costa et al., 2019) and lentiviruses in small ruminants (Pinheiro et al., 2001; Alves et al., 2017b), and may also justify the result of this study; however, further studies are necessary to prove this scenario.

Rams acquired from animal expositions were 0.192 times more likely to be seropositive, i.e., there was an 19.2% increase in prevalence. Similar result was found in sheep from the State of Minas Gerais (Guimarães et al., 2009). It is known that animals that were in contact with a large group of animals favour the transmission of diseases such as CL (Alves et al., 2018), leptospirosis, and lentiviruses for small ruminants (Alves et al., 2017a; 2017b), as well as other parasitic diseases (Limeira et al., 2018). Animal care during acquisition, such as clinical examination for CL, isolation and monitoring measures in sheep before introducing them into the flock may have been neglected by the producers. In this survey, 70.40% (138/196) of the owners refer they use no quarantine or any type of treatment in newly acquired sheep, which may facilitate the introduction of the disease into the flocks. Newly acquired animals should be kept isolated and tested for CL before introducing, using tests such as the ELISA, which has proven efficacy in research conducted in Brazil and other countries as Australia, Netherlands, Germany, UK and Canada (Dercksen et al, 2000; Kaba et al., 2001; Menzies et al., 2004; Binns et al., 2007; Paton, 2010; Sting et al., 2012; Rebouças et al., 2013). The access of producers to CL diagnosis is currently difficulty due to a reduced number of laboratories working with these techniques, and those that work are focused on scientific research. The implementation of laboratories in strategic locations and with adequate logistics are

essential conditions that must be met and directed to producers through incentives aimed at easier access to this tool.

In flocks where the offspring is separated from the ewes, animals presented 0.132 times more likely to be seropositive (13.2% increase in prevalence), however, there are recommendations to separate ewes from offspring as a measure to control the disease (Guimarães et al., 2011a). The frequency of seropositivity observed in young animals was 16.75% (129/770), reflecting a high level of infection in this category considering that this is a chronic disease (Alves et al., 2007; Paton, 2010; Faccioli-Martins et al., 2014). Separating the offspring from the ewes may stress the lambs (Barros et al., 2009; Peruzzi et al., 2015) and affect their immune system, increasing the animal's susceptibility to infections (Chartier and Paraud, 2012), which may justify the association found in this survey. Thus, some alternatives are recommended, such as continuous weaning and the use of differentiated feeding for lambs, as well as directing these animals to *C. pseudotuberculosis*-free areas, minimising the risk of early infection in lambs.

Animals that consumed water from ponds were 0.365 times more likely to be seropositive than animals that consumed water from other sources (36.5% increase in prevalence), highlighting the environmental importance on the disease transmission cycle. This was the main source of drinking water for sheep, present in 57.14% (112/196) of the farms. Pond water is accumulated rainfall, which generally drains organic matter from the environment, with the risk of carrying the etiological agent along with other environmental waste, contaminating the water reservoirs. *C. pseudotuberculosis* can survive for approximately 63 days in the soil, 78 days in wood, 93 days in wire, and 54 days in typical Caatinga thorns (Sá et al., 2018), proving this hypothesis. In general, the animals have direct access to these sites, which may increase the risk of water source contamination and the risk of infection for sheep. There is a possibility of zoonotic transmission of CL through contaminated water. CL cases in humans occurred mainly in people who had direct contact with infected animals (Peel et al., 1997). Drinking water was considered a risk factor for disease transmission in farms due to microbiological patterns above the potability limit (Amaral et al., 2003). Problems in accessing drinking water lead to public health impacts (Razzolini and Günther, 2008) and water scarcity in the semiarid of Northeastern Brazil favours non-potable water consumption. There are tools such as simplified filtration systems that improve the quality of drinking water in rural farms in the region (Pinto and Hermes, 2006). Some measures are recommended to decrease the risk of environmental contamination by *C. pseudotuberculosis*, such as to

isolate water sources by blocking the direct access of flocks to the ponds and the use of water treatment systems that reduce the risk of CL in animals and humans.

The elimination of infected animals was pointed out as a factor associated with seropositivity to disease (26.3% increase in prevalence). Eliminating animals with the clinical form of the disease may be contributing to the intra-flock transmission of CL during the period in which the infected animal remains in contact with the others before being discarded, as well as inadequate disposal through the commercialisation of these animals to other farms, which may justify this association. Early diagnosis of CL in flocks should be prioritised in order to reduce the risk of *C. pseudotuberculosis* infection, and serological techniques such as indirect ELISA (Baird and Malone, 2010; Faccioli-Martins et al., 2014; Oreiby, 2015; Farias et al., 2018) and intradermal tests (Alves and Olander, 1999) are recommended as they allow the diagnosis of the disease before the onset of clinical signs.

Immediate sacrificing or slaughtering of the infected animals in a refrigerated facility is recommended as a control measure in cases where the treatment is not effective and the clinical form of the disease is recurrent (Ribeiro et al., 2013; Faccioli-Martins et al., 2014; Farias et al., 2019a). Animals with clinical signs should be separated from the flock and treated for a period that can avoid environmental contamination by *C. pseudotuberculosis*, with subsequent disposal of the animal. In both recommendations, disposal should be done in such a way that the infected animal is not sent to other farms. These sheep should be slaughtered in establishments under sanitary inspection, which allow the proper destination of the meat considering specific standards (Brazil, 2017) and eliminating possible risks of transmission to consumers.

According to the data reported in this study, the implementation of an efficient CL control programme in the Northeastern Brazil would be possible through the use of strict sanitary control, vaccination and management practices that avoid environmental and flock contamination (Guimarães et al., 2011a; Faccioli-Martins et al., 2014). The identification of positive animals and the periodic vaccination of the flock, associated with a rational disposal programme of the affected animals and treatment of abscesses with appropriate disposal of infective material should be implemented. Information and improvement policies for producers regarding disease control and monitoring should be encouraged.

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**CONFLIT OF INTEREST STATEMENT**

The authors declare that they have no competing interests.

**DATA AVAILABILITY STATEMENT**

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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## Figure captions

**FIGURE 1** Geographical distribution of municipalities with sheep raising farms tested for caseous lymphadenitis in the states of Paraíba, Rio Grande do Norte, Ceará, Piauí, and Sergipe, Northeastern Brazil.

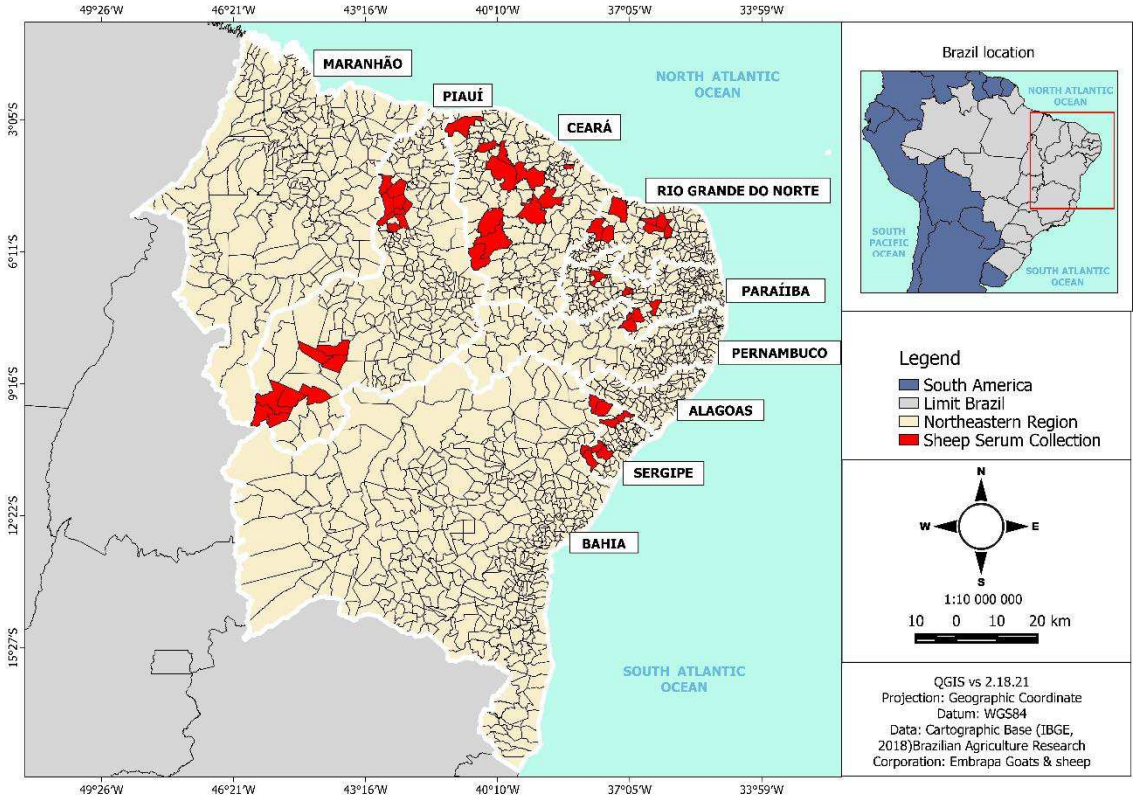

**TABLE 1** Flock-level and animal-level seroprevalences for caseous lymphadenitis in sheep from the Northeast region of Brazil.

| State | Flock-level prevalence | | | Animal-level prevalence | | |
|---|---|---|---|---|---|---|
| | No. of flocks | No. of positive flocks (%) | 95% CI (%) | No. of animals | No. of positive animals (%) | 95% CI |
| Ceará | 34 | 32 (94.12) | 80.91-98.37 | 403 | 158 (39.21) | 34.56-44.05 |
| Paraíba | 23 | 21 (91.30) | 73.20-97.58 | 269 | 83 (30.86) | 25.64-36.61 |
| Piauí | 43 | 41 (95.35) | 84.54-98.72 | 495 | 209 (42.22) | 37.95-46.62 |
| Rio Grande do Norte | 46 | 43 (93.48) | 82.50-97.76 | 552 | 227 (41.12) | 37.09-45.28 |
| Sergipe | 50 | 47 (94.00) | 83.78-97.94 | 593 | 189 (31.87) | 28.25-35.73 |
| Total | 196 | 184 (93.88) | 89.60 - 96.46 | 2,312 | 866 (37.46) | 35.51-39.45 |

**TABLE 2** Univariable analysis with the variables selected (P ≤ 0.2) for the robust Poisson regression.

| Variable/category | Total number of animals | No. of positive animals (%) | *P* |
|---|---|---|---|
| Farm size/<100 ha | 1,258 | 442 (35.13) | 0.000 |
| Presence of sheep pens/no | 621 | 257 (41.38) | 0.020 |
| Flock size/< 100 animals | 1,771 | 620 (35.00) | 0.000 |
| Consorted rearing of sheep and goats/yes | 1,330 | 522 (39.24) | 0.041 |
| Animals are separated by sex/yes | 345 | 155 (44.92) | 0.002 |
| Offspring is separated from ewes/yes | 1,568 | 609 (38.83) | 0.048 |
| Ram acquisition from animal expositions/yes | 409 | 196 (47.92) | 0.000 |
| Ram acquisition from neighbour's farms/no | 673 | 285 (42.34) | 0.002 |
| Predominant breed/crossbred | 1,763 | 691 (39.19) | 0.002 |
| Care for lambs at birth/no | 1,742 | 677 (38.86) | 0.015 |
| Lamb suckle as soon as born/yes | 786 | 337 (42.87) | 0.000 |
| Lump is treated as soon as it bursts/no | 1,923 | 739 (38.42) | 0.034 |
| Elimination of infected animals/yes | 131 | 66 (50.38) | 0.002 |
| Use of lime at the corral entrances/no | 1,124 | 452 (40.21) | 0.009 |
| Water supplied to sheep from ponds/yes | 171 | 79 (46.19) | 0.017 |

**TABLE 3** Factors associated with the seroprevalence of caseous lymphadenitis in sheep from the Northeast region of Brazil.

| Variable | Estimated coefficient | Standard error | Wald's Chi-square | Prevalence ratio (PR) | 95% confidence interval for PR | *P* |
|---|---|---|---|---|---|---|
| Purebred sheep | 0.173 | 0.0728 | 5.678 | 1.189 | 1.031; 1.372 | 0.017 |
| Ram acquisition from animal expositions | 0.176 | 0.0753 | 5.446 | 1.192 | 1.001; 1.279 | 0.020 |
| Offspring is separated from ewes | 0.124 | 0.0625 | 3.906 | 1.132 | 1.029; 1.382 | 0.048 |
| Water supplied to sheep from ponds | 0.311 | 0.1013 | 9.423 | 1.365 | 1.119; 1.665 | 0.002 |
| Elimination of infected animals | 0.233 | 0.1051 | 4.922 | 1.263 | 1.028; 1.551 | 0.027 |

Goodness of fit: Pearson Chi-square value = 1,441.964; degrees of freedom - df = 2,295; value/df = 0.628

## 3. CAPÍTULO III: Seroprevalence of caseous lymphadenitis in goats sold in an animal fair in the northeastern semi-arid region of Brazil

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**Seroprevalence of caseous lymphadenitis in goats sold in an animal fair in the northeastern semi-arid region of Brazil**

**Soroprevalência da linfadenite caseosa em caprinos comercializados em feira de animais no Semiárido nordestino**

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**Abstract:** Goat farming in the northeastern regions of Brazil plays an important role in the socio-economic functions of the country. However, high rates of morbidity and mortality occur in the animals, caused primarily by infectious diseases like caseous lymphadenitis, which is widespread in goat herds; this causes serious economic losses to the farming business. Although events such as animal fairs are common in the region, wherein most of the goats and sheep are sold, the risk of disease transmission remains, since the health condition of the animal is unknown. The aim of this study was to determine the frequency of anti-*Corynebacterium pseudotuberculosis* antibodies present in the goats sold at the animal fair of Tabira - PE, Northeastern semi-arid. Serum samples from 233 goats were collected from the period of November 2014 to June 2015. The diagnosis of infection by *C. pseudotuberculosis* was made using the indirect ELISA technique. The number of goats seropositive for *C. pseudotuberculosis* was identified as 87 (37.34%; 95% CI = 31.38 - 43.71%). Of the nine flocks evaluated, five (55.55%) were positive. Thus, it is suggested that infection by *C. pseudotuberculosis* is widespread in the goats sold at the animal fairs in the semi-arid Northeastern, reinforcing the need for disease diagnosis in goat herds in the region for the possible implementation of disease control programs.

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**Key words**: Agglomerations. Caseous lymphadenitis. Goats. Serology.

**Resumo**: A caprinocultura se destaca na região Nordeste do Brasil por desempenhar importante função socioeconômica. Contudo altas taxas de morbidade e mortalidade ocorrem nas propriedades causadas principalmente por doenças infecciosas, destacando-se a linfadenite caseosa, que se encontra amplamente difundida nos rebanhos caprinos, acarretando sérios prejuízos econômicos à atividade. Eventos do tipo feira de animais são comuns na região, onde é comercializada grande parte da produção de caprinos e ovinos, porém existe um risco de transmissão de doenças nesses locais quando a condição sanitária dos animais é desconhecida. O objetivo desse estudo foi determinar a frequência de anticorpos anti -*Corynebacterium pseudotuberculosis* em caprinos comercializados na feira de animais do município de Tabira-PE, Semiárido nordestino. Foram coletadas amostras de soro de 233 caprinos no período de novembro de 2014 a junho de 2015. O diagnóstico da infecção por *C. pseudotuberculosis* foi realizado pela técnica de ELISA-indireto. Foram identificados 87 (37,34%; IC 95% = 31,38 - 43,71%) caprinos soropositivos para *C. pseudotuberculosis*. Dos nove rebanhos avaliados, cinco (55,55%) resultaram positivos. Sugere-se que a infecção por *C. pseudotuberculosis* encontra-se disseminada nos caprinos comercializados em feiras de animais do Semiárido nordestino, reforçando a necessidade do diagnóstico da enfermidade em rebanhos caprinos da região para possível implementação de programas de controle da doença.

**Palavras-chave**: Aglomerações. Caprinos. Linfadenite Caseosa. Sorologia.

## Introduction

Goat farming is an extremely important activity of the northeastern region of Brazil, where 92.9% and 59.8% of the goat and sheep population, respectively (IBGE, 2016) are concentrated. Goat farming is estimated to be present in more than one million of the rural establishments of Brazil, playing an important socio-economic role (MOREIRA; GUIMARÃES FILHO, 2011). However, high rates of morbidity and mortality occur in the caprine producing system of the region, caused mainly by the infectious diseases. This in turn results in serious economic losses to the productive chain, thus hindering the activity (PINHEIRO et al., 2000; MEDEIROS et al., 2005; ALENCAR et al., 2010).

Among these infectious diseases, we highlighted caseous lymphadenitis (CL), caused by *Corynebacterium pseudotuberculosis*, which is recognized by the formation of

abscesses in the superficial or internal lymph nodes and organs (FACCIOLI-MARTINS et al., 2014). The damages caused by the disease are related to the impairment of organic functions, drop in productivity, reproductive problems, mastitis, skin depreciation, carcass condemnation, and even animal death. In Brazil, it is estimated that most of the goats and sheep are infected, mainly in the northeast region of Brazil, which contains majority of the national goat and sheep herds (ALVES et al., 2007).

There are several serological tests available for the diagnosis, such as ELISA (*enzyme-linked immunosorbent assay*), which has adequate sensitivity and specificity, and is very useful in studies of CL prevalence in herds, presenting as a diagnostic option in the disease control programs (FACCIOLI-MARTINS et al., 2014). Nonetheless, only a few serological surveys have been carried out in Brazil so far, probably due to the difficulties in obtaining supplies and the lack of necessary infrastructure to carry out the study (MARTINS et al., 2010).

The commercialization of the small ruminants in trade fairs is one of the main strategies of production distribution in the northeastern region of Brazil (NOGUEIRA FILHO et al., 2010). There are places where a substantial part of the goat and sheep production commerce occurs, by the farmers themselves or by the intermediary merchants, which involves acquiring the animals in the region's breeding farms and reselling them in these places. Animal fairs occur frequently, contributing to the growth and economic development of the interior cities of the Northeast (MAIA, 2007). Nevertheless, these agglomerations represent a serious problem related to the transmission of the infectious diseases to the herds due to the stringent contact and movement of the infected animals, vectors, and fomites (THRUSFIELD, 2007).

Thus, information about CL epidemiology and the knowledge of disease transmission dynamics in the goat herds of the northeastern region is required, especially in the segments of higher risk, such as in animal agglomerations. Hence, the objective of this study was to determine the frequency of anti-*C. pseudotuberculosis* antibodies generated in the goats sold at the Tabira-PE animal fair of the northeastern semi-arid region, using the indirect ELISA technique.

**Material and Methods**

The study was conducted at the animal fair of the municipality of Tabira (latitude 07°35'31"S and longitude 37°32'24"W), located in the Sertão do Pajeú, State of Pernambuco. The fair takes place biweekly, on Tuesdays and Wednesdays, receiving animals from Pernambuco and other states of the northeast. Cattle and small ruminants

are the main animals sold, and due to the significant amount of sales, it is considered an important commercial center of the region.

The studied population comprised adult goats (> 6 months of age), of both genders, and without any defined racial pattern. To calculate the number of animals sampled, the following parameters were considered: (a). expected prevalence of 50% (used in order to maximize the sample); (b). absolute error of 7%; and (c). 95% confidence interval, according to the formula for simple random samples. According to these parameters, although the minimum sample "n" was 196 animals, 233 were used. The owners who participated in the survey were chosen by the convenience sampling method (THRUSFIELD, 2007).

The number of samples per collection was based on the total number of goats that participated in the fair during this period, which summed up to 16,434. These data were obtained from the report on the entry of animals, through the Animal Transit Guides (GTAs) presented by the owners. The data were provided by the Agricultural Protection and Inspection Agency of the State of Pernambuco (ADAGRO). Nine owners accepted to participate in the research, signing the respective Informed Consent Form. The animals were selected randomly from the facilities where the herd commercialization was practiced. The collections were made from November 2014 to June 2015. Blood was collected from 233 goats. Of these, 206 originated from the State of Pernambuco and 27 from the State of Bahia.

The serological test used for the detection of anti-*C. pseudotuberculosis* antibodies was the indirect ELISA, according to the methodology described by Carminati (2005), using antigen produced from the sheep-strain in brain-heart broth with 0.1% Tween 80 and purified by three-phase partitioning (TPP), according to Paule et al. (2004). ELISA was carried out with the modifications developed by the Brazilian Agricultural Research Corporation (Embrapa Goats and Sheep, Sobral - CE).

The 96-well flat bottom polystyrene plate (NUNC®) was sensitized with 100 µl of antigen per well, diluted in 0.05 M carbonate-bicarbonate buffer (pH 9.6) to a final concentration of 1.25 ng/µl and incubated at 4° C overnight. Following this, the plate was washed twice with wash buffer (PBS with 0.05% Tween 20). The blocking step was then performed with 100 µl of 2% casein solution (in PBS) per well, with subsequent incubation for 1 hour at 37° C. After the washing step, 100 µl of the 1:100 diluted serum (in PBS-T20 + 0.05% casein) was added, and the plate was incubated for 1 hour at 37° C. For the positive and negative controls, goats' sera known to be positive (confirmed by microbiological examination) and negative (disease-free herds) for *C.*

*pseudotuberculosis,* respectively were used. A blank containing only the incubation buffer was used. Each of the serum and control was tested in duplicate. After the washing step, 100 µl/well of rabbit total immunoglobulin goat anti-immunoglobulin conjugate (SIGMA® A5420), marked with peroxidases and diluted in the ratio of 1: 10,000 was added and incubated for 45 minutes at 37° C. After the washing step, 100 µl /well of developing solution (15 ml of citric acid solution at pH 5.0 + 3 mg of orthophenylenediamine + 3 µl of 30% hydrogen peroxide) was added. The plate was incubated for 15 minutes at room temperature, in the dark. The reaction was stopped by adding 30 µl of 5% sulfuric acid. The ELISA reader (Multiskan™ FC Microplate Photometer) was then run at 492-nm wavelength filter. This test had a sensitivity of 78.57% and specificity of 98.75%.

The project was approved by the Research Ethics Committee (CEP) of the Rural Health and Technology Center (CSTR/UFCG) under the protocol number 098/2016.

**Results**

A total of 233 goat sera were examined for the presence of antibodies against *C. pseudotuberculosis* secreted antigens by the indirect ELISA method. Of the sera tested, 87 samples (37.34%, 95% CI = 31.38 - 43.71%) revealed a positive result. Of a total of 73 samples of male goats, 12 (16.44%) were seropositive and of the 160 samples of female goats tested, 75 (46.88%) presented positive serology (Table 1). Five of the nine studied properties (5/9, 55.55%) were positive, presenting a proportion higher than 30% of the seropositive samples per property (Table 2).

**Discussion**

The diagnosis of *C. pseudotuberculosis* infection can be performed mainly through bacterial isolation and identification, serological techniques, and polymerase chain reaction (OREIBY, 2015). The isolation and identification of the agent in caseous material drained from the abscesses or organs is considered as the gold standard for the diagnosis of CL (BAIRD; FONTAINE, 2007). Nevertheless, some infected animals may not show the clinical signs such as abscess formation, which may be a limiting factor in performing the technique. Hence, serological techniques that have adequate sensitivity and specificity are indicated for the disease diagnosis, since they have the advantage of identifying the infection in situations where the animals present compatible clinical symptomatology (BINNS et al., 2007).

Among the serological techniques for CL diagnosis, ELISA is an important

technique. According to Faccioli-Martins et al. (2014), various improvements have made it possible for the use of this assay in disease-control programs. The advantages are cost-benefit, ease of application, and acceptable sensitivity and specificity (OREIBY, 2015). Nassar et al. (2014) reported other advantages like shorter test time, ease of antigen preparation, and the use of small quantity of inputs, make the use of this test possible for disease diagnosis in the epidemiological studies and for commercial purposes. Thus, in addition to facilitating CL diagnosis in herds, ELISA contributes to disease control in situations where clinical symptoms are not evident, in turn becoming an important emerging tool to be used in the implementation of disease-control programs in Brazil. In this study, the use of this test for the diagnosis of CL allowed the epidemiological diagnosis of the disease at the site of agglomeration and commercialization of small ruminants, proving that the use of this technique as a diagnostic tool in these situations is useful and effective.

The use of ELISA for the diagnosis of CL in Netherlands (DERCKSEN et al., 2000), Germany (KABA et al., 2001; STING et al., 2012), and Canada (MENZIES et al., 2004) demonstrated sensitivity and specificity variations between 81% - 97% and 96% - 99%, respectively, due to the different standardizations of antigens produced from bacterial cells, exotoxins, and interferon-γ. In Brazil, Carminati et al. (2003) developed and standardized an indirect ELISA using the antigens obtained from culture supernatant of *C. pseudotuberculosis*, with sensitivity and specificity of 93.5% and 100%, respectively. Zerbinati et al. (2007) standardized an indirect ELISA with sensitivity and specificity of 99.8% and 98.0%, respectively, using the purified bacterial protein antigen. The indirect ELISA test used in this study, using a TPP-derived antigen of secreted proteins, had a better efficiency as compared to the other tests such as the sandwich ELISA. Additionally, the indirect ELISA using antigens not obtained by TPP, as described by Carminati (2005), showed a greater reliability in the use of this technique for disease diagnosis.

Few serological surveys for *C. pseudotuberculosis* in small ruminants have been conducted in Brazil using indirect ELISA. In Minas Gerais, Guimarães et al. (2009) observed seropositivity of 70.9% in sheep. Later, seropositivity of 78.9% in goats (SEYFFERT et al., 2010) and 43.7% in sheep was found (GUIMARÃES et al., 2011a). In the State of Ceará, Carmo et al. (2009) found seropositivity of 26.2% in goats. In the Federal District, Carmo et al. (2012) observed a prevalence of 44.0% in sheep. In Bahia, studies by Martins et al. (2010) showed 27.54% of seropositivity in sheep. In Pernambuco, Martins et al. (2011) observed 54.98% of seropositive sheep. Despite the

significant differences between the results found in the above-mentioned studies as well as the other studies, it is noted that the disease is indeed widespread in the country, with a high frequency of seropositive animals.

The seropositive properties observed in this study (55.55%) are similar to the results obtained by Carmo et al. (2012) in the Federal District and by Martins et al. (2010) in Bahia, who observed a seropositivity of 50% and 56%, respectively. Nevertheless, it was lower than the result obtained by Carmo et al. (2009), who found that seropositivity was 82.7% in Ceará and 95.9% to 100% in Minas Gerais (GUIMARÃES et al., 2009; SEYFFERT et al., 2010). However, the results from the study reveal that a high percentage of positive properties with a high number of seropositive goats are found in each property studied.

Studies using the isolation agent were performed in Paraíba by Andrade et al. (2012), which evaluated goats and sheep in the semi-arid region of Brazil, where they observed 7.7% of the animals with clinical signs of CL and isolated the agent in 68.2% of the samples. Souza et al. (2011) evaluated sheep slaughtered in a municipality in the Cariri region of Paraíba, originated in Bahia, Pernambuco, and Paraíba; the macroscopic lesions like CL were identified in 15.9% of the inspected animals and *C. pseudotuberculosis* was isolated in 74.5% of the cultured samples. These results reinforce the need for more comprehensive serological studies, making it possible for the development of control measures for this disease in goats and sheep in the region.

In this study, the frequency of seropositivity in females (46.88%) was significantly higher than that found in males (16.44%). In another study, Souza et al. (2011) demonstrated a higher frequency of agent isolation in the females (17.9%) than in males (13.8%). The authors attributed this fact to the greater slaughter age of females due to their long stay in the farms as compared to that of the males. Thus, the former would have greater chances of acquiring the infection, in addition, the infected ewes could contribute to the maintenance and heightened the risk of disease transmission in the herd.

The infected animals, with or without clinical symptoms, are considered the main source of infection and can contaminate soil, water, food, pastures, and facilities, through the nasal secretions, feces, and pus from the contaminated abscesses (GUIMARÃES et al., 2011b). The introduction of sick animals into the herds facilitates the disease transmission. An important control measure by the breeders would be to avoid the acquisition of these animals. It is common practice to buy goats at animal fairs in the northeast, where the owners acquire them, and later introduce them to the farm herds (NOGUEIRA FILHO et al., 2010). In this case, the commercialization of infected animals

in such events can act as a facilitator of disease spread. Therefore, control measures as a requirement for health certification or absence of clinical signs of the disease at the entrance of small ruminants at events should be implemented.

One aspect that should be considered is the breeding system practiced by the animal traders. They acquire species such as goats, sheep, and cattle from the region's owners through purchase and keep them confined in small spaces until the time of sale. This breeding system presents peculiarities such as intensive breeding, in contrast to the extensive and semi-intensive systems that are generally practiced by most of the owners of the Brazilian Northeast (GUILHERME et al., 2017). It should be emphasized that intensive breeding is a determinant condition for high CL prevalence due to the confinement of animals in a small space, particularly when there is inadequate control of the disease in such spaces (SOUZA et al., 2011). Therefore, this study suggested that the confinement condition of intensive breeding may favor CL transmission among herd animals.

Studies have cited that animal traffic is an important risk factor for disease dissemination (BIGRAS-POULIN et al., 2007; CAPANEMA et al., 2012). This raises the possibility of CL transmission through movement and close contact between the infection sources, susceptible vectors, and interferences at the agglomeration site, as well as the traffic between region’s goat and sheep breeding farms to the animal fairs. Alves et al. (2017) emphasized the epidemiological importance of the Tabira - PE animal fair of the northeastern semi-arid region, since the animals traded in that place originate mainly in the states such as Bahia, Pernambuco, and Paraíba and are moved to several municipalities of these and other states of the northeast region. In this way, this event can act as a facilitator of spread of diseases such as CL, since infected animals can be bought by owners of several regions, becoming disseminators of this disease after being introduced to the healthy herds.

## Conclusion

The *C. pseudotuberculosis* infection is widespread in goats sold at the animal fairs. This study highlights the importance of indirect ELISA in disease diagnosis in goat herds, mainly due to the rapidity and ease of the technique, which allows the implementation of this method in disease-control programs in the region's breeding farms, animal transportation inspection sites, and in animal agglomerations.

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**Table 1.** Frequency of goats tested for *C. pseudotuberculosis* by gender, sold at the animal fairs in the northeastern semi-arid region between November 2014 and June 2015

| | Goats | | |
|---|---|---|---|
| | **Males** | **Females** | **Total** |
| Positives | 12 (16.4) | 75 (46.88) | **87 (37.34)** |
| Negatives | 61 (83.56) | 85 (53.12) | **146 (62.66)** |
| **Total** | **73 (100.0)** | **160 (100.0)** | **233 (100.0)** |

**Table 2.** Frequency of municipalities and source properties of goats tested for *C. pseudotuberculosis*, sold at an animal fair in the municipality of Tabira - PE in the northeastern semi-arid region between November 2014 and June 2015.

| Municipality/Origin | Tested Properties | Positives | Tested Animals | Positives (%) |
|---|---|---|---|---|
| Casa Nova - BA | 1 | 1 | 25 | 11 (44.00) |
| Remanso - BA | 1 | - | 2 | - |
| Tabira - PE | 2 | 1 | 63 | 26 (41.26) |
| Calumbi - PE | 1 | - | 2 | - |
| Sertânia - PE | 1 | - | 6 | - |
| São José do Egito - PE | 1 | 1 | 55 | 18 (32.72) |
| Brejinho - PE | 1 | 1 | 30 | 15 (50.00) |
| Santa Terezinha - PE | 1 | 1 | 50 | 17 (30.91) |
| **Total** | **9** | **5** | **233** | **87 (37.34)** |

## CONCLUSÃO GERAL

De acordo com os resultados observados nos estudos conduzidos para composição dessa Tese conclui-se que:

O teste de ELISA-indireto utilizado nesse estudo apresentou sensibilidade e especificidade de 91,84% e 97,01%, com elevada confiabilidade, ressaltando a possibilidade de utilização da técnica para diagnóstico da LC em rebanhos de ovinos e caprinos, bem como para embasamento na certificação para o controle de trânsito e para as aglomerações de pequenos ruminantes.

A infecção por *C. pseudotuberculosis*, determinada por sorologia, encontra-se amplamente disseminada nas propriedades criadoras de ovinos e em rebanhos caprinos comercializados em feiras de animais da região Nordeste. Ressalta-se a necessidade de implementação de medidas de controle eficazes, com a detecção de animais positivos por meio da utilização de testes sorológicos e práticas de manejo que evitem a disseminação do agente no ambiente e contaminação dos animais.

Com base nos fatores associados à soropositividade para *C. pseudotuberculosis* em ovinos, recomenda-se a implementação de medidas de controle em animais de raças puras e que participam de eventos com aglomerações animais, minimizar a contaminação com material infectante no ambiente de criação, reduzir o estresse do manejo de desmama dos cordeiros, descartar adequadamente animais infectados, isolar e monitorar animais recém adquiridos, em conjunto com políticas de incentivo da utilização de técnicas de diagnóstico precoce da doença e da vacinação periódica dos rebanhos.