Brazilian Journal of Microbiology (2021) 52:431–438
https://doi.org/10.1007/s42770-020-00403-0

VETERINARY MICROBIOLOGY - RESEARCH PAPER

Bacteriological, cytological, and molecular investigation of *Corynebacterium pseudotuberculosis*, mycobacteria, and other bacteria in caseous lymphadenitis and healthy lymph nodes of slaughtered sheep

Thiago de Oliveira Zamprogna[1] · Dayana Ribeiro[2] · Vasco A.C. Azevedo[2] · Gustavo Henrique Batista Lara[1] · Rodrigo Garcia Motta[1] · Rodrigo Costa da Silva[3] · Amanda Keller Siqueira[1] · Geraldo de Nardi Júnior[4] · Fernando José Paganini Listoni[1] · Lorrayne de Souza Araújo Martins[1] · Aristeu Vieira da Silva[5] · Fábio Vinícius Ramos Portilho[1] · André da Rocha Mota[1] · Carolina Aparecida Rodrigues[1] · Beatriz Oliveira de Almeida[1] · Márcio Garcia Ribeiro[1]

Received: 26 February 2020 / Accepted: 4 November 2020 / Published online: 13 November 2020

Abstract

Caseous lymphadenitis (CL) in sheep is a chronic contagious disease caused by *Corynebacterium pseudotuberculosis*, commonly characterized by abscess formation in peripheral lymph nodes and disseminated infections. Nonetheless, other microorganisms, including with zoonotic relevance, can be isolated from CL-resembling lymph nodes. Currently, mycobacteria have been reported in visceral granulomatous lesions in small ruminants, a fact that poses a public health issue, particularly in slaughtered sheep intended for human consumption. Cytology using fine needle aspiration and microbiological culturing are suitable tests for routine diagnostic, whereas present drawbacks and molecular methods have been confirmatory. Data about the occurrence of mycobacteria in both lymph nodes with aspect of CL and apparently healthy visceral nodes of sheep slaughtered for human consumption are scarce. In this study, 197 visceral lymph nodes of sheep showed lymphadenitis and 202 healthy visceral lymph nodes of slaughtered sheep intended for human consumption were submitted to conventional bacteriological diagnosis, mycobacteria culturing, and cytological evaluation. Compatible *Corynebacterium* isolates were subjected to multiplex PCR targeting 16S rRNA, *rpo*B, and *pld* genes to detect *C. pseudotuberculosis*. Based on microbiological identification, *C. pseudotuberculosis* (86/197; 43.7%), streptococci γ-hemolytic (17/197; 8.6%), and *Trueperella pyogenes* (12/197; 6.1%) were prevalent in lymph nodes with abscesses, as opposed to staphylococci (53/202; 26.2%) in apparently healthy lymph nodes. No mycobacteria were isolated. Cytology identified 49.2% (97/197) Gram-positive pleomorphic organisms (coryneform aspect). Multiplex PCR confirmed genetic material of *C. pseudotuberculosis* in 74.4% (64/86) of the samples with *C. pseudotuberculosis* isolation and 66% (64/97) samples with cytological coryneform aspect (κ = 86.78%; 95% CI = 79.87–93.68%). These findings emphasize the prevalence of *C. pseudotuberculosis* in abscess formation among peripheral lymph nodes of sheep. Other bacteria were also identified in lymph nodes sampled that resembling *C. pseudotuberculosis*–induced infections that may difficult the diagnosis. Multiplex PCR revealed a valuable assay to detect *C. pseudotuberculosis*, in addition to routine methods applied to CL-diagnosis. No mycobacteria were identified in lymph nodes sampled, with and without apparent lesions. Nonetheless, due to

Responsible Editor: Miliane Moreira Soares de Souza

✉ Márcio Garcia Ribeiro
marcio.ribeiro@unesp.br

[1] Department of Animal Production and Preventive Veterinary Medicine, School of Veterinary Medicine and Animal Science, São Paulo State University (UNESP), Botucatu, SP, Brazil

[2] Laboratory of Cellular and Molecular Genetics, Institute of Biologic Sciences, Federal University of Minas Gerais (UFMG), Belo Horizonte, MG, Brazil

[3] School of Agrarian Sciences, University of Western São Paulo (UNOESTE), Presidente Prudente, SP, Brazil

[4] Technology Faculty, FATEC, Botucatu, SP, Brazil

[5] Zoonosis and Public Health Research Group, Department of Biologial Sciences, Feira de Santana State University (UEFS), Feira de Santana, BA, Brazil

public health impacts, this pathogen should be considered as a differential diagnosis of *C. pseudotuberculosis*–induced infections during inspection procedures of slaughtered sheep intended for human consumption.

Keywords Ovine lymphadenitis · *Corynebacterium* spp. · Multiplex PCR · Slaughterhouses

Introduction

Corynebacterium pseudotuberculosis is a well-recognized intracellular pleomorphic Gram-positive bacterium; opportunistic in nature; and able to infect humans, livestock, companion animals, and wildlife. Biovar *Ovis* infects sheep and goats, whereas biovar *Equi* infects cattle, horses, camels, and buffaloes [1–3]. This pathogen belongs to the CMNR group (*Corynebacterium*, *Mycobacterium*, *Nocardia*, and *Rhodococcus*), which is a heterogeneous group of pathogenic bacteria characterized by diverse pyo- or granulomatous clinical infections that affect animals and humans [4].

Corynebacterium pseudotuberculosis is a primary agent of caseous lymphadenitis (CL) in both sheep and goats, a chronic pyogranulomatous disease characterized by abscess formation in peripheral lymph nodes (extern form) and visceral organs (intern form), although the external form is most common in goats, whereas the internal form is more frequently found in sheep [5]. The primary route of entry of the pathogen is represented by skin trauma after an injury caused by shearing, tail docking, tagging, castration, or other environmental hazards that may result in cutaneous trauma. The pathogen is also transmitted through ingestion, direct contact with aerosol, and may infect mucous membranes and wounds. *C. pseudotuberculosis* survives in the environment for several months, likely contributing to its spread and persistence within farms or flocks [1, 5].

The major virulent factors of the organism are represented by phospholipase D and a lipid component of the bacterial structure. Phospholipase D, an exotoxin, encoded by the *pld* gene, possibly enhances the spread of bacteria by increasing vascular permeability as well as damaging endothelial cells, whereas the lipid component of the bacterial wall protects against hydrolytic enzymes of phagocytic cells, enabling intracellular permanence of the pathogen and development of chronic pyogranulomatous reactions [1–3].

Submandibular, parotid, prescapular, and prefemoral nodes are the main lymph nodes affected, although supramammary, inguinal, and other nodes may be affected as well [1, 5–7]. The microorganism may spread by hematogenous route from a primary site of the infection and develop organ abscesses, the "thin ewe syndrome," because of progressive weight loss [1, 5]. Purulent nasal secretion, cough, fever, chronic weight loss, and failure to thrive are the main clinical signs of internal *C. pseudotuberculosis* infections, which show poor prognosis or outcome. This internal form of the disease poses a diagnostic challenge because initial *C. pseudotuberculosis* organ infections require imaging (radiography or ultrasonography) or transtracheal lavage for early diagnosis, and possibly, diseased animals are neglected or unnoticed on endemic farms. Nonetheless, these animals represent a source of infection to susceptible hosts through *C. pseudotuberculosis* shedding, mainly by nasal secretions [5].

Although *C. pseudotuberculosis* is a primary cause of CL [1, 7–9], a group of pyogenic microorganisms, including of zoonotic impact, have also been isolated from sheep with abscess formation on peripheral lymph nodes, e.g., staphylococci (*Staphylococcus aureus*), *Trueperella pyogenes*, *Pasteurella multocida*, and streptococci [5, 9]. These organisms are opportunistic in nature, inhabit the skin and mucous membranes, and are transmitted mainly by traumatic skin inoculation or cutaneous wounds, causing lymph nodes aspect that resembles *C. pseudotuberculosis*–induced infections [5, 10], reinforcing the importance of proper diagnosis of the agents that cause lymph node abnormalities in sheep or flocks.

Caseous lymphadenitis remains a source of significant economic losses in many countries, including reduced yields of meat, milk, and wool, carcass, and skin condemnation; early disposal or culling of infected animals; and death caused by systemic spread of bacteria [1, 7, 8].

In the last decade, mycobacterial infections have been reported as a cause of granulomatous (caseous) pulmonary and lymphatic clinical disorders in small ruminants around the world [11, 12], including in Brazil [13]. The genus *Mycobacterium* includes a set of species with human and animal relevance classified into three groups, i.e., (i) obligatory pathogens of humans and animals (e.g., *M. tuberculosis*, *Mycobacterium bovis*, *Mycobacterium africanum*, and *Mycobacterium caprae*, the so-called *Mycobacterium tuberculosis* complex), (ii) potentially pathogenic microorganisms to animals and humans (e.g., *Mycobacterium avium* subsp. *avium*, *Mycobacterium avium* subsp. *paratuberculosis*, *Mycobacterium fortuitum*, and *Mycobacterium scrofulaceum*), and (iii) saprophytic or ubiquitous species (e.g., *Mycobacterium gordonae*, *Mycobacterium terrae*, *Mycobacterium smegmatis*, and *Mycobacterium phlei*). Mycobacteria cause pulmonary and extrapulmonary human infections, especially among people living with HIV/AIDS [14].

In humans, there is a marked increase of *C. pseudotuberculosis*–induced infections. The pathogen has been reported causing lymphadenitis and abscesses in people [6, 15]. Likewise, some species of staphylococci, streptococci, actinomycetes, and enterobacteria [10], which have been

isolated from lymph nodes with and without lesions in sheep may be pathogenic to humans. In addition, *M. caprae* isolated from livestock have showed able to infect humans as well [16]. The presence of pathogens with zoonotic relevance, able to infect lymph nodes and organs of sheep, represent a public health issue due to risks of direct transmission from sheep-to-humans, particularly among animals intended for human consumption.

Traditionally, routine diagnosis of CL in small ruminants has focused on clinical signs, bacteriological cultures, serological approaches, and postmortem examination [5, 9], although serological approaches show drawbacks, particularly to orient individual animals for culling programs due to the inability to distinguish between early exposed and sick animals [5, 17]. In the last decades, different molecular methods (conventional, real-time, and multiplex PCR, sequencing, DNA-DNA hybridization, matrix-assisted laser desorption-ionization time-off flight mass spectrometry-MALDI-TOF MS) have been used to confirm conventional diagnosis methods, in addition to investigate the virulence profile, genetic diversity, and geographical distribution of *C. pseudotuberculosis* strains worldwide [1, 2, 7, 8].

In Brazil, clinical manifestations, bacteriological culture, and cytology are apparently the main procedures used in the routine diagnosis of CL among herds/flocks of small ruminants [18, 19], although molecular diagnosis has been used to confirm *C. pseudotuberculosis* in comprehensive studies [1, 6, 17]. Nevertheless, a few studies in Brazil have focused on the postmortem prevalence of *C. pseudotuberculosis*, mycobacteria, and other agents able to infect lymph nodes, with and without lesions, from livestock slaughtered intended for human consumption [20–22], particularly among small ruminants [23, 24].

In this scenario, we investigated bacterial culturing, cytological, and molecular identification of *C. pseudotuberculosis*, mycobacteria, and other opportunistic bacteria in lymph nodes compatible with CL and healthy lymph nodes of slaughtered sheep intended for human consumption.

Methods

Sample size

The sample size was calculated using the Open-Epi site based on the following parameters: an estimated population of 500,000 sheep in the State of Sao Paulo, Brazil; a prevalence of caseous lymphadenitis of about 70% in herds; a 95% confidence level; and a 6% absolute error. Thus, a minimum sample of 188 sheep with peripheral lymph nodes compatible with CL was estimated to compare the results of microbiological diagnosis, cytology, and multiplex PCR. Since there is no study in Brazil regarding the identification of *C. pseudotuberculosis* and mycobacteria in apparently healthy lymph nodes from slaughtered sheep, a convenience sampling, using similar minimum number described above to CL was also sampled from apparently healthy lymph nodes.

Animals, farms, and slaughterhouses

A cross-sectional study was performed where the animals were subdivided into two groups, i.e., G1 and G2. G1 consisted of 197 sheep with enlarged peripheral lymph nodes, compatible with clinical CL, while G2 consisted of 202 healthy visceral lymph nodes collected in a slaughterhouse in São Paulo State, Brazil, sampled in 2013. The animals slaughtered were up to 100 days old and had a weight of approximately 30 kg. They came from 14 farms located in São Paulo State, the southeastern region of Brazil, from small (< 50 animals) to medium (50–300 animals) scale farms, presenting similar conditions in terms of general management, health measures, facilities, and feed (pasture). The animals were randomly enrolled in the study regarding age, breed, and gender. Just one lymph node was sampled from each animal from both studied groups. Except for enlargement of peripheral lymph nodes, animals from G1 and G2 had no signs of other diseases.

Cytology and microbiological culturing of specimens

Caseous lymphadenitis

Enlarged peripheral lymph nodes (submandibular, parotid, prefemoral, popliteal, prescapular lymph nodes) were subjected to aseptic fine needle aspiration (FNA) in duplicate, and samples were collected using a Valeri™ (MPJ Equipamentos Médicos Ltda., Brazil) cytoaspirator with hypodermic needles (30 × 7 mm) and individual disposable syringes (10 mL) [19]. All specimens were transported to the laboratory on ice (4–8 °C). One sample of each sheep was submitted for cytological examination by Gram and Diff-Quik staining. The other sample was subjected to microbiological culturing on 5% sheep blood agar (Oxoid™, Hampshire, UK) and MacConkey agar (Oxoid™, Hampshire, UK), and incubated aerobically at 37 °C for 72 h.

Healthy lymph nodes

Healthy lymph nodes (mediastinal, pre-hepatic, and mesenteric lymph nodes) were aseptically collected at the slaughterhouse during meat inspections by a Brazilian official veterinarian. All lymph nodes sampled were collected using individual sterile gloves, immediately placed in individual and hermetically sealed commercial plastic Zip lock® bags (CBC Embalagens Plásticas Ltda, São Paulo, Brazil), transported to the laboratory on ice (4–8 °C), and stored frozen at − 20 °C. In

the laboratory, the surface of each lymph node was subjected to antisepsis (5% iodine plus 70% alcohol). Subsequently, the lymph nodes were seared by a red hot spatula, and with support of sterile scissors and tweezers, a deep fragment of each intact lymph node was collected for bacterial cultivation [20–22] and cytological examination as described above.

C. pseudotuberculosis and other bacterial isolates

All samples from CL and apparent healthy lymph nodes were subjected to culturing in defibrinated sheep blood agar (5.0%) and MacConkey agar (Oxoid, Hampshire, UK), and incubated at 37 °C under aerobic conditions for 72 h [10]. Small, white-to-cream and dry colonies, surrounded by a hemolytic zone of *C. pseudotuberculosis*–resembling cells, where pleomorphic Gram-positive rods were seen (Gram staining), were further tested for biochemical properties (e.g., catalase, urease, and glucose fermentation) and synergistic hemolysis (CAMP test) with a standard strain of *Rhodococcus equi* (ATCC 33.701+) [1]. Colonies suspected of enterobacteria in MacConkey agar were submitted to biochemical tests (motility, lysine decarboxylase, urease, phenylalanine deaminase, indole, glucose, H_2S, citrate production/reduction). Other microorganisms isolated from sheep blood agar were identified based on Gram staining, type of hemolysis, catalase, and other conventional phenotypic tests [10]. Lymph nodes infection was defined as the presence of at least 20 colony-forming units (exuberant isolation).

Mycobacteria identification

For mycobacteria isolation, healthy lymph node fragments and peripheral lymph node contents were subjected to Petroff decontamination [10, 20], with some modifications. They were subsequently cultured on Löwenstein-Jensen (Difco™, Becton, Dickinson and Company, France) and Stonebrink-Lesslie media (in-house), and incubated aerobically at 25 and 37 °C, respectively, for 90 days. Suggestive colonies of mycobacteria were stained using the Ziehl-Neelsen technique, classified by conventional methods [10] and subjected to further molecular analyses [21, 22].

Molecular detection of *C. pseudotuberculosis*

The molecular identification of *C. pseudotuberculosis* and *C. pseudotuberculosis*–like isolates was based on multiplex polymerase chain reaction as described previously [17]. This multiplex assay detected 16S rRNA, *rpo*B (RNA polymerase ribosomal β-subunit), and *pld* (phospholipase D exotoxin) genes and used the following primers: 16S-F (5′-ACCG CACTTTAGTGTGTGTG-3′), 16S-R (5′-CTCT ACGCCGATCTTGTAT-3′), C2700F (5′-CGTA TGAACATCGGCCAGGT-3′), C3130R (5′-TCCA TTTCGCCGAAGCGCTG-3′), PLD-F (5′-ATAA GCGTAAGCAGGGAGCA-3′), and PLD-R2 (5′-ATCA GCGGTGATTGTCTTCCAGG-3′) [8, 17, 25]. The *C. pseudotuberculosis* standard strain 1002 was used as positive control.

Data analysis

The relationship between the microbiological and cytological results was analyzed by the agreement using the kappa (κ) test, as well as the standard deviation and the 95% confidence interval (95% CI). Descriptive statistics were used to report the frequency of isolation of different microorganisms and its relation to the cytological results. As multiplex PCR was assayed to confirm *C. pseudotuberculosis* isolation by the phenotypic tests, the percentage of success was reported. All analyses were performed using BioEstat 5.0 [26].

Results

Positive bacterial isolation was observed in 75.1% ($n = 148/197$) of enlarged peripheral lymph node samples compatible with CL. *Corynebacterium pseudotuberculosis* (86/197 = 43.7%), *Streptococcus* γ-hemolytic (17/197 = 8.6%), and *Trueperella pyogenes* (12/197 = 6.1%) were the prevalent microorganisms in microbiological culturing and phenotypic identification. Considering the enlarged peripheral lymph nodes (G1), 49.2% (97/197) revealed Gram-positive pleomorphic organisms (coryneform aspect). In the same way, 6.1% (12/197) of the samples showed coryneform organisms in cytology, but resulted in *Trueperella pyogenes* isolation (Table 1).

Staphylococci and streptococci were prevalent in mono and mixed coinfections among 202 healthy visceral lymph nodes sampled from slaughtered sheep (G2). In contrast, no mycobacteria and *C. pseudotuberculosis* isolates were observed from G2 (Table 1).

Cytology identified 49.2% (97/197) Gram-positive pleomorphic organisms (coryneform aspect).

Multiplex PCR confirmed *C. pseudotuberculosis* in 74.4% (64/86) of enlarged peripheral lymph node samples with phenotypic identification of the microorganisms in the microbiological culture. In contrast, 66% (64/97) of the samples of caseous lymphadenitis with the presence of Gram-positive pleomorphic organisms (coryneform aspect) in cytology were positive to *C. pseudotuberculosis* detected by multiplex PCR (Table 2). These results showed agreement $\kappa = 86.78\% \pm 3.52\%$ (95%), CI 79.87–93.68%.

Table 1 Mono- and co-occurrence of microorganisms in caseous lymphadenitis (enlarged lymph nodes) and healthy lymph nodes of slaughtered sheep

Microorganisms	Caseous lymphadenitis Number of isolates (%)	Healthy apparent visceral lymph nodes Number of isolates (%)
Corynebacterium pseudotuberculosis	86/197 (43.7)	--
Streptococcus γ-hemolytic	17/197 (8.6)	7/202 (3.5)
Staphylococcus γ-hemolytic	12/197 (6.1)	53/202 (26.2)
Trueperella pyogenes	12/197 (6.1)	--
Staphylococcus β-hemolytic	6/197 (3.0)	--
Enterobacter spp.	3/197 (1.5)	--
Streptococcus β-hemolytic	3/197 (1.5)	7/202 (3.5)
Escherichia coli	3/197 (1.5)	4/202 (2.0)
Bacillus spp.	3/197 (1.5)	3/202 (1.5)
Streptococcus α-hemolytic	2/197 (1.0)	3/202 (1.5)
Hafnia alvei	1/197 (0.5)	--
Aspergillus spp.	1/197 (0.5)	--
Staphylococcus γ-hemolytic + *Streptococcus* β-hemolytic	--	13/202 (6.4)
Staphylococcus γ-hemolytic + *Streptococcus* γ-hemolytic	--	10/202 (4.9)
Staphylococcus γ-hemolytic + *Streptococcus* α-hemolytic	--	4/202 (2.0)
Streptococcus α-hemolytic + *Streptococcus* β-hemolytic	--	2/202 (1.0)

% , percentage

Discussion

In the current study, multiplex PCR confirmed a 74.4% *C. pseudotuberculosis* in the abscess formation in peripheral lymph nodes of sheep with phenotypic identification of the microorganisms in the microbiological culture. In addition, other bacteria (staphylococci, streptococci, enterobacteria, and other actinomycetes) were identified in CL samples resembling the clinical aspect of the disease, reinforcing laboratory diagnosis as an important tool to control the disease. In addition to conventional methods applied to the routine laboratory diagnosis of CL (e.g., aerobic bacterial culture, cytology), multiplex PCR was a valuable assay to confirm *C. pseudotuberculosis* etiology. Apart from no identification of mycobacteria in lymph nodes sampled, with and without lesions, due to public health relevance, this pathogen should be considered as a differential diagnosis of *C. pseudotuberculosis*–induced infections during inspection procedures of slaughtered sheep intended for human consumption.

Despite current reports of mycobacterial infections in small ruminants worldwide, including in Brazil, data on the isolation of mycobacteria from lymphadenitis and healthy lymph node content are scarce. Most studies involving the diagnosis

Table 2 Microbiological, cytological, and multiplex PCR diagnosis of *C. pseudotuberculosis* from enlarged peripheral lymph nodes from animals with clinical signs of caseous lymphadenitis and from healthy visceral lymph nodes of slaughtered sheep

Test	Results	Caseous lymphadenitis	Healthy visceral lymph nodes
Cytology	+*	97	0
	−	100	202
	Total	197	202
Microbiological	+	86	0
	−	111	202
	Total	197	202
Multiplex PCR‡	+	64	0
	−	22	0
	Total	86	0

+, positive; −, negative

*Gram-positive pleomorphic organisms

‡ 16S rRNA, *rpo*B, and *pld* genes (confirmation test for positive results for cytological and microbiological tests)

of *C. pseudotuberculosis*–induced infections in small ruminants have focused on peripheral caseous lymphadenitis [2, 7, 8] or lymph nodes with a tuberculosis-like aspect by the inspection service in slaughterhouses [12, 13]; conversely, few studies have investigated apparently healthy visceral lymph nodes from sheep intended for human consumption. In addition, mycobacterial infections have not been routinely investigated as a differential diagnosis to *C. pseudotuberculosis* infection, which was one of the most important motivations of the present study.

Several bacterial species are able to infect lymph nodes of small ruminants, although *C. pseudotuberculosis* has been described as the primary pathogen of CL in small ruminants worldwide [1, 2, 7, 25, 27, 28]. In the same way, the present study identified, phenotypically, 43.7% (86/197) *C. pseudotuberculosis* strains from enlarged lymph nodes of sheep, which reinforces the predominance of this pathogen as the causative agent of CL in this species.

Staphylococci, streptococci, and some enterobacteria species were also identified among both caseous lymphadenitis and healthy visceral lymph node samples. These pathogens are opportunistic in nature and may be found in soil, feces, water, and farm tools (e.g., enterobacteria); they are also commensal inhabitants of the skin and conjunctive and oral mucosae (e.g., staphylococci and streptococci) of livestock [9, 10]. This finding reinforces the need for differential diagnosis of *C. pseudotuberculosis*–induced infections and other agents that may infect lymph nodes of sheep.

The genus *Mycobacterium* contains several species classified into three groups of relevance for animal and human health, namely, (i) obligate animal and human pathogens; (ii) potentially pathogenic mycobacteria to animals and humans, "nontuberculous" or opportunistic mycobacteria; and (iii) ubiquitous, saprophytic, or environmental mycobacteria [14]. Obligate pathogens comprehend the *Mycobacterium tuberculosis* complex (e.g., *Mycobacterium tuberculosis*, *M. bovis*, *M. africanum*, *M. caprae*), whereas the potentially pathogenic mycobateria group comprehends the *M. avium-intracellulare* complex (i.e., *M. avium* subsp. *avium*, *M. avium* subsp. *paratuberculosis*), *M. fortuitum*, *M. scrofulaceum*, and others; while the saprophytic mycobacteria group includes *M. gordonae*, *M. terrae*, *M. smegmatis*, and others [14, 29].

Mycobacterium tuberculosis and *M. bovis* are the main causative agents of tuberculosis in humans and cattle, respectively, a well-known life-threatening disease worldwide that causes significant economic losses and public health concerns [12, 14]. Small ruminants are susceptible to *M. bovis* and *M. caprae*, which cause granulomatous/caseous lesions mainly in pulmonary and lymphatic tracts. Nevertheless, mycobacterial infections in sheep are usually neglected, unnoticed, or restricted to case reports [11]. In addition, individual cases are observed during routine postmortem inspection at the slaughterhouse [12], including in Brazil [13].

No mycobacteria isolation was observed in this study. This finding emphasizes the low impact of mycobacterial infections to the etiology of CL or even in healthy visceral lymph nodes from slaughtered sheep. Nevertheless, herein, lymph node sampling was restricted to just one geographical region of São Paulo State, Brazil, a fact that limited the considerations of the present study.

Routine diagnosis of caseous lymphadenitis in small ruminants has been based on clinical aspects, bacteriological culture, as well as serological and cytological examinations of lymph node samples [1, 5, 9, 19]. In the current study, 43.7% of enlarged peripheral lymph nodes were positive in the phenotypic identification of *C. pseudotuberculosis*, although 49.2% showed coryneform aspect (Gram-positive pleomorphic organisms) in cytology. In addition, 6.1% (12/197) *T. pyogenes* also showed coryneform aspect. This limitation of the cytology was expected due to the similar morphotintorial aspect between *C. pseudotuberculosis* and *T. pyogenes* [10, 19]. In the same way, the biochemical tests used to identify *C. pseudotuberculosis* produces variable results, i.e., nitrite reduction, glucose and lactose fermentation, motility, urease, and gelatin liquefaction [1, 3, 25], allowing distinct biochemical profiles [18]. These findings require molecular methods to confirm the diagnosis of *C. pseudotuberculosis* infections [2, 8, 17, 18, 25, 30].

At present, molecular methods, e.g., PCR, genome sequencing, and MALDI-TOF MS enable definitive diagnosis, along with geographical distribution studies, virulence profile, and genetic diversity investigation of *Corynebacterium* species from domestic animals worldwide, including *C. pseudotuberculosis* strains [2, 7, 8, 31–34]. In the coming years, large-scale sequencing has seen used to investigate the microbiota of lymph nodes from humans [35] and animals (pigs) [36], with the aim to diagnose and differentiate agents/diseases; however, this next generation sequencing method has not yet been applied in the investigation of lymph nodes from sheep with and without lesions compatible with CL.

On a global level, studies have used distinct molecular approaches of PCR in the diagnosis of *C. pseudotuberculosis* in CL. In Turkey, 55.1% (81/147) of *C. pseudotuberculosis* were isolated in enlarged mediastinal and prescapular lymph nodes from carcasses of slaughtered sheep, whereas relative PCR (*pld* gene) sensitivity and specificity to culturing were 98.8 and 92.4%, respectively [37]. In another study in Turkey, PCR targeting the 16S rRNA gene detected *C. pseudotuberculosis* in 96.9% (93/96) of lymph node samples, with microbiological isolation of the microorganism [25]. This study identified 86 *C. pseudotuberculosis* isolates based on aerobic bacteriological culturing and phenotypic procedures, and 74.4% (64/86)

isolates were confirmed by multiplex PCR. These discrepancies between microbiological identification and multiplex PCR among CL cases may be attributed to the discriminatory power of *rpo*B and *pld* genes, able to differentiate *C. pseudotuberculosis* from other *Corynebacterium* species, e.g., *Corynebacterium ulcerans* [1, 8, 25, 31]. *Corynebacterium pseudotuberculosis* and *C. ulcerans* show similar morphotintorial aspects, as well as biochemical and phenotypic properties, including a positive CAMP test with standard *Rhodococcus equi* strains [31]. Herein, amplification of multiple loci in a single PCR reaction is a tool for the rapid and valuable speciation of bacterial pathogens, since our multiplex PCR targets the genes 16rRNA and *rpoB*, which has been used for taxonomic and phylogenetic studies, and the *pld* gene that encodes PLD exotoxin related to the virulence of *C. pseudotuberculosis* [17].

No speciation by MALDI-TOF MS or other automatic system diagnosis method of non-*C. pseudotuberculosis* isolates, a convenient sampling of apparently healthy lymph nodes (because of lack of studies with healthy lymph nodes of slaughtered sheep in Brazil), and no investigation of pathogens from healthy peripheral lymph nodes may be considered limitations of the current study.

The intracellular nature of *C. pseudotuberculosis* in phagocytic cells, the environmental resistance of the organism, and the nonresponsive behavior of the pathogen to conventional antimicrobials represent factors that possibly contribute to the chronic persistence of the disease in herds and flocks, requiring the adoption of rigorous biosecurity measures to prevent and control the disease [5]. Thus, culling of diseased animals, disinfection of shearing equipment and other instruments, removal of hazards in the environment that may potentially cause skin injuries, periodic clinical and laboratory examination of suspected animals, quarantine of new animals prior to introduction on the farms, vaccination, and/or establishment of a veterinarian-owner-animal relationship are mandatory to prevent and control LC in the herds [5, 10].

Overall, a 74.4% *C. pseudotuberculosis* was detected by a multiplex PCR in peripheral lymph nodes of sheep compatible with CL where the microorganism was also identified by bacteriological methods. Also, other bacteria were identified in CL-resembling nodes, which may limit the routine diagnosis using conventional methods. The multiplex PCR revealed a valuable and rapid molecular assay to confirm *C. pseudotuberculosis* as a primary agent of the disease. No mycobacteria were recovered from lymph nodes sampled, with and without lesions. Nonetheless, due to public health impacts, this pathogen should be considered as a differential diagnosis of *C. pseudotuberculosis*–induced infections during inspection procedures of slaughtered sheep intended for human consumption.

Acknowledgments The authors thank the National Council for Scientific and Technological Development (CNPq), Brazil, for research productivity fellowships (PQ-1D) given to Márcio Garcia Ribeiro.

Funding The authors thank the São Paulo Research Foundation (FAPESP) for the financial support (#2011/03120-1) and

Compliance with ethical standards

This study was carried out in accordance with the guidelines for the ethical use of animals approved by the Ethic Committee on Animal Use (CEUA) of the School of Veterinary Medicine and Animal Sciences, São Paulo State University, UNESP, Botucatu, SP, Brazil (protocol number 062/19).

Conflict of the interest The authors declare that they have no conflict of the interest.

References

1. Dorella FA, Pacheco LG, Oliveira SC, Miyoshi A, Azevedo V (2006) *Corynebacterium pseudotuberculosis*: microbiology, biochemical properties, pathogenesis and molecular studies of virulence. Vet Res 37:201–218
2. Dorneles EM, Santana JA, Ribeiro D, Dorella FA, Guimarães AS, Moawad MS, Selim SA, Guaraldi ALM, Miyoshi A, Ribeiro MG, Gouveia AM, Azevedo V, Heinemann MB, Lage AP (2014) Evaluation of ERIC-PCR as genotyping method for *Corynebacterium pseudotuberculosis* isolates. PLoS One 9:e98758
3. Soares SC, Silva A, Trost E, Blom J, Ramos R, Carneiro A, Ali A, Santos AR, Pinto AC, Diniz C, Barbosa EGV, Dorella FA, Aburjaile F, Rocha FS, Nascimento KKF, Guimarães LC, Almeida S, Hassan SS, Bakhtiar SM, Pereira UP, Abreu VAC, Schneider MPC, Miyoshi A, Tauch A, Azevedo V (2013) The pangenome of the animal pathogen *Corynebacterium pseudotuberculosis* reveals differences in genome plasticity between the biovar *Ovis* and *Equi* strains. PLoS One 8:e53818
4. Tauch, A., Sandbote, J (2014) The family Corynebacteriaceae. In: The prokaryotes: Springer Berlin Heidelberg
5. Williamson LH (2001) Caseous lymphadenitis in small ruminants. Vet Clin N Am: Food Anim Pract 17:359–371
6. Torres LFC, Ribeiro D, Hirata R Jr, Pacheco LGC, Souza MC, Santos LS, Santos CS, Salah M, Costa MM, Ribeiro MG, Selim SA, Azevedo VAC, Mattos-Guaraldi AL (2013) Multiplex polymerase chain reaction to identify and determine the toxigenicity of *Corynebacterium* spp. with zoonotic potential and an overview of human and animal infections. Mem Inst Oswaldo Cruz 108:272–279
7. Abdallauh DA, Abdelkareem AM, Adel MK, Tharwat ME, Amir HA (2016) *Corynebacterium pseudotuberculosis*: disease prevalence, lesion distribution, and diagnostic comparison through microbiological culture and molecular diagnosis. Alex J Vet Sci 51: 189–198
8. Parise D, Parise MTD, Viana MVCV, Muñoz-Bucio AV, Cortés-Pérez YA, Arellano-Reynoso B, Díaz-Aparicio E, Dorella FA, Pereira F, Carvalho AF, Figueiredo HCP, Ghosh PDB, Gomide ACP, Azevedo VAC (2018) First genome sequencing and comparative analyses of *Corynebacterium pseudotuberculosis* strains from Mexico. Stand Genomic Sci:13–21
9. Constable PD, Hinchliff KW, Done S, Gruenberg W (2016) Veterinary medicine: a textbook of the diseases of cattle, horses, sheep, pigs, and goats. 11th Edition, (Saunders, U.S.A)

10. Quinn PJ, Markey BK, Leonard FC, Fitzpatrick ES, Fanning S, Hartigan PJ (2011) Veterinary microbiology and microbial disease. 2nd ed. Wiley-Blackwell, Chichester
11. Marianelli C, Cifani N, Capucchio MT, Fiasconaro M, Russo M, La Mancusa F, Pasquali P, Di Marco V (2010) A case of generalized bovine tuberculosis in a sheep. J Vet Diagn Investig 22:445–448
12. Pesciaroli M, Alvarez J, Boniotti MB, Cagiola M, Di Marco V, Marianelli C, Pacciarini M, Pasquali P (2014) Tuberculosis in domestic animal species. Res Vet Sci 97:S78–S85
13. Higino SSS, Pinheiro SR, Rocha VCM, Souza GO, Portela RA, Alves CJ, Vasconcellos SA, Dib CC, Rosário TR, Melville PA, Azevedo SS (2013) Tuberculose em caprinos e ovinos abatidos no semiárido da Paraíba, Brasil. Arquivos Inst Biol 80:281–287
14. Stanford J, Stanford C (2012) Mycobacteria and their world. Int J Mycobacteriol 1:3–12
15. Trost E, Ott L, Schneider J, Schröder J, Jaenicke S, Goesmann A, Husemann P, Stoye J, Dorella FA, Rocha FS, Soares SC, D'Afonseca V, Miyoshi A, Ruiz J, Silva A, Azevedo V, Burkovski A, Guiso N, Join-Lambert OF, Kayal S, Tauch A (2010) The complete genome sequence of *Corynebacterium pseudotuberculosis* FRC41 isolated from a 12-year-old girl with necrotizing lymphadenitis reveals insights into gene-regulatory networks contributing to virulence. BMC Genomics 11:728
16. Prodinger WM, Eigentler A, Allerberger F, Schönbauer M, Glawischnig W (2002) Infection of red deer, cattle, and humans with *Mycobacterium bovis* subsp. *caprae* in western Austria. J Clin Microbiol 40:2270–2272
17. Pacheco LGC, Pena RR, Castro TLP, Dorella FA, Bahia RC, Carminati R, Frota MNL, Oliveira SC, Meyer R, Alves FSF, Miyoshi A, Azevedo V (2007) Multiplex PCR assay for identification of *Corynebacterium pseudotuberculosis* from pure cultures and for rapid detection of this pathogen in clinical samples. J Med Microbiol 56:480–486
18. Nassar AFC, Daniel GT, Ruiz R, Miyashiro S, Scannapieco EM, Souza Neto J, Gregory L (2015) Diagnostic comparison of *Corynebacterium pseudotuberculosis* through microbiological culture and PCR in sheep samples. Arquivos Instit Biol 82:1–6
19. Ribeiro MG, Dias Junior JG, Paes AC, Barbosa PG, Nardi Júnior G, Listoni FJP (2001) Punção aspirativa com agulha fina no diagnóstico do *Corynebacterium pseudotuberculosis* na linfadenite caseosa caprina. Arquivos Instit Biol São Paulo 68:23–28
20. Lara GHB, Ribeiro MG, Leite CQF, Paes AC, Guazzelli A, Silva AV, Santos ACB, Listoni FJP (2011) Occurrence of *Mycobacterium* spp. and other pathogens in lymph nodes of slaughtered swine and wild boars (*Sus scrofa*). Res Vet Sci 90: 185–188
21. Franco MMJ, Ribeiro MG, Pavan FR, Miyata M, Heinemann MB, Souza Filho AF, Cardoso R, Almeida A, Sakate R, Paes AC (2017) Genotyping and rifampicin and izoniazid resistance in *Mycobacterium bovis* strains isolated from the lymph nodes of slaughtered cattle. Tuberculosis 104:30–37
22. Morais ABC, Bolaños CAD, Alves AC, Ikuta CY, Lara GHB, Heinemann MB, Giuffrida R, Listoni FJP, Mioni MSR, Motta RG, Takai S, Ribeiro MG (2018) Identification of *Mycobacterium* species and *Rhodococcus equi* in peccary lymph nodes. Trop Anim Health Prod 50:1319–1326
23. Souza MF, Carvalho AQ, Garino JRF, Riet-Correa F (2011) Caseous lymphadenitis in slaughtered hair sheep in northeastern Brazil. Pesqui Vet Bras 31:224–230
24. Unanian MM, Silva AEDF, Pant KP (1985) Abscesses and caseous lymphadenitis in goats in tropical semi-arid north-east Brazil. Trop Anim Health Prod 17:57–62
25. Çetinkaya B, Karahan M, Atil E, Kalin R, De Baere T, Vaneechoutte M (2002) Identification of *Corynebacterium pseudotuberculosis* isolates from sheep and goats by PCR. Vet Microbiol 88:75–83
26. Ayres M, Ayres M Jr, Ayres DL, Santos AS (2007) BioEstat 5.0. Aplicações estatísticas nas áreas das Ciências Biológicas e Médicas. Belém, Instituto de Desenvolvimento Sustentável Mamirauá
27. Spier SJ, Toth B, Edman J, Quave A, Habasha F, Garrick M, Byrne BA (2012) Survival of *Corynebacterium pseudotuberculosis* biovar equi in soil. Vet Rec 170:7–8
28. Latif NAA, Abdullah FFJ, Othman AM, Rina A, Chung ELT, Zamri-Saad M, Lila MAM (2015) Isolation and detection of *Corynebacterium pseudotuberculosis* in the reproductive organs and associated lymph nodes of non-pregnant does experimentally inoculated through intradermal route in chronic form. Vet World 8: 924–927
29. Wagner D, Young LS (2004) Nontuberculous mycobacterial infections: a clinical review. Infection 32:257–270
30. Khamis A, Raoult D, La Scola B (2005) Comparison between *rpoB* and 16S rRNA gene sequencing for molecular identification of 168 clinical isolates of *Corynebacterium*. J Clin Microbiol 43:1934–1936
31. Eisenberg T, Kutzer P, Peters M, Sing A, Contzen M, Rau J (2014) Nontoxigenic tox-bearing *Corynebacterium ulcerans* infection among game animals, Germany. Emerg Infect Dis 20:448–452
32. Pinto AC, de Sá PH, Ramos RT, Barbosa S, Barbosa HP, Ribeiro AC, Silva WM, Rocha FS, Santana MP, Castro TLP, Miyoshi A, Schneider MPC, Silva A, Azevedo V (2014) Differential transcriptional profile of *Corynebacterium pseudotuberculosis* in response to abiotic stresses. BMC Genomics 15:14
33. Almeida S, Tiwari S, Mariano D, Souza F, Jamal SB, Coimbra N, Raittz RT, Dorella FA, Carvalho AF, Pereira FL, Soares SC, Leal CA, Barh D, Ghosh P, Figueiredo H, Moura-Costa LF, Portela RW, Meyer R, Silva A, Azevedo V (2016) The genome anatomy of *Corynebacterium pseudotuberculosis* VD57 a highly virulent strain causing Caseous lymphadenitis. Stand Genomic Sci 8:11–29
34. Guerrero JAV, De Oca JRM, Acosta DJ, León FH, Morales-Erasto V, Salazar HGM (2018) Isolation and molecular characterization of *Corynebacterium pseudotuberculosis* from sheep and goats in Mexico. Microb Pathog 117:304–309
35. Kiernan, M.G., Coffey, J.C., McDermott, K., Cotter, P.D., Cabrera-Rubio, R., Kiely, P.A., Dunne, C.P (2019). The human mesenteric lymph node microbiome differentaites between Crohn's disease and ulcerative colitis. J Crohn's Colitis 58–66
36. Zwirzitz B, Pinior B, Metzler-Zabeli B, Handler M, Gense K, Knecht C, Landing A, Dzieciol M, Wetzels SU, Wagner M, Schmitz-Esser S, Mann E (2019) Microbiota of the gut-lymph node axis: depletion of mucosa-associated segmented filamentous bacteria and enrichment of Methanobrevibacter by colistin sulfate and linco-spectin in pigs. Front Microbiol 40(e10):599
37. Ilhan Z (2013) Detection of *Corynebacterium pseudotuberculosis* from sheep lymph nodes by PCR. Rev Méd Vét 164:60–66