Veterinary Dermatology

DOI: 10.1111/j.1365-3164.2010.00934.x

# Epidemiology, clinical signs, histopathology and molecular characterization of canine leproid granuloma: a retrospective study of cases from Brazil

**Lissandro Gonçalves Conceição*, Livia Maria Rosa Acha†, Alexandre Secorun Borges‡, Fernanda G. Assis‡, Fabricia Hallack Loures§ and Fabyano Fonseca e Silva¶**

*Departamento de Veterinária, Universidade Federal de Viçosa, Viçosa, Minas Gerais, Brazil
†Clinica Veterinária São Francisco de Assis, Muriaé, Minas Gerais, Brazil
‡Universidade Estadual Paulista, Faculdade de Medicina Veterinária e Zootecnia, Botucatu, São Paulo, Brazil
§Diagnóstico em Dermatopatologia Veterinária, Condomínio Bosque Acamari, 126, Viçosa, Minas Gerais, Brazil
¶Departamento de Bioestatísca, Universidade Federal de Viçosa, Viçosa, Minas Gerais, Brazil
Correspondence: Lissandro Gonçalves Conceição, Departamento de Veterinária, Universidade Federal de Viçosa, Viçosa, Minas Gerais, Brazil 36570-000. E-mail: lissandro@ufv.br

**Sources of Funding**
The study was self-funded; however, CNPq (Conselho Nacional de Pesquisa e Desenvolvimento–National Council of Research and Development–Brazil) provided a postgraduate scholarship to Livia Maria Rosa Acha.
**Conflict of Interest**
No conflicts of interest have been declared.

## Abstract

**Thirty-eight cases of canine leproid granuloma were diagnosed between 2000 and 2008. Diagnosis was based upon clinical and histopathological findings and the presence of acid-fast bacilli in skin sections. The clinical lesions were localized predominantly on the pinnae and included papules, plaques and nodules, with or without ulceration. Boxer dogs were the breed most affected. Histopathological findings included nodular to diffuse pyogranulomatous, lymphoplasmocytic inflammatory infiltrates, with or without necrosis, localized in the dermis or subcutaneous tissue. The bacillary loading and morphology were variable among the lesions analysed. There was no significant correlation between bacterial load and histopathological pattern, dominant type of inflammatory infiltration or the amount of necrosis or giant cells. No correlation was observed between giant cells and histopathological pattern. In the majority of cases where a PCR-based assay was done, a novel mycobacterium species as the main aetiological agent was identified, as reported in previous studies.**

Accepted 20 August 2010

## Introduction

Cutaneous diseases caused by mycobacteria are rare in small animals and, in general, they comprise four groups of diseases: atypical cutaneous mycobacteriosis, canine leproid granuloma, feline leprosy and skin tuberculosis.[1–3]

Canine leproid granuloma (CLG), also known as canine leprosy,[2,4,5] is characterized by nodular lesions in the skin and subcutaneous tissues of dogs. The aetiological agent is a recently discovered nonculturable mycobacterium, which was partly sequenced genetically in 2000.[5–7] In Brazil, identification of the mycobacterium that causes CLG has not yet been carried out.

Canine leproid granuloma was originally described in 1973, in Zimbabwe, Africa, but the disease is well documented in Australia and New Zealand.[2,4,5] In Brazil, the first description of CLG was in 1990;[8] this disease was probably under-recognized, and it is believed to be relatively common in Brazil because of the climatic similarities between Brazil and Australia.

The objective of the present study was to describe the epidemiological, clinical and histopathological findings of CLG, as well as the PCR-based analysis and genetic sequencing of this novel mycobacterium, in affected Brazilian dogs.

## Materials and methods

Thirty-eight histopathological records and paraffin blocks from cases diagnosed with CLG were obtained from two pathology laboratories: the Brazilian Institute of Veterinary Diagnosis and Specialties (Provet, Moema, São Paulo, Brazil) and the Veterinary Department of the Federal University of Viçosa (UFV, Minas Gerais, Brazil) from July 2000 to December 2008. All clinical cases (data and samples) were from southeastern Brazil, specifically from the cities of São Paulo, Juiz de Fora, Viçosa and Belo Horizonte (Figure 1). Molecular evaluation was performed at the Veterinary Clinical Science and Molecular Biology Laboratory, São Paulo State University (Unesp), School of Veterinary Medicine and Animal Science (FMVZ), Botucatu, São Paulo, Brazil.

### Clinical and epidemiological studies

Clinical and epidemiological data were collected for all 38 cases of CLG. Veterinarians who submitted tissues from patients were contacted by mail, telephone or e-mail, and the following information was collected: breed, sex, age, hair length, month in which the lesions first appeared, clinical progression of disease, gross description and anatomical site of the lesions, overall health of patient and laboratory test abnormalities, concurrent diseases (if any), and description of the dog's environment.

### Histopathological studies

Cutaneous samples were obtained from either an incisional or an excisional skin biopsy. Tissues were fixed in 10% neutral buffered formalin, routinely processed and embedded in paraffin. Histological

© 2011 The Authors. Veterinary Dermatology

**Figure 1.** Map of South America and Brazil illustrating all the Federation States and a closer view of the southeastern region and its respective states. The CLG cases were sent mainly from the cities of Belo Horizonte (1), Viçosa (2), Juiz de Fora (3) and São Paulo (4). SP, São Paulo; MG, Minas Gerais; ES, Espírito Santo; RJ, Rio de Janeiro.

sections (4–6 μm thick) were stained with haematoxylin and eosin (H&E), periodic acid–Schiff (PAS) for fungal structures and Ziehl–Neelsen for acid-fast bacilli. The study included cases in which the clinical signs were consistent with CLG and histological sections showed positive results for acid-fast bacilli and negative PAS staining for fungal structures.

Forty histopathological slides, obtained from 35 CLG cases, were examined using routine light optical microscopy. In three cases, the slides were not appropriate for histopathological examination or the paraffin block was not available for further sectioning. The inflammatory histopathological pattern,[9,10] predominant inflammatory cell infiltration type (granulomatous versus pyogranulomatous), presence of tissue necrosis, involvement of nerve fibres, and the amount and morphology of acid-fast bacilli were recorded.

Tissue necrosis was classified as follows: absent (0); mild (+) when approximately 20% of the lesion was affected; moderate (++) when necrosis occurred in 20–50% of the lesion; or intense (+++) when more than 50% of the lesion showed necrosis.

Giant cells were quantified as follows: absent (0); mild (+) when cells were rarely visualized throughout the entire slide; or moderate (++) when they were observed in every second or third high-power field (HPF; ×40 magnification).

The amount of acid-fast bacilli was classified as follows: mild (+) when bacilli were rarely identified on the slide; moderate (++) when bacilli were present in approximately every third HPF; or intense (+++) when the bacilli were visualized in almost every HPF.

Statistical correlation analyses were performed for the following parameters: the number of giant cells and the histopathological pattern; the number of bacilli and the histopathological pattern; the number of bacilli and the predominant inflammatory cell infiltration; the number of bacilli and the number of giant cells; and the number of bacilli inside and outside necrotic areas in the same lesion.

## Statistical analysis

The statistical methods were chosen according to the characteristics of the variables analysed, so that the assumptions imposed by the employed tests could be met.

The chi-squared test was employed to evaluate the following relations: (i) the histopathological pattern (diffuse or multinodular) and the number of giant cells; (ii) the score of the bacilli found and the histopathological pattern; (iii) the bacillus score and the predominant inflammatory cell infiltration type (granulomatous or pyogranulomatous); (iv) the bacillus score and the number of giant cells; and (v) the bacillus score and localization of bacilli (inside or outside necrotic tissue).

However, for certain variables, the number of samples was not large enough, and Fisher's exact test with Monte Carlo correction was used. These tests were performed using the PROC FREQ from the SAS® statistical software system (SAS Institute, Cary, NC, USA),[11] with a level of significance of 5%.

The relative risk (RR) was calculated to evaluate the predisposition of certain breeds to develop CLG. For these analyses, the total number of animals affected by CLG, the total number of cases and the total number of animals of each breed affected by CLG during the investigation period were compared.[12]

For the evaluation of hair-type predisposition, size of dogs and anatomical sites, 95% confidence intervals were calculated. Ratios were considered statistically similar when overlapping of these intervals was observed. This procedure was also used to evaluate whether a seasonal predisposition for the disease exists. These intervals were calculated by the PROP.TEST function of the statistical R software system (R Development Core Team, Vienna, Austria).[13]

## Polymerase chain reaction (PCR) and DNA sequencing

*DNA isolation*

Twenty-five samples with confirmed histopathological diagnoses of leproid granuloma from a total of twenty-two dogs (three samples represented different lesions from the same animal and two samples represented different lesions from another animal) were evaluated for the presence of mycobacterial DNA by PCR and DNA sequencing. All handling, extraction and amplification procedures were performed in separated laboratories using disposable tips with barrier protection. A control skin sample from tissue with no diagnosis of leproid granuloma was analysed without prior knowledge (blind control). Genomic DNA was extracted from formalin-fixed, paraffin-embedded tissue with a genomic DNA purification kit (QIAamp® DNA FFPE tissue kit; Qiagen, Valencia, CA, USA). Briefly, 5-μm-thick sections were obtained (with a different disposable microtome blade for each sample) and immediately placed into 1.5 mL sterile plastic microtubes (DNA, RNA and DNase free; Axygen, CA, USA) with xylol (analytical grade reagent; Merck, Darmstadt, Germany) to remove paraffin. Residual xylol was removed by the addition of 100% ethanol (analytical grade reagent; Merck). Extraction was performed following kit instructions, and 80 μL of low-EDTA elution buffer was used for elution. The yield and quality of RNA were spectrophotometrically evaluated at 260/280 nm (NanoDrop 2000™ Spectrophotometer; Thermo Scientific©, Wilmington, DE, USA). The DNA samples were stored at –20 °C prior to amplification.

*DNA amplification*

16S ribosomal RNA (rRNA) gene region amplification was performed in duplicate using a previously described nested PCR with slight modifications.[6] Forward primer (Integrated DNA Technologies, Coralville, IA, USA) 246 (5′-AGA GTT TGA TCC TGG CTC AG-3′) and reverse primer 247 (5′-TTT CAC GAA CAA GCC CAG AA-3′) were used for the first amplification round of the 16S rRNA region (producing a 590 bp fragment). DNA (2 μL) was added to 23 μL of the PCR mix (GoTaq® Master Mix; Promega, WI, USA), with a final volume of 25 μL and a final concentration of 1 × DNA polymerase, 400 mM dATP, 400 mM dGTP, 400 mM dCTP, 400 mM dTTP and 3 mM $MgCl_2$. Each oligonucleotide primer was used at a final concentration of 0.6 μm. The amplification was performed using a Mastercycler® Ep gradient S machine (Eppendorf, Hamburg, Germany). Cycling parameters were as follows: 95 °C for 2 min (initial denaturation) and 30 cycles at 95 °C for 30 s (denaturation), 60 °C for 1 min (annealing) and 72 °C for 1 min (extension), followed by a final extension at



© 2011 The Authors. Veterinary Dermatology
© 2011 ESVD and ACVD, 

72 °C for 7 min. A negative control sample lacking template was used in each reaction for both rounds. The nested primers were as follows: forward M1 (5′-AGT GGC GAA CGG GTG AGT AAC-3′) and reverse R7 (5′-TTA CGC CCA GTA ATT CCG GAC AA-3′), with an expected 455 bp product. Ten microlitres from the first PCR at a 1:100 dilution was added to 40 μL of GoTaq mix. The final PCR mixture, with a 50 μL volume, contained DNA polymerase, 400 mM dATP, 400 mM dGTP, 400 mM dCTP, 400 mM dTTP, 3 mM $MgCl_2$ and primers at a final concentration of 0.6 μm. Cycling parameters were 95 °C for 2 min (initial denaturation), 25 cycles at 95 °C for 30 s (denaturation), 68 °C for 1 min (annealing) and 72 °C for 30 s (extension), with a final extension for 5 min at 72 °C. Samples were kept frozen at −20 °C. In this second reaction, a negative control was also prepared, using the first blank sample. The PCR products (for both the first-round and nested reactions) were analysed by 1.0% agarose gel electrophoresis (Invitrogen, Carlsbad, CA, USA) and visualized with ethidium bromide. Molecular weights were estimated by comparison with a known molecular weight marker (100 bp ladder; Norgen Biotek, Ontario, Canada). Gels were documented with an Eagle Eye II (Stratagene, TX, USA). Samples that resulted in sequences different from *Mycobacterium murphy* were submitted to a new extraction using only the dermis and subcutaneous tissue after the epidermis had been manually dissected.

*PCR purification, sequencing and analysis*

The PCR products were purified using the Illustra GFX™ PCR DNA and Gel Band Purification Kit (GE® Healthcare, Piscataway, NJ, USA). After purification, DNA yield and quality were evaluated spectrophotometrically at 260/280 nm (NanoDrop 2000™ Spectrophotometer; Thermo Scientific©) and analysed by agarose gel electrophoresis. Automated direct sequence analysis (MegaBACE 1000; Amersham Biosciences; Dyenamic ET Dye Terminator kit using software Sequence Analyzer 3.0, Amersham Place, Little Chalfont, England) was performed in duplicate using the same nested primers described in the previous subsection (M1 and R7). The sequences and electropherograms obtained were analysed using Sequencher® 4.7 software and aligned (Clustal multiple analysis software; http://www.clustal.org/) to verify sequence homology against previously available sequences from the National Center for Biotechnology Information (NCBI) GenBank (http://www.ncbi.nlm.nih.gov/), NCBI GenBank bacterial genomic database (http://www.ncbi.nlm.nih.gov/sutils/genom_tree.cgi), European Molecular Biology Laboratory Nucleotide Sequence Database (EMBL; http://www.ebi.ac.uk/embl/) and Ribosomal Differentiation of Medical Microorganisms (RIDOM; http://rdna.ridom.de/). Five representative 16S ribosomal RNA fragments from five different dogs were submitted to GenBank. The accession numbers of *Mycobacterium* sequences from GenBank and EMBL used for comparison in this study are as follows: *M.murphy* (AF144747), *M. tilburgii* (Z50172), *M. triplex* (AJ535505), *M. genavense* (EF107701), *M. interjectum* (AF547937), *M. simiae* (EU022520), *M. madagascariense* (AF547943), *M. goodii* (DQ447773), *M. kubicae* (AF547941), *M. lentiflavum* (AB362381), *M. heidelbergense* (X70960), uncultured *Mycobacterium* sp. (AF325531), *M. duvalii* (AF547918), *M. mageritense* (AJ699399), *M. moriokaense* (AY859686), *M. smegmatis* (AJ536041), *Mycobacterium* XN10-17 (FJ358417), uncultured *Mycobacterium* ARFS-3 (AJ277836), *Mycobacterium* sp. L47 (DQ249999), *M. immunogenum* strain CIP 106684T (AY457080), *M. genavense* (EU742187) and uncultured bacterium (EU132651).

## Results

### Clinical and epidemiological study

From a total of 38 CLG cases, epidemiological and clinical data were retrieved from 32 dogs. Complete information was not available on six dogs, and in other cases specific data points were not available. This is the reason for the variation in the numbers of cases cited in the text (e.g. 29 of 32, 19 of 31 or 18 of 30).

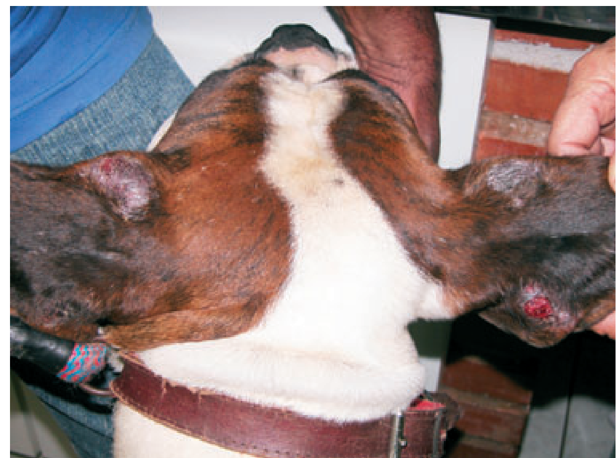

**Figure 2.** Dorsal view of head and both pinna of boxer dog affected by canine leproid granuloma (CLG). Note the nodular ulcerative lesions on the convex surface of both pinnae.

In the period between 2000 and 2008, CLG represented 0.51% (38 cases of 7403) of all skin biopsies received for histopathological examination at the two institutions.

Boxer dogs were the most affected breed (19), followed by rottweiler (4), mixed breed (4), Labrador retriever (3), dachshund (2), Dogue de Bordeaux (1), Fila Brasileiro (1), American Staffordshire bull terrier (1), Brazilian terrier (1), weimaraner (1) and long-haired Brussels Griffon (1). Boxer dogs represented 50% of all cases. Only boxer and rottweiler dogs showed high relative risks for the development of the disease ($P < 0.05$). The disease affected mainly short-coated dogs (28 of 36 or 77.8%), followed by those with intermediate coat hair length (rottweiler and Labrador retriever; 7 of 36 or 19.4%). Long-haired dogs were represented by only one dog (1 of 36 or 2.8%). Thirty cases (30 of 36 or 83.3%) were represented by large-sized dogs. Short-coated and large-sized dogs were predisposed to CLG development ($P < 0.05$).

Skin lesions included papules, plaques, nodules and tumours, measuring from 2 mm to 7 mm in diameter. The lesions were localized exclusively on the pinna in 77.8% of the cases (28 of 36; Figure 2), but other body parts were also affected, with or without pinna involvement: pinna and forelegs (1 of 36); pinna, hindlegs and head (1 of 36); pinna and face (4 of 36); pinna and hindlegs (1 of 36); and pinna and nasal planum (1 of 36). In total, the pinna was the most affected site (34 of 36 or 94.4%; $P < 0.05$). Lesions were localized on the concave surface of the pinna in only one dog (1 of 34). In all other dogs (33 of 34), lesions presented on the convex surface of the pinna.

Canine leproid granuloma occurred in animals from 1 to 11 years old, with an average age of 5.8 years and median of 7 years. In this study, the disease affected both sexes equally (19 of 38).

One stray animal (3.1%) was included in the study group, and it was reported to be emaciated. Submandibular lymphadenopathy was described in this emaciated dog and in another dog with a large ulcerated lesion (2 of 30).

The evolution of lesions was known in 26 cases. It varied from 7 days to 2 years. Nevertheless, in most cases (88%), the diagnosis was made within 2 months after the appearance of the lesions.

© 2011 The Authors. Veterinary Dermatology
© 2011 ESVD and ACVD, 

Canine leproid granuloma was more frequently observed during September to March (17 of 26 or 65.4%); these are the warmest months of the year in Brazil. Although the seasons are not well defined in Brazil, a greater incidence of CLG was observed in summer and spring (16 of 26 or 61.5%; 9 in summer and 7 in spring), in comparison with winter and autumn (10 of 26 or 38.5%; 7 in winter and 3 in autumn). However, this difference was not statistically significant ($P > 0.05$).

Twenty-nine animals (29 of 32) showed multiple lesions ($P < 0.05$), and only three dogs (9.4%) presented with one single nodule. In most dogs, the lesions had a bilateral distribution (19 of 31), while 12 animals presented with unilateral lesions (12 of 31). All lesions were well circumscribed, and ulceration was observed in 18 animals (18 of 30), mostly in the larger lesions.

Mild signs of pain or pruritus were reported by owners in seven animals (7 of 27).

Flies and mosquitoes were present in the environment of most affected dogs (20 of 23). In general, their dwelling places were near parks or vacant lots, and the dogs lived outdoors. Some animals lived or frequently visited rural areas. Previous skin wounds were reported before the development of CLG lesions in three dogs (3 of 13). Fly bites were the putative cause of these wounds and in one case it appeared to be caused by a bird peck. Two animals shared the same environment. The follow-up period was known for 23 animals and varied greatly; from 2 months to 7 years, with an average of 19 months. Lesion recurrences were not observed in any of the cases.

### Histopathological studies

Histopathological examinations were performed on 40 lesions from 35 affected dogs. In a few cases, owing to the nature of the lesion (e.g. superficial biopsy), it was not possible to analyse all the histopathological variables. Owing to the highly suggestive clinical and histopathological findings, microbiological culture was performed in only one case, and the result was negative for *Mycobacterium* species.

Histopathological findings included multinodular (17 of 39) and diffuse inflammatory patterns (22 of 39; Figure 3). Granulomatous and pyogranulomatous inflammatory reactions occurred in the same proportions (20 of 40).

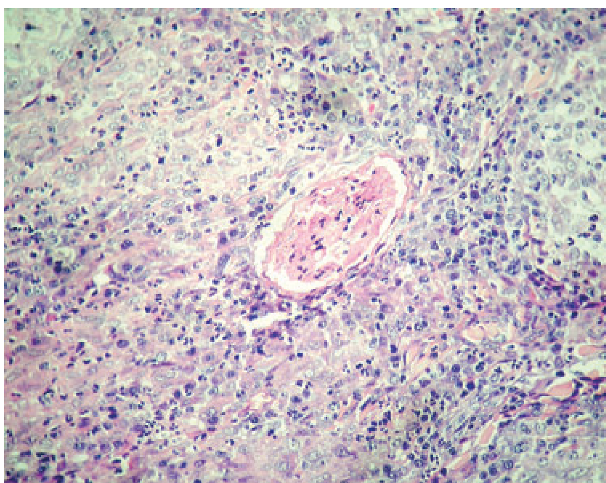

**Figure 3.** Photomicrograph of CLG. The nerve fibre remains undisturbed by dense mononuclear inflammatory infiltrate (H&E; original magnification ×400).

The cellularity was similar for all cases, and the granulomas/pyogranulomas were mainly composed of epithelioid macrophages, neutrophils, lymphocytes and plasmocytes in different proportions.

Multinucleated giant cells were present in 23 cases (23 of 40), with a mild (15 of 23) or moderate level of occurrence (eight of 23).

The inflammatory infiltration was vertically oriented, mostly in the periphery of some lesions (14 of 40), surrounding adnexal appendages.

Inflammatory reactions involved the dermis (25 of 39) and also the subcutaneous tissue (two of 39) or the dermal–epidermal interface (five of 39). Inflammation restricted to the deep dermis was also observed (seven of 39). Ulceration (17 of 40), acanthotic epidermis (16 of 40) or normal epidermis (seven of 40) occurred with different frequencies.

Tissue necrosis occurred in approximately half of the lesions (21 of 40), with the following distribution: mild (three of 21), moderate (12 of 21) and intense (six of 21). Oedema and vascular proliferation foci occurred in several cases (seven of 40 and 31 of 40, respectively). Vascular proliferation was present in most of the lesions, usually with a uniform distribution within the inflammatory infiltrate.

When visible and within the inflammatory process (17 of 40 or 42.5%), nerve fibres did not exhibit significant histopathological changes (Figure 3).

The number of acid-fast bacilli was not uniform; it varied considerably among the dogs, among different lesions in the same animal and even inside the same lesion. Bacillary load was low (11 of 36 or 30.5%), moderate (15 of 36 or 41.7%) or intense (10 of 36 or 27.8%). There was no statistical difference ($P > 0.05$) among the following variables: (i) load of bacilli and histopathological pattern (multinodular or diffuse); (ii) load of bacilli and type of inflammatory infiltrate (granulomatous or pyogranulomatous); (iii) load of bacilli and number of giant cells; and (iv) number of giant cells and histopathological pattern (multinodular or diffuse). All of these parameters were evaluated in the same lesion.

Bacillary morphology was not uniform. In several cases, both preserved and fragmented bacillary morphologies could be found in the same lesion. However, the preserved bacillary morphology predominated (26 of 36 or 72.2%). Although not statistically significant ($P > 0.05$ by confidence interval overlapping test), higher numbers of acid-fast bacilli (fragmented bacilli) were observed in necrotic tissue (nine of 16 or 56.3%).

### Polymerase chain reaction and genetic sequencing

Using PCR analysis in combination with agarose gel electrophoresis, the product of the first amplification, predicted to be 590 bp, was not observed in any of the samples evaluated. In the second (nested) amplification reaction, using primers M1 and R7, positive results were achieved, with a fragment of approximately 450 bp.



© 2011 The Authors. Veterinary Dermatology
© 2011 ESVD and ACVD, *Veterinary Dermatology*, **22**, 249–256.

The amplification reaction was positive for the presence of *Mycobacterium* spp. in the majority of samples (18 of 22 animals or 81.8%). Three samples represented different lesions in the same animal, and two other samples from another animal were positive. Only four samples (four of 22 or 18.2%) did not produce a band. Negative controls in all reactions presented negative results, confirming the absence of contamination. The blind control sample, originating from a distinct type of cutaneous lesion, was also negative.

Samples from the eighteen positive animals were compared with the sequences of *Mycobacterium* spp. already described. The first analysis was carried out with *M. murphy*, accession number AF144747, described by Hughes *et al.*[6] The product of the amplification was 100% identical to *M. murphy* in 13 animals (13 of 18 or 72.2%). Partial sequencing of the 16S rRNA gene was as follows:

ACGTGGGTAATCTGCCCTGCACTTCGGGATAAGC
CTGGGAAACTGGGTCTAATACCGGATATGACCAC
GAAGCGCATGCTTTGTGGTGGAAAGCTTTTGCGG
TGTGGGATGGGCCCGCGGCCTATCAGCTTGTTGG
TGGGGTGATGGCCTACCAAGGCGACGACGGGTA
GCCGGCCTGAGAGGGTGTCCGGCCACACTGGGA
CTGAGATACGGCCCAGACTCCTACGGGAGGCAG
CAGTGGGGAATATTGCACAATGGGCGCAAGCCT
GATGCAGCGACGCCGCGTGGGGGATGACGGCC
TTCGGGTTGTAAACCTCTTTCAGCAGGGACGAAG
CGCAAGTGACGGTACCTGCAGAAGAAGCACCGG
CCAACTACGTGCCAGCAGCCGCGGTAATACGTA
GGGTGCGAGCG

Mycobacterial sequences from different lesions in the same animal were identical. The amplification products from samples from five other dogs (five of 18 or 27.8%) did not show 100% homology with the *M. murphy* sequence and were also different when compared with each other. Representative fragments were submitted to GenBank (accession numbers GQ340424, GQ340425, GQ340426, GQ340427 and GQ340428). These sequences were compared with other *Mycobacterium* species using the databases at http://rdna.ridom.de/, http://www.ncbi.nlm.nih.gov/sutils/genom_tree.cgi, EMBL (http://www.ebi.ac.uk/embl/) and GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Of five mycobacteria identified, one (GQ340427) presented 100% homology with the sequence of *Mycobacterium* XN10-17, accession number FJ358417.1. The other four species identified did not present 100% homology with any of the mycobacteria with genetic sequences available in these data sets. The best homology values were found for the following micro-organisms: uncultured bacterium ARFS-3, accession number AJ277836.1 (99% homology with GQ340428); *M. immunogenum* strain CIP 106684T 16S ribosomal RNA gene, partial sequence (AY457080.1) and *M. chelonae* partial 16S rRNA gene, strain ITG 95-0026 (AJ419969.1; 99% homology with GQ340426); uncultured *Mycobacterium* sp. clone K3840/1 16S ribosomal RNA gene, partial sequence (AY148216.1) and *M. llatzerense* strain MC10 16S ribosomal RNA gene, partial sequence (FJ770983.1; 98% homology with GQ340424); uncultured bacterium clone FFCH10753 16S ribosomal RNA gene, partial sequence (EU132651.1) and uncultured *Mycobacterium* sp. clone DOS72 16S ribosomal RNA gene, partial sequence (EF438354.1; 99% homology with GQ340425). These paraffin-embedded samples had their epidermis dissected to enable only the dermis and subcutaneous tissue to be used for a new DNA extraction and amplification. All PCRs were negative for these five animals after this procedure was performed.

## Discussion

In the present study, short- or medium-length coated, large breeds (i.e. boxer dogs and rottweilers) were found to be predisposed to developing CLG. The reason for the predisposition of boxer dogs to CLG is not yet known, but a similar predisposition has been reported in many parts of the world.[2,4,7] Short coat, body size and pendulous ears do not adequately explain this predisposition, since other phenotypically similar breeds are not affected to the same degree. It is possible that boxer dogs may have a deficiency in their immunological response to mycobacteria. This subject requires further studies.

While CLG predominantly affected middle-aged dogs in the present study, no particular age trend has been reported in dogs from Australia.[4] It is interesting to note that as an infectious disease, the question arises as to why younger dogs were not affected as often as older dogs, since a weaker (less well-developed) immune system should be expected in young animals. A long incubation period may be one explanation, but this hypothesis needs confirmation. Similar to previous reports, no gender bias was found in this study.

As most of the dogs were otherwise healthy, CLG does not appear to be favoured in cases of concomitant debilitating diseases.

The lesions typically occurred on the pinna, as previously described.[2,4,5,7] Such anatomical preference supports the speculative theory about the probable mode of transmission of the disease. Although the aetiology of CLG is not well understood, it is believed that susceptible animals are inoculated via insect bites.[2,3,10,14] The lesions occur preferentially on anatomical regions that are more susceptible to insect bites, such as the ears and the cephalic segment. In particular, the skin of the pinna generally has a lower hair density and more superficial vascularization, which may favour blood feeding. Additionally, large dogs usually spend more time outdoors.[7,10] Another hypothesis about the pathogenesis of CLG is that the micro-organism has a preference for developing in colder body regions, such as the pinna.[4,5]

It is also believed that mycobacterial infection may occur due to previous cutaneous wounds or scratches inflicted by cats.[5] In the present study, three dogs had histories of prior lesions on the pinna before the development of leproid lesions, but a history of cat exposure was not documented. Additionally, in most cases, the presence of flies and other insects in the dog's environment was definitively reported by owners, and the dogs were predominantly outdoors.

© 2011 The Authors. Veterinary Dermatology

Ulceration was more frequently observed in larger lesions. Trauma and vascular injury due to severe inflammatory reaction may be the cause. Despite the worrisome clinical aspect of the lesions, the disease did not seem to bother the majority of the animals, since pain and pruritus were noted in only a few dogs.

Typically, the disease had a rapid clinical evolution, with owners seeking early medical assistance, partly because skin tumours were the main differential diagnosis. All dogs except one were otherwise healthy, in agreement with previous studies.[2,4,5,7,10] Submandibular lymphadenopathy was described in two dogs. In one of the cases, this finding probably occurred due to the extensive ulceration of large lesions. The other dog was homeless, emaciated and suffering from chronic ehrlichiosis. These findings strongly suggest a low pathogenic potential for the infective agent, since there was no significant spreading of lesions or systemic involvement.[5,7,15]

Approximately two-thirds of the cases (65.4%) occurred during the hottest months of the year (September to March). Another study found the opposite result, with a higher incidence during the coldest months of the year, in autumn and winter.[4] It was considered that the agent may have a long incubation period and that the inoculation of the micro-organism may have occurred during the hotter months, when the number of possible vectors would be greater. Consequently, the disease appeared a few months later. However, such differences in results cannot easily be explained. In our region, in the southeastern portion of Brazil, there is an increase in flies, mosquitoes and other potential vectors during the hotter months of the year. These vectors are known to disturb dogs and can eventually transmit the infective agent. In addition, it is interesting to note that some Brazilian cities (e.g. São Paulo, Belo Horizonte, Viçosa and Juiz de Fora), where the majority of the cases arose, have latitudes and climatic characteristics somewhat similar to certain Australian regions (e.g. Sydney).[16] Therefore, further studies are necessary to explain the seasonal difference between Brazilian and Australian cases.

The differential diagnosis of CLG includes other infectious, parasitic or sterile granulomatous diseases and neoplastic disorders.[5,10,17] A definite diagnosis of CLG was based on histopathological findings from H&E staining and Ziehl–Neelsen staining for acid-fast bacilli. So far, it has not been possible to cultivate or isolate the mycobacterium causing CLG using protocols that work for other species of mycobacteria.[2,4,7] Owing to the strong evidence, based on both clinical and histopathological findings, only one case was submitted for microbial culture, yielding a negative result. However, the negative result is important because it allows the exclusion of other relevant zoonotic mycobacterioses.[7]

The absence of recurrence in the cases reported here reinforces the good prognosis for this disease. Occasionally, however, some dogs may develop chronic and severe lesions that are refractory to treatment.[7,18]

Histopathological findings were similar to those previously described.[7,15] Granulomatous infiltrate affected the dermis and, sometimes, the dermal–epidermal interface and subcutaneous tissue. The subcutaneous region was affected in a smaller number of cases, since most lesions occurred on the pinna, which has no significant amount of adipose panniculus.[10]

The more intense vascularization of the periadnexal dermis may explain the frequent perifollicular arrangement of granulomas in CLG. This finding was identified mainly in the periphery of the most diffuse lesions. However, in a few cases, the inflammatory infiltrate surrounded only adnexal units. This inflammatory pattern was considered a very strong indication of sterile granuloma/pyogranuloma syndrome in dogs.[19,20] However, molecular (PCR-based) and immunohistochemical studies of cases of canine sterile granuloma syndrome were positive for *Leishmania* species.[21] Therefore, CLG, as verified in the present study by positive bacilloscopy and PCR results, must also be included in differential diagnoses of periadnexal granulomatous inflammation in dogs.

'Cutaneous tuberculosis' and 'mycobacterial granuloma of the canine skin' were the terms initially used to describe CLG, which first appeared in Zimbabwe.[22,23] The name 'canine leproid granuloma syndrome' was introduced in 1998.[4] However, the term 'leproid' implies something that closely resembles leprosy. Although understandable in some aspects, since it is a cutaneous disease caused by an unculturable mycobacterium, there are considerable clinical differences between CLG and human leprosy.[24–28]

Furthermore, the absence of any associations between the number of giant cells and the histopathological pattern, between giant cells and bacillus load and between the histopathological pattern and bacillus load also constitutes a major difference from human leprosy. The involvement of nerve bundles, either by inflammatory cells or bacillus infiltration, which is a characteristic of human leprosy,[25–27] was not verified in any of the CLG cases. Therefore, the term 'leproid' may not be appropriate to describe this disease.

Polymerase chain reaction is very useful for the aetiological diagnosis of mycobacterial infections in animals, especially for nonculturable agents or those that are difficult to culture.[6] In our study, PCR assays were positive for most tested cases, confirming the presence of mycobacterial DNA in CLG lesions. The number of base pairs amplified was similar to that previously described which validates the technique applied.[6]

Negative results could have occurred because of inadequate material conservation, which might have caused DNA degradation. Sample analysis with a small amount of bacilli, as visualized histopathologically, does not explain the negative results observed. Other paucibacillary samples showed positive results with PCR, providing a precise diagnosis even with little genetic material.

Amplification and sequencing of regions V2 and V3 of the16S rRNA gene have been recommended for the diagnosis and identification of *Mycobacterium* species.[5,6,29] These regions have a great heterogeneity, which distinguishes and identifies species.[29]

This novel mycobacterial species, which causes the CLG, was first sequenced from Australian cases.[6] Another study showed 99–100% homology with the previous report.[5] In the present study, we have also corroborated these findings. However, other sequenced mycobacteria were not homologous to this novel myco-



© 2011 The Authors. Veterinary Dermatology
© 2011 ESVD and ACVD, *Veterinary Dermatology*, **22**, 249–256.

bacterial species or to those described in the literature. They were also different from each other, suggesting that probably saprophytic mycobacterium could be detected on our first initial amplification in five samples. This is more reasonable than associating these cases with new species responsible for CLG, especially after the unsuccessful amplification using DNA from tissues without the epidermis. However, different aetiological agents responsible for the same clinical presentation have also been described for other canine cases[6] and for feline leprosy.[30] Other similarities between feline leprosy and CLG include the difficulty in culturing mycobacterium and possible transmission by insect vectors or trauma.[1,30]

Despite the possible involvement of new species in a few cases, the novel and still unnamed mycobacterium sequenced in the present study is identical to previous reports[5,6] and represents an important aetiological agent of the disease also in Brazil.

Homology between the unclassified mycobacterium identified in this study and fast-growing mycobacterium was low (below 94%). Only *M. smegmatis* presented a homology of 97% with one of the samples analysed. Mycobacteria induce a granulomatous inflammatory response,[31,32] and several species, including saprophytic ones, have been involved in granulomatous dermatitis of dogs, cats and humans. These other mycobacterioses are caused by saprophytic micro-organisms commonly found in water and soil, and animals are inoculated through entry of contaminated material into the skin. The majority of mycobacteria involved are classified as fast growing, including *M. fortuitum*, *M. chelonei*, *M. phlei*, *M. smegmatis* and *M. thermoresistible*.[1,2] In addition to these, there is a recent report of *M. goodii* infection in a dog.[33] Atypical mycobacterioses are characterized by the presence of nodules, ulcers and recurrent or chronic draining tracts with fistulae, which appear mostly on the ventral abdomen and inguinal region, differing from the typical clinical signs of CLG.

In conclusion, short-coated and large-sized dogs, predominantly boxer dogs, were predisposed to CLG. No gender predisposition was observed, and most occurrences were reported in middle-aged animals. The lesions were characterized by multiple firm nodules, either ulcerated or not, especially on the pinna. Otherwise, the dogs were reported to be healthy, and the prognosis was good. Histopathologically, CLG was characterized by a nodular to diffuse granulomatous or pyogranulomatous dermatitis. Acid-fast bacilli were present with different numbers and morphologies. No significant association between the number of bacilli and the histopathological pattern, type of inflammatory infiltrate, level of tissue necrosis or number of giant cells was found. The novel species of mycobacteria identified in most cases of CLG was similar to previous studies.

## Acknowledgements

The authors acknowledge the CNPq for the postgraduate scholarship, Jana Tessarolo Clemente and Fabiano Hipolito Silvestre for their assistance in collecting some data, Renato de Lima Santos, Vidal Haddad Jr and Adriane Pimenta da Costa Val for their critical review of the manuscript, Aline Elisa Santana for editing some pictures, and all veterinary practitioners who sent the samples for histopathological diagnosis and Raul Negrão Fleury for all the teaching in comparitive dermatopathology.

## References

1. Mundell AC. Mycobacterial skin diseases in small animals. In: Bonagura JD, Kirk RW, eds. Kirk's Current Veterinary Therapy XII Part A. Philadelphia, W. B. Saunders, 1995: 622–5.
2. Lemarie SL. Mycobacterial dermatitis. Veterinary Clinics of North America: Small Animal Practice 1999; 29: 1291–301.
3. Twomey LN, Wuerz JA, Alleman AR. A ''down under'' lesion on the muzzle of a dog. Veterinary Clinical Pathology 2005; 34: 161–3.
4. Malik R, Love DN, Wigney DI *et al.* Mycobacterial nodular granulomas affecting the subcutis and skin of dogs (canine leproid granuloma syndrome). Australian Veterinary Journal 1998; 76: 403–7.
5. Foley JE, Borjesson D, Gross TL *et al.* Clinical, microscopic and molecular aspects of canine leproid granuloma in the United States. Veterinary Pathology 2002; 39: 234–9.
6. Hughes MS, James G, Ball N *et al.* Identification by 16S rRNA gene analyses of a potential novel mycobacterial species as an etiological agent of canine leproid granuloma syndrome. Journal of Clinical Microbiology 2000; 3: 953–9.
7. Malik R, Hughes MS, Martin P *et al.* Canine leproid granuloma syndrome (canine leprosy). In: Greene CE, ed. Infectious Diseases of the Dog and Cat, 3rd edn. St Louis, Saunders Elsevier, 2006: 480–2.
8. Larsson CE, Michalany N, Pinheiro SR *et al.* Mycobacteriosis in domestic dogs. Report of two cases in São Paulo. Revista Faculdade de Medicina Veterinária Zootecnia da Universidade de São Paulo 1994; 31: 35–41.
9. Ackerman AB, Chongchitnant N, Sanchez J *et al.* Histologic Diagnosis of Inflammatory Skin Diseases – an Algorithmic Method Based on Pattern Analysis, 2nd edn. Baltimore: Williams Wilkins, 1997: 944.
10. Scott DW, Miller WH, Griffin CE. Small Animal Dermatology, 6th edn. Philadelphia: W.B. Saunders, 2001: 1528.
11. SAS Institute Users guide, version 9. Cary: SAS Institute, 2006: 501.
12. Thrusfield MV. Veterinary Epidemiology, 3rd edn. Oxford: Blackwell, 2007: 266–88.
13. R Development Core Team. R: a language and environment for statistical computing. Vienna: R Foundation for Statistical Computing, 2008. . ISBN 3-900051-07-0, http://www.R-project.org
14. Malik R. Mycobacterial diseases affecting the skin or subcutis of cats and dogs. Veterinary Dermatology 2004; 15 (suppl): 16 (abstract).
15. Charles J, Martin P, Wigney DI *et al.* Cytology and histopathology of canine leproid granuloma syndrome. Australian Veterinary Journal 1999; 77: 799–803.
16. Allmetsat. Weather reports and forecasts, satellite images, tropical cyclones, world climate data. Available at: http://pt.allmetsat.com/clima. Accessed Jan 20, 2009.
17. Gross TL, Ihrke PJ, Walder EJ *et al.* Skin Diseases of the Dog and Cat: Clinical and Histopathological Diagnosis, 2nd edn. Oxford: Blackwell Publishing, 2005: 932.
18. Malik R, Martin P, Wigney D *et al.* Treatment of canine leproid granuloma syndrome: preliminary findings in seven dogs. Australian Veterinary Journal 2001; 79: 30–6.
19. Panich R, Scott DW, Miller WH. Canine cutaneous sterile pyogranuloma/granuloma syndrome: a retrospective analysis of 29 cases (1976 to 1988). Journal of the American Animal Hospital Association 1991; 27: 519–28.
20. Santoro D, Prisco M, Ciaramella P. Cutaneous sterile granulomas/pyogranulomas, leishmaniasis and mycobacterial infections. Journal of Small Animal Practice 2008; 49: 552–61.
21. Cornegliani L, Fondevila D, Vercelli A *et al.* PCR technique detection of *Leishmania* spp. but not *Mycobacterium* spp. in canine cutaneous 'sterile' pyogranuloma/granuloma syndrome. Veterinary Dermatology 2005; 16: 233–8.

© 2011 The Authors. Veterinary Dermatology

22. Smith RIE. Canine skin tuberculosis. Rhodesian Veterinary Journal 1973; 3: 63–4.
23. Smith RIE. Canine mycobacterial skin granuloma. Rhodesian Veterinary Journal 1979; 10: 24.
24. Neves RG, Guilhon RMP. Hanseníase. In: Diógenes MJN, Guilhon RMP, Gonçalves HS *et al.*, eds. Atlas de Dermatopatologia Tropical. Fortaleza, Inova, 1997: 13–51.
25. Ridley DS, Jopling WH. Classification of leprosy according to immunity A five-group system. International Journal of Leprosy 1966; 34: 255–73.
26. Fleury RN. Critérios para o emprego da classificação de Ridley e Jopling na rotina dos laboratórios de anatomia patológica (Primeira parte). Hansenologia Internacionalis 2005; 30: 201–5.
27. Ridley DS, Job CK. The pathology of leprosy. In: Hastings RC, ed. Leprosy, 1st edn. New York, Churchill-Livingstone, 1985: 100–33.
28. Ridley DS, Jopling WH. A classification of leprosy for research purposes. Leprosy Review 1962; 33: 119–28.
29. Chakravorty S, Helb D, Burday M *et al.* A detailed analysis of 16S ribosomal RNA gene segments for the diagnosis of pathogenic bacteria. Journal of Microbiological Methods 2007; 69: 330–9.
30. Fyfe JA, McCowan C, O'Brien CR *et al.* Molecular characterization of a novel fastidious mycobacterium causing lepromatous lesions of the skin, subcutis, cornea, and conjunctiva of cats living in Victoria, Australia. Journal of Clinical Microbiology 2008; 46: 618–26.
31. Collins T. Acute and chronic inflammation. In: Cotran RS, Kumar V, Collins TR, eds. Robbins Pathologic Basis of Disease, 6th edn. Philadelphia, W.B. Saunders Co., 1999: 50–88.
32. Ringler DJ. Inflamação e Reparo. In: Jones TC, Hunt RD, King NW, eds. Patologia Veterinária, 6th edn. São Paulo, Manole, 2000. 119–65.
33. Bryden SL, Burrows AK, O'Hara AJ. *Mycobacterium goodii* infection in a dog with concurrent hyperadrenocorticism. Veterinary Dermatology 2004; 15: 331–8.

**Résumé** Trente-huit cas de syndrome granulomateux léproïde canin (CLG) ont été diagnostiqué entre 2000 et 2008. Le diagnostic repose sur les signes cliniques, histopathologiques et la présence de bacilles acido-résistants dans les coupes de peau. Les lésions cliniques étaient localisées principalement sur les pavillons auriculaires et regroupaient papules, plaques et nodules, avec ou sans ulcérations. Le boxer était la race la plus représentée. Les lésions histopathologiques regroupaient des infiltrats inflammatoires nodulaires à diffus pyogranulomateux, lymphoplasmocytaires, avec ou sans nécrose, localisés dans le derme ou le tissu sous-cutané. La charge bacillaire et la morphologie étaient variables en fonction des lésions analysées. Il n'y avait aucune corrélation significative entre la charge bactérienne et le patron histopathologique, le type d'infiltrat inflammatoire, la quantité de nécrose ou de cellules géantes. Aucune corrélation n'a été observée entre les cellules géantes et le patron histopathologique. Dans la majorité des cas où une PCR a été réalisée, l'agent étiologique principalement isolé était d'une espèce de mycobactérie atypique comme déjà rapporté dans de précédentes études.

**Resumen** Se diagnosticaron 38 casos de granuloma leproide canino (CLG) entre los años 2000 y 2008. El diagnostico se basó en los hallazgos clínicos e histopatológicos y en la presencia de bacilos positivos a tinción de ácido-resistencia en lesiones de la piel. Las lesiones clínicas se localizaron predominantemente en las orejas e incluían papulas, placas y nódulos con o sin ulceración. Los perros Boxer fueron la raza mas afectada. Los hallazgos histopatológicos incluyeron infiltrados nodulares o difusos piogranulomatosos, linfoplasmaciticos, con o sin necrosis, localizados en la dermis o el tejido subcutáneo. La cantidad de bacilos y la morfología fueron variables entre las lesiones analizadas. No hubo correlación significativa entre la carga bacteriana y el patrón histopatológico, tipo de inflamación dominante o la cantidad de necrosis o de células gigantes. No hubo correlación entre la presencia de células gigantes y el patrón histopatológico. En la mayoría de los casos donde se hizo PCR se aisló una nueva especie de micobacteria como el principal agente etiológico, como se ha indicado en estudios previos.

**Zusammenfassung** Zwischen 2000 und 2008 wurden achtunddreißig Fälle von caninem leproiden Granulom (CLG) diagnostiziert. Die Diagnose basierte auf klinischen und histopathologischen Befunden und auf dem Vorkommen von säurefesten Stäbchenbakterien (Bacilli) in Hautschnitten. Die klinischen Veränderungen befanden sich hauptsächlich an den Ohrmuscheln und bestanden aus Papeln, Plaques und Knoten, mit und ohne Ulzeration. Boxer waren die am häufigsten repräsentierte Hunderasse. Die histopathologischen Befunde bestanden aus nodulären bis pyogranulomatösen, lymphoplasmazellulären entzündlichen Infiltraten, mit oder ohne Nekrose, die in der Dermis und im subkutanen Gewebe lokalisiert waren. Das Ausmaß und die Morphologie der Bacilli waren bei den untersuchten Veränderungen verschieden. Es bestand keine signifikante Korrelation zwischen der Anzahl der Bakterien und dem histopathologischen Muster, dem hauptsächlichen Typ der entzündlichen Infiltration oder dem Ausmaß der Nekrose oder der Riesenzellen. Es wurde keine Korrelation zwischen den Riesenzellen und dem histopathologischen Muster beobachtet. In der Mehrzahl der Fälle, wo ein auf PCR-basierender Assay durchgeführt wurde, wurde eine neue Spezies eines Mycobakterium als das hauptsächlich auslösende Agens isoliert, was schon in früheren Studien beschrieben worden war.

**要約** 2000 年から 2008 年の間に 38 例の犬のレプラ様肉芽腫（ＣＬＧ）が診断された。診断は臨床所見と病理組織学所見、皮膚切片中の抗酸菌の存在に基づき行われた。病変は主に耳介に存在する、ときに潰瘍を伴うあるいは伴わない、限局性の膿庖、局面および結節であった。ボクサーが最も罹患しやすい犬種であった。病理組織学的な所見は、壊死を伴うあるいは伴わない、真皮または皮下組織で限局する、結節状からび漫性の化膿性肉芽腫およびリンパ球プラズマ細胞性炎症細胞浸潤であった。桿菌の量や形態は検討した病変の間で様々であった。細菌の量と病理組織学的なパターン、主要な浸潤細胞のタイプ、壊死の範囲や巨細胞の数には有意な相関は認められなかった。PCR に基づいた分析が行われた大部分の症例で、すでに今までの研究で報告された新種のマイコバクテリウムが主な病因として分離された。

© 2011 The Authors. Veterinary Dermatology