

PCR-based identification of *Mycobacterium murphy* causing Canine Leproid Granuloma Syndrome in Niterói, southeast Brazil - case report

[Identificação baseada em PCR de *Mycobacterium murphy* causando Síndrome do Granuloma Lepróide Canino em Niterói, sudeste do Brasil] - Relato de Caso

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ABSTRACT

Canine Leproid Granuloma Syndrome (CLGS), also known as canine leprosy, is a cutaneous nodular infectious disease caused by *Mycobacterium* sp.. Despite being reported worldwide, it is still quite unknown and underdiagnosed. Diagnosis may be achieved by cytopathology or histopathology of skin lesions, but identification of the infectious agent is complex, since bacterial *in vitro* growth is not possible, relying upon molecular techniques such as PCR to confirm *Mycobacterium* DNA in the sample. We report a CLGS case in Niterói, Rio de Janeiro state, Brazil, diagnosed by cytopathology and submitted to molecular identification of the agent. PCR amplification of *hsp65* gene was performed and revealed 100% genetic homology to *M. murphy* strain. This is the first CLGS report with molecular identification in Rio de Janeiro state, and this finding should raise awareness about CLGS as a differential diagnosis among granulomatous skin diseases in this region.

Keywords: CLGS, *hsp65* sequence, isolate Metcalfe, molecular methods

RESUMO

A síndrome de granuloma leproide canino (SGLC), também conhecida como lepra canina, é uma doença infecciosa cutânea nodular causada por *Mycobacterium* sp. Apesar de ser relatada mundialmente, ainda é bastante desconhecida e subdiagnosticada. O diagnóstico pode ser conseguido por citopatologia ou histopatologia de lesões cutâneas, mas a identificação do agente infeccioso é complexa, uma vez que o crescimento *in vitro* bacteriano não é possível, dependendo de técnicas moleculares como a PCR para confirmar o DNA de *Mycobacterium* na amostra. Relatou-se um caso da SGLC em Niterói, estado do Rio de Janeiro, Brasil, diagnosticado por citopatologia e submetido à identificação molecular do agente. Foi realizada amplificação por PCR do gene *hsp65*, que revelou 100% de homologia genética com a cepa *M. murphy*. Este é o primeiro relato da SGLC com identificação molecular no estado do Rio de Janeiro, o que mostra a importância de se acrescentar a SGLC ao diagnóstico diferencial das doenças granulomatosas de pele nessa região.

Palavras-chave: CLGS, sequência *hsp65*, isolado Metcalfe, métodos moleculares

INTRODUCTION

Canine Leproid Granuloma Syndrome (CLGS) is a cutaneous disease caused by *Mycobacterium*

infection. It presents itself as single or multiple nodules commonly appearing on the dorsal ear fold (Malik *et al.*, 2006; Malik *et al.*, 2013). Cytological and histologic examination reveal medium-sized negative stained bacilli with

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Romanowski-type stains that are positive in acid-fast stains such as Ziehl-Nielsen (Twomey *et al.*, 2005). *In vitro* growth requirements for these bacteria have not been determined due to its fastidious growth, but negative culture is useful for excluding other clinically relevant Mycobacteria (Malik *et al.*, 2006). Since isolation is still not possible, molecular diagnosis based on the 500bp of the 16S rRNA gene (Hughes *et al.*, 2000) or the ITS1 region (Fyfe *et al.*, 2008) are useful for its etiological diagnosis, also giving information about genetic correlation with isolated *Mycobacterium* species (Hughes *et al.*, 2000). CLGS may be underdiagnosed, since its etiological identification is difficult and other granulomatous diseases are reported in dogs (Santoro *et al.*, 2008; Malik *et al.*, 2013). There are few reports in Brazil and even fewer using molecular characterization. This report focuses on a confirmed case of CLGS with molecular identification of species in the city of Niterói, Rio de Janeiro, Brazil.

CASE REPORT

A 3-year-old mixed-breed female dog was admitted at Hospital Universitário de Medicina Veterinária Professor Firmino Mársico Filho, Universidade Federal Fluminense, with a tumor on the right ear fold (Figure 1). The nodule measured 3,0 x 2,5 x 1,5cm. Fine Needle Cytology (FNC) was obtained and stained in Giemsa, revealing pyogranulomatous inflammation, with negative stained bacilli inside macrophages (Figure 2A). *Mycobacterium* was suspected and a new sample by FNC was stained in acid-fast stain, revealing positive stained bacilli inside and outside macrophages (Figure 2B). Excisional biopsy was performed, and samples were submitted to histopathology, culturing, and molecular analysis (Figure 3). Histopathology revealed pyogranulomatous inflammation with extensive necrosis. Bacterial growth in MGIT-960 and in Löwestein-Jensen medium were negative. Molecular analysis results were as follows. The patient was treated with systemic and topical antibiotic therapy and did not present any signs involving the disease after healing the wound. Genotyping analyses

were performed at Laboratório de Biologia Molecular Aplicada a Micobactérias and at Laboratório de Genética Molecular de Microorganismos, Instituto Oswaldo Cruz in Rio de Janeiro city, Brazil. Samples were submitted to DNA extraction and purification using the Qiagen kit DNeasy Blood & Tissue (Invitrogen do Brasil). Summarily, 180µl of ATL buffer and 20µl of proteinase K from the kit were added to the biopsy, mixed by vortex and after overnight incubation at 56°C, DNA was purified using spin-column from the kit as described by the manufacturer. Amplification of part of *hsp65* was performed using the primer sets Tb11 (F ACC AAC GAT GGT GTG TCC AT) and Tb12 (R CTT GTC GAA CCG CAT ACC CT) and using amplification conditions as described in earlier studies (Devulder *et al.*, 2005; Fyfe *et al.*, 2008), yielding a fragment of 441bp. The PCR product was analyzed on agarose gel, purified with the Illustra PCR and Gel Band Purification Kit (GE Healthcare, Little Chalfont, BUX, UK) and submitted to sequencing using the same primers, using the ABI Big Dye 3.1 Terminator Ready Reaction Kit (Applied Biosystems do Brasil). Sequences were generated on an ABI 3730 Genetic Analyzer (Applied Biosystems) and compared with sequences available on NCBI. Our sequence was 100% identical to that of EF611178.1, derived from *Mycobacterium* sp. strain CLGS, isolate Metcalfe.



Figure 1. Dog presenting a granuloma on right ear fold; The nodule measured 3.0x25 x 1.5cm. Niterói, 2014.

PCR-based identification...

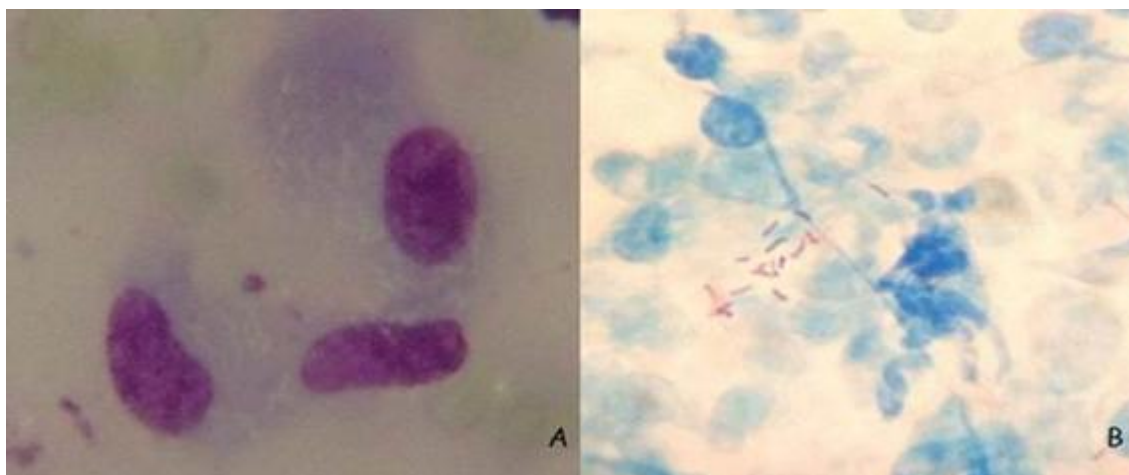


Figure 2. Fine needle cytology slides from canine granuloma sample. A. Macrophages presenting negative stained bacilli in its cytoplasm. Pyogranulomatous inflammation wa diagnosed. Giemsa stain, 1000x. B. Acid-fast staining shows positive stained bacilli outside macrophages. Acid-fast stain, 1000x. Niterói, 2014.

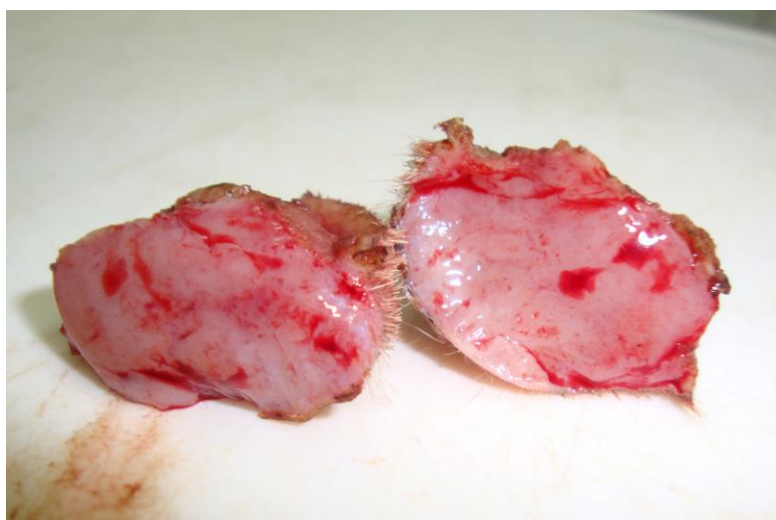


Figure 3. Tissue specimen of granuloma obtained by excisional biopsy. Specimens were then submitted to histopathology, culturing and molecular analysis. Niterói, 2014.

DISCUSSION

There have been reports of Leprosy-like *Mycobacterium* species in several animal hosts, but mycobacterial infections are considered uncommon in dogs (Malik *et al.*, 2006). First description of CLGS was from Zimbabwe, in a boxer and a bullmastiff (Smith, 1973), with reports from Australia appearing soon afterwards. Our report shows similarities with other Brazilian and worldwide reports, such as the patient presenting a single tumor in the ear pinna with no further systemic compromise (Maruyama, 2010; Conceição *et al.*, 2011;

Tabanez e Arrais, 2013). Mostly, such species are difficult to grow *in vitro* and therefore need molecular confirmation on the DNA level. About 15 years ago, the *Mycobacterium* species was described in several dogs from Australia and 16S rDNA reference sequence was determined (Hughes *et al.*, 2000). In a later study, part of the hsp65 sequence of the isolate *Mycobacterium* sp. strain CLGS, isolate Metcalfe (also called *M. murphy*) specific for this organism was reported (Fyfe *et al.*, 2008). In this report, PCR result was positive for *M. murphy*. This isolate presents high nucleotide homology to pathogenic species *M. simiae*, *M. interjectum*, and *M. genavense*

(Hughes *et al.*, 2000) but since its requirements for *in vitro* growth are not known, it remains as a not fully described species. Sequence analysis of the 16S rDNA is used for identification to the species level, and concatenated analysis of 16S rDNA and *hsp65* sequences allowed phylogenetic classification of this species, being very close to *M. simiae* and the cat isolate *M. sp tarwin* (Fyfe *et al.*, 2008). Because we use sequencing of part of *hsp65* as the routine procedure in our laboratory for identification of *Mycobacterium* to the species level, we applied this system for the identification of the acid-fast bacilli and 100% homology was observed with the *hsp65* sequence of *M. murphy*. Few reports exist on the occurrence of canine leprosy in Brazil and even less on molecular confirmation of the identity of the causative agent. A review of histopathological records and direct PCR sequence analysis on samples from 38 dogs with CLGS between 2000 and 2008 was described, confirming the presence of *M. murphy* in 13 dogs from the states of São Paulo and Minas Gerais (Conceição *et al.*, 2011). We now extend these observations on a possibly emerging infectious disease to another state of Southeast Brazil.

CONCLUSION

CLGS, even though being common in Australia, is still quite unknown worldwide and in Brazil. This could be due to its benign presentation, with few symptoms and sometimes a self-limited course of disease. Since negative-stained bacilli in routine cytology and histopathology stains can be hard to find and *in vitro* growth requirements for these bacteria have not yet been established, molecular diagnosis is extremely useful for CLGS diagnosis. This report raises questions about CLGS's role as a possible emerging infectious disease in Rio de Janeiro state, since it is the first case of CLGS identified by molecular analysis as *M. murphy* in the municipal district of Niterói. Since it is believed that CLGS is underdiagnosed, veterinary physicians should be aware of it as a differential diagnosis among other common nodular cutaneous diseases, such as neoplastic, inflammatory, and infectious conditions.

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