










Pneumonia by *Cryptococcus neoformans* in a goat in the Southern region of Brazil

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ABSTRACT: This report describes the clinical, pathological, and molecular aspects of a pneumonia by *Cryptococcus neoformans* in a goat in the Southern region of Brazil. A goat with a history of prolonged transportation presented dyspnea, nasal discharge and cough, and was subjected to necropsy, histopathology, and mycological evaluation. Grossly, cranio-ventral pulmonary consolidation was observed, characterized by firm and grayish areas interspersed with dark red foci. Histopathology of the lungs showed areas of parenchymal necrosis, containing blastoconidia with a slightly basophilic central cell, surrounded by an unstained capsule. It was associated with moderate granulomatous inflammatory infiltrate and peripheral fibrosis. The capsule and fungus cell exhibited marked Alcian Blue and periodic acid-Schiff staining, respectively. Diagnosis of fungal pneumonia by *C. neoformans* was based on clinical, pathological, and molecular findings.

Key words: goats, respiratory diseases, pathology, mycology, molecular diagnosis.

Pneumonia por *Cryptococcus neoformans* em um caprino na região Sul do Brasil

RESUMO: Este relato objetiva descrever os aspectos clínicos, patológicos e moleculares de pneumonia por *Cryptococcus neoformans* em um caprino na região Sul do Brasil. Um caprino com histórico de transporte prolongado apresentou dispnéia, secreção nasal e tosse e foi encaminhado para necropsia, análise histopatológica e micológica. Macroscopicamente, observou-se consolidação pulmonar cranioventral, caracterizada por áreas firmes e acinzentadas, entremeadas por focos vermelho-escuros. Na análise histopatológica dos pulmões foram evidenciadas áreas de necrose do parênquima, que continham blastoconídeos com célula central levemente basofílica, circundada por cápsula não corada, associados a moderado infiltrado inflamatório granulomatoso e fibrose periférica. A cápsula e a célula do fungo foram fortemente coradas pelo azul alciano e pelo ácido periódico de Schiff, respectivamente. O diagnóstico de pneumonia fúngica por *C. neoformans* foi baseado nos achados clínicos, patológicos e moleculares.

Palavras-chave: caprinos, doenças respiratórias, patologia, micologia, diagnóstico molecular.

Cryptococcosis is a localized or systemic mycosis that affects animals and humans (BOVERS et al., 2008). The disease is caused by fungi belonging to the *Cryptococcus* genus. Phylogenetic analysis and genotyping studies have revealed significant genetic diversity within the pathogenic *Cryptococcus neoformans/Cryptococcus gattii* species complex. According to the current classification, *Cryptococcus neoformans* variety *grubii* has been renamed as

Cryptococcus neoformans, while the other variety *Cryptococcus neoformans* variety *neoformans* has been renamed to *Cryptococcus deneoformans* (HAGEN et al., 2015).

In animals, cryptococcosis mainly affects cats (GALIZA et al., 2014). However, it is also described in other domestic species, including dogs (PAULA et al., 2014), horses (KOMMERS et al., 2005), cattle (RIET-CORREA et al., 2011), goats

(ROSA et al., 2016), and sheep (LE MOS et al., 2007). In goats, lungs appear to be the main organ affected by the fungus (BARÓ et al., 1998). Although, there are few studies confirming this (GUTIÉRREZ & MARÍN, 1999; LUVIZOTTO et al., 2009). Hence, the aim of this study was to describe the clinical, pathological, and molecular aspects of granulomatous pneumonia by *Cryptococcus neoformans* in a goat in the Southern region of Brazil.

A one-year-old mixed-breed, male goat, exhibited dyspnea, nasal discharge, and cough, lasting three days. The animal had been recently transported on a five-day trip from the Northeast to the Southern region of Brazil, with scarce supply of water and food, and in close proximity to other goats from different origins. The goat died and was subjected to necropsy.

Grossly, moderate hydrothorax was observed. Lungs were heavy, did not collapse, and showed marked cranio-ventral consolidation, predominantly involving the right lung. This was characterized by multifocal to coalescent, firm, grayish areas, intercalated with dark red foci (Figure 1A), which were associated with a moderate amount of foam inside the trachea.

Fragments of different organs were collected, fixed in 10% neutral buffered formalin solution, processed routinely, and stained with hematoxylin and eosin (H&E). Lung sections were also stained with Alcian blue (AB), periodic acid-Schiff

(PAS) and Ziehl-Neelsen (ZN). In addition, lung samples were collected, kept under refrigeration, and subjected to bacteriological, mycological, and molecular evaluations.

Histopathology of the lungs showed extensive areas of parenchymal necrosis, containing clear spaces with blastoconidia, composed by a slightly basophilic central cell, ranging from 3 to 10µm in diameter, surrounded by an unstained capsule. They were associated with a moderate pyogranulomatous inflammatory infiltration, composed of epithelioid macrophages, multinucleated giant cells, lymphocytes, plasma cells, and occasional neutrophils, fibrin deposition and proliferation of fibrous connective tissue at the periphery (Figure 2B). In the AB stain, the capsule was strongly bluish with a radiated appearance (Inset in the Figure 2B). The central cell was positive in PAS stain, with a higher intensity of coloration in the cell wall. In ZN stain no acid-fast bacilli were identified. No morphological changes were observed in the other systems and organs analyzed.

Tissue fragments were subjected to aerobic culture at 37°C on blood agar and MacConkey agar for 72 hours, and incubated at 30°C on Sabouraud dextrose agar for seven days. There was no bacterial nor mycological culture growth.

The PureLink™ Genomic DNA Mini Kit (Invitrogen) was used for DNA extraction from the lung sample. Molecular identification was

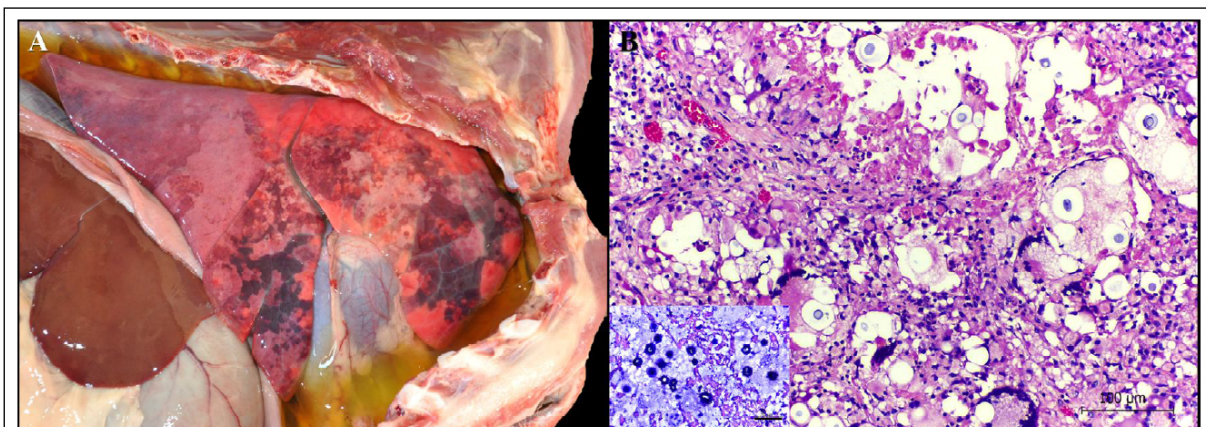
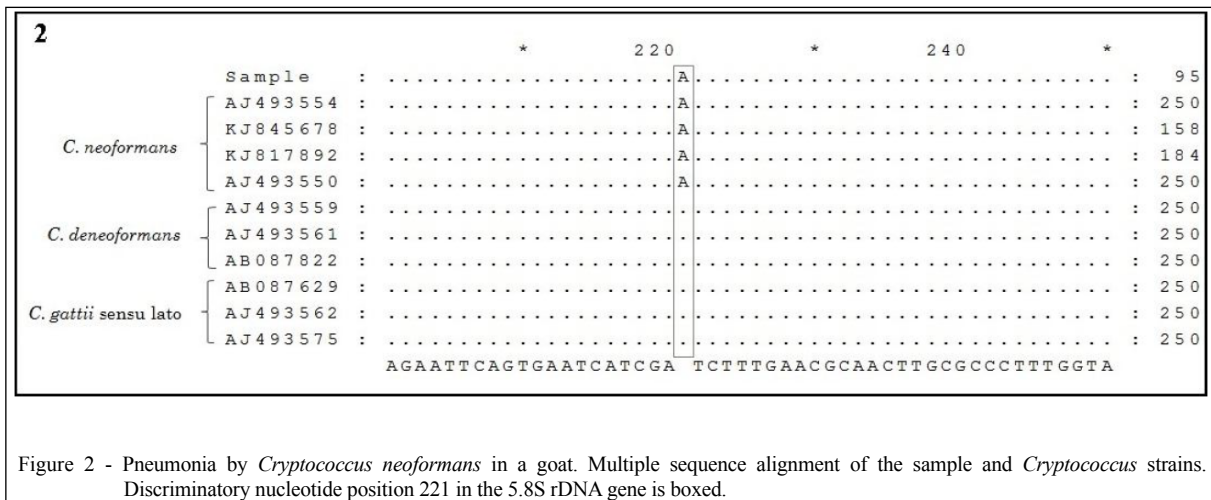


Figure 1 - Pneumonia by *Cryptococcus neoformans* in a goat. (A) Severe cranio-ventral pulmonary consolidation, characterized by multifocal to coalescing, firm, and grayish areas interspersed with dark red foci. Additionally, severe hydrothorax is observed. (B) Numerous blastoconidia are observed in the middle of the pulmonary parenchyma, with a slightly basophilic central cell, surrounded by an unstained capsule. These are associated with moderate granulomatous inflammatory infiltrate, fibrin deposition and proliferation of fibrous connective tissue at the periphery. H&E, magnification 20×. Inset, fungal capsule exhibits marked Alcian Blue stain positivity. Alcian Blue, magnification 40×.



performed using panfungal primers (ITS3-F /ITS4-R) (WHITE et al., 1990). The PCR product obtained includes the 5.8S rDNA and the ITS2 region. It was purified using the PureLink™ PCR Purification Kit (Invitrogen) and sequenced by ABI-Prism 3500 Genetic Analyzer (Applied Biosystems). Analysis were performed using BIOEDIT software. Multiple sequence alignments were performed using Clustal W in order to compare our sample to other *Cryptococcus* species sequences present in the GenBank database (Access numbers AJ493554, KJ845678, KJ817892, AJ493550, AJ493559, AJ493561, AB087822, AB087629, AJ493562, AJ493575) (KATSU et al., 2004; KANG et al., 2009). The comparison revealed similar ITS sequences, showing a characteristic base “A” at nucleotide position 221 (KANG et al., 2009) (Figure 2). Although, our sequence showed only one variable nucleotide, specifically at the position 221, an “A” is attributed to *Cryptococcus neoformans*, while in all other ITS types of *C. deneoformans* as well as *C. gattii* strains, the nucleotide is “G”. To reinforce this signature, the sequencing of the ITS1 region would contribute to differentiate between *C. neoformans* and the two others species.

The diagnosis of pulmonary cryptococcosis due to *C. neoformans* was based on histomorphological characteristics and molecular methods. Caprine cryptococcosis is considered uncommon in Brazil. Although, previous studies had associated it mainly with respiratory and neurological disorders (LUVIZOTTO et al., 2009; ROSA et al., 2016).

The epidemiology of cryptococcosis depends of the fungal species involved; although, it

is known that *C. neoformans* causes disease mainly in immunosuppressed individuals (RODRIGUES et al., 1999). Cryptococcosis in goats is occasionally reported in association with other agents, such as *Mycobacterium bovis* (GUTIÉRREZ & MARÍN, 1999) and *Corynebacterium pseudotuberculosis* (LUVIZOTTO et al., 2009). However, consistent lesions with those caused by these agents were not observed in this case. It is also known that stressful activities, such as transportation, can cause immune system impairment (ROSA et al., 2016). Although the immune status of this goat was not known, it had been subjected to prolonged transportation under conditions of food and water shortage, which probably facilitated the disease outcome. In addition, the hypothesis that the goat was infected before the trip and the stress reactivated the infection should be considered (BOLTON et al., 1999).

The clinical signs presented by the goat, as well as the gross and histological findings observed in the lungs, are similar to those described by other authors in domestic animals (GUTIÉRREZ & MARÍN, 1999; KOMMERS et al., 2005; LEMOS et al., 2007; LUVIZOTTO et al., 2009; GALIZA et al., 2014). In goats, the lungs appeared to be the major organs affected by cryptococcosis (BARÓ et al., 1998), and the lesions observed in this goat explain the dyspnea, cough and nasal discharge. Hydrothorax is probably associated with pneumonia. Inflammatory processes lead to an increased vascular permeability and intravascular hydrostatic pressure which may result in an active increase flow of blood into the microvasculature (hyperemia) and cause fluid

leakage (MOSIER, 2017). In contrast to other mycotic infections, the inflammatory reaction produced against *Cryptococcus* is usually minimal, because the capsule masks the yeast from recognition by phagocytes (CASWELL & WILLIAMS, 2016). However, some authors indicated that the inflammatory process may vary from scarce to accentuated (BOLTON et al., 1999; GALIZA et al., 2014), as observed in this case.

Morphological pattern of the fungus observed in the histopathological examination, characterized by blastoconidia with a slightly basophilic central cell, surrounded by an unstained capsule, gives the lesion a characteristic “soap bubble” appearance. This, associated with the histochemical characteristics obtained through the AB and PAS stains, are typical of fungi of the *Cryptococcus* genus (GUARNER & BRANDT, 2011; RIET-CORREA et al., 2011; GALIZA et al., 2014). These findings allowed the differentiation of other yeast fungi such as *Histoplasma capsulatum* and *Blastomyces dermatitidis*, as well as parasitic infections by *Mullerius capillaris* and *Dictyocaulus filaria* (CASWELL & WILLIAMS, 2016). Interstitial pneumonia, commonly associated with maedi-visna and caprine arthritis-encephalitis, was not observed in this goat as well as suppurative bronchopneumonia, classically associated with bacterial infections (CASWELL & WILLIAMS, 2016). It should be noted that there was no bacterial growth in the microbiological culture.

In our study, the comparative analysis between the nucleotide sequence of the fungal sample and the sequences deposited in Genbank revealed similarity with *C. neoformans*. *Cryptococcus* was not isolated and a panfungal PCR assay was necessary to confirm the identity. Negative samples (not containing the fungal elements or containing nonviable fungi) in mycological culture of tissue can occur in walled-off infections with endemic yeasts, such as in cryptococcosis, histoplasmosis or coccidioidomycosis (WHITE et al., 1990).

The identification of *C. neoformans* is described for the first time in Brazil as the cause of caprine cryptococcal pneumonia. This condition is uncommonly reported in this species; however, it should be considered as a differential diagnosis for goats with respiratory disease.

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DECLARATION OF CONFLICTING INTERESTS

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

AUTHORS' CONTRIBUTIONS

The authors contributed equally to the manuscript.

REFERENCES

- BARÓ, T. et al. First Identification of autochthonous *Cryptococcus neoformans* var. *gattii* isolated from goats with predominantly severe pulmonary disease in Spain. **Journal of Clinical Microbiology**, v.36, p.458-461, 1998. Available from: <<http://jcm.asm.org/content/36/2/458.full.pdf+html>>. Accessed: Nov. 10, 2017.
- BOLTON, L. A. et al. Cryptococcosis in captive cheetah (*Acinonyx jubatus*): two cases. **Journal of the South African Veterinary Association**, v.70, p.35-39, 1999. Available from: <<https://pdfs.semanticscholar.org/13f7/06190e444ed54f149a3f65c5c8c3f4434dbd.pdf>>. Accessed: Aug. 20, 2018.
- BOVERS, M. et al. Diversity of the *Cryptococcus neoformans-Cryptococcus gattii* species complex. **Revista Iberoamericana de Micología**, v.25, S4-S12, 2008. Available from: <<https://www.ncbi.nlm.nih.gov/pubmed/18338917>>. Accessed: Nov. 10, 2017.
- CASWELL, J. L. & WILLIAMS, K. J. Respiratory System. In: MAXIE, M. G. **Jubb, Kennedy, and Palmer's pathology of domestic animals**. 6th. Vol.2. St. Louis, Missouri: Elsevier, 2016. Cap.5, p.495-591.
- GALIZA, G. J. N. et al. Determining histomorphological and histochemical characteristics in the diagnosis of cryptococcosis in companion animals. **Pesquisa Veterinária Brasileira**, v.34, p.261-269, 2014. Available from: <<http://www.scielo.br/pdf/pvb/v34n3/11.pdf>>. Accessed: Nov. 10, 2017. doi: 10.1590/S0100-736X2014000300011.
- GUARNER, J.; BRANDT, M. E. Histopathologic diagnosis of fungal infections in the 21st century. **Clinical Microbiology Reviews**, v.24, p.247-280, 2011. Available from: <<https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3122495/pdf/zcm247.pdf>>. Accessed: Nov. 10, 2017. doi: 10.1128/CMR.00053-10.
- GUTIÉRREZ, M.; MARÍN, J. F. G. *Cryptococcus neoformans* and *Mycobacterium bovis* causing granulomatous pneumonia in a goat. **Veterinary Pathology**, v.36, p.445-448, 1999. Available from: <<https://www.ncbi.nlm.nih.gov/pubmed/10490212>>. Accessed: Nov. 10, 2017. doi: 10.1354/vp.36-5-445.
- HAGEN, F. et al. Recognition of seven species in the *Cryptococcus gattii/Cryptococcus neoformans* species complex. **Fungal Genetics and Biology**, v.76, p.16-48, 2015. Available from: <<https://www.sciencedirect.com/science/article/pii/S1087184515000328?via%3Dihub>>. Accessed: Nov. 10, 2017. doi: 10.1016/j.fgb.2015.02.009.
- KANG, Y. et al. New ITS genotype of *Cryptococcus gattii* isolated from an AIDS patient in Brazil. **Microbiology**

- and Immunology**, v.53, p.112-116, 2009. Available from: <<https://onlinelibrary.wiley.com/doi/epdf/10.1111/j.1348-0421.2008.00101.x>>. Accessed: Jan. 27, 2018. doi: 10.1111/j.1348-0421.2008.00101.x.
- KATSU, M. et al. The internal transcribed spacers and 5.85 rRNA gene show extensive diversity among isolates of the *Cryptococcus neoformans* species complex. *FEMS Yeast Research*, v.4, p.377-388, 2004. Available from: <<https://www.ncbi.nlm.nih.gov/pubmed/14734018>>. Accessed: Jan. 27, 2018. doi: 10.1016/S1567-1356(03)00176-4.
- KOMMERS, G. D. et al. Granulomatous cryptococcal pneumonia in a horse. *Ciência Rural*, v.35, p.938-940, 2005. Available from: <<http://www.scielo.br/pdf/cr/v35n4/a32v35n4.pdf>>. Accessed: Nov. 10, 2017. doi: 10.1590/S0103-84782005000400032.
- LEMOS, L. S. et al. Pulmonary cryptococcosis in slaughtered sheep: anatomopathology and culture. *Veterinary Microbiology*, v.125, p.350-354, 2007. Available from: <<https://www.ncbi.nlm.nih.gov/pubmed/17646062>>. Accessed: Nov. 10, 2017. doi: 10.1016/j.vetmic.2006.11.028.
- LUVIZOTTO, M. C. R. et al. Brain and lung cryptococcoma and concurrent *Corynebacterium pseudotuberculosis* infection in a goat: a case report. *Journal of Venomous Animals and Toxins including Tropical Diseases*, v.15, p.553-561, 2009. Available from: <<http://www.scielo.br/pdf/jvatitd/v15n3/a15v15n3.pdf>>. Accessed: Nov. 10, 2017. doi: 10.1590/S1678-191992009000300015.
- MOSIER, D. A. Vascular disorders and thrombosis. In: ZACHARY, J. F. **Pathologic basis of veterinary disease**. 6th. St. Louis, Missouri: Elsevier, 2017. Cap.2, p.44-72.
- PAULA, D. A. J. et al. Occurrence and molecular characterization of cryptococcosis in dogs and cats in Mato Grosso, Brazil. *Pesquisa Veterinária Brasileira*, v.34, p.167-172, 2014. Available from: <<http://www.scielo.br/pdf/pvb/v34n2/v34n2a12.pdf>>. Accessed: Nov. 10, 2017. doi: 10.1590/S0100-736X2014000200012.
- RIET-CORREA, F. et al. Bovine cryptococcal meningoencephalitis. *Journal of Veterinary Diagnostic Investigation*, v.23, p.1056-1060, 2011. Available from: <<http://journals.sagepub.com/doi/pdf/10.1177/1040638711416624>>. Accessed: Nov. 10, 2017. doi: 10.1177/1040638711416624.
- RODRIGUES, M. L. et al. Pathogenicity of *Cryptococcus neoformans*: virulence factors and immunological mechanisms. *Microbes and Infection*, v.1, p.293-301, 1999. Available from: <<https://www.ncbi.nlm.nih.gov/pubmed/10602663>>. Accessed: Nov. 10, 2017.
- ROSA, F. B. et al. Granulomatous leptomeningitis in a goat associated with infection by *Cryptococcus neoformans*. *Brazilian Journal of Veterinary Pathology*, v.9, p.98-102, 2016. Available from: <<https://pdfs.semanticscholar.org/3704/d70888738c81acf664917a193874571fcb2c.pdf>>. Accessed: Nov. 10, 2017.
- WHITE, T. J. et al. Amplication and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: INNIS, M. A. et al. (Eds). **PCR protocols: a guide to methods and applications**. Londres: Academic Press, 1990.