Systemic infection by *Spencermartinsiella* sp. in a Nile crocodile (*Crocodylus niloticus*)

Infecção sistêmica por Spencermartinsiella sp. em um crocodilo do Nilo (Crocodylus niloticus)

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Abstract
A male adult crocodile (*Crocodylus niloticus*) was diagnosed with systemic yeast infection. Histologically, there were extensive areas of necrosis in the lung, which were associated with a diffuse severe lympho-plasmo-histiocytic inflammatory infiltrate, with numerous multinucleated giant cells, and myriads of intralesional pseudo-hyphae and yeast like organisms within distended foveolae. Necrotic foci were also observed in the mucosa of the digestive tract, trachea, tunica intima of arteries, liver, and heart, with a marked inflammatory lympho-histiocytic infiltrate, with large numbers of epithelioid macrophages and giant cells, and intralesional and intravascular pseudo-hyphae and yeast-like organisms. Oval yeast structures with 4 to 6 µm in diameter and 5 to 8 µm thick paralleled-wall pseudo-hyphae were observed in PAS or GMS stained sections. PCR with DNA template extracted from paraffin embedded tissues amplified the D1/D2 domains of the large subunit rRNA gene, which was sequenced and found to be identical to sequences of a new species, isolated from rotting wood in Brazil, of the genus *Spencermartinsiella*, which its closest relative is *Spencermartinsiella cellulosicola*.
Keywords: Crocodile. *Crocodylus niloticus*. Candidiasis. *Spencermartinsiella* sp.

Resumo
Um crocodilo macho adulto (*Crocodylus niloticus*) foi diagnosticado com infecção fúngica sustêmica. Histologicamente, havia extensas áreas de necrose no pulmão, que estavam associadas com infiltrado inflamatório linfo-plasmo-histiocitário, com numerosas células gigantes multinucleadas e miríade de pseudo-hifas e organismos leveduriformes intralesionais, dentro de favéolas distendidas. Focos necróticos também foram observados na mucosa do trato digestório, traquéia, túnica íntima de artérias, fígado e coração, com acentuado infiltrado inflamatório linfo-histiocitário, com grande número de macrófagos epitelioides e células gigantes e hifas e organismos leveduriformes intralesionais e intravasculares. Cortes corados por PAS e GMS evidenciaram estruturas leveduriformes ovais com 4 a 6 µm de diâmetro e pseudo-hifas de paredes espessas e paralelas com 5 a 8 µm. PCR realizado com DNA extraído de material parafinizado amplificou os domínios D1/D2 da subunidade maior do gene rRNA, cuja sequencia foi idêntica a sequências de uma nova espécie, isolada no Brasil de madeira em decomposição, do gênero *Spencermartinsiella*, cuja espécie mais próxima é *Spencermartinsiella cellulosicola.*
Palavras-chave: Crocodilo. *Crocodylus niloticus*. Candidíase. *Spencermartinsiella* sp.

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Several yeast species are considered opportunistic pathogens of humans and animals. These yeasts are saprophytic and found on wet outer surfaces or mucosae of men and animals, including the skin, ear canal, conjunctival sac, mouth, digestive tract, as well as perianal, genital, and oral mucosae (CLEFF et al.,

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2005; JADHAV; PAL, 2006; BRITO et al., 2007). However, impairment of physical and/or immunological barriers may allow these opportunistic fungi to become pathogenic (MORETTI et al., 2004; BRITO et al., 2009). Fungal diseases are common in captive reptiles, particularly snakes, lizards, turtles, and crocodiles. These infections are often associated with environmental predisposing factors such as inadequate temperature, poor hygiene, and high humidity, but may also be associated with host primary conditions including immunosuppression, stress, parasitic or bacterial infections, and prolonged antibiotic therapy (MILDE et al., 2000; HARKEWICZ, 2002; SCHUMACHER, 2003, 2006; PARÉ; JACOBSON, 2007). Mycoses in reptiles commonly affect the skin, gastrointestinal tract and respiratory tract (SCHUMACHER, 2011). Conversely, systemic mycoses are rare in reptiles, often originating from the respiratory or gastrointestinal tract, and they are usually associated with opportunistic saprophytic fungi (SCHUMACHER, 2003), resulting in high mortality rates (MILDE et al., 2000; SCHUMACHER, 2006). Mycotic pneumonia is rarely reported in crocodiles, lizards, and snakes (SCHUMACHER, 2003). In crocodiles, *Fusarium* spp., *Candida* spp., and *Aspergillus* spp. are the main fungal pathogens causing dermatitis and pneumonia (SCHUMACHER, 2003). This report describes the pathological findings and diagnosis of systemic candidiasis in a Nile crocodile (*Crocodylus niloticus*) kept in captivity in Brazil.

A male 40-year-old Nile crocodile (*Crocodylus niloticus*), kept in captivity at the Zoological Garden in Belo Horizonte (Brazil), developed hyporexia by the end of 2013, and in February 2014, a material measuring 5 x 15 cm, predominantly whitish with red areas, which resembled a fibrinous exudate, was found on the premises. Samples of this material were processed for histopathology and it was confirmed to be an inflammatory and hemorrhagic exudate, presumably from the respiratory tract since there were no ingesta or fecal matter, and it was not heavily colonized by bacteria. The crocodile was then treated with penicillin (6,000,000 IU, IM, three doses every 72 h). Ten days after the treatment, the animal presented flotation difficulty. The animal was then treated with ceftazidime (1 gram, IM, three doses every 72 h), but did not respond well to treatment and died on April 30, 2014. Fragments of the lung, large vessels, digestive tract, trachea, liver, heart, testis, pancreas, thyroid gland, kidney and spleen were collected, fixed by immersion in 10% buffered formalin, and processed for histopathology. Four µm-thick sections were stained with hematoxylin and eosin. Additional selected tissue sections were stained with Gomori-Grocott methenamine silver (GMS) stain or periodic acid–Schiff (PAS).

Histologically, there were extensive areas of necrosis in the lung, associated with a granulomatous inflammatory reaction, consisting of a diffuse and severe lympho-plasmo-histiocytic infiltrate with numerous multinucleated giant cells (Figure 1A and 1B), and myriad intralesional pseudo-hyphae and yeast-like organisms (Figure 1C) within distended foveolae containing cellular debris and large numbers of giant cells, macrophages, and some heterophils. The trachea had diffuse mucosal necrosis with a heterophilic and histiocytic inflammatory infiltrate, and numerous giant cells, with numerous intralesional yeast and pseudo-hyphae (Figure 1D).

In the liver and large blood vessels there were multiple extensive areas of necrosis associated with granulomatous inflammation characterized by a lympho-plasmo-histiocytic infiltrate with numerous multinucleated giant cells. Similar focal and mild lesions were observed in the heart. Additionally, there were extensive areas of necrosis and ulceration in the digestive tract, with lympho-histiocytic infiltrate containing some multinucleated giant cells, which was associated with numerous intravascular pseudo-hyphae and yeast-like organisms and thrombosis.

Hepatocytes were diffusely vacuolated, with well-delimited cytoplasmic empty vacuoles that displaced the nuclei to the periphery of the cell, which was compatible with diffuse hepatic lipidosis. Macrophages containing intracytoplasmic brown pigment

(interpreted as hemossiderin) were observed in the liver and spleen. No microscopic lesions were observed in the pancreas, testes, thyroid gland, and kidneys.

GMS and PAS staining evidenced oval yeasts with 4 to 6 µm in diameter and pseudo-hyphae with parallel wall and 5 to 8 µm in diameter, which were abundant in areas with necrosis and granulomatous reaction (Figure 2).

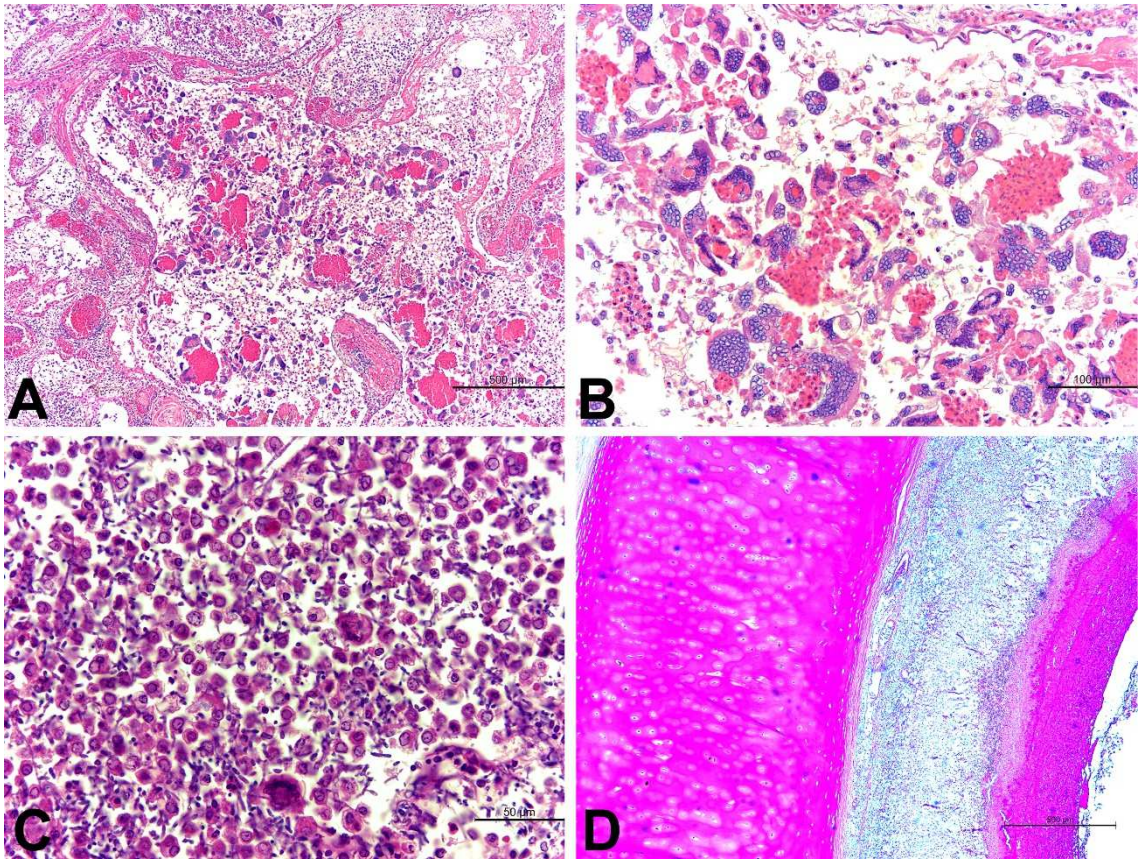

Figure 1 – Systemic infection by *Spencermartinsiella* sp. in a Nile crocodile (*Crocodylus niloticus*). (A) Severe granulomatous pneumonia; faveolae are filled with inflammatory cells and erythrocytes, bar = 500 µm. (B) Higher magnification of A with numerous histiocytes and multinucleated giant cells, and hemorrhage, bar = 100 µm. (C) Histiocytic infiltrate in the lung with a few multinucleated giant cells and myriad of pseudo-hyphae and yeast-like organisms, bar = 50 µm. (D) Trachea with extensive ulceration of the mucosa, which is covered with a fibrinous exudate, bar = 500 µm. Hematoxylin and eosin

DNA samples were extracted from formalin-fixed paraffin-embedded tissues, including lung, trachea, liver, and digestive tract. The ribosomal cluster region spanning the internal transcribed spacers (ITS), including the 5.8S rRNA gene, and the D1/D2 domains of the large subunit rRNA gene was amplified by PCR using the primers ITS1 and NL4 (TCCGTAGGTGAACCTGCGG and GGTCCGTGTTTCAAGACGG, respectively), as previously described (LACHANCE et al., 1999). Sequencing was performed using an ABI3130 capillary electrophoresis apparatus, using BigDye v3.1 and POP7 polymer. The sequences were assembled, edited and aligned with the program MEGA6 (TAMURA et al., 2013). The sequences obtained were compared with those included in the GenBank database using the Basic Local Alignment Search Tool (BLAST at http://www.ncbi.nlm.nih.gov/) (ALTSCHUL et al., 1990). After alignments with sequences in the EMBL GenBank database, was found to be identical (100% identity) to sequences of the new yeast species *Spencermartinsiella* sp. UFMG-CM-Y454 (GenBank

accession number KT033723). The closest relative of this new species is *Spencermartinsiella cellulosicola* (GenBank accession number HM151016). These species differed by four substitutions in D1/D2 sequences and six substitutions and 32 indels in the ITS sequences. This new *Spencermartinsiella* species was isolated from rotting wood collected in an Atlantic rainforest site of the Ecological Reserve of the Rio Doce State Park, in State of Minas Gerais, Brazil (M. R. Lopes and C. A. Rosa, unpublished data). This species and the other *Spencermartinsiella* species are frequently isolated from rotting wood samples, and are considered saprophytic yeasts living from the sugars released during wood decomposition (MORAIS et al., 2016). *Spencermartinsiella* spp. have pseudo-hyphae, blastoconidia and budding cells (yeast). In histological sections, this organism is morphologically identical to *Candida* sp. Indeed, *Candida cellulosicola* has recently been reassigned to the genus *Spencermartinsiella* (MORAIS et al., 2016). Importantly, *Spencermartinsiella* spp. have not been previously recognized as human or animal pathogens. The crocodile was likely infected by this saprophytic yeast when in contact with rotting plant materials.

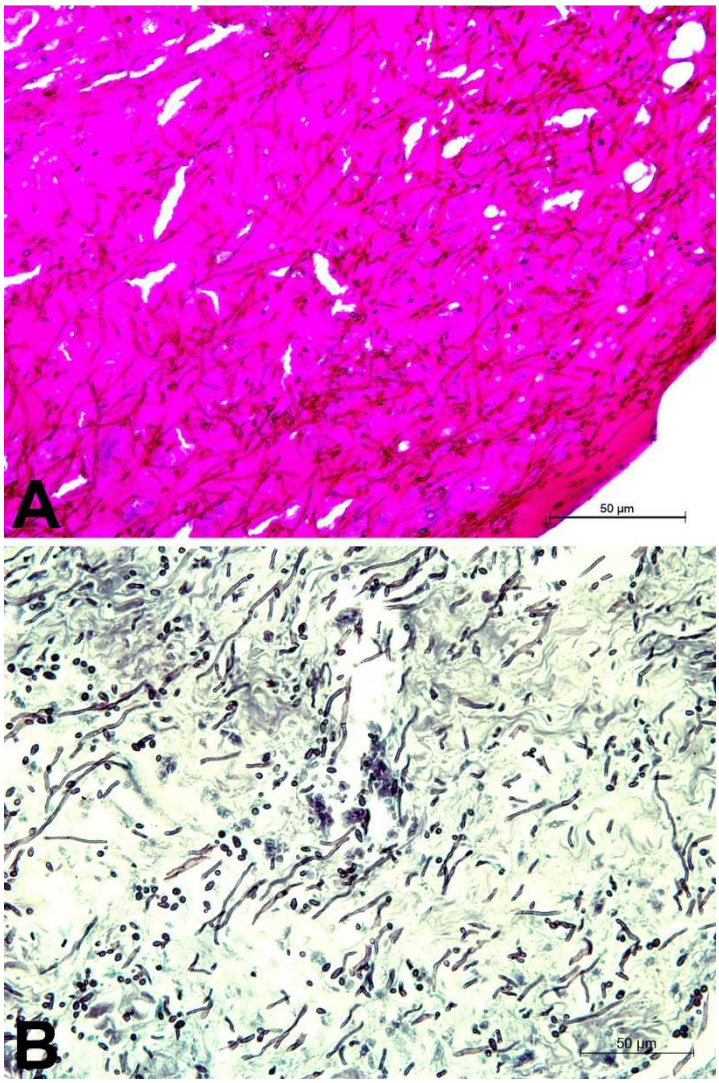

Figure 2 – Systemic infection by *Spencermartinsiella* sp. in a Nile crocodile (*Crocodylus niloticus*). PAS (A) and GMS (B) stained tracheal exudate demonstrating numerous pseudo-hyphae and yeast-like organisms, bar = 50 µm

In this case, morphological and molecular findings clearly demonstrated that the crocodile developed systemic infection with *Spencermartinsiella* sp. According to Dupont (2002), detection of fungal organisms in histological sections requires special staining techniques, including GMS, Gridley's fungus, and PAS. However, although these staining techniques favor morphological observation of mycotic organisms, they do not suffice for species identification. Therefore, molecular methods are a suitable alternative for species identification in formalin-fixed and paraffin-embedded tissues. Particularly, PCR amplification of ribosomal DNA genes (rDNA) and/or intervening internal transcriber spacer (ITS) regions followed by sequencing in most cases allow identification of the organism associated with the lesion (HENDOLIN et al., 2000; CHEN et al., 2001; SCUPHAM et al., 2006; LAU et al., 2007; MANTER; VIVANCO, 2007). The ITS region sequences, especially the ITS2 region, are of great importance to differentiate clinically relevant yeast species (LEAW et al., 2006). Identification of the etiologic agent is essential for a correct diagnosis and, whenever possible, establishment of a proper antifungal therapy (MILDE et al., 2000; HARKEWICZ, 2002). Culture and isolation tests are time-consuming, but most importantly, fungal isolation is not doable when only formalin-fixed samples are available, as in this case. Indeed, ribosomal DNA amplification and sequencing has been successfully applied in this case to confirm the etiologic diagnosis, as previously reported (MILDE et al., 2000; CHANG et al., 2001; KHLIF et al., 2007).

Fungal infections in reptiles are considered more common in herbivore rather than carnivore reptiles (KOSTKA et al, 1997; MILDE et al, 2000). Under captive conditions, fungal diseases are common among snakes, lizards, turtles and crocodiles (SCHUMACHER, 2003; SCHUMACHER, 2006). Systemic yeast infections have been previously reported in reptiles, particularly in turtles (turtles, tortoises, terrapins) (ORÓS et al., 2004), chameleons and lizards (SCHUMACHER, 2006). *Candida albicans* have been isolated from a case of esophagitis and muscle necrosis in a *Crocodilus lacerinus* (ZWART et al., 1968). The histopathological findings in this case, namely granulomatous pneumonia and ulcerative enteritis, are consistent with fugal lesions in reptiles as previously reviewed (KOSTKA et al., 1997).

Yeasts are part of the skin and mucosal microbiota of various hosts, but it may become pathogenic due to impairment of physical or immune host barriers (MORETTI et al., 2004; BRITO et al., 2009). In this case, advanced age and a previous respiratory infection/inflammation may have likely resulted in immunosuppression. Furthermore, repeated antibiotic treatments may also have favored infection in this case due to suppression of the commensal microbiota. Indeed, systemic yeast infection usually affects immunocompromised individuals (BARBEDO; SGARBI, 2010). Histologically, yeast and hyphae forms were both observed in various tissues in this case. The yeast form is present in colonization in a healthy host, whereas hyphae tend to develop in cases of deficiency of the immune system, although both forms are important in the pathogenesis (BROWN; GOW, 1999; BARBEDO; SGARBI, 2010).

In conclusion, histological and molecular findings in this case confirmed the diagnosis of necrotizing and granulomatous pneumonia, tracheitis, arteritis, hepatitis, myocarditis, gastritis/enteritis, associated with *Spencermartinsiella* sp. infection. This is the first report of a yeast species of the genus *Spencermartinsiella* as the causative agent of infection in reptiles.

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