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ERICK PLATINÍ FERREIRA DE SOUTO

ESTUDO COMPARATIVO DE CASOS DE PITIOSE EM EQUÍDEOS, RUMINANTES, CARNÍVOROS E AVE NO NORDESTE DO BRASIL

PATOS/PB
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Tese apresentada ao Programa de Pós-Graduação em Ciência e Saúde Animal da Universidade Federal de Campina Grande como requisito parcial para a obtenção do título de Doutor em Ciência e Saúde Animal

Prof. Dr. Antonio Flávio Medeiros Dantas
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RESUMO

Descrevem-se nesta tese quatro artigos científicos que abordam a pitiose em animais domésticos no Nordeste do Brasil. O primeiro capítulo reúne e comparativamente descreve os casos de pitiose em equídeos, ruminantes, carnívoros e aves acometidos na região Nordeste do Brasil durante um período de 35 anos de diagnóstico no Laboratório de Patologia Animal da Universidade Federal de Campina Grande. Durante o período de estudo foram recebidas 13.542 amostras teciduais provenientes de necropsias e biópsias. Das quais, 306 foram diagnosticados como pitiose: 195 casos em equinos, 75 em ovinos, 19 em cães, 6 em muares, 4 em bovinos, 3 em gatos, 2 em caprinos, 1 em asinino e 1 em avestruz. Os equídeos foram as espécies mais afetadas, com lesões na pele, glândula mamária e fossas nasais. Dentre os ruminantes, os ovinos foram os mais afetados, com lesões cutâneas, nasais e digestivas, enquanto os bovinos e caprinos apresentaram lesões cutâneas. Os carnívoros desenvolveram lesões principalmente no trato alimentar, suficientemente graves para determinar a morte ou eutanásia dos animais. O único caso em ave afetou o trato alimentar e a excisão cirúrgica determinou a remissão. Para a maioria dos animais, a doença teve um curso clínico crônico e potencialmente grave, exceto para os bovinos que apresentaram cura espontânea das lesões. Os sinais clínicos estavam diretamente relacionados à localização das lesões, que sempre apresentavam evidência histológica de inflamação crônica associada a hifas intralesionais. O segundo capítulo caracteriza os aspectos epidemiológicos, clínicos, patológicos e os principais métodos de diagnóstico da pitiose em equinos, muares e asininos. Foram recebidas 1.331 amostras teciduais de equídeos, das quais 202 (15,17%) foram diagnosticadas como pitiose. Equídeos de ambos os sexos e com idades variando de 4 meses a 25 anos foram afetados. A maioria dos animais era mestiço (79,7%) e criado em sistema extensivo (73,26%). A doença ocorreu durante todo o ano, mas a maior incidência (70,29%) foi observada após o período das chuvas. O curso clínico foi sempre crônico. As lesões localizavam-se preferencialmente nos membros e parede toracoabdominal ventral e caracterizavam-se por nódulos ou massas com ulcerações e secreção serossanguinolenta. A superfície de corte demonstrou tratos fistulosos contendo *kunkers*. A histopatologia revelou acentuado infiltrado inflamatório de eosinófilos com áreas multifocais bem definidas de necrose de eosinófilos e colagenólise e imagens negativas de hifas intralesionais. No asinino, observou-se infiltrado inflamatório piogranulomatoso circundando essas áreas. A imuno-histoquímica para *Pythium insidiosum* revelou forte imunomarcação das hifas. O terceiro capítulo descreve três casos de pitiose em gatos, caracterizando os aspectos epidemiológicos, clínicos e patológicos. Foram recebidas 1.928 amostras teciduais de gatos, das quais três foram diagnosticadas como pitiose. Macroscopicamente, os gatos apresentavam uma massa multinodular na cavidade oral associada a deformidade facial (caso 1), uma grande massa multinodular espessando a parede do jejuno (caso 2) e um nódulo cutâneo ulcerado na base da cauda (caso 3). Histologicamente, observou-se inflamação piogranulomatosa e necrose, com hifas intralesionais, predominantemente não coradas. A imuno-histoquímica para *Pythium insidiosum* revelou forte imunomarcação das hifas. O quarto capítulo relata um caso de pitiose esofágica em um avestruz, caracterizando os aspectos epidemiológicos, clínicos, patológicos e imuno-histoquímicos. Uma avestruz-do-pescoço-vermelho, com dois anos de idade, apresentava um nódulo no terço médio do esôfago. Na histologia observou-se uma área focalmente extensa de necrose estendendo-se da túnica mucosa à adventícia. Circundando a área de necrose, observou-se uma reação inflamatória composta principalmente por granulócitos e macrófagos, associada à fibroplasia e neovascularização. Em meio às áreas de necrose e inflamação, verificavam-se numerosas imagens negativas de hifas em seções longitudinais e transversais, melhor apreciadas pela coloração de metenamina nitrato de prata de Grocott. O diagnóstico definitivo de infecção por *Pythium insidiosum* foi confirmado por imuno-histoquímica. A pitiose é uma doença endêmica no Nordeste do Brasil. Equinos, ovinos e cães parecem ser os mais comumente afetados, mas é importante estar ciente de que a doença pode afetar outras espécies animais. Os médicos veterinários, clínicos e patologistas, devem estar familiarizados com as características clinicopatológicas da doença e com a ampla variedade de espécies animais suscetíveis.

Palavras-chaves: Doenças infecciosas; oomiceto; *Pythium insidiosum.*

ABSTRACT

We describe in this thesis four scientific papers that address pythiosis in domestic animals in northeastern Brazil. The first chapter gathers and comparatively describes pythiosis cases in equidae, ruminants, carnivores and birds affected in the northeastern region of Brazil over a 35-year period of diagnosis at the Animal Pathology Laboratory of the Federal University of Campina Grande. During the study period, 13,542 tissue samples from necropsies and biopsies were received. Among these samples, 306 were diagnosed as pythiosis: 195 cases in horses, 75 in sheep, 19 in dogs, 6 in mules, 4 in cattle, 3 in cats, 2 in goats, 1 in a donkey, and 1 in an ostrich. The equidae were the most affected species, with lesions in the skin, mammary glands and nasal cavities. Among ruminants, sheep were the most affected, with cutaneous, nasal and digestive lesions, while cattle and goats had cutaneous lesions. Carnivores developed lesions mainly in the alimentary tract, severe enough to determine the animals' death or euthanasia. The single case in a bird affected the alimentary tract and surgical excision determined remission. For most animals, the disease had a long-term and life-threatening clinical course, except for cattle that had spontaneous healing. The clinical signs were directly related to the location of the lesions, which always presented histological evidence of chronic inflammation associated with intralesional hyphae. The second chapter characterizes the epidemiological, clinical and pathological aspects and the main diagnostic techniques for pythiosis in horses, mules and donkeys. Were received 1,331 tissue samples of equidae, 202 (15.17%) of which were diagnosed as pythiosis. Equidae of both sexes with ages varying from 4 months to 25 years were affected. Most animals were mixed breed (79.7%) and reared in an extensive system (73.26%). The disease occurred throughout the year but the highest incidence (70.29%) was noted after the rainy season. The clinical course was always chronic. The lesions were preferentially located on the limbs and ventral thoracoabdominal wall and characterized by nodules or tumor-like masses with ulcerations and serosanguineous discharge. The cut surface showed fistulous tracts containing *kunkers*. Histopathology revealed a marked inflammatory infiltrate of eosinophils with multifocal well-defined areas of eosinophil necrosis and collagenolysis and intralesional negatively-stained hyphal profiles; in the donkey, a pyogranulomatous inflammatory infiltrate was noted surrounding these areas. Immunohistochemistry for *Pythium insidiosum* revealed strong immunolabelling of the hyphae. The third chapter describes three cases of pythiosis in cats, characterizing the epidemiological, clinical, and pathological findings. Were received 1,928 tissue samples of cats, three of which were diagnosed as pythiosis. Grossly, the cats showed a multinodular mass in the oral cavity associated with facial deformity (case 1), a large multinodular mass thickening the jejunum wall (case 2), and an ulcerated nodule in the skin at the base of the tail (case 3). Histologically, pyogranulomatous inflammation and necrosis, with intralesional predominantly negatively stained hyphae, were observed in all cases. Immunohistochemistry for *Pythium insidiosum* revealed strong immunolabelling of the hyphae. The diagnosis of pythiosis was based on the epidemiological, clinical and anatomopathological findings, and was confirmed by immunohistochemistry. The fourth chapter reports a case of esophageal pythiosis in an ostrich, characterizing the epidemiological, clinical, pathological, and immunohistochemical aspects. A 2-year-old female red-necked ostrich presented with a nodular mass in the middle third of the esophagus. Histologically, there was a focally extensive area of necrosis extending from the tunica mucosa to the adventitia. Surrounding the necrotic area were a dense inflammatory reaction composed mainly of granulocytes and macrophages and associated with fibroplasia and neovascularization. In some inflammatory and necrotic areas, there were numerous longitudinal and cross sections of negatively stained hyphae, which were best visualized using Grocott methenamine silver stain. Definitive diagnosis of infection by *Pythium insidiosum* was confirmed by immunohistochemistry. Pythiosis is an endemic disease in northeastern Brazil. Horses, sheep, and dogs seem to be most commonly affected, but it is important to be aware that the disease can affect other animal species. Veterinary clinicians and pathologists should be familiar with the clinicopathological features of the disease and the wide range of susceptible animal species.

Keywords: Infectious diseases; oomycete; *Pythium insidiosum.*

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1. INTRODUÇÃO

O gênero *Pythium*, pertencente à classe dos oomicetos, possui mais de 120 espécies distribuídas em todo planeta, sendo a maioria patógenos de plantas (Santurio et al., 2006; Gaastra et al., 2010). As espécies *Pythium insidiosum* e, mais recentemente, *Pythium aphanidermatum* (Calvano et al., 2011), são as únicas reconhecidamente capazes de causar doença em organismos animais.

Considera-se que para haver a manutenção do ciclo biológico do agente são necessários um ambiente aquático, contendo substrato orgânico, e temperaturas variando entre 30 e 40º C (Chaffin et al., 1995). Nessas condições, desenvolvem-se os zoósporos biflagelados móveis, forma infectante capaz de invadir plantas e tecidos de animais (Alexopoulos et al., 1996). A porta de entrada utilizada pelo agente determina a localização das lesões e resulta em diferentes formas clínicas da doença (Gaastra et al., 2010).

A pitiose já foi descrita em muitas espécies animais, domésticas e silvestres, e também no homem (Gaastra et al., 2010). Dentre os animais domésticos, a espécie equina é a mais frequentemente acometida (Leal et al., 2001; Sallis et al., 2003; Tabosa et al., 1999). Observam-se principalmente lesões cutâneas que acometem, mais frequentemente, os membros e a porção ventral da parede tóracoabdominal (Santurio et al., 2006). As lesões caracterizam-se por grandes massas teciduais, de aspecto tumoral, com bordos irregulares e ulcerações, que usualmente drenam secreção serosanguinolenta. No interior dessas massas observam-se trajetos fistulosos contendo estruturas amarelo-acinzentadas, irregulares, ramificadas e com aspecto arenoso (*kunkers*), que consistem em áreas centrais de necrose de eosinófilos contendo hifas intralesionais (Gaastra et al., 2010). Nessa forma cutânea da doença, já foi observado o envolvimento secundário dos linfonodos (Chaffin et al., 1995; Martins et al., 2012) e do tecido ósseo (Mendoza et al., 1988; Alfaro e Mendoza, 1990; Eaton, 1993) adjacentes. Tendo sido descrita ainda, nessa espécie, as formas intestinal (Allison e Gillis, 1990; Morton et al., 1991; Purcell et al., 1994), pulmonar (Goad, 1984) e nasal (Souto et al., 2016).

Os caninos são a segunda espécie mais comumente afetada. A forma gastrintestinal é a mais comum nessa espécie e caracteriza-se pela formação de grandes massas nas paredes gastrintestinais, manifestadas clinicamente como distúrbios digestivos (Miller et al., 1983; Fischer et al., 1994; Martins et al., 2012). A forma cutânea da doença, em contraste ao que se observa em equinos, ocorre menos frequentemente nessa espécie (Gaastra et al., 2010; Dykstra et al., 1999). As lesões consistem em inflamações granulomatosas ou piogranulomatosas associadas à necrose e hifas intralesionais (Martins et al., 2012).

Os ovinos têm mais frequentemente as cavidades nasais comprometidas. As lesões usualmente se estendem da região mucocutânea do plano nasal às cavidades nasais, podendo ocorrer deformidade nasal, parcial oclusão das narinas, fistulas oro-nasais e drenagem de secreções serossanguinolentas (Ubiali et al., 2013). As lesões consistem em massas necróticas que na histopatologia são vistas como infiltrado inflamatório piogranulomatoso com áreas centrais de necrose contendo hifas intralesionais circundadas por reação de *Splendore-Hoeppli* (Ubiali et al., 2013; Santurio et al., 2008). Em surtos de

pitiose ocorridos em ovinos no Sertão paraibano, alguns animais apresentaram lesões cutâneas nos membros, abdome e região pré-escapular (Tabosa et al., 2004). Tendo sido também descrita a infecção do trato digestivo de cordeiros (Pessoa et al., 2012).

Nos bovinos a pitiose manifesta-se como doença cutânea, geralmente na região distal dos membros. As lesões cutâneas caracterizam-se por áreas de ulcerações, espessamento da derme e edema na região afetada (Santurio et al., 1998; Pérez et al., 2005; Gabriel et al., 2008; Grecco et al., 2009). Na histopatologia, as lesões apresentaram-se como granulomas ou piogranulomas circundados por tecido conjuntivo fibroso. As hifas apresentam-se predominantemente confinadas ao interior dessas reações e, por vezes, desintegradas (Martins et al., 2012). Os casos observados apresentaram cura espontânea das lesões (Gabriel et al., 2008; Grecco et al., 2009).

A pitiose é ocasionalmente descrita em outras espécies domésticas, como gatos (Thomas e Lewis, 1998; Rakich et al., 2005), asininos (Maia et al., 2016; Alvarez et al., 2013), muares (Tabosa et al., 1999) e caprinos (Do Carmo et al., 2015), e, de forma ainda menos frequente, em animais silvestres (Gaastra et al., 2010). No homem, a maioria dos casos da doença foi observada na Tailândia, com casos pontuais nos Estados Unidos da América, Austrália, Haiti, Nova Zelândia e Brasil (Santurio et al., 2006).

Os muitos relatos de casos de pitiose em animais, sob a forma de casos individuais e surtos, comprovam a provável existência da doença em todas as regiões do Brasil (Santurio et al., 2006). Especificamente na região Nordeste, a doença vem sendo diagnosticada em diferentes espécies animais e, por vezes, sob apresentações clinicopatológicas incomuns ou nunca previamente descritas. Diante dessa excepcional e diversificada casuística faz-se necessário reunir e descrever comparativamente os casos de pitiose em equídeos, ruminantes, carnívoros e aves, com o objetivo de caracterizar os principais aspectos epidemiológicos, clínicos e anatomopatológicos.

Desta forma, esta tese está dividida em quatro capítulos com os artigos científicos formatados de acordo com as normas dos periódicos aos quais foram submetidos, conforme o que estabelece a NORMA Nº 01/2013 de 09 de julho de 2013 do Programa de Pós-Graduação em Ciência e Saúde Animal da Universidade Federal de Campina Grande, como parte dos requisitos para obtenção do título de Doutor em Ciência e Saúde Animal. O primeiro capítulo reúne e comparativamente descreve os casos de pitiose em equídeos, ruminantes, carnívoros e aves acometidos na região Nordeste do Brasil durante um período de 35 anos de diagnóstico. O artigo foi submetido à revista *Research in Veterinary Science*. O segundo capítulo caracteriza a pitiose em equinos, muares e asininos abordando os aspectos epidemiológicos, clínicos, patológicos e os principais métodos de diagnóstico. O artigo foi aceito para publicação no *Journal of Equine Veterinary Science.* O terceiro capítulo descreve três casos de pitiose em gatos, caracterizando os aspectos epidemiológicos, clínicos e patológicos. O artigo foi publicado no *Journal of Medical Mycology*. O quarto capítulo relata um caso de pitiose esofágica em um avestruz na mesorregião do Sertão, Nordeste do Brasil, caracterizando os aspectos epidemiológicos, clínicos, patológicos e imuno-histoquímicos. O artigo foi publicado no Arquivo Brasileiro de Medicina Veterinária e Zootecnia.

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CAPÍTULO I

Pythiosis in equidae, ruminants, carnivores, and bird in northeastern Brazil: 306 cases

O presente trabalho foi submetido à revista *Research in Veterinary Science* (anexo 1).

Pythiosis in equidae, ruminants, carnivores, and bird in northeastern Brazil: 306 cases

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ABSTRACT: The epidemiological, clinical and pathological findings in naturally occurring cases of pythiosis in domestic animals in northeastern Brazil are described. From January 1985 to December 2020 the Laboratory of Animal Pathology of the Federal University of Campina Grande examined 13,542 tissue samples from necropsies examinations and biopsies. Among these samples, 306 were diagnosed as pythiosis: 195 cases in horses, 75 in sheep, 19 in dogs, 6 in mules, 4 in cattle, 3 in cats, 2 in goats, 1 in a donkey, and 1 in an ostrich. The affected animals were allocated into four groups: equidae, ruminants, carnivores and birds. The equidae were the most affected species, with lesions in the skin, mammary glands and nasal cavities. Among ruminants, sheep were the most affected, with cutaneous, nasal and digestive lesions, while cattle and goats rarely had cutaneous lesions. Carnivores developed lesions mainly in the alimentary tract, severe enough to determine the animals' death or euthanasia. The single case in a bird affected the alimentary tract and surgical excision determined remission. The disease had a long-term and life-threatening clinical course for most animals, except for cattle that had spontaneous healing. The clinical signs were directly related to the location of the lesions, which always presented histological evidence of chronic inflammation associated with intralesional hyphae. Pythiosis is an endemic disease in northeastern Brazil. Horses, sheep, and dogs seem to be most commonly affected, but it is important to be aware that the disease can affect other animal species. Veterinary clinicians and pathologists should be familiar with the clinicopathological features of the disease and the wide range of susceptible animal species.

Key words: Domestic animals, infectious disease, oomycete, hyphae, *Pythium insidiosum*.

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1. Introduction

Pythiosis is an inflammatory and frequently life-threatening disease caused by the oomycete *Pythium insidiosum* (kingdom Stramenopila, family Pythiaceae) that affects several animal species and occasionally humans (Gaastra et al., 2010).

The infection occurs mainly in tropical, subtropical, and temperate areas of the world and clinical cases have been observed in several countries of Southeast Asia, South America, North America, Central America and the Caribbean islands. Only a few cases have been reported in Europe and Africa (Gaastra et al., 2010; Santurio et al., 2006).

In South America, Brazil accounts for the highest incidence of pythiosis in animals. In fact, the Brazilian Pantanal area has the highest incidence and prevalence of equine pythiosis in the world (Santurio et al., 2006). Similarly, northeastern Brazil has numerous cases of equine pythiosis (Tabosa et al., 1999) but the disease has been diagnosed frequently in other animal species, including sheep (Tabosa et al., 2004; Portela et al., 2010), dogs (Frade et al., 2017), cattle (Maia et al., 2020), goat (Do Carmo et al., 2014), donkey (Maia et al., 2012) and ostrich (Souto et al., 2019).

In addition to the diversity of susceptible animals stands out the multiple clinical forms, manifestations and outcomes of the disease. Therefore, the present paper gathers and comparatively describes the epidemiological, clinical, and anatomopathological findings of pythiosis in different animal species affected in northeastern Brazil.

2. Material and methods

A retrospective study was carried out in all biopsy and necropsy samples of equidae, ruminants, carnivores and birds, from January 1985 to December 2020, at the Animal Pathology Laboratory of the Federal University of Campina Grande, Patos, Paraiba, northeastern Brazil.

Epidemiological data, clinical signs and gross lesions were reviewed from the reports. Samples of the skin, lymph nodes, central nervous system and organs from the thoracic and abdominal cavities were fixed in 10% buffered formalin, processed routinely, embedded in paraffin wax and cut into 3 µm sections. The sections were stained with hematoxylin and eosin (HE), periodic acid-Schiff (PAS) and Grocott's methenamine silver (GMS).

Immunohistochemistry (IHC) was performed for the identification of the agent. The IHC protocol proposed by Martins et al. (2012) with modifications was performed using a rabbit polyclonal anti–*P. insidiosum* antibody (non-commercial). Briefly, sections were dewaxed and rehydrated and endogenous peroxidase activity was blocked with H2O2 3% in distilled water. Antigen retrieval was by microwaving (10 min at full power) in TRIS-EDTA (pH 9.0). Sections were incubated at 37º C for 60 min with the primary antibody diluted at 1:800 (equidae) or 1:1,000 (carnivores, ruminants and bird). The secondary reagent was a polymer-HRP followed by chromogen 3.3′-diaminobenzidine (DAB; Sigma-Aldrich, Saint Louis, Missouri, USA). Sections were counterstained with Harris hematoxylin and coverslipped. As a positive control, histological sections from a confirmed case of equine pythiosis were used. Sections from the same equine were used as negative controls, with the primary antibody replaced by phosphate buffered saline containing 0.5% polysorbate 20.

Samples of the lesions were collected for microbiological culture and identification of *P. insidiosum*. The samples were washed in distilled water, implanted into 2% dextrose Sabouraud agar plates, and incubated at 37° C for 48 hours under anaerobic conditions. The slides were stained with lactophenol cotton-blue with subsequent visualization of hyphae by light microscopy (LM).

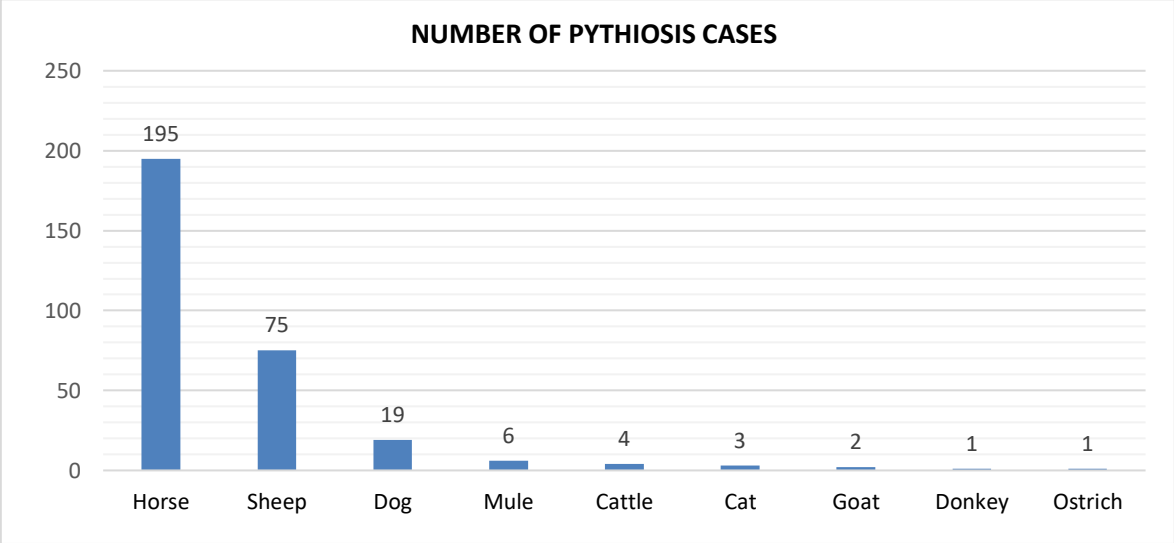
DNA extraction was performed using paraffin embedded tissue samples, after heating in alkaline pH buffer, followed by phenol–chloroform extraction, ethanol precipitation, and

resuspension in ultrapure water, as previously described (Shi et al. 2004). PCR was performed using primers targeting PI1 (5′-TTCGTCGAAGCGGACTGCT-3′) and PI2 (5′-GCCGTACAACCCGAGAGTCATA-3′), which amplified a 105-bp fragment for the first ribosomal internal transcribed spacer region (ITS1) DNA of *P. insidiosum*. DNA extracted from paraffin-embedded tissue samples from a confirmed case of equine pythiosis was used as positive control. Ultrapure water was used as negative control.

3. Results

During the study period, we received 13,542 tissue samples from necropsy examinations and biopsy sampling. Among these, 306 were diagnosed as pythiosis. The number of pythiosis cases per affected animal species is available in Graph 1.

Graph 1. Pythiosis cases in different animal species in northeastern Brazil (1985-2020).



The affected animals were allocated into four groups: equidae, ruminants, carnivores, and birds; and the main epidemiological, clinical and anatomopathological findings are summarized in Table 1.

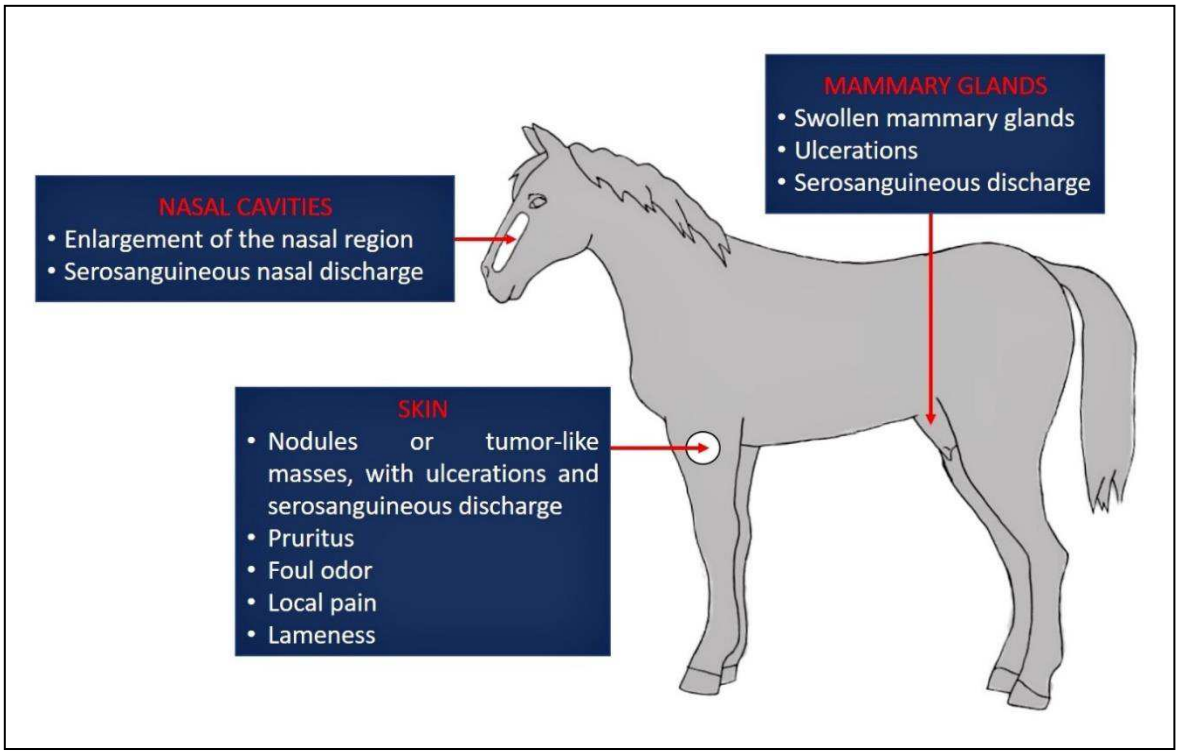
3.1 Equidae

During the study period, we received 1,331 tissue samples from necropsy examinations and biopsy sampling in equidae, of which 1,051 of horses, 218 of donkeys and 62 of mules. Among these samples, 202 were diagnosed as pythiosis (195 cases in horses, 6 in mules and 1 in donkey), which represents 15.17% of the diseases diagnosed in equidae.

Equidae of both sexes, female (114 [56.4%]) and male (88 [43.6%]), were affected. The age ranged from 4 months to 25 years, mean of 6.3 years old. Most animals were mixed breed (161 [79.7%]) but purebred (41 [20.3%]) were also affected. Regarding the breeding system, most animals were raised in an extensive system (148 [73.26%]), with free access to local dams (80 [39.6%]). The disease had the highest incidence (142 cases [70.29%]) after the rainy season (May to October).

The clinical course was always chronic and ranged from 14 days to 24 months, with an average of 68 days. Affected animals had lesions in the skin (197/202), mammary glands (3/202) and nasal cavities (2/202). The clinical signs were directly related to the location of the lesions, as shown in Illustration 1.

Illustration 1. Clinical forms and corresponding clinical signs in equidae with pythiosis



Cutaneous lesions were preferentially located on the limbs and the ventral aspect of the abdomen and chest, in most cases as single lesions (169 cases [83.66%]). Gross lesions were characterized by nodules or tumor-like masses, measuring 4 to 50 cm in diameter, with ulcerations, irregular margins and serosanguineous discharge (Fig. 1A and B). Lesions in the mammary glands were characterized by udder enlargement with multifocal areas of ulceration, measuring 1 to 3.5 cm in diameter, and serosanguineous discharge. The cut surface showed fistulous tracts and irregular cavitations containing several yellow-gray irregular firm necrotic masses (*kunkers*), which easily detached from the lesion (Fig. 1C and D).

Lesions in the nasal cavities were characterized by bilateral enlargement of the nasal region and serosanguineous nasal discharge. The mid-sagittal section of the head revealed *kunkers* in the nasal vestibule (rhinofacial form) or nasal turbinates and meatuses (rhinopharyngeal form) (Fig. 1E and F).

Histopathology of lesions in horses and mules revealed a marked inflammatory infiltrate of eosinophils, with multifocal to coalescent well-defined areas of eosinophil necrosis and collagenolysis, surrounded by intact eosinophils, macrophages, neutrophils, and occasional lymphocytes and plasma cells. Within the necrotic areas, often distributed at the periphery, there were longitudinal and transverse sections of tubuliform structures not stained by HE (negatively-stained hyphal profiles). Peripherally, marked proliferation of fibroblasts and collagen fibers and neovascularization (immature granulation tissue) was observed. The epidermis was often ulcerated and covered by cellular debris, dried fibrin, and bacteria.

In the donkey, there were multifocal pyogranulomas, with central areas of eosinophil necrosis surrounded by inflammatory infiltrate of eosinophils, lymphocytes, plasma cells, macrophages, and multinucleated giant cells, occasionally delimited by proliferation of fibroblasts and collagen fibers. Within the necrotic areas, negatively-stained hyphal profiles, sometimes surrounded by small

amounts of *Splendore-Hoeppli* reaction, were noted. The epidermis was ulcerated and covered by cellular debris.

Of the 202 affected equidae, 182 were treated with surgical excision of lesions, and 168 out of 182 (92.3%) achieved complete remission; in 14 cases out of 182 (7.7%) the lesions were severe enough to make the treatment unfeasible and the animal was euthanized. In 10 animals, complete remission of the lesions was achieved with the corticosteroid triamcinolone acetonide; and in 10 cases, no data were available regarding treatment and clinical outcome.

3.2 Ruminants

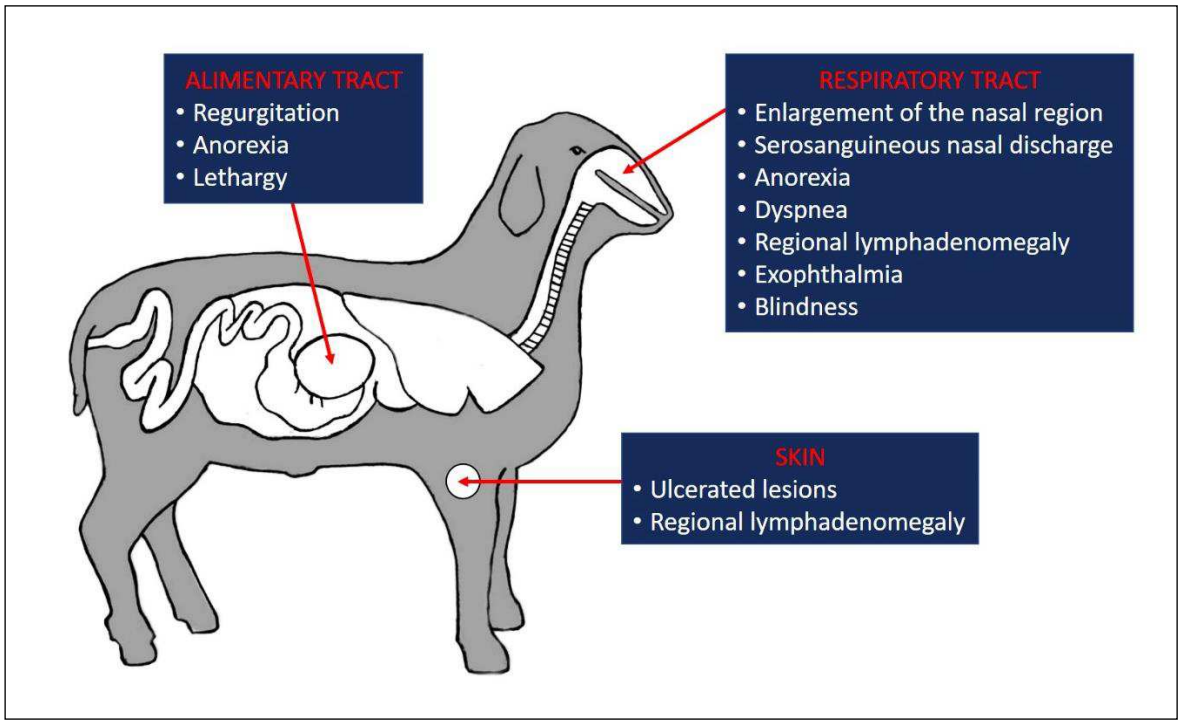
During the study period, we received 4,476 tissue samples from necropsy examinations and biopsy sampling in ruminants, of which 1,995 of cattle, 1,407 of goats, and 1,074 of sheep. Among these samples, 81 were diagnosed as pythiosis (75 cases in sheep, 4 in cattle and 2 in goats), which represents 1.8% of the diseases diagnosed in ruminants.

3.2.1 Sheep

Sheep of both sexes (43 females and 32 males) were affected, with ages ranging from 21-day- to 5-year- old, mean of 3,4 years old. Regarding the breed, 65 (86.66%) animals were mixed breed and 10 (13.33%) Santa Ines. All animals were raised in an extensive system, with occasional access to water reserves. The disease occurred in the form of individual cases (24 animals) and outbreaks (4 outbreaks affecting 51 animals), whose morbidity rates in the herds ranged from 7.5% to 40%. All cases had a chronic clinical course (30 to 90 days).

The affected sheep had lesions in the skin (55/75), respiratory (22/75) or alimentary (2/75) tracts (Fig. 2A, B, C and D). Some animals had cutaneous and nasal lesions simultaneously (4/75). The clinical signs were directly related to the location of the lesions, as shown in Illustration 2.

Illustration 2. Clinical forms and corresponding clinical signs in sheep with pythiosis



The cutaneous lesions (55/75) were characterized by ulcerations with moist or dry reddish surface, measuring 4 to 29 cm in diameter, and sometimes covered by thick crusts or serosanguineous exudate. The lesions were located on the limbs (46/55), mucocutaneous junction of the nasal planum (5/55), ventral abdomen (2/55) and scapular region (2/55). Dissemination of the infection to regional lymph nodes was noted in some cases (10/55).

Lesions in the respiratory tract were located primarily in the nasal cavities (22/75) and were characterized by unilateral or bilateral enlargement of the nasal region (22/22), often with serosanguineous nasal discharge (18/22). The mid-sagittal section of the head revealed necrotic yellow-red irregular friable masses extending from the mucocutaneous region of the nostrils (rhinofacial form) to the ethmoidal region of the nasal cavities (rhinopharyngeal form). These masses often caused partial occlusion of the nostrils and had a prominent fetid smell.

Oronasal fistula (9/22), ulcers on the hard palate (4/22), destruction of the nasal septum (4/22), exophthalmia (4/22), and bone rarefaction in the ethmoid region (2/22) were seen as the most common complications. Dissemination of the infection to the lungs (13/22), submandibular lymph nodes (11/22) and brain (1/22) was observed.

Lesions in the digestive tract (2/75) were seen in the prestomachs and abomasum of two lambs. A 21-day-old nursing Santa Ines crossbred sheep presented with a history of food regurgitation, lethargy, and anorexia. The lesions were characterized by necrotizing brownish multifocal areas on the serous surface of the rumen and reticulum. The lesions in the prestomachs extended from the mucosa to the serosa and, on cut surface, showed a yellowish granular appearance interspersed with dark reddish areas. The second case was a 30-day-old crossbred lamb that was found dead. Lesions were characterized by adhesions between the serosa of the omasum and abomasum by multiple nodules measuring approximately 0.1 cm in diameter. Focally extensive areas covered by a yellowish caseous granular exudate were seen in the mucosa of the reticulum, omasum, and abomasum.

Regardless of the injury site, histopathological examination revealed pyogranulomas associated with negatively-stained hyphal profiles. The lesions were characterized by multifocal to coalescing areas of necrosis surrounded by dense infiltrates of eosinophils and neutrophils, macrophages, multinucleated giant cells and a few lymphocytes and plasma cells. Within the necrotic areas and inflammatory response, transverse and longitudinal sections of negatively-stained hyphal profiles, sometimes surrounded by granular and eosinophilic *Splendore-Hoeppli* reaction, were seen. The lymph nodes, lungs and brain that were secondarily affected showed similar hyphae and pyogranulomatous inflammation.

Of the 75 affected animals, 4 achieved remission with treatment. Of which, 3 had skin lesions and were treated with surgical excision, and 1 had nasal lesions and was treated with oral fluconazole (10mg/kg, QD, for 42 days), broad-spectrum antibiotics, vitamin complex, and cleaning the nostrils with saline and 2% chlorhexidine.

3.2.2 Cattle

Adult cattle of both sexes (3 females and 1 male) and raised extensively or semi-intensively were affected. The lesions were exclusively cutaneous and characterized by two gross patterns: nodular or flat ulcers (Fig. 3A, B, C and D).

Nodular lesions were characterized by raised edges and ulcerated irregular reddish surface. Some nodules showed partially covered by skin with depressed and alopecic fistulous tracts. On the cut surface the lesion extends into the skin, subcutaneous tissue and sometimes infiltrated the adjacent

musculature, and was characterized by irregular yellow-reddish areas, delimited by whitish, smooth and shiny tissue.

Flat ulcerated lesions were characterized by slightly depressed, moist and reddish center with elevated irregular borders. On the cut surface, these lesions were restricted to the epidermis and dermis and were characterized by a whitish and firm tissue.

The lesions measured 8 to 15 cm in diameter, and were located on the limbs, gluteal and cervical regions. In one case the regional lymph nodes were enlarged and at the cut showed multifocal to coalescent yellow nodules interspersed with discrete reddish areas. In all cases, no treatment was prescribed, but spontaneous remission of the lesions occurred between 1-7 months.

Histopathological examination revealed ulcerative pyogranulomatous dermatitis associated with negatively-stained hyphal profiles. There were multifocal areas of necrosis associated with a marked inflammatory response composed by epithelioid macrophages, multinucleated giant cells, lymphocytes, plasma cells, neutrophils and eosinophils, extending from the superficial dermis to the deep dermis, subcutaneous tissue and, sometimes, to the adjacent musculature. In the nodular pattern, marked fibroplasia surrounding the inflammatory response was observed. In some cases, pyogranulomas were characterized by a central area containing neutrophils and degenerated eosinophils and surrounded by macrophages, epithelioid macrophages and multinucleated giant cells, peripherally delimited by fibrous connective tissue.

In all cases, within the pyogranulomas and multinucleated giant cells transverse and longitudinal sections of negatively-stained hyphal profiles, sometimes surrounded by sparse *Splendore-Hoeppli* reaction, were seen. The lymph nodes that were grossly altered showed similar hyphae and pyogranulomatous inflammation. As a distinctive feature, hyphae were often degenerate or fragmented in response to inflammation.

3.2.3 Goats

Two goats were affected. An 8-month-old crossbred female goat presented with a 1-month history of lameness and weight loss associated with a focally extensive, ulcerative, draining and pruriginous cutaneous lesion in the metatarsal-phalangeal region of the left hindlimb. The second case was a 2-year-old Savana female goat that presented with a 1-month history of lameness and enlargement of the metacarpophalangeal region of the right forelimb associated with an ulcerated and draining cutaneous lesion in the interdigital space (Fig. 4A and B). Complete surgical excision of the cutaneous lesion (case 1) and limb amputation (case 2) were performed and proved to be effective.

Histopathological examination revealed well-circumscribed pyogranulomas in the superficial and deep dermis. The pyogranulomas were characterized by central areas of neutrophils and eosinophils with negatively-stained hyphal profiles surrounded by sparse *Splendore-Hoeppli* reaction. The central zone was surrounded by a large number of macrophages, epithelioid macrophages and multinucleated giant cells.

3.3 Carnivores

During the study period, we received 7,727 tissue samples from necropsy examinations and biopsy sampling of domestic carnivores, of which 5,339 of dogs and 2,388 of cats. Among these samples, 22 were diagnosed as pythiosis (19 cases in dogs and 3 in cats), which represents 0.28% of the diseases diagnosed in domestic carnivores.

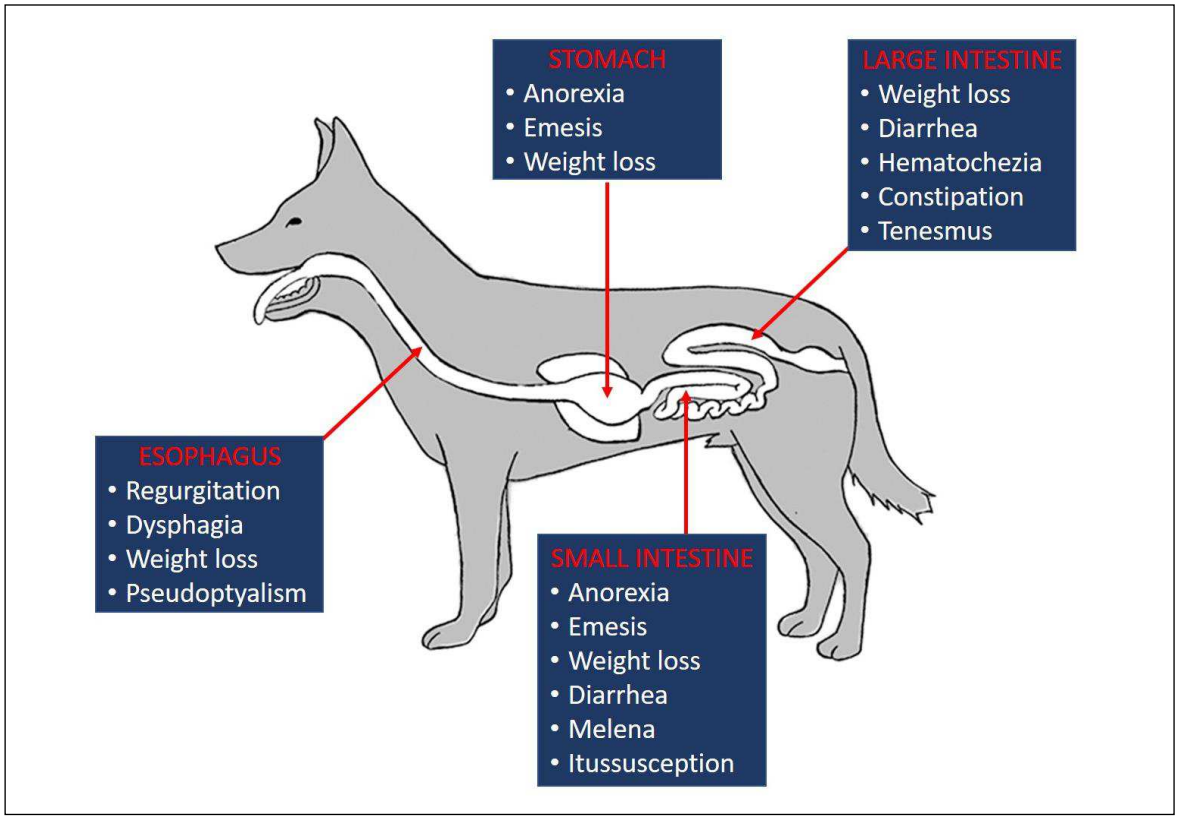
The diagnosis was established at postmortem examination in 13 cases (11 dogs and 2 cats) and by biopsy in 9 cases, but when the biopsy was performed, the lesions were very large, making the treatment unfeasible.

3.3.1 Dogs

Of the affected dogs, 13 (68.4%) were females and 6 (31.6%) males. The age ranged from 1 year to 7 years, mean of 2,6 years old. Mixed-breed (11 [57.8%]) and purebred dogs (8 [42.2%]) were affected. Regarding the breeding system, all 19 (100%) dogs were semi-domiciliated, with occasional access to the street (14 [73.6%]), countryside (9 [47.4%]) or local dams (3 [15.8%]).

The lesions were predominantly located in the alimentary tract (17/19 [89.5%]), with primary involvement of the small intestine (8 [42.1%]), large intestine (6 [31.6%]), esophagus (2 [10.5%]), and stomach (1 [5.3%]) (Fig. 5A, B, C, D and E). The lesions present in the intestines often extended to the mesentery, omentum, mesenteric lymph nodes, and pancreas, sometimes causing a generalized adhesion of the abdominal cavity organs. There was also 1 (1/19 [5.25%]) case with lesions primarily on the skin (Fig. 5F) and 1 (1/19 [5.25%]) case with lesions exclusively on the trachea. The clinical signs of pythiosis in the alimentary tract were directly related to the location of the lesions, as shown in Illustration 3.

Illustration 3. Correlation between the location of lesions in the alimentary tract and clinical signs in dogs with pythiosis.



Lesions located in the alimentary tract were characterized by transmural thickening of the affected segments by a firm irregular multinodular mass. The cut surface was shiny, whitish and interspersed by granular yellowish areas. The cutaneous lesion, located at the base of the tail, was characterized by areas of alopecia, ulceration and small cavitations that drained serosanguineous exudate. At cut, there was a thickening of the subcutaneous tissue by multifocal yellowish areas mottled red. The tracheal lesion was characterized by an irregular yellowish mass, measuring 2 x 1 x 0,5 cm, adhered to the wall and partially obstructing the tracheal lumen. At cut, the mass was slightly yellow, compact, firm and irregular.

Histopathology revealed granulomatous or pyogranulomatous and eosinophilic inflammatory infiltrate admixed with areas of necrosis. The granulomatous pattern was characterized by central areas of necrosis delimited by an inflammatory infiltrate composed of macrophages, multinucleated giant cells and occasional lymphocytes and plasma cells. Peripherally, there were reactive fibroblasts producing abundant collagenous matrix and neovascularization. Some cases displayed a pyogranulomatous pattern, characterized by central areas of necrosis, containing degenerated neutrophils and eosinophils, and surrounded by intact eosinophils, macrophages and rare multinucleated giant cells.

Within the necrotic areas and through inflammatory response, numerous negatively-stained hyphal profiles (some with weakly basophilic walls) were observed in HE-stained sections. Inflammation, necrosis and intralesional hyphae were also seen in the adjacent lymph nodes in 4 out of 19 cases.

3.3.2 Cats

All affected cats were raised indoor/outdoor with free access to the street. The lesions were located in the oral cavity (case 1), small intestine (case 2), and skin at the base of the tail (case 3).

Case 1 was a 1-year-old Persian male cat presented with a one-week history of right cheek volume increase, with asymmetry and deformity of the face. On physical examination there was a whitish firm poorly delimited and multinodular mass located on the soft palate and extending to the facial region. The animal had difficulty eating and drinking and weight loss. At necropsy, the oral mass extended transmurally and irregularly to the cheek, periorbital and infra-auricular regions of the right antimere (Fig. 6A and B).

Case 2 was an 8-year-old mixed breed female cat presented with a one-week history of apathy, anorexia, weight loss, intermittent diarrhea and vomiting. Necropsy revealed a yellowish soft multinodular mass, measuring approximately 10 x 8 x 6 cm, thickening the wall of the proximal jejunal segment. At the opening of the affected segment, it was observed that the mass partially obstructed the intestinal lumen and had a central fistulous tract extending from the mucosa to the muscular layer and, in some areas, to the serosa; in such a way that the intestinal contents leaked into the peritoneal cavity causing fibrinosuppurative peritonitis. On the cut surface, the mass was yellowish and contained multifocal to coalescing reddened cavities (Fig. 6C and D).

Case 3 was a 2-year-old mixed breed female cat presented with a two-week history of nodular volume increase with ulcerated surface, indistinct borders, and measuring approximately 3 cm diameter in the skin around the base of the tail. On the cut surface, the nodule was whitish and firm.

Histopathology revealed multifocal pyogranulomas, characterized by central areas of necrosis admixed with numerous degenerate neutrophils and surrounded by marked inflammatory infiltrate of eosinophils, macrophages, and multinucleated giant cells. At the periphery, reactive fibroblasts producing abundant collagenous matrix and neovascularization were observed. Within the necrotic areas, inflammatory response and fibroplasia, numerous negatively-stained hyphal profiles (some with weakly basophilic walls) were observed, in HE-stained sections. As peculiar findings of case 2, there was fibrinoid necrosis on the wall of some blood vessels associated with hyphal invasion and spread of infection to the mesenteric lymph nodes.

3.4 Birds

A single case of pythiosis in a bird was diagnosed. A 2-year-old female red-necked ostrich presented with hyporexia for one week. On clinical examination, the bird showed dysphagia and partial esophageal obstruction by a mass in the middle third of the esophagus. Surgery was

successfully performed and the bird recovered without complications. A transmural thickening area, measuring approximately 10 x 5 x 3 cm, and partially obstructing the esophageal lumen was found. The mucosa was blackened and friable and showed discrete ulcerations. The area was removed and, on cut surfaces, there were irregular pale areas extending through the wall, from the mucosa to the muscular layer and, in some areas, to the adventitia (Fig. 7A and B).

Histopathology revealed a focally extensive area of necrosis surrounded by heterophils, eosinophils, macrophages and lymphocytes, associated with fibroplasia and neovascularization. Numerous longitudinal and cross sections of negatively-stained hyphal profiles, in HE-stained sections, were detected throughout the necrotic areas and inflammatory response. Additionally, hyphal invasion of blood vessel walls, hemorrhage, congestion and presence of granulocytes in the lamina propria were observed.

3.5 Common histochemical, immunohistochemical, microbiological, and molecular features

In histological sections stained by PAS the walls of the hyphae were weakly stained, but when GMS technique was used, the hyphae were intensely stained in black and characterized by tubular structures with almost parallel walls, irregular branching, rare septation and measuring approximately 2-8 µm.

IHC for *P. insidiosum* revealed strong immunolabelling of the cytoplasm and the wall of hyphae.

Microbiological culture revealed the growth of whitish mold-like submerged colonies, with an irregular radiate pattern and short aerial mycelium. Microscopy revealed sparsely septate hyaline hyphae with occasional branches at right angles, morphological findings compatible with *P. insidiosum.*

The sequences of DNA were 97 to 99% identical to multiple GenBank isolates of *P. insidiosum* (identified by using Blastn).

4. Discussion

The diagnosis of pythiosis was based on the epidemiological, clinical and anatomopathological findings, and was confirmed by microbiological, immunohistochemical or molecular methods over the 35-year of diagnosis. It is well-known that *P. insidiosum* performs its biological cycle in an aquatic environment containing organic substrate and temperatures between 30º C and 40º C (Gaastra et al. 2010); in these environments, the microorganism reproduces asexually, yielding infective biflagellate zoospores (Gaastra et al. 2010).

This study draws attention to the high frequency of pythiosis in different animal species in the area of influence of the Federal University of Campina Grande, in the state of Paraiba, northeastern Brazil. It is likely that this status reflects the establishment of a diagnostic laboratory with the development of diagnostic techniques for pythiosis in this institution and not an increase in the frequency of the disease. The high frequency of pythiosis probably also occurs in other states in the region where there are no regularly functioning diagnostic laboratories carrying out systematic pythiosis diagnoses. The northeastern region of Brazil, with a territory of 1.554.257 km^2, is characterized by having a large area of semiarid climate, with high temperatures and a long period of drought. The need for water reservoirs during the dry period has been mentioned as an important factor in the development of the disease (Tabosa et al. 1999). During the drought, equidae and ruminants spend more time inside these reservoirs or in their surroundings in search of forage, which, associated with high water temperature, apparently favors infection by *P. insidiosum*.

A large number of pythiosis cases have been reported in several animal species in this region, including cases in an ostrich (Souto et al. 2019) and in a goat (Do Carmo at al. 2014), which represented the only cases described in these species to date.

The variation in the frequency of the disease among animal species in this retrospective study stands out. In fact, pythiosis appears to be particularly common in horses and sheep and rare in cats, goats, and donkeys. The expressive lower incidence of the disease in these animal species may be partly explained by the behavioral characteristic of aversion to aquatic environments, which decreases the chance of contact with the pathogen (Pessoa et al., 2014; Souto et al. 2020).

Moreover, these pythiosis cases notably do not share the same presentation, clinical course and outcome. In most animals, pythiosis is a long-term and life-threatening illness (Gaastra et al. 2010; Santúrio et al. 2006), especially when it affects the respiratory and alimentary tracts.

Lesions in the respiratory tract are more frequent in the nasal cavities of sheep, and tend to affect the rhinofacial region (Ubiali et al. 2013). The incidence of the disease in the form of outbreaks stands out, sometimes affecting dozens of animals (Tabosa et al. 2004). Similar lesions in the nasal cavities have also been described in horses (Souto et al. 2016) and cats (Bissonnette et al. 1991), invariably causing the animals' death or euthanasia.

Lesions in the alimentary tract are more frequent in dogs (Gaastra et al. 2010), and have rarely been reported in horses (Silva et al. 2020; Bezerra Júnior et al. 2010), cats (Souto et al. 2020; Rakich et al., 2005) and sheep (Pessoa et al. 2012). Dogs show a wide range of clinical signs, according to the location of the lesions in the alimentary tract (Frade et al. 2017; Berryessa et al. 2008). Horses generally present the classical clinical signs of colic (Silva et al. 2020; Bezerra Júnior et al. 2010).

Even the cutaneous lesions that currently are successfully treated in equidae (Álvarez et al. 2016; Maia et al. 2016), often have a poor prognosis in dogs (Dykstra et al. 1999) and cats (Dowst et al. 2019; Soares et al. 2019). It seems to be a frequent finding that when dogs and cats show clinical signs and undergo clinical evaluations, they already have very extensive lesions (Berryessa et al. 2008; Rodrigues et al. 2006; Souto et al. 2020). In fact, the diagnosis was made antemortem by incisional biopsies in some of these cases, but due to the severity of the lesions, euthanasia was performed.

In contrast, cattle show spontaneous healing of the lesions, which have been seen exclusively in the skin (Grecco et al. 2009; Gabriel et al. 2008) and regional lymph nodes (Maia et al. 2020). The self-limiting characteristic of the lesions is likely determined by several factors related to the inflammatory response, including the type of antigen-presenting cell, the cytokines involved at the time of antigen presentation, the dose and affinity of the antigen for the T cell receptor; the timing and level of co-stimulatory signals (Maia et al. 2020).

A key feature of equidae pythiosis was the presence of the gross concretions termed "*kunkers*". These structures correspond histologically to dense accumulations of eosinophils with intralesional hyphae (Mendoza, 2005), and are likely caused by the participation of mast cells and eosinophils in a self-perpetuating complex cycle within the inflammatory site (Munitz and LeviSchaffer, 2004; Martins et al., 2012). In chronic cases, the only place where the hyphae can be found is within *kunkers* (Gaastra et al., 2010), which means these concretions are the ideal material to isolate the agent.

On the other hand, ruminants and carnivores had a granulomatous to pyogranulomatous inflammatory pattern, in which eosinophils and neutrophils were present in smaller numbers. For most animals, the immune response cannot prevent the propagation of the agent but interestingly cattle are able to mount an effective immune response (Maia et al. 2020; Grecco et al. 2009; Gabriel

et al. 2008). As a matter of fact, hyphae are usually seen disintegrated and confined to pyogranulomas (Martins et al. 2012), which demonstrates the effectiveness of the inflammatory response.

In turn, the ostrich had a necroheterophilic and granulomatous inflammation, similar to a previously described case of cutaneous pythiosis in a White-faced Ibis (Pesavento et al. 2008).

Considering all these animal species and the multiple clinical forms, pythiosis has many differential diagnoses, as summarized in Table 2.

Table 2. Main differential diagnoses of pythiosis according to animal species and clinical forms.

Animal	Clinical form	Main differential diagnosis
Equidae	Cutaneous *Souto et al. 2021*	Exuberant granulation tissue, habronemosis, sarcoid, squamous cell carcinoma, eosinophilic granuloma and multisystemic eosinophilic epitheliotropic disease
	Respiratory *Souto et al. 2016*	Infections by *Conidiobolus* spp. or other zygomycosis, rhinosporidiosis, neoplasms
	Alimentary *Silva et al. 2020*	Gastrointestinal disorders (colic) by mass-like lesions such as neoplasms, foreign bodies, enteroliths, phytobezoars, impactions, intussusceptions, and severe intestinal inflammation
	Mammary glands *Souto et al. 2019*	Infections caused by bacteria and, rarely, by fungi, and traumas
Ruminants	Cutaneous *Tabosa et al. 2004*	Granulomas caused by bacterial or fungal infections, and neoplasms
	Respiratory *Portela et al. 2010*	Infections by *Conidiobolus* spp. or other zygomycosis, *Cryptococcus* spp., *Aspergillus* spp., protothecosis and neoplasms
	Alimentary *Pessoa et al. 2016*	Alimentary tract disorders
Carnivores	Alimentary *Souto et al. 2020* *Rodrigues et al. 2006*	Gastrointestinal tract disorders by mass-like lesions such as neoplasms (lymphoma, adenocarcinomas and feline gastrointestinal eosinophilic sclerosing fibroplasia), tussusception, and infections caused by zygomycete fungi and the oomycete *Lagenidium* spp.
	Cutaneous *Souto et al. 2020* *Frade et al. 2017*	Neoplasms and infections caused by zygomycete fungi, such as *Conidiobolus* spp. and *Basidiobolus* spp., and the oomycetes *Lagenidium* spp.
Birds	Cutaneous *Pesavento et al. 2008*	Granulomas caused by bacterial or fungal infections
	Alimentary *Souto et al. 2019*	Gastrointestinal tract disorders by mass-like lesions such as neoplasms, foreign bodies, impactions, and granulomatous inflammation

In general, based on the histological evidence of intralesional hyphae, the differential diagnosis should include infections caused by filamentous fungi and the oomycete *Lagenidium* spp. (Grooters, 2003). Histochemical staining may be an important tool in the recognition of the agent. The histomorphological characteristics of the *P. insidiosum* are easily visualized in sections stained with GMS but not with PAS, allowing to rule out of most fungal organisms, which strongly stain with this technique (Grooters, 2003). Notwithstanding, definitive identification relies on microbiological

culture, immunohistochemistry or molecular diagnosis, since these agent's morphological features are similar (Grooters, 2003).

The culture-based diagnoses have been problematic, since veterinarians often fail to submit samples for fungal culture because the gross lesions associated with pythiosis are easily confused with neoplasia or bacterial infection (Rodrigues et al. 2006). In addition, many diagnostic laboratories are unfamiliar with culture techniques for oomycetes.

Immunohistochemistry is an option to confirm the diagnosis when microbiological culture is not possible or only paraffin-embedded tissues are available, as occurred in most of the cases reported here. The primary anti-*P. insidiosum* antibody utilized in these cases was also utilized in previous cases of pythiosis in horses (Souto et al. 2019; Souto et al. 2016), sheep (Tabosa et al., 2004), dogs (Frade et al., 2017) and cats (Souto et al. 2020), supporting its sensitivity and specificity.

An alternative to culture and immunohistochemistry that has become more routinely available in recent years is extraction, amplification, and sequencing of DNA from paraffin-embedded tissues. Panfungal polymerase chain reaction (PCR), targeting the internal transcribed spacer 2 (ITS-2), is now available to identify fungal and fungal-like organisms, such as *P. insidiosum* (Meason-Smith et al. 2017).

Finally, it is important to consider that although several cases of pythiosis have been reported in animals only one case was reported in a human in Brazil (Bosco et al., 2005). That said, it is possible that the disease is being underdiagnosed in humans and it is recommended that it should be included in the differential diagnosis of cutaneous and systemic illnesses, especially in endemic areas where the disease is often diagnosed in animals.

Conclusion

Pythiosis is an endemic disease in northeastern Brazil. Horses, sheep, and dogs seem to be most commonly affected, but it is important to be aware that the disease can occasionally (or rarely) affect other animal species. The skin, the upper respiratory and the alimentary tracts are the most commonly involved sites, and the clinical signs reflect the location of the lesions. The clinical outcome is highly variable, usually life-threatening for most species, but cattle can present spontaneous healing.

The disease should be included in the differential diagnosis whenever there is histological evidence of chronic inflammation with intralesional hyphae, considering the usual inflammatory pattern seen in each animal species. The definitive diagnosis can be established based on the epidemiological, clinical, and anatomopathological findings associated with techniques for identification of the agent, such as microbiological culture, immunohistochemistry, and molecular techniques. Veterinary clinicians and pathologists should be familiar with the clinicopathological features of the disease and the wide range of susceptible animal species.

Disclosure of interest

The authors declare that they have no competing interest.

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Table 1. Epidemiological, clinical and anatomopathological findings of pythiosis in affected animal species in northeastern Brazil (1985-2020).

Animal group	Specie	Pythiosis / Samples / %	Clinical findings			Pathological findings
			Primary site	Main clinical signs	Outcome	
Equidae	Horse	195/1051 (18.55%)	Skin 190/195 (97.43%)	Ulcerated nodules or tumor-like masses with serosanguineous discharge	Remission after treatment or euthanasia	*Kunkers* / Multifocal areas of eosinophil necrosis (horse and mule), sometimes surrounded by pyogranulomatous inflammatory infiltrate (donkey); both patterns with intralesional negatively-stained hyphal profiles
			Mammary glands 3/195 (1.53%)	Swollen mammary glands with multifocal ulcerations and serosanguineous discharge	Remission after surgical excision	
			Nasal cavities 2/195 (1.0%)	Swelling of the rhinofacial region and serosanguineous nasal discharge	Euthanasia	
	Mule	6/62 (9.67%)	Skin 6/6 (100%)	Ulcerated nodules with serosanguineous discharge	Remission after surgical excision	
	Donkey	1/218 (0.46%)	Skin 1/1 (100%)	Ulcerated nodules with serosanguineous discharge	Remission after surgical excision	
Ruminants	Sheep	75/1074 (6.98%)	Skin 55/75 (73.33%)	Ulcerations with moist or dry reddish surface, sometimes covered by crusts or exudate	Remission after excision or death	Pyogranulomas with negatively-stained hyphal profiles, often surrounded by *Splendore-Hoeppli* reaction
			Nasal cavities 22/75 (29.33%)	Unilateral or bilateral enlargement of the nasal region and serosanguineous nasal discharge	Death, euthanasia or Remission after treatment	
			Alimentary tract 2/75 (2.66%)	Regurgitation, lethargy and anorexia	Death	
	Cattle	4/1995 (0.20%)	Skin 4/4 (100%)	Reddish, alopecic, nodular or ulcerated lesions	Self-healing	
	Goat	2/1407 (0.14%)	Skin 4/4 (100%)	Ulcerative and draining lesions on the limbs and lameness	Remission after excision or amputation	
Carnivores	Dog	19/5,339 (0.35%)	Alimentary tract 17/19 (89.5%)	Anorexia, weight loss, emesis, regurgitation, dysphagia, constipation, blood in the stool	Death or euthanasia	Granulomas or pyogranulomas with negatively-stained hyphal profiles, sometimes hyphae may be weakly basophilic
			Skin 1/19 (5.25%)	Reddish ulcerated lesion with serosanguineous discharge	Euthanasia	
			Respiratory tract 1/19 (5.25%)	Coughing, choking, persistent nausea	Death	
	Cat	3/2388 (0.12%)	Alimentary tract 2/3 (66.6%)	Anorexia, weight loss, emesis, diarrhea, and palpable masses on the soft palate and intestines	Death or euthanasia	
			Skin 1/3 (33.3%)	Ulcerated nodular lesion	Death	
Birds	Ostrich	1/8 (12.5%)	Alimentary tract 1/1 (100%)	Hyporexia, dysphagia and palpable mass in the esophagus	Remission after surgical excision	Necroheterophilic and granulomatous inflammation with negatively-stained hyphae

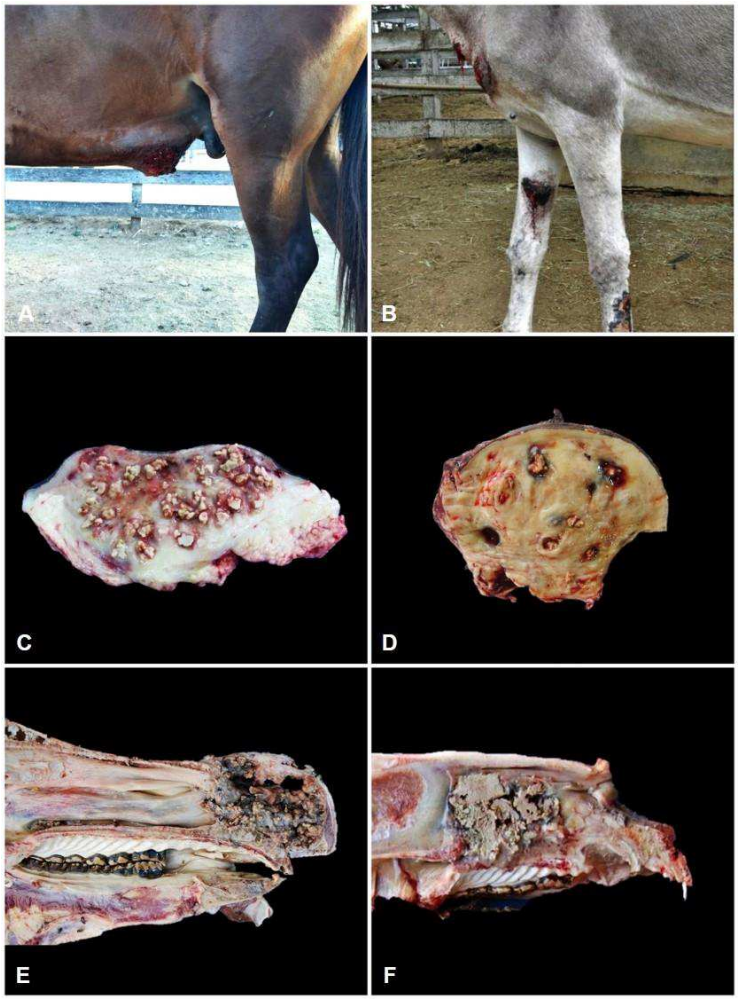

Figure 1 - Pythiosis in equidae. A) Horse. Ulcerated cutaneous nodule with serosanguineous discharge in the ventral aspect of the abdominal wall. B) Donkey. Ulcerated cutaneous nodules with serosanguineous discharge on the forelimbs and chest. C) Cutaneous nodule, cut surface. Numerous intralesional *kunkers* (arrows). D) Mammary gland, cut surface. Fistulous tracts with intralesional *kunkers* (arrows). E) Mid-sagittal section of the head, nasal cavity. Areas of necrosis and cavitation containing *kunkers* in the nasal vestibule (rhinofacial form). F) Mid-sagittal section of the head, nasal cavity. Areas of necrosis containing *kunkers* in the region of the nasal turbinates and meatuses (rhinopharyngeal form).

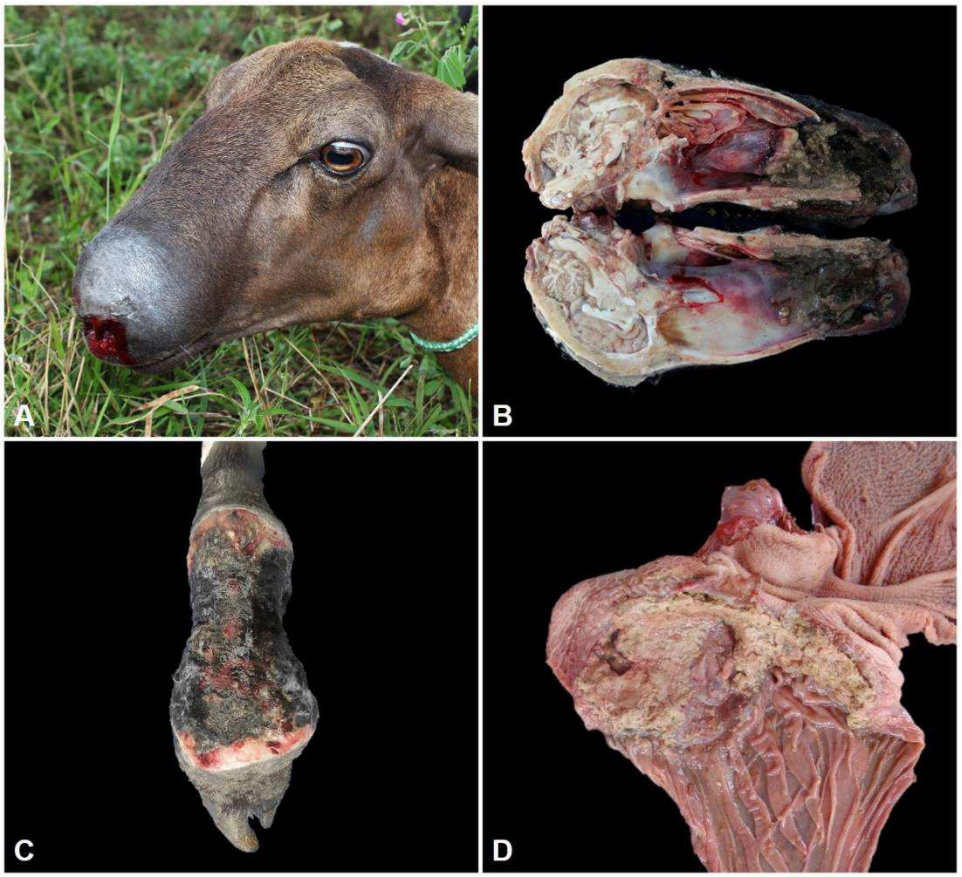

Figure 2 - Pythiosis in sheep. A) Enlargement of the nasal region and serosanguineous nasal discharge. B) Mid-sagittal section of the head, nasal cavities. A necrotic yellow-brown irregular friable mass extending from the mucocutaneous region of the nostrils to the initial portion of the nasal cavities (rhinofacial form). C) Forelimb. Enlargement of the metacarpophalangeal region associated with an ulcerative cutaneous lesion with reddish moist surface covered by blackened thick crusts. D) Abomasum and prestomachs. A yellow caseous granular exudate covering the ulcerated mucosa of the abomasum (A), omasum (O) and reticulum (R).

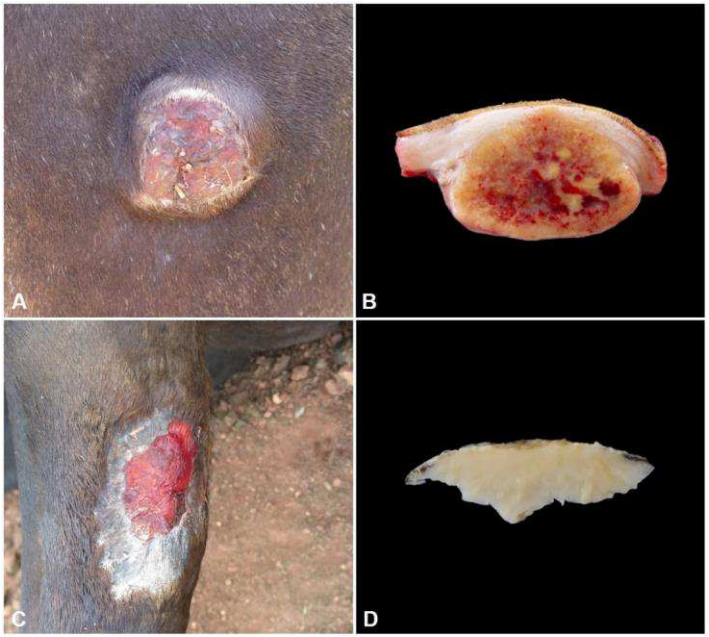

Figure 3 - Pythiosis in cattle. A) Skin, gluteal region. Nodular lesion with reddish ulcerated surface and raised edges. B) Nodular lesion, cut surface. Irregular yellowish and reddish areas delimited by whitish, smooth and shiny tissue. C) Skin, humeral region. Ulcerated lesion with depressed moist and reddish center and slightly elevated irregular borders. D) Flat ulcer lesion, cut surface. Whitish and firm tissue restricted to the epidermis and dermis.

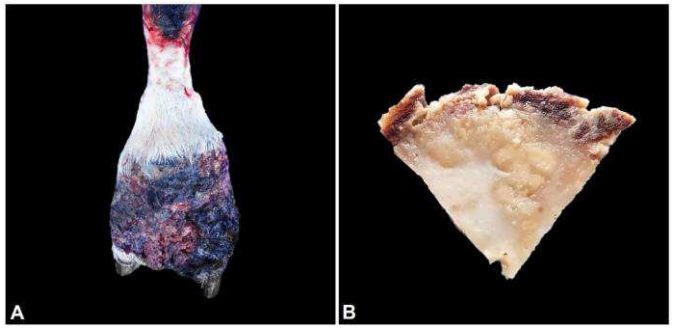

Figure 4 - Pythiosis in goats. A) Forelimb. Enlargement of the interdigital region associated with an ulcerative cutaneous lesion with reddish moist surface covered by blackened thick crusts and serosanguineous exudate. B) Skin, cut surface. Irregular yellowish areas associated with extensive ulceration of the epidermis.

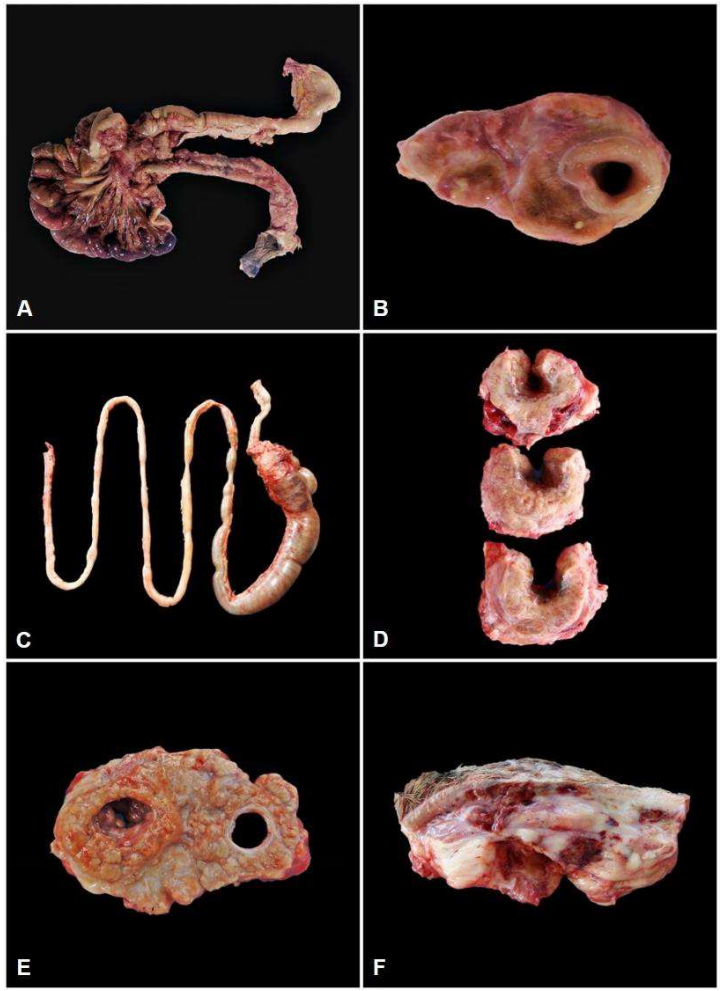

Figure 5 - Pythiosis in dogs. A) Alimentary tract. Irregular yellow-reddish mass involving the small intestine and mesentery. B) Duodenum, cross section. Thickening of the intestinal wall by an irregular yellow-reddish compact mass. C) Large intestine. Irregular yellowish multinodular mass thickening the wall of the cecocolic segment. D) Colon, cross section. Thickening of the intestinal wall by an irregular yellowish mass. Note the stenosis of the lumen. E) Cross-section of the esophagus and trachea. A yellowish multinodular mass circumferentially thickening the esophageal wall (left) and surrounding the tracheal cartilages (right). F) Skin, cut surface. Thickening of the subcutaneous tissue by multifocal yellowish areas mottled red.

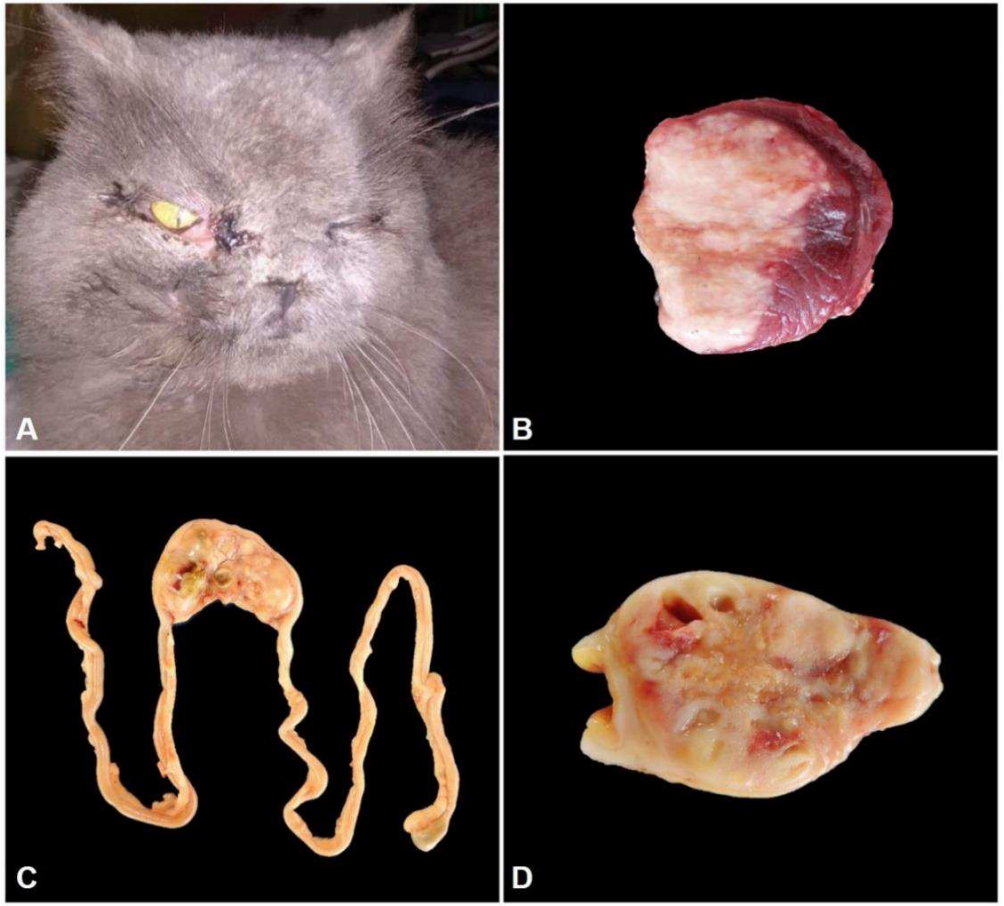

Figure 6 - Pythiosis in cats. A) Case 1. Right cheek enlargement, with asymmetry and deformity of the face. B) Case 1. Facial musculature, cut surface. An irregular whitish multinodular mass infiltrating the musculature. C) Case 2. Intestine. Yellowish multinodular mass thickening the wall of the proximal jejunal segment. D) Case 2. Intestinal mass, cut surface. A yellowish compact mass with multifocal reddened cavities.

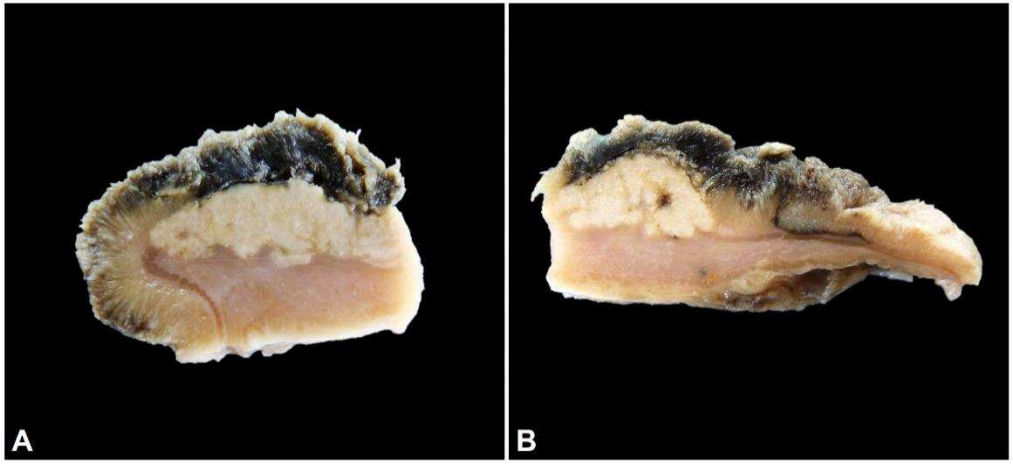

Figure 7 - Pythiosis in ostrich. A) Esophagus, cross section. An irregular yellowish area thickening the esophageal wall and extending through the mucosa to the muscular layer. Note the blackened and friable mucosa. B) Esophagus, longitudinal section. An irregular yellowish area thickening the esophageal wall and extending through the mucosa to the muscular layer. Note the blackened and friable mucosa.

CAPÍTULO II

Pythiosis in equidae in northeastern Brazil: 1985-2020

O presente trabalho foi aceito para publicação no *Journal of Equine Veterinary Science* (anexo 2).

Pythiosis in equidae in northeastern Brazil: 1985-2020

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ABSTRACT: The epidemiological, clinical, pathological, microbiological and immunohistochemical findings of pythiosis in equidae in northeastern Brazil are described. From January 1985 to December 2020 the Laboratory of Animal Pathology of the Federal University of Campina Grande received 1,331 tissue samples of equidae, 202 (15.17%) of which were diagnosed as pythiosis. Equidae of both sexes with ages varying from 4 months to 25 years were affected. Most animals were mixed breed (79.7%) and reared in an extensive system (73.26%). The disease occurred throughout the year but the highest incidence (70.29%) was noted after the rainy season. The clinical course was always chronic. The lesions were preferentially located on the limbs and ventral thoracoabdominal wall and characterized by nodules or tumor-like masses with ulcerations and serosanguineous discharge. The cut surface showed fistulous tracts containing *kunkers*. The direct examination of the *kunkers* and microbiological culture revealed sparsely septate and branched hyaline hyphae. Histopathology revealed a marked inflammatory infiltrate of eosinophils with multifocal well-defined areas of eosinophil necrosis and collagenolysis and intralesional negatively-stained hyphal profiles; in the donkey, a pyogranulomatous inflammatory infiltrate was noted surrounding these areas. Immunohistochemistry for *Pythium insidiosum* revealed strong immunolabelling of the hyphae. Pythiosis occurs endemically in equidae in northeastern Brazil, with seasonal variation in the incidence. The intralesional *kunkers* establishes an accurate presumptive diagnosis, but confirmation should preferably be performed through histopathology associated with immunohistochemistry, culture-based or molecular methods. The early surgical treatment proves to be efficient in most cases. Triamcinolone acetonide emerges as a non-invasive and efficient therapeutic alternative.

Key words: Equidae disease; infectious disease; hyphae; *Pythium insidiosum*.

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Introduction

Pythiosis is a chronic inflammatory disease caused by the oomycete *Pythium insidiosum*, which affects both domestic and wild animals as well as humans. Among domestic animals, horses are the most commonly affected [1]. In this species, the main clinical presentation of the disease is cutaneous/subcutaneous [2] with occasional involvement of adjacent lymph nodes [3], bones [4], and mammary glands [5]. The primary involvement of the nasal cavities [6], lungs [7, 8], soft palate [9] and intestine [10] are also reported. Far less commonly, cutaneous pythiosis has also been reported in mules [11] and donkeys [12, 13].

The disease occurs in regions of tropical, subtropical and temperate climate, and has been reported in the Americas, some European countries, Southeast Asia, Oceania and Africa [14]. In Brazil, it is likely that the first cases of pythiosis in horses were diagnosed in Rio Grande do Sul, South region [15], with subsequent case reports in the Midwest [16, 17] and Southeast [18, 19] regions. Due to the marked scarcity and irregularity of rainfall during most of the year, the disease was not believed to occur in the Northeastern region; when Tabosa et al. (1999) [11] described the first cases of cutaneous pythiosis in horses and mules. Since then, many cases have been diagnosed in equidae in this region, sometimes under rare or unprecedented clinical-pathological presentations [5, 6]. Therefore, the present report describes the epidemiological, clinical, anatomopathological, microbiological and immunohistochemical findings of pythiosis in equidae in northeastern Brazil.

Material and methods

A retrospective study was carried out in all biopsy samples and necropsy examinations of equidae, from January 1985 to December 2020, at the Animal Pathology Laboratory of the Federal University of Campina Grande, Patos, Paraiba, northeastern Brazil. Epidemiological data, clinical signs and gross lesions were reviewed from the diagnostic laboratory reports.

Samples of the intralesional necrotic concretions (*kunkers*) were collected for direct examination and microbiological culture. For direct examination, the samples were macerated and clarified in 20% Potassium Hydroxide (KOH), with subsequent visualization of hyphae by light microscopy (LM). For culture and microbiological identification of *P. insidiosum*, the samples were washed in distilled water, implanted into 2% dextrose Sabouraud agar plates, and incubated at 37 ° C for 48 hours under anaerobic condition. The slides were stained with lactophenol cotton-blue with subsequent visualization of hyphae by light microscopy (LM).

Samples of the organs of the thoracic and abdominal cavities, lymph nodes, skin and central nervous system were fixed in 10% buffered formalin, processed routinely for histopathology, embedded in paraffin wax and cut into 4 µm sections. The sections were stained with hematoxylin and eosin (HE), periodic acid-Schiff (PAS) and Grocott's methenamine silver stain (GMS).

Immunohistochemistry (IHC) was performed for identification of the agent. The IHC protocol, Martins et al. (2012) [20] modified method, was performed using a rabbit polyclonal anti–*P. insidiosum* antibody (non-commercial). Briefly, sections were dewaxed and rehydrated and endogenous peroxidase activity was blocked with oxygen peroxide (H2O2) 3%. Antigen retrieval was done by microwaving (10 min at full power) in TRIS-EDTA (pH 9.0). Sections were incubated at 37º C for 60 min with the primary antibody diluted at 1:800. The secondary antibody was a polymer-HRP (EasyLink One; EasyPath) followed by chromogen 3.3′-diaminobenzidine (DAB; DakoCytomation). Sections were counterstained with Harris hematoxylin and coverslipped. As a positive control, histological sections from a confirmed case of equine pythiosis were used. Sections from the same equine were used as negative controls, with the primary antibody replaced by phosphate buffered saline containing 0.5% polysorbate 20 (Tween 20).

Results

During the study period, we received 1,331 tissue samples from necropsy examinations and biopsy sampling in equidae, of which 1051 of horses, 218 of donkeys and 62 of mules. Among these samples, 202 were diagnosed as pythiosis (195 cases in horses, 6 in mules and 1 in donkey), which represents 15.17% of the diseases diagnosed in equidae.

Of the affected equidae, 111 (54.95%) were females, 85 (42.07%) males and in 6 (2.97%) cases sex was not informed. The age ranged from 4 months to 25 years, mean of 6,3 years old. From all animals, 161 (79.7%) were mixed breed, 12 (5.94%) Quarter Horse, 5 (2.47%) Mangalarga, 2 (1.0%) Criollo and 2 (1.0%) Breton. There was no information about breed of 20 (9.90%) horses. Regarding the rearing system, 148 (73.26%) animals were raised in an extensive and 36 (17.82%) in a semi-intensive farming system, and in 18 (8.91%) cases these data were not available. In 80 (39.6%) cases the animals' access to local dams was recorded in the clinical history. All equidae came from states in the Northeastern of Brazil, including Paraiba (161), Rio Grande do Norte (17), Pernambuco (8), Bahia (1) and Maranhao (1). In 14 cases the origin was not properly informed. The disease occurred throughout the year, although the highest incidence, 142 cases (70.29%), was noted after the end of the rainy season (May to October).

The clinical course was always chronic and ranged from 14 days to 24 months, with an average of 68 days. The lesions were often associated with serosanguineous discharge (125 [61.88%]), pruritus (51 [25.24%]), foul odor (12 [5.94%]), local pain (11 [5.44%]) and lameness (4 [1.98%]). Severe weight loss or emaciation was reported in 24 (11.88%) cases. The owners usually treated the wound empirically before taking the animal to clinical care and in 44 (21.78%) cases they reported that the “wound did not heal”.

Gross lesions were characterized by nodules or tumor-like masses, measuring 4 to 50 cm in diameter, with ulcerations and irregular margins (Fig.1A, B and C). In 9 (4.45%) cases, the

lesions were located in the deep dermis and subcutaneous tissue, with no secretion or ulceration of the overlying epidermis, which was sometimes blackened and alopecic (this was considered an atypical presentation).

In all cases the cut surface of the lesion showed fistulous tracts and irregular cavitations containing several yellow-gray irregular firm necrotic masses (*kunkers*), which easily detached from the lesion. The *kunkers* ranged from 1 to 4 cm in diameter and had a characteristic coral-like appearance (Fig.1D).

Data regarding the location of the lesions are listed, in decreasing order of frequency, in table 1. In most cases (169/202 [83.66%]) the lesions were focal, but in 33 animals (16.33%) there were multifocal lesions, totaling 246 injuries.

Table 1. Location of the lesions caused by *P. insidiosum* in equidae, diagnosed from January 1985 to December 2020.

Anatomical site	**Nº**	**%**
Limbs	91	37.0%
Abdomen	73	29.7%
Chest	36	14.6%
Cervical	6	2.4%
Inguinal	5	2.0%
Shoulder blade	5	2.0%
Penis / Foreskin	5	2.0%
Mammary glands	3	1.2%
Armpits	3	1.2%
Face	3	1.2%
Nostrils	2	0.8%
Nasal cavities	2	0.8%
Lip	1	0.4%
Ear	1	0.4%
Perianal	1	0.4%
Uninformed	9	3.6%
	246	

The direct examination of the *kunkers* showed myriads of filamentous hyaline hyphae occasionally branched and with rare septa (Fig.1E). Microbiological culture revealed the growth of whitish mold-like submerged colonies, with an irregular radiate pattern and short aerial mycelium. Microscopy revealed sparsely septate hyaline hyphae with occasional branches at right angles (Fig.1F), morphological findings compatible with *P. insidiosum.*

Histopathology of lesions in horses and mules revealed a marked inflammatory infiltrate of eosinophils spreading diffusely through the superficial and deep dermis, with multifocal to coalescent well-defined areas of eosinophil necrosis and collagenolysis (Fig.2A); surrounding these areas, intact eosinophils, macrophages, neutrophils, and occasional lymphocytes and plasma cells were noted. Within the necrotic areas, often distributed at the periphery, there were

longitudinal and transverse sections of tubuliform structures not stained by HE (negatively-stained hyphal profiles) (Fig.2B and C). Peripherally, marked proliferation of fibroblasts and collagen fibers and neovascularization (immature granulation tissue) was observed. The epidermis was often extensively ulcerated and covered by cellular debris, dried fibrin, and bacteria.

In the donkey, there were multifocal pyogranulomas, with central areas of necrotic eosinophils surrounded by inflammatory infiltrate of eosinophils, lymphocytes, plasma cells, macrophages, and multinucleated giant cells (Fig.2D), occasionally delimited by proliferation of fibroblasts and collagen fibers. Within the necrotic centers, transverse and longitudinal sections of negatively-stained hyphae, sometimes surrounded by small amounts of *Splendore-Hoeppli* reaction, were also noted. In the deep dermis, infiltration of macrophages, lymphocytes and eosinophils was seen in the wall of some hair follicles (mural folliculitis), which were sometimes ruptured and partially replaced by areas of eosinophil necrosis and intralesional hyphae (furunculosis). The epidermis was ulcerated and covered by cellular debris.

In histological sections stained by PAS the walls of the hyphae were weakly stained, but when GMS technique was used, the hyphae were intensely stained in black and characterized by tubular structures with almost parallel walls, irregular branching, rare septation and measuring approximately 2-8 µm (Fig.2E). IHC for *P. insidiosum* revealed strong immunolabelling of the cytoplasm and the wall of hyphae in brown (Fig.2F).

In the last three decades, the treatment employed consisted of surgical excision of lesions with a safety margin; associated with periodic wound cleaning, use of antiseptic solutions and administration of anti-tetanus serum and penicillinic broad-spectrum antibiotic. Of the 202 cases, 182 were treated with this protocol and 168 out of 182 (92.3%) achieved complete remission; in 14/182 (7.7%) cases the lesions were severe enough to make the surgical procedure unfeasible and the animal was euthanized.

In 10 out of 202 (4.9%) cases, no data were available regarding treatment and clinical outcome. In recent years, treatment with the corticosteroid triamcinolone acetonide has been tested in 10 out of 202 (4.9%) horses and a 100% remission rate was obtained.

Discussion

The diagnosis of pythiosis was based on the epidemiological, clinical, anatomopathological and microbiological findings, and was confirmed by immunohistochemistry. It is well-known that for the maintenance of the biological cycle of the agent, an aquatic environment containing organic substrate and temperatures between 30º C and 40º C is necessary; in these circumstances, the motile biflagellate zoospores, the infective form of the agent, develop [3].

These epidemiological conditions notoriously occur in northeastern Brazil because of the high environmental temperatures recorded throughout the year and the practice of storing water in reservoirs, in the form of dams and ponds [11]. The equidae have access to these reservoirs, formed during the rainy season and maintained for most of the year, for bathing, water intake and consumption of the plants that sprout or are grown along the banks.

Therefore, animals in extensive or semi-intensive rearing systems are presumably more susceptible, particularly after the rainy season due to progressive food shortages during the dry season [11]. Predisposition by race, sex, or age is not recognized [14].

Horses were far more affected (96.53%) than mules (2.97%) and donkeys (0.49%). Similar data were obtained from epidemiological surveys in the Pantanal region and were attributed to the predominance of horses compared to equine hybrids [21]. However, in the northeastern region, there is greater equity in the equidae herd [22] and even so the horses were more affected. It is important to take into account that mules, and especially donkeys, have the behavioral characteristic of aversion to aquatic environments [23]; therefore, they are less exposed to the infectious agent.

The lesions were located mainly on the distal extremities and the ventral aspect of the thoracoabdominal wall, body parts that are most often in prolonged contact with contaminated water [11, 24]. Direct contact with contaminated water also determined the occasional incidence of lesions in unusual anatomical sites, such as the ears, nasal cavities [6], and mammary glands [5].

In most animals (83.66%) the lesions were solitary, but multifocal lesions and at different anatomical sites were also noted and have been previously documented [17, 25]. The involvement of adjacent structures and dissemination of the infection to distant organs were rarely seen in this survey. In one case, in which the lesions were primary in the nasal cavities, there was involvement of the maxillary bone and dissemination to the lungs [6]. The spread of the infection to internal organs has been rarely described and is believed to be associated with vascular invasion of detached hyphal segments [26].

In most cases, the lesions were characterized by ulcerated tumor-like masses or nodules, which are the usual aspect of the lesion [1]. The course of this clinical presentation is typically fast and accompanied by a progressive increase in the size of the lesions, weight loss, weakness, and death of the animals [17, 24], if treatment is not performed. In a few cases, the lesions were restricted to the deep dermis and subcutaneous tissue and no skin ulceration was seen, which is considered an atypical presentation characterized by its peculiar prolonged clinical course (over 1 year) [17].

The discharge of serosanguineous exudate from ulcerated lesions was the most common clinical sign, and the presence of this exudation pattern in tumoral lesions of equidae can be suggestive of the disease. In some cases, the exudative pattern can vary from serosanguineous to

mucopurulent [25], certainly due to secondary bacterial infections. Generally, ulcerated lesions with abundant secretion present a high degree of secondary contamination, which represents an additional obstacle in the treatment of the disease [17]. The pruritus was also seen with relative frequency. Equines can experience intense itching, even promoting auto-mutilation in an attempt to relieve discomfort [1, 3]. In addition, lameness can be seen in animals that develop large lesions on the limbs [3]. The involvement of adjacent bone tissue has been described and establishes a poor prognosis [4, 25].

The disease has a recognized chronic clinical course, and clinical cases with an evolution of more than one year have been described [17]. In this survey, cases with evolution ranging from a few days to two years were verified. Moreover, it is likely that there was late identification of the onset of the lesions in some cases, especially in animals of low zootechnical value and raised in extensive systems.

The intralesional concretions known as *kunkers* have been described in horses, mules [11], donkeys [12] and camels [27], being absent in other animal species [1]. *Kunkers* are always present in equine pythiosis and provide an accurate presumptive diagnosis. The direct examination of these structures, macerated and clarified with Potassium Hydroxide, allows the visualization of hyphae and strengthens the presumptive diagnosis of pythiosis [3].

The definitive diagnosis should preferably be established through anatomopathological examination [3]. The histomorphological characteristics of the intralesional hyphae can be better evaluated using special histochemical staining techniques and it is recognized that hyphae are easily visualized in sections stained with GMS but not with PAS [28]. Notwithstanding, definitive identification relies on microbiological culture, immunohistochemistry and molecular diagnosis [28].

Microbiological culture should be performed with sterile mycelium on Sabouraud medium, preferably not supplemented with chloramphenicol [29]. Due to the fact that *P. insidiosum* in culture does not develop sporangia, the induction of zoosporogenesis should be performed in order to precisely identify the pathogen [1].

Immunohistochemistry has good specificity and sensitivity [20, 28] and it is an excellent option to confirm the diagnosis when microbiological culture is not possible or only paraffin-embedded tissues are available.

Serological methods have also been used to diagnose pythiosis, including complement fixation tests, immunodiffusion tests, florescent antibodies, immunoblots, and enzyme-linked immunosorbent assays (ELISA) [30]. However, molecular techniques, such as Polymerase Chain Reaction (PCR), stand out for accurately identify various *Pythium* species, as well as for performing phylogenic and taxonomic studies [30].

It is worthy of remark that in cases of pythiosis in horses and mules, macrophages and multinucleated giant cells, even if present, they do not predominate at the inflammatory site, so the term “granulomatous” may be inaccurate. Therefore, the terms "eosinophilic and necrotizing" are proposed, as they better describe the changes noted in most animals.

However, in the donkey, the inflammatory response was eosinophilic and pyogranulomatous, sometimes surrounded by proliferation of fibroblasts and collagen fibers; similar to that seen in other species, such as dogs and ruminants [20]. A detailed report of this case was previously described by Maia et al. (2016) [12].

The differential diagnosis of pythiosis in equidae should include exuberant granulation tissue, cutaneous habronemosis, sarcoid, squamous cell carcinoma, and particularly eosinophilic granuloma and multisystemic eosinophilic epitheliotropic disease. Histopathology is sufficient to differentiate from these conditions in most cases. However, in the last two conditions the microscopic lesions are very similar, so it is advisable to perform histochemical stains to confirm the presence or absence of intralesional hyphae [31].

An outstanding feature of the lesions is the irresponsiveness to alternative treatments adopted by the owners, such as periodic cleaning of wounds and topical solutions containing antibiotics or fipronil. Numerous treatments have been employed using antifungal drugs, immunotherapy and surgical resection [1].

The use of antifungal agents does not show satisfactory results due to the morphological characteristics of the agent, which lacks ergosterol in its cytoplasmic membrane. Most commercial antifungals interfere with the biosynthesis of this compound [1].

Immunotherapy has been used as a single therapeutic agent or adjuvant to surgical exeresis, with success rates ranging from 53% to 75%, when associated with surgical exeresis [32, 33]. However, its efficiency remains variable and dependent on individual factors. In addition, its applicability is questionable in animals of low zootechnical value.

Surgical excision with a safety margin represents the most traditional measure for the treatment of pythiosis [14]. In this study, it proved to be a resolutive measure in most cases (92.3%), which can be attributed to the immediate recognition of the disease by local veterinarians and surgical procedure before the lesions acquire large dimensions or extend to vital structures, when the excision becomes infeasible and the prognosis is poor.

Promising results have been achieved with the use of the corticosteroid triamcinolone acetonide, which proved to be a non-invasive, faster and lower cost alternative [34, 35].

Conclusion

Pythiosis occurs endemically in northeastern Brazil, with seasonal variation in the incidence. Affects equidae of all ages, both sexes, reared predominantly in extensive systems and with access to water reservoirs. The clinical course is always chronic. The lesions are

preferentially located on the limbs and ventral thoracoabdominal wall and characterized by nodules or tumor-like masses with ulcerations and serosanguineous discharge. The intralesional *kunkers* establishes an accurate presumptive diagnosis, but confirmation should preferably be performed through histopathology associated with immunohistochemistry, microbiological culture, or molecular techniques. The traditional early surgical treatment proves to be efficient in most cases. Triamcinolone acetonide emerges as a non-invasive and efficient therapeutic alternative.

Disclosure of interest

The authors declare that they have no competing interest.

Acknowledgments.

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Figures

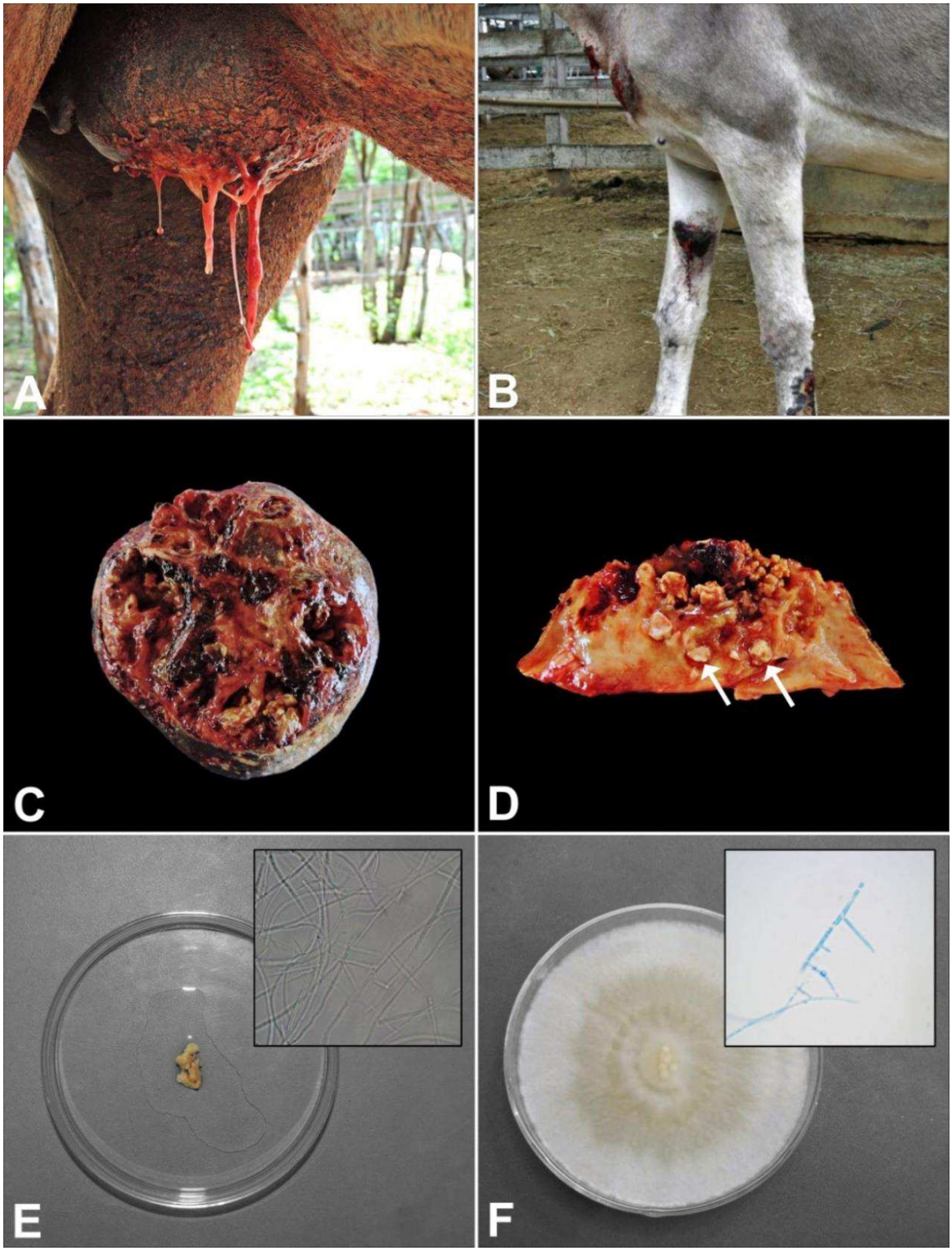

Figure 1 - Pythiosis in equidae. A) Horse with an ulcerated tumor-like mass in the ventral abdominal region draining profuse serosanguineous secretion. B) Donkey with multifocal ulcerated cutaneous lesions on the forelimbs and chest. C) Cutaneous nodule with multiple ulcerations and fistulous tracts. D) Cutaneous nodule, cut surface. Numerous intralesional *kunkers* (arrows). E) *Kunker*, direct examination. Inset, multiple filamentous hyaline hyphae occasionally branched and with rare septa. LM. Obj. 1000x. F) Microbiological culture. Inset, sparsely septate hyphae with occasional branches at right angles. Lactophenol cotton-blue, LM. Obj. 1000x.

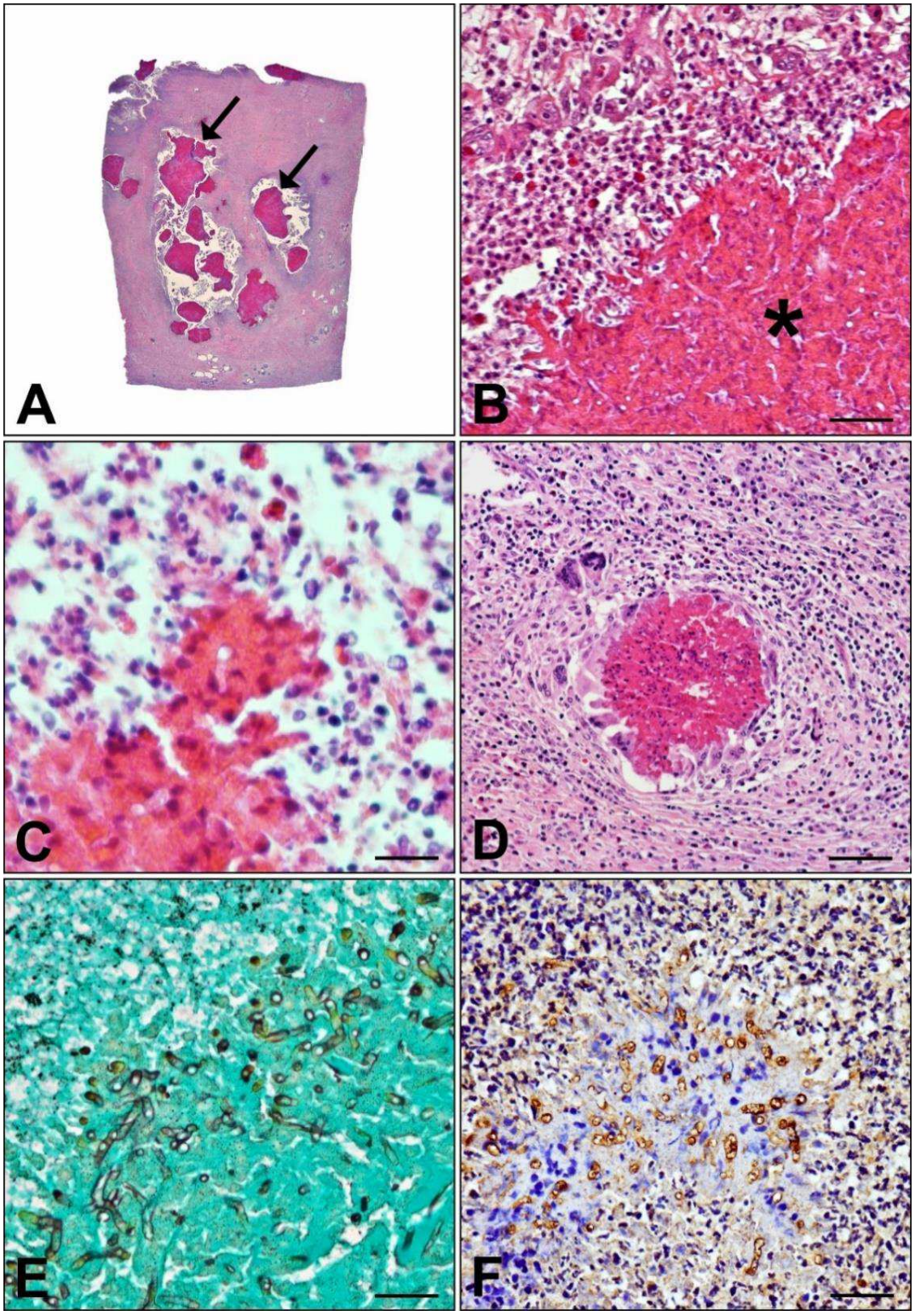

Figure 2 - Pythiosis in equidae. A) Skin, deep dermis. Multifocal to coalescent areas of eosinophil necrosis and collagenolysis (arrows), grossly corresponding to *kunkers*. HE. Subgross. B) Area of eosinophil necrosis (asterisk), with intralesional negatively-stained hyphal profiles, and surrounded by inflammatory infiltrate of eosinophils, neutrophils, macrophages, lymphocytes and plasma cells. HE. Bar = 20 µm. C) Intralesional negatively-stained hyphae. HE. Bar = 40 µm. D) Pyogranulomas with central areas of eosinophils necrosis and intralesional negatively-stained hyphal profiles. Note the multinucleated giant cells. HE. Bar = 20 µm. E) Hyphae impregnated in black. GMS. Bar = 20 µm. F) Strong immunolabelling of the hyphae (in brown) for *P. insidiosum*. IHC, DAB. Bar = 20 µm.

CAPÍTULO III

Pythiosis in cats in northeastern Brazil

O presente trabalho foi publicado no *Journal of Medical Mycology* (anexo 3).

Pythiosis in cats in northeastern Brazil

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ABSTRACT: The epidemiological, clinical and anatomopathological aspects of pythiosis in cats in northeastern Brazil are described. From January 2000 to December 2018 the Laboratory of Animal Pathology of the Federal University of Campina Grande received 1,928 tissue samples of cats, three of which were diagnosed as pythiosis. Grossly, the cats showed a multinodular mass in the oral cavity associated with facial deformity (case 1), a large multinodular mass thickening the jejunum wall (case 2), and an ulcerated nodule in the skin at the base of the tail (case 3). Histologically, pyogranulomatous inflammation and necrosis, with intralesional predominantly negatively stained hyphae, were observed in all cases. Immunohistochemistry for *Pythium insidiosum* revealed strong immunolabelling of the hyphae. The diagnosis of pythiosis was based on the epidemiological, clinical and anatomopathological findings, and was confirmed by immunohistochemistry. Although uncommon in cats, pythiosis should be readily considered as a differential diagnosis of chronic pyogranulomatous infections of the gastrointestinal tract and skin, especially in endemic areas, where the disease is often diagnosed in other animal species.

Key words: Cat disease; pyogranulomas; hyphae; oomycete; pythiosis.

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Introduction

Pythiosis is an inflammatory and frequently life-threatening infection caused by the oomycete *Pythium insidiosum*. This agent develops mycelium like fungi, but it is not a true fungus, since its cell walls do not contain chitin but are composed of cellulose and β-glucans, its cytoplasmic membrane lacks ergosterol, the thallus is diploid and coenocytic, the sexual process is oogamy and the organism develops infective biflagellate zoospores in wet environments [10]. The infection occurs mainly in tropical, subtropical and temperate regions, and affects several animal species and humans, but most cases occur in immunocompetent horses and dogs [10, 30].

Only a few cases of pythiosis have been reported in cats, affecting skin and subcutaneous tissue [6, 12, 23, 29], nasal and retrobulbar regions [4], oral cavity [7] and intestines [11, 20]. Definitive diagnosis relies on microbiological culture, immunohistochemistry or molecular technics [13]. The present report describes the epidemiological, clinical and anatomopathological aspects of pythiosis in cats in northeastern Brazil.

Material and methods

A retrospective study was carried out in all biopsy samples and necropsy examinations of cats, from January 2000 to December 2018, at the Animal Pathology Laboratory of the Federal University of Campina Grande, Patos, Paraiba, northeastern Brazil.

Epidemiological data, clinical signs and gross lesions were reviewed from the reports. Samples of the organs of the thoracic and abdominal cavities, lymph nodes, skin and central nervous system were fixed in 10% buffered formalin, processed routinely, embedded in paraffin wax and cut into 3 µm sections. The sections were stained with hematoxylin and eosin (HE), periodic acid-Schiff (PAS) and Grocott's methenamine silver stain (GMS).

Immunohistochemistry (IHC) was performed for identification of the agent. The IHC protocol, Martins et al. (2012) [17] modified method, was performed using a rabbit polyclonal anti–*P. insidiosum* antibody (non-commercial). Briefly, sections were dewaxed and rehydrated and endogenous peroxidase activity was blocked with oxygen peroxide (H_2O_2) 3%. Antigen retrieval was done by microwaving (10 min at full power) in TRIS-EDTA (pH 9.0). Sections were incubated at 37ºC for 60 min with the primary antibody diluted at 1:1,000. The secondary antibody was a polymer-HRP (EasyLink One; EasyPath) followed by chromogen 3.3′-diaminobenzidine (DAB; DakoCytomation). Sections were counterstained with Harris hematoxylin and coverslipped. As a positive control, histological sections from a confirmed case of equine pythiosis were used. Sections from the same equine and tested cats were used as negative controls, with the primary antibody replaced by phosphate buffered saline containing 0.5% polysorbate 20 (Tween 20).

The sensitivity and specificity of the primary antibody is according to Martins et al (2012) [17]. The antibody was tested in a case of canine gastrointestinal pythiosis that was confirmed by

microbiological culture and zoosporogenesis [21]. The anti-*P. insidiosum* antibody was also tested by IHC with samples of tissue from known cases of zygomycosis (bovine and avian), aspergillosis (avian), candidiasis (feline) and cryptococcosis (equine). All of these reactions were negative, supporting the specificity of the reagent [17].

Results

During the study period, we received 1,928 tissue samples from necropsy examinations and biopsy sampling in cats. Among these samples, 3 (0,1%) were diagnosed as pythiosis. The lesions were located in the oral cavity (case 1), small intestine (case 2) and skin at the base of the tail (case 3). The main epidemiological and clinical findings are summarized in Table 1.

Table 1. Epidemiological and clinical findings of pythiosis in cats in northeastern Brazil.

Case	Age	Sex	Breed	Clinical aspects		
				Main clinical signs	Course	Presumptive diagnosis
1	1 year	Male	Persian	Asymmetry and facial deformity	1 week	Neoplasm
2	8 years	Female	Mixed breed	Anorexia, weight loss, diarrhea, vomiting and abdominal mass	1 week	Gastrointestinal disorder/ Neoplasm
3	2 years	Female	Mixed breed	Ulcerated nodular skin lesion	2 weeks	Cutaneous infection

Case 1. A 1-year-old male Persian cat was presented with a one-week history of right cheek volume increase, with asymmetry and deformity of the face (Fig. 1A). The owner reported that the animal was raised indoor/outdoor with free access to a pond located near its residence. On physical examination there was halitosis and a whitish firm poorly delimited and multinodular mass located on the soft palate (Fig. 1B) and extending to the facial region. The animal had difficulty eating and drinking and weight loss. Due to the progression of the clinical condition and lack of response to treatment with anti-inflammatory drugs and antibiotics, euthanasia was performed.

At necropsy, the oral mass extended transmurally and irregularly to the cheek, periorbital and infra-auricular regions of the right antimere (soft tissues). Histological examination revealed the marked expansion of the oral mucosa, musculature, subcutaneous and deep dermis by multifocal pyogranulomas, characterized by central areas of necrosis surrounded by marked inflammatory infiltration of plasma cells, eosinophils and occasional lymphocytes. These areas were externally delimited by macrophages, epithelioid macrophages and rare Langhans-type giant cells associated with reactive fibroblasts producing abundant collagenous matrix. Within the necrotic areas and through inflammatory response, numerous negatively-stained hyphal profiles were observed in HE-stained sections.

Case 2. An 8-year-old female mixed breed cat was presented with a one-week history of apathy, anorexia, weight loss, intermittent diarrhea and vomiting. The owner reported that the animal was raised indoor/outdoor in a suburban area and complains it was progressively losing weight. Physical examination revealed cachectic body condition, pale mucous membranes and a mass measuring approximately 10 cm was palpated within the abdomen. The cat died and necropsy was performed.

Gross examination of the abdomen revealed adhesions between omentum, mesentery, and intestinal segments, approximately 30 ml of yellowish fibrinosuppurative exudate and deposition of fibrin filaments on the surface of the liver and intestinal serosa. There was a yellowish, soft, multinodular mass, measuring approximately 10 x 8 x 6 cm, thickening the wall of the proximal jejunal segment (Fig. 1C). At the opening of the affected segment, it was observed that the mass partially obstructed the intestinal lumen and had a central fistulous tract extending from the mucosa to the muscular layer and, in some areas, to the serosa; in such a way that the intestinal contents leaked into the peritoneal cavity. On the cut surface, the mass was yellowish and contained, multifocal to coalescing, reddened cavities (Fig. 1D). The mesenteric lymph nodes were enlarged.

Histological examination of multiple intestinal sections revealed the marked expansion of the intestinal wall, with the replacement of the normal architecture, by multifocal pyogranulomas (Fig. 2A), characterized by central areas of necrosis admixed with numerous degenerate neutrophils. Surrounding the necrotic areas, there were numerous epithelioid macrophages, foamy macrophages, eosinophils, neutrophils and rare Langhans-type giant cells; and neovascularization and marked fibroplasia, characterized by branching trabeculae of dense collagen separated and surrounded by accentuated inflammatory infiltrate of eosinophils, with many of them degenerate, and a few macrophages (Fig. 2B).

Within the necrotic areas and spreading through fibroplasia, numerous negatively-stained hyphae (some with weakly basophilic walls) were observed in HE-stained sections (Fig. 2C). The foamy macrophages and Langhans-type giant cells often had hyphae in their cytoplasm.

In some sections the overlying mucosa was multifocally ulcerated and had a moderate inflammatory infiltrate of lymphocytes, plasma cells and macrophages from the lamina propria to the muscular layer. Fibrinoid necrosis on the wall of some blood vessels associated to hyphal invasion was also observed.

Pyogranulomatous inflammation and necrosis with intralesional hyphae were also seen in the mesenteric lymph nodes. These organs exhibited diffuse infiltration of macrophages, foamy macrophages and Langhans-type giant cells mixed with occasional eosinophils and neutrophils. The multinucleated giant cells often contained hyphae in their cytoplasm.

Case 3. A 2-year-old female mixed breed cat, raised indoor/outdoor, was presented with a two-week history of nodular volume increase with ulcerated surface, indistinct borders, and

measuring approximately 3 cm diameter in the skin around the base of the tail. Incisional biopsy was performed. On the cut surface, the nodule was firm and whitish.

Histopathologic examination revealed a multifocal pyogranulomatous and ulcerative dermatitis, characterized by central areas of necrosis admixed with numerous degenerate neutrophils and surrounded by marked inflammatory infiltrate of eosinophils, macrophages, epithelioid macrophages and rare neutrophils and Langhans-type giant cells. At the periphery, reactive fibroblasts producing abundant collagenous matrix and neovascularization were observed. Within the necrotic areas and through inflammatory response, numerous negatively-stained profiles of hyphae were observed in HE-stained sections. The lesions extend beyond the limits of the histological sections and the surgical margins. When the owner was sought, he reported that the cat had died a few days ago.

In histological sections of all cases stained by PAS the walls of the hyphae were weakly stained, but when impregnated with GMS the hyphae were intensely stained and characterized by tubular structures with almost parallel walls, irregular branching, rare septation and measuring approximately 2-8 µm (Fig. 2D). IHC for *P. insidiosum* revealed strong immunolabelling of the cytoplasm and the wall of hyphae in brown (Fig. 2D, inset).

Discussion

The diagnosis of pythiosis was based on the epidemiological, clinical and anatomopathological findings, and was confirmed by immunohistochemistry. The northeastern of Brazil is considered endemic for pythiosis because of the high environmental temperatures recorded during most of the year and the practice of storing water in reservoirs, like dams and ponds, to which animals have access. A large number of cases have been described in several animal species in the region, such as equines [24, 25, 27], sheep [19, 28] dogs [8], goat [5], donkey [16] and ostrich [26]; however, no case of pythiosis in cats has been described. As a matter of fact, pythiosis is extremely rare in cats, probably due to the behavioral characteristic of aversion to aquatic environments, which decreases the chance of contact with the pathogen.

The infective stage of *P. insidiosum* is a biflagellate aquatic zoospore that is released into warm water environments and causes infection by encysting in damaged skin or gastrointestinal mucosa [13]. In these cases, the cats spent an extensive amount of time outdoors, which may have exposed it to water containing these infective zoospores.

Clinical signs of pythiosis vary according to the infection site. The skin is the main anatomical site affected in horses, while dogs have the gastrointestinal tract more often affected [10].

In horses, the lesions reach especially the limbs and ventral portions of the thoracoabdominal wall, probably due to increased contact with contaminated water [10]. And, as

seen in horses, cutaneous lesions in cat 3 presumably developed when the animal had prolonged access to contaminated water.

In dogs, in which gastrointestinal lesions are far more frequent, clinical signs depend on the affected site in the digestive tract. Since the lesions are most commonly located in the intestine, it is usually observed vomiting, diarrhea, progressive weight loss and abdominal pain [3, 8]. Lesions located on the esophagus may manifest as regurgitation and vomiting [3], and in the large intestine may lead to hematochezia [9]. In cat 2, the lesion was located in the small intestine and clinical signs evidenced the chronic impairment of the bowel function. In cat 1, the primary site of injury was the oral cavity and the main clinical sign was the deformity of the face, caused by contiguous spread of infection to adjacent soft tissues. Lesions in the oral cavity have been previously reported in a cat [7], horse [22] and sheep [1]; in the latter, also leading to facial deformity.

In the previously reported cases of pythiosis in cats, the lesions were located in the skin, digestive or respiratory tracts. Cutaneous lesions were seen as nodular or plaque-like ulcerative dermatitis [6, 12] or non-ulcerated deep dermal and subcutaneous masses (panniculitis) [23, 29]. In the digestive tract, lesions were seen as a nodule in the oral cavity [7] or palpable intestinal masses accompanied by bowel disorders [11, 20]. In the single report in which the nasal cavities and nasopharynx were the primary site of infection, the lesion extended to the retrobulbar region with consequent protrusion of the eyeball [4]. Therefore, depending on the site of entry, the infection might result in a diverse range of clinical signs.

Because of the chronic and sometimes “insidious” course, most cases of pythiosis are advanced when the diagnosis is made, precluding the adoption of some treatment [10]. In cat 2, the formation of a transmural fistulous tract allowed that the intestinal contents leaked into the peritoneal cavity causing acute fibrinosuppurative peritonitis and consequently death. Lesions on the gastrointestinal tract may cause segmental thickening and ulceration, sometimes leading to transmural necrosis, peritonitis, and omental adhesions [30].

Histological evidence of pyogranulomatous inflammation with intralesional hyphae allows the distinction between pythiosis and other conditions, such as tumors of the feline digestive tract (e.g. alimentary lymphoma, adenocarcinomas and feline gastrointestinal eosinophilic sclerosing fibroplasia) and infections caused by other microorganisms (which do not grow as mycelium in tissues).

Thus, differential diagnoses should include infections caused by filamentous fungi and the oomycete *Lagenidium* spp. [12]. Histochemical staining may be an important tool in the recognition of the etiologic agent. The histomorphological characteristics of the *P. insidiosum* were easily visualized in sections stained with GMS but not with PAS, allowing to rule out of most fungal organisms, which strongly stain with this technique [12]. Notwithstanding, definitive

identification relies on microbiological culture, immunohistochemistry and molecular diagnosis, since these agent's morphological features are similar [12].

Microbiological culture should be performed with sterile mycelium on Sabouraud medium, preferably not supplemented with chloramphenicol [2]. Reference spectrum for *P. insidiosum,* on the Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS), is currently available on open-access platforms and this should allow immediate identification of the pathogen [2].

Immunohistochemistry is an option to confirm the diagnosis when microbiological culture is not possible or only paraffin-embedded tissues are available, as occurred in the three cases reported here. The primary anti-*P. insidiosum* antibody utilized in these cases was also utilized in previous cases of pythiosis in horses [24, 25], sheep [19], dogs [8], donkey [16] and ostrich [26], supporting its sensitivity and specificity.

An alternative to culture and immunohistochemistry that has become more routinely available in recent years is extraction, amplification, and sequencing of DNA from paraffin-embedded tissues. Panfungal polymerase chain reaction (PCR), targeting the internal transcribed spacer 2 (ITS-2), is now available to identify fungal and fungal-like organisms, such as *P. insidiosum* [18].

Intralesional hyphae, predominantly negatively-stained, were seen in all cases. Interestingly, in cat 2 some hyphae were seen weakly basophilic, in HE-stained sections, as previously reported in some cases of canine pythiosis [17]. However, the only difference we can notice between this case and the others, besides the intestinal location, was the abundance of hyphae.

In cat 2 was also observed hyphal angioinvasion and dissemination to mesenteric lymph nodes. Although angioinvasion by hyphae was a prominent feature in a previously reported case of intestinal pythiosis in a cat, dissemination of infection was not confirmed [20].

Treatment options for pythiosis traditionally consist of antifungal drugs, immunotherapy or radical surgery [13]. Antifungal treatment is difficult because microorganism's cell membrane lacks ergosterol, the target of most antifungal drugs [10]. Immunotherapy in cats and dogs has a lower cure rate than in horses, besides in cats it has only been tested three times with no response whatsoever [10, 29]. The most effective treatment is radical surgical resection of the affected tissue [13], yet in some cases, because of the extensiveness of tissue involved or the involvement of vital structures, the prognosis for recovery is guarded or poor. Interestingly, recent in vitro [15] and in vivo [14] studies have highlighted the possible activity of antibiotics against *P. insidiosum*. In particular, macrolides (azithromycin) and tetracyclines (minocycline) have been shown to have synergistic activity in vitro [2].

Conclusion

Although uncommon in cats, pythiosis should be readily considered as a differential diagnosis of chronic pyogranulomatous infections of the digestive tract and skin, especially in endemic areas, where the disease is often diagnosed in other animal species.

Disclosure of interest

The authors declare that they have no competing interest.

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Figures

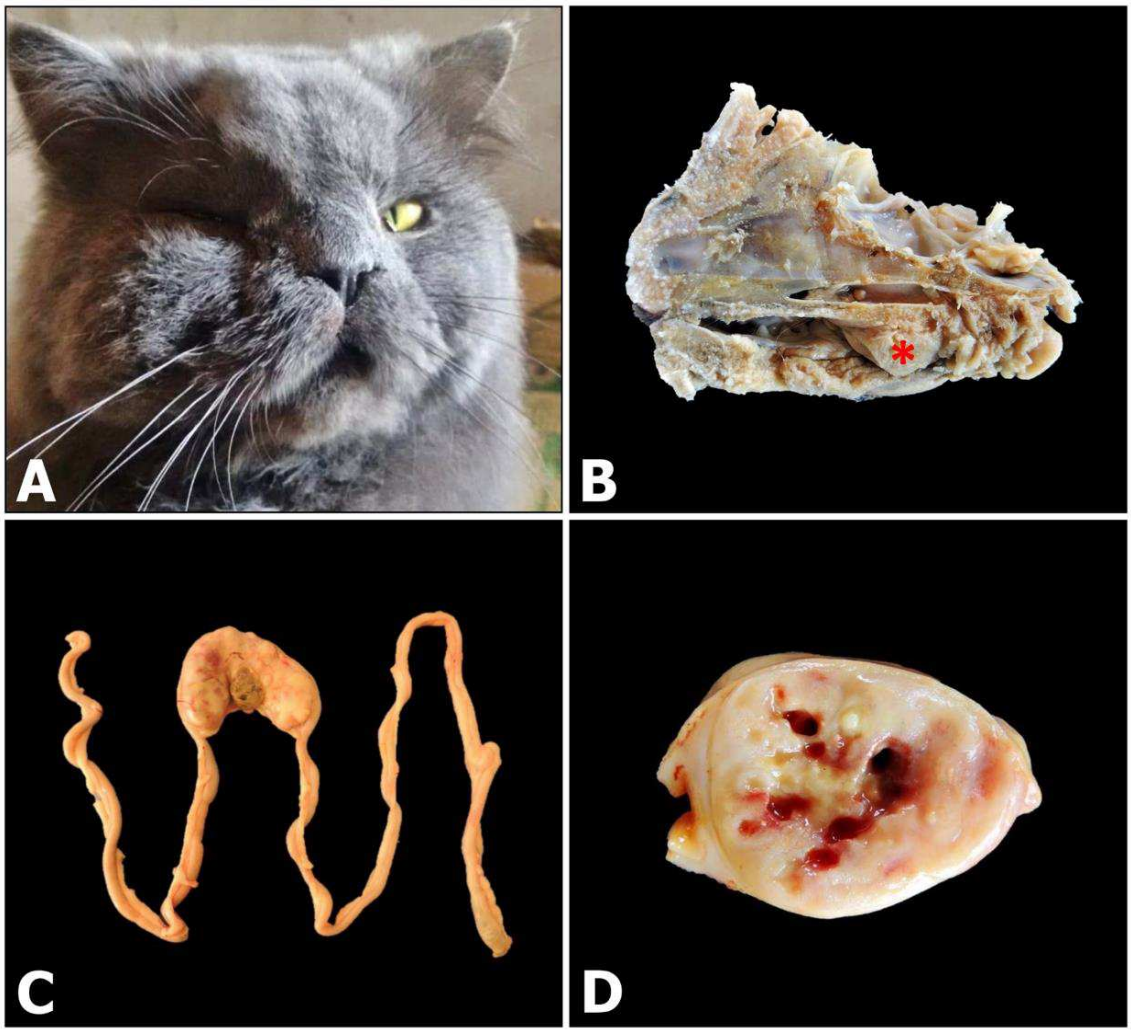

Figure 1 - Pythiosis in cats. A) Case 1. Right cheek volume increase, with asymmetry and deformity of the face. B) Case 1. Right sagittal section of the head. Whitish nodular mass (red asterisk) located on the soft palate. C) Case 2. Intestine. Yellowish multinodular mass thickening the wall of the proximal jejunal segment. D) Case 2. Intestinal mass. Cut surface revealed multifocal to coalescing reddened cavities.

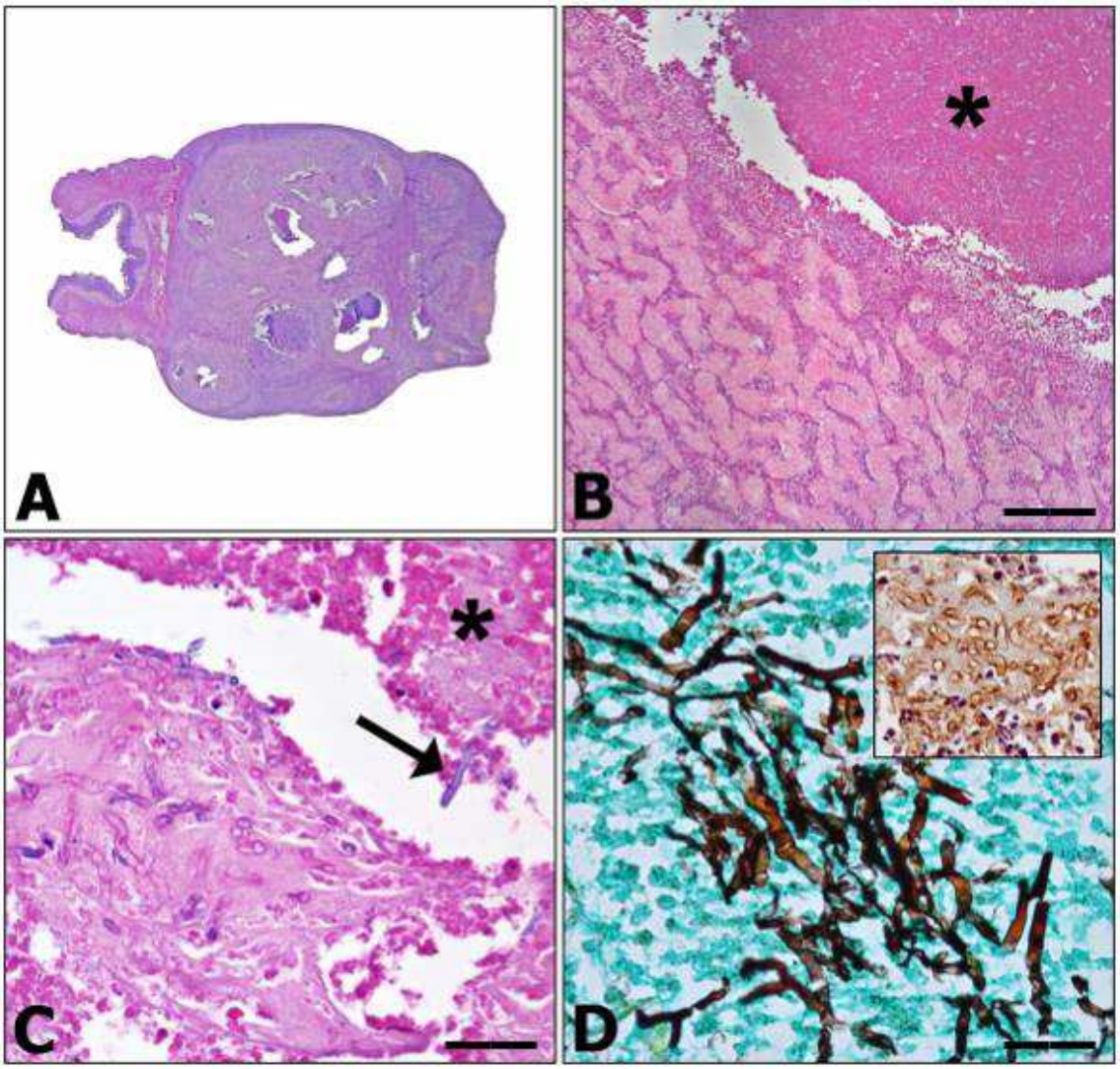

Figure 2 - Pythiosis in cats. A) Case 2. Marked expansion of the intestinal wall, with the replacement of the normal architecture, by multifocal pyogranulomas and fibroplasia. HE. Subgross. B) Case 2. Intestinal mass. Central area of necrosis (asterisk) surrounded by an accentuated inflammatory infiltrate and fibroplasia. HE. Bar = 50 µm. C) Case 2. Hyphae spreading through areas of necrosis (asterisk) and fibroplasia, some with weakly basophilic walls (arrow). HE. Bar = 20 µm. D) Case 3. Hyphae intensely stained in black. GMS. Bar = 20 µm. Inset, strong immunolabelling of the hyphae (in brown) for *Pythium insidosum*. IHQ, DAB. Bar = 20 µm.

CAPÍTULO IV

Esophageal pythiosis in an ostrich (*Struthio camelus*)

O presente trabalho foi publicado no *Arquivo Brasileiro de Medicina Veterinária e Zootecnia* (anexo 4).

Esophageal pythiosis in an ostrich (*Struthio camelus*)

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ABSTRACT: A 2-year-old female red-necked ostrich presented with a nodular mass in the middle third of the esophagus and was submitted to surgery. Histologically, there was a focally extensive area of necrosis extending throughout the mucosa and muscular layer and, in some sections, through the adventitia. Surrounding the necrotic area were a dense inflammatory reaction composed mainly of granulocytes and macrophages and associated with fibroplasia and neovascularization. In some inflammatory and necrotic areas, there were numerous longitudinal and cross sections of negatively stained hyphae, which were best visualized using Grocott methenamine silver stain. Definitive diagnosis of infection by *Pythium insidiosum* was confirmed by immunohistochemistry. The ostrich was drinking water from a pond located in a paddock where some horses developed cutaneous pythiosis previously.
Key words: Avian diseases, esophagitis, hyphae.

RESUMO: Uma avestruz-do-pescoço-vermelho, com dois anos de idade, apresentava um nódulo no terço médio do esôfago e foi submetida a procedimento cirúrgico. Histologicamente, observou-se uma área focalmente extensa de necrose estendendo-se da túnica mucosa à muscular, e, em algumas secções, à túnica adventícia. Circundando a área de necrose, observou-se uma reação inflamatória composta principalmente por granulócitos e macrófagos, associada à fibroplasia e neovascularização. Em meio às áreas de necrose e inflamação, verificavam-se numerosas imagens negativas de hifas em seções longitudinais e transversais, melhor apreciadas pela coloração de metenamina nitrato de prata de Grocott. O diagnóstico definitivo de infecção por *Pythium insidiosum* foi confirmado por imuno-histoquímica. A avestruz recebia água para consumo de um lago localizado em uma área de pastagem, no qual alguns cavalos haviam desenvolvido pitiose cutânea anteriormente.
Palavras-chave: Doença de ave, esofagite, hifas.

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INTRODUCTION

Pythiosis is a chronic inflammatory disease caused by the oomycete *Pythium insidiosum* (kingdom Stramenopila, family Pythiaceae), which occurs mainly in tropical, subtropical and temperate regions and can affect several animal species (Chaffin *et al*., 1995). In domestic mammals, this disease is well documented in horses, dogs, cats, sheep and cattle (Martins *et al*., 2012; Rakich *et al*., 2005; Tabosa *et al*., 2004); and occasionally reported in other species, such as goat (Carmo *et al*., 2015) and donkey (Maia *et al*., 2016). In wild mammals, pythiosis was reported in camels, spectacled bears, Bengal tiger and American jaguar (Gaastra *et al*., 2010).

In avian species, there is a single record of the cutaneous form in a white-faced ibis (*Plegadis chihi*) (Pesavento *et al*., 2008). The present report describes a case of esophageal pythiosis in an ostrich (*Struthio camelus*), which to our knowledge is the first characterization of the disease in this species.

MATERIAL AND METHODS

Epidemiological data, clinical signs and pathological findings were reviewed from the reports of the Animal Pathology Laboratory of the Federal University of Campina Grande, Patos, Paraiba, Brazil. An anesthetic protocol for the surgical procedure was performed with xylazine 2% (0,7mg/kg IV) and subcutaneous infiltration of local anesthesia with 30 mL of 2% lidocaine with vasoconstrictor.

Tissue samples were fixed in 10% neutral buffered formalin, processed routinely, embedded in paraffin wax and cut into 3 µm sections. The sections were stained with hematoxylin and eosin (HE), periodic acid–Schiff (PAS) and Grocott's methenamine silver stain (GMS).

Immunohistochemistry (IHC) was performed for identification of the agent. The IHC protocol was performed according to Martins *et al*. (2012) using a polyclonal antibody (anti-*P. insidiosum*) produced in rabbits (Gabriel *et al*., 2008). Briefly, the primary antibody at a dilution of 1 in 1,000 was detected by use of streptavidin-biotin-alkaline phosphatase complex and labelling was 'visualized' with Permanent Red (Dako, Glostrup, Denmark) as a substrate chromogen. We analyzed a previously confirmed positive control (equine cutaneous pythiosis) simultaneously with the tested samples. The negative control included tissue samples incubated with phosphate buffered saline instead of primary antibody.

RESULTS

In a farm located in the semiarid region of Paraiba's state, northeastern Brazil, eight ostriches were raised, separated in couples, in four paddocks of a half hectare each. They were supplemented with chopped barbary fig (*Opuntia ficus-indica*) and wheat bran. The drinking water came from a pond located in a neighboring paddock, where it was pumped through pipes

into a tank and then distributed to drinkers. In previous years, nine horses from the farm that consumed water and fed on aquatic plants in this pond developed cutaneous pythiosis.

A 2-year-old female red-necked ostrich presented with hyporexia for one week. On clinical examination, the bird showed dysphagia and partial esophageal obstruction by a mass in the middle third of the esophagus. Surgery was successfully performed and the bird recovered without complications. A transmural thickening area, measuring approximately 10 x 5 x 3 cm, and partially obstructing the esophageal lumen was found. The mucosa was blackened and friable and showed discrete ulcerations. The area was removed and, on cut surfaces, there were irregular pale areas extending through the wall from the mucosa to the muscular layer and, in some areas, to the adventitia (Fig. 1A).

Histologically, there was a focally extensive area of necrosis extending from the mucosa to the muscular layer and, in some sections, to the adventitia (Fig. 1B). Surrounding the necrotic area were heterophils, eosinophils, macrophages and lymphocytes, associated with fibroplasia and neovascularization. Numerous longitudinal and cross sections of negatively stained hyphae, in HE-stained sections, were detected throughout the inflammatory and necrotic areas. Additionally, hyphal invasion of blood vessel walls, hemorrhage, congestion and presence of granulocytes in the lamina propria were observed.

In histological sections stained by PAS the walls of the hyphae were weakly stained, but when impregnated with GMS the hyphae were intensely stained and characterized by tubular structures with almost parallel walls, sparsely septate and measuring approximately 3-8 µm in diameter (Fig. 1C). The hyphae revealed strong immunolabelling of the cytoplasm and wall in red for *P. insidiosum* (Fig. 1D).

DISCUSSION

The diagnosis was based on the epidemiological, clinical, anatomopathological, histochemical and immunohistochemical findings, which were consistent with esophageal infection by *P. insidiosum.* In the farm where the ostriches were raised, there were nine cases of cutaneous pythiosis in horses previously diagnosed. These horses had unrestricted access to the local pond, which also was used as the only drinking water source for the ostriches. Thus, it is likely that the infection occurred by intake of water contaminated by the mobile zoospores of *P. insidiosum*.

The pathogenesis of infection in digestive tract is uncertain. It is unclear if pre-existing mucosal lesions are essential for the penetration of *P. insidiosum* zoospores (Mendoza *et al*., 1996; Grooters *et al*., 2003). However, the habit of ostriches ingesting foreign objects, such as rocks and grains of sand, and fibrous or rough foods are important causes of traumatic lesions in their digestive tract (Carrer *et al*., 2004). In this case, the ostrich was supplemented with chopped

barbary fig, a species of cactus covered by sharp spines, which could have damaged the esophageal mucosa and favoured the penetration of the agent.

Injury to the digestive tract by *P. insidiosum* has been described previously in dogs (Frade *et al.,* 2017), horses (Bezerra Júnior *et al*., 2010), cats (Rakich *et al*., 2005) and lambs (Pessoa *et al*., 2012). In dogs, this is the main presentation of the disease and the intestinal segments are particularly affected, with clinical signs of vomiting, weight loss, intermittent diarrhea, hematochezia and palpable masses in the abdomen (Frade *et al.*, 2017). In this case was affected the esophagus (upper digestive tract) and the clinical signs consequently reflected the partial luminal stenosis.

The esophageal lesion was characterized by a pyogranulomatous reaction with infiltration mainly by heterophils, eosinophils, lymphocytes, and macrophages, and it is similar to gastrointestinal pythiosis in other species (Frade *et al.,* 2017; Martins *et al*., 2012; Pessoa *et al*., 2012).

The histochemical staining was an important auxiliary tool in the recognition of the etiologic agent. The PAS stain is known to promote weak staining of oomycetes such as *P. insidiosum*, allowing rule out of most fungal organisms, which stain strongly; while GMS stain allows clear visualization of the histomorphological characteristics of the agent (Grooters *et al*., 2003). Nevertheless, these histomorphological characteristics are not distinctive and important differential diagnoses may include Zygomycetes and the oomycete *Lagenidium* spp (Grooters *et al*., 2003). Identification of the agent should be confirmed by immunohistochemical techniques.

In conclusion, *P. insidiosum* affects the ostriches digestive tract and should be considered as a cause of dysphagia. We believe this paper represents the first characterization of the disease in this species.

ACKNOWLEDGMENT

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Figures

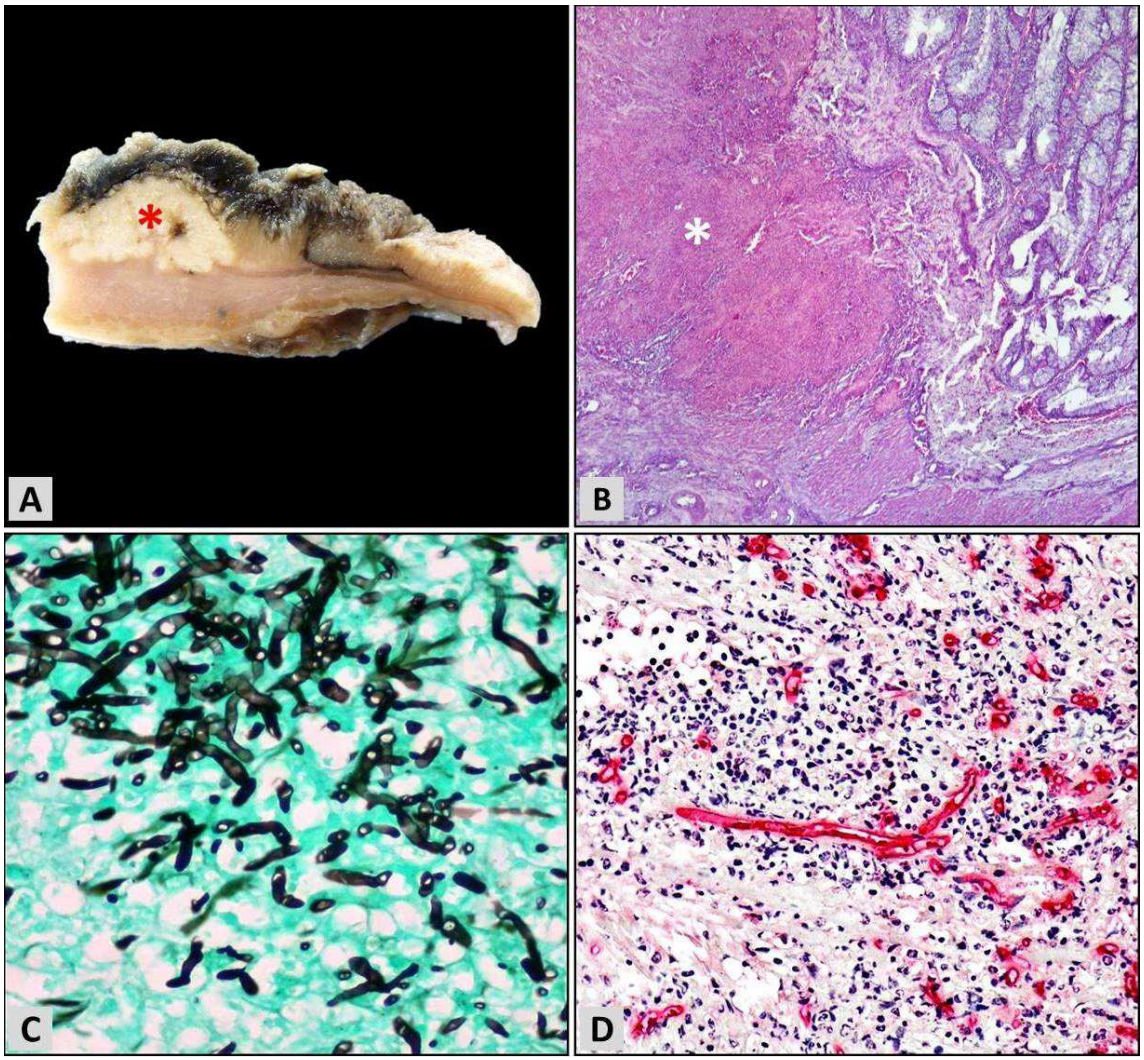

Figure 1 - Esophageal pythiosis in an ostrich (*Struthio camelus*). A) Cross section of the esophagus showing a thickened and irregular pale area (asterisk) extending through the mucosa to the muscular layer. B) Focally extensive area of necrosis (asterisk) extending from the submucosa to the muscular layer. HE. Bar, 100 µm. C) Numerous intralesional hyphae intensely impregnated in black. GMS. Bar, 20 µm. D) IHC showing strong immunolabelling of the hyphae in red. Bar, 20 µm.

CONSIDERAÇÕES FINAIS

O Hospital Veterinário Universitário da Universidade Federal de Campina Grande presta um importante serviço de diagnóstico veterinário na mesorregião do Sertão da Paraíba. Com frequência, recebendo também materiais biológicos de animais provenientes de outras regiões e estados do Nordeste do Brasil.

O diagnóstico das doenças de animais, domésticos e selvagens, e a subsequente divulgação à sociedade e à comunidade científica possibilitam a conscientização local e fornecem subsídios para que sejam adotadas medidas de tratamento, controle e profilaxia. Nesse contexto e considerando a excepcional e diversificada casuística, a pitiose representa uma doença de grande relevância local.

Desta forma, podemos caracterizar a pitiose como uma doença endêmica no Nordeste do Brasil. Os equinos, ovinos e cães parecem ser os mais comumente afetados, mas é importante estar ciente de que a doença pode ocasionalmente (ou raramente) afetar outras espécies animais. A pele, o trato respiratório superior e o trato alimentar são os locais mais comumente acometidos, e os sinais clínicos refletem a localização das lesões. O desfecho clínico é muito variável, sendo potencialmente grave para a maioria das espécies animais, mas os bovinos podem apresentar cura espontânea das lesões.

A doença deve ser incluída no diagnóstico diferencial sempre que houver evidência histológica de inflamação crônica com hifas intralesionais, considerando o habitual padrão inflamatório visto em cada espécie animal. O diagnóstico definitivo pode ser estabelecido com base nos achados epidemiológicos, clínicos e anatomopatológicos associados às técnicas de identificação do agente, como a cultura microbiológica, imuno-histoquímica e testes moleculares.

Os médicos veterinários, clínicos e patologistas, devem estar familiarizados com as características clinicopatológicas da doença e a ampla variabilidade de espécies animais suscetíveis.

ANEXO I

Normas para a revista *Research in Veterinary Science*

Use of Word Processing Software

It is important that the file be saved in the native format of the word processor used. The text should be in single-column format. Keep the layout of the text as simple as possible. Most formatting codes will be removed and replaced on processing the article. In particular, do not use the word processor's options to justify text or to hyphenate words. However, do use bold face, italics, subscripts, superscripts etc. When preparing tables, if you are using a table grid, use only one grid for each individual table and not a grid for each row. If no grid is used, use tabs, not spaces, to align columns. The electronic text should be prepared in a way very similar to that of conventional manuscripts (see also the Guide to Publishing with. Elsevier: https://www.elsevier.com/guidepublication). Note that source files of figures, tables and text graphics will be required separate file submissions. See also the section on Electronic artwork. To avoid unnecessary errors you are strongly advised to use the 'spell-check' and 'grammar-check' functions of your word processor.

Introduction

State the objectives of the work and provide an adequate background, avoiding a detailed literature survey or a summary of the results.

Material and methods

Provide sufficient details to allow the work to be reproduced by an independent researcher. Methods that are already published should be summarized, and indicated by a reference. If quoting directly from a previously published method, use quotation marks and also cite the source. Any modifications to existing methods should also be described.

Results

Results should be clear and concise.

Discussion

This should explore the significance of the results of the work, not repeat them. A combined Results and Discussion section is often appropriate. Avoid extensive citations and discussion of published literature.

Conclusions

The main conclusions of the study may be presented in a short Conclusions section, which may stand alone or form a subsection of a Discussion or Results and Discussion section.

Appendices

If there is more than one appendix, they should be identified as A, B, etc. Formulae and equations in appendices should be given separate numbering: Eq. (A.1), Eq. (A.2), etc.; in a subsequent appendix, Eq. (B.1) and so on. Similarly for tables and figures: Table A.1; Fig. A.1, etc.

•***Title.*** Concise and informative. Titles are often used in information-retrieval systems. Avoid abbreviations and formulae where possible.

•***Author names and affiliations.*** Please clearly indicate the given name(s) and family name(s) of each author and check that all names are accurately spelled. You can add your name between parentheses in your own script behind the English transliteration. Present the authors' affiliation addresses (where the actual work was done) below the names. Indicate all affiliations with a lower-case superscript letter immediately after the author's name and in front of the appropriate address. Provide the full postal address of each affiliation, including the country name and, if available, the e-mail address of each author.

•***Corresponding author.*** Clearly indicate who will handle correspondence at all stages of refereeing and publication, also post-publication. This responsibility includes answering any future queries about Methodology and Materials.

Ensure that the e-mail address is given and that contact details are kept up to date by the corresponding author.

•***Present/permanent address.*** If an author has moved since the work described in the article was done, or was visiting at the time, a 'Present address' (or 'Permanent address') may be indicated as a footnote to that author's name. The address at which the author actually did the work must be retained as the main, affiliation address. Superscript Arabic numerals are used for such footnotes.

Highlights

Highlights are mandatory for this journal as they help increase the discoverability of your article via search engines. They consist of a short collection of bullet points that capture the novel results of your research as well as new methods that were used during the study (if any). Please have a look at the examples here: example Highlights.

Highlights should be submitted in a separate editable file in the online submission system. Please use 'Highlights' in the file name and include 3 to 5 bullet points (maximum 85 characters, including spaces, per bullet point).

Abstract

A concise and factual abstract is required. The abstract should state briefly the purpose of the research, the principal results and major conclusions. An abstract is often presented separately from the article, so it must be able to stand alone. For this reason, References should be avoided, but if essential, then cite the author(s) and year(s). Also, non-standard or uncommon abbreviations should be avoided, but if essential they must be defined at their first mention in the abstract itself. Abstract, self-contained and embodying the main conclusions. It should note the relevance to veterinary

science as well as the aims and objectives of the work. Sentences such as 'the results are discussed', which merely describe the paper, are not allowed.

Keywords
Immediately after the abstract, provide a maximum of 6 keywords, using American spelling and avoiding general and plural terms and multiple concepts (avoid, for example, 'and', 'of'). Be sparing with abbreviations: only abbreviations firmly established in the field may be eligible. These keywords will be used for indexing purposes.

Abbreviation and symbols
Authors are asked to explain each scientific abbreviation at it first occurrence in their papers; for example, complement fixations test (CFT). The policy of the journal with respect to units and symbols is that SI (System International) symbols should be used.

Acknowledgements
All contributors who do not meet the criteria for authorship as defined above should be listed in an acknowledgements section. Examples of those who might be acknowledged include a person who provided purely technical help, writing assistance, or a department chair who provided only general support. Authors should disclose whether they had any writing assistance and identify the entity that paid for this assistance.

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List funding sources in this standard way to facilitate compliance to funder's requirements:
Funding: This work was supported by the National Institutes of Health [grant numbers xxxx, yyyy]; the Bill & Melinda Gates Foundation, Seattle, WA [grant number zzzz]; and the United States Institutes of Peace [grant number aaaa].
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Nomenclature
1. Authors and Editors are, by general agreement, obliged to accept the rules governing biological nomenclature, as laid down in the International Code of Botanical Nomenclature, the International Code of Nomenclature of Bacteria, and the International Code of Zoological Nomenclature. Virologists should consult the latest Report of the International Committee on Taxonomy of Viruses for proper nomenclature and spelling.
2. All biotica (crops, plants, insects, birds, mammals, etc.) should be identified by their scientific names when the English term is first used, with the exception of common domestic animals.
3. All biocides and other organic compounds must be identified by their Geneva names when first used in the text. Active ingredients of all formulations should be likewise identified.
4. For chemical nomenclature, the conventions of the International Union of Pure and Applied Chemistry and the official recommendations of the IUPAC-IUB Combined Commission on Biochemical Nomenclature should be followed.

Footnotes
Footnotes should be used sparingly. Number them consecutively throughout the article. Many word processors can build footnotes into the text, and this feature may be used. Otherwise, please indicate the position of footnotes in the text and list the footnotes themselves separately at the end of the article. Do not include footnotes in the Reference list. 1. Footnotes should only be used if absolutely essential. In most cases it should be possible to incorporate the information in normal text. 2. If used, they should be numbered in the text, indicated by superscript numbers, and kept as short as possible.

Artwork
General points
•Make sure you use uniform lettering and sizing of your original artwork.
•Embed the used fonts if the application provides that option.
•Aim to use the following fonts in your illustrations: Arial, Courier, Times New Roman, Symbol, or use fonts that look similar.
•Number the illustrations according to their sequence in the text.
•Use a logical naming convention for your artwork files.
•Provide captions to illustrations separately.
•Size the illustrations close to the desired dimensions of the published version.

You are urged to visit this site; some excerpts from the detailed information are given here.
Formats
If your electronic artwork is created in a Microsoft Office application (Word, PowerPoint, Excel) then please supply 'as is' in the native document format.
Regardless of the application used other than Microsoft Office, when your electronic artwork is finalized, please 'Save as' or convert the images to one of the following formats (note the resolution requirements for line drawings, halftones, and line/halftone combinations given below): EPS (or PDF): Vector drawings, embed all used fonts. TIFF (or JPEG): Color or grayscale photographs (halftones), keep to a minimum of 300 dpi.TIFF (or JPEG): Bitmapped (pure black & white pixels) line drawings, keep to a minimum of 1000 dpi. TIFF (or JPEG): Combinations

bitmapped line/half-tone (color or grayscale), keep to a minimum of 500 dpi.

Color artwork

Please make sure that artwork files are in an acceptable format (TIFF (or JPEG), EPS (or PDF), or MS Office files) and with the correct resolution. If, together with your accepted article, you submit usable color figures then Elsevier will ensure, at no additional charge, that these figures will appear in color online (e.g., ScienceDirect and other sites) regardless of whether or not these illustrations are reproduced in color in the printed version. **For color reproduction in print, you will receive information regarding the costs from Elsevier after receipt of your accepted article**. Please indicate your preference for color: in print or online only. Further information on the preparation of electronic artwork.

Tables

Please submit tables as editable text and not as images. Please ensure each table is submitted as a separate file. Number tables consecutively in accordance with their appearance in the text and place any table notes below the table body. Be sparing in the use of tables and ensure that the data presented in them do not duplicate results described elsewhere in the article. Please avoid using vertical rules and shading in table cells.

1. Authors should take notice of the limitations set by the size and lay-out of the journal. Large tables should be avoided. Reversing columns and rows will often reduce the dimensions of a table.
2. If many data are to be presented, an attempt should be made to divide them over two or more tables.
3. Tables should be numbered according to their sequence in the text. The text should include references to all tables.
4.Please ensure each table is submitted as a separate file. Tables should never be included in the text.
5. Each table should have a brief and self-explanatory title.

Manuscript Formatting

Manuscripts should have **numbered lines**, with wide margins and **double spacing**, throughout, i.e. also for abstracts, footnotes and references. **Every page of the manuscripts, including the tile page, references, tables, etc., should be numbered.** However, in the text no reference should be made to page numbers; if necessary one may refer to sections. Avoid excessive usage of italics to emphasize part of the text.

References

Data references

This journal encourages you to cite underlying or relevant datasets in your manuscript by citing them in your text and including a data reference in your Reference List. Data references should include the following elements: author name(s), dataset title, data repository, version (where available), year, and global persistent identifier. Add [dataset] immediately before the reference so we can properly identify it as a data reference. The [dataset] identifier will not appear in your published article.

Reference management software

When preparing your manuscript, you will then be able to select this style using the Mendeley plug-ins for Microsoft Word or LibreOffice.

Text: All citations in the text should refer to:
1. *Single author:* the author's name (without initials, unless there is ambiguity) and the year of publication;
2. *Two authors:* both authors' names and the year of publication;
3. *Three or more authors:* first author's name followed by 'et al.' and the year of publication.

Citations may be made directly (or parenthetically). Groups of references should be listed first alphabetically, then chronologically.

Examples: 'as demonstrated (Allan, 2000a, 2000b, 1999; Allan and Jones, 1999). Kramer et al. (2010) have recently shown ....'

List: References should be arranged first alphabetically and then further sorted chronologically if necessary. More than one reference from the same author(s) in the same year must be identified by the letters 'a', 'b', 'c', etc., placed after the year of publication.

Examples:

Reference to a journal publication: Foster, N., Berndt, A., Lalmanach, A.C., Methner, U., Pasquali, P., Rychlik, I., Velge, P., Zhou, X., Barrow, P., 2012. Emergency and therapeutic vaccination–is stimulating innate immunity an option? Res. Vet. Sci. 93, 7–12. Reference to a book: Strunk Jr., W., White, E.B., 2000.

ANEXO II

Normas para o *Journal of Equine Veterinary Science*

Use of word processing software

It is important that the file be saved in the native format of the word processor used. The text should be in single-column format. Keep the layout of the text as simple as possible. Most formatting codes will be removed and replaced on processing the article. In particular, do not use the word processor's options to justify text or to hyphenate words. However, do use bold face, italics, subscripts, superscripts etc. When preparing tables, if you are using a table grid, use only one grid for each individual table and not a grid for each row. If no grid is used, use tabs, not spaces, to align columns. The electronic text should be prepared in a way very similar to that of conventional manuscripts (see also the Guide to Publishing with Elsevier). Note that source files of figures, tables and text graphics will be required whether or not you embed your figures in the text. See also the section on Electronic artwork. To avoid unnecessary errors you are strongly advised to use the 'spell-check' and 'grammar-check' functions of your word processor.

Manuscript types and word count

Please adhere to the following maximum word and reference counts when submitting manuscripts. The word count excludes References, Tables, Figures captions and Supplementary data. Editorials. Editorials do not have abstract, but include 2 or 3 keywords. They typically should be no longer than 1500 words and include no more than 20 references.

Reviews and Minireviews Review articles typically should be fewer than 4000 words and 100 references, and should include an unstructured abstract of no more than 250 words and 6 keywords. Minireviews follow the same general instructions as those for "general" Reviews, but take an in-depth look at the recent developments in the chosen subject. Apart from a few essential references, publications analysed must have been published in the past five years. Their less exhaustive character makes it different from general Reviews. They should not exceed 2500 words and 50 references, and should include an unstructured abstract of no more than 250 words and 6 keywords.

•Original articles. Research reports should not exceed 3000 words of text and 30 references. They should include a structured abstract of no more than 250 words and 6 keywords, and the following sections: Introduction, Material and methods, Results, Discussion.

•Case reports and Technical notes. These articles should not exceed 2000 words of text and 20 references, and should include an unstructured abstract of no more than 250 words and 6 keywords.

•Letters to the editor. Letters to the editor do not have abstract, but 2 or 3 keywords. They typically should be no longer than 1500 words for fewer than 10 references.

Article structure

Subdivision - unnumbered sections

Divide your article into clearly defined sections. Each subsection is given a brief heading. Each heading should appear on its own separate line. Subsections should be used as much as possible when cross-referencing text: refer to the subsection by heading as opposed to simply 'the text'.

Introduction

State the objectives of the work and provide an adequate background, avoiding a detailed literature survey or a summary of the results.

Material and methods

Provide sufficient details to allow the work to be reproduced by an independent researcher. Methods that are already published should be summarized, and indicated by a reference. If quoting directly from a previously published method, use quotation marks and also cite the source. Any modifications to existing methods should also be described.

Results

Results should be clear and concise.

Discussion

This should explore the significance of the results of the work, not repeat them. A combined Results and Discussion section is often appropriate. Avoid extensive citations and discussion of published literature.

Glossary

Please supply, as a separate list, the definitions of field-specific terms used in your article.

Appendices

If there is more than one appendix, they should be identified as A, B, etc. Formulae and equations in appendices should be given separate numbering: Eq. (A.1), Eq. (A.2), etc.; in a subsequent appendix, Eq. (B.1) and so on. Similarly for tables and figures: Table A.1; Fig. A.1, etc.

Essential title page information

• ***Title.*** Concise and informative. Titles are often used in information-retrieval systems. Avoid abbreviations and formulae where possible.

• ***Author names and affiliations.*** Please clearly indicate the given name(s) and family name(s) of each author and check that all names are accurately spelled. You can add your name between parentheses in your own script behind the English transliteration. Present the authors' affiliation addresses (where the actual work was done) below the names. Indicate all affiliations with a lower-case superscript letter immediately after the author's name and in front of the appropriate address. Provide the full postal address of each affiliation, including the country name and, if available, the e-mail address of each author.

•***Corresponding author.*** Clearly indicate who will handle correspondence at all stages of refereeing and publication, also post-publication. This responsibility includes answering any future queries about Methodology and Materials.

Ensure that the e-mail address is given and that contact details are kept up to date by the corresponding author.

•***Present/permanent address.*** If an author has moved since the work described in the article was done, or was visiting at the time, a 'Present address' (or 'Permanent address') may be indicated as a footnote to that author's name. The address at which the author actually did the work must be retained as the main, affiliation address. Superscript Arabic numerals are used for such footnotes.

Highlights

Highlights are optional yet highly encouraged for this journal, as they increase the discoverability of your article via search engines. They consist of a short collection of bullet points that capture the novel results of your research as well as new methods that were used during the study (if any). Please have a look at the examples here: example

Highlights. Highlights should be submitted in a separate editable file in the online submission system. Please use 'Highlights' in the file name and include 3 to 5 bullet points (maximum 85 characters, including spaces, per bullet point).

Structured abstract
A structured abstract, by means of appropriate headings, should provide the context or background for the research and should state its purpose, basic procedures (selection of study subjects or laboratory animals, observational and analytical methods), main findings (giving specific effect sizes and their statistical significance, if possible), and principal conclusions. It should emphasize new and important aspects of the study or observations.

Acknowledgements
Collate acknowledgements in a separate section at the end of the article before the references and do not, therefore, include them on the title page, as a footnote to the title or otherwise. List here those individuals who provided help during the research (e.g., providing language help, writing assistance or proof reading the article, etc.).

Formatting of funding sources
List funding sources in this standard way to facilitate compliance to funder's requirements:
Funding: This work was supported by the National Institutes of Health [grant numbers xxxx, yyyy]; the Bill & Melinda Gates Foundation, Seattle, WA [grant number zzzz]; and the United States Institutes of Peace [grant number aaaa].
It is not necessary to include detailed descriptions on the program or type of grants and awards. When funding is from a block grant or other resources available to a university, college, or other research institution, submit the name of the institute or organization that provided the funding.
If no funding has been provided for the research, please include the following sentence:
This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Footnotes
Footnotes should be used sparingly. Number them consecutively throughout the article. Many word processors can build footnotes into the text, and this feature may be used. Otherwise, please indicate the position of footnotes in the text and list the footnotes themselves separately at the end of the article. Do not include footnotes in the Reference list.

Artwork *Electronic artwork*
General points
•Make sure you use uniform lettering and sizing of your original artwork.
•Embed the used fonts if the application provides that option.
•Aim to use the following fonts in your illustrations: Arial, Courier, Times New Roman, Symbol, or use fonts that look similar.
•Number the illustrations according to their sequence in the text.
•Use a logical naming convention for your artwork files.
•Provide captions to illustrations separately.
•Size the illustrations close to the desired dimensions of the published version.
•Submit each illustration as a separate file.
•Ensure that color images are accessible to all, including those with impaired color vision.
A detailed guide on electronic artwork is available.

Please do not:
•Supply files that are optimized for screen use (e.g., GIF, BMP, PICT, WPG); these typically have a low number of pixels and limited set of colors;
•Supply files that are too low in resolution;
•Submit graphics that are disproportionately large for the content.

Color artwork
Please make sure that artwork files are in an acceptable format (TIFF (or JPEG), EPS (or PDF) or MS Office files) and with the correct resolution. If, together with your accepted article, you submit usable color figures then Elsevier will ensure, at no additional charge, that these figures will appear in color online (e.g., ScienceDirect and other sites) in addition to color reproduction in print. Further information on the preparation of electronic artwork.

Illustration services
Elsevier's Author Services offers Illustration Services to authors preparing to submit a manuscript but concerned about the quality of the images accompanying their article. Elsevier's expert illustrators can produce scientific, technical and medical-style images, as well as a full range of charts, tables and graphs. Image 'polishing' is also available, where our illustrators take your image(s) and improve them to a professional standard. Please visit the website to find out more.

Figure captions
Ensure that each illustration has a caption. Supply captions separately, not attached to the figure. A caption should comprise a brief title (**not** on the figure itself) and a description of the illustration. Keep text in the illustrations themselves to a minimum but explain all symbols and abbreviations used.

Tables
Please submit tables as editable text and not as images. Tables can be placed either next to the relevant text in the article, or on separate page(s) at the end. Number tables consecutively in accordance with their appearance in the text and place any table notes below the table body. Be sparing in the use of tables and ensure that the data presented in them do not duplicate results described elsewhere in the article. Please avoid using vertical rules and shading in table cells.

References

Citation in text

Please ensure that every reference cited in the text is also present in the reference list (and vice versa). Any references cited in the abstract must be given in full. Unpublished results and personal communications are not recommended in the reference list, but may be mentioned in the text. If these references are included in the reference list they should follow the standard reference style of the journal and should include a substitution of the publication date with either 'Unpublished results' or 'Personal communication'.

Reference links

Increased discoverability of research and high quality peer review are ensured by online links to the sources cited. In order to allow us to create links to abstracting and indexing services, such as Scopus, CrossRef and PubMed, please ensure that data provided in the references are correct. Please note that incorrect surnames, journal/book titles, publication year and pagination may prevent link creation. When copying references, please be careful as they may already contain errors. Use of the DOI is highly encouraged.

Reference style

Text: Indicate references by number(s) in square brackets in line with the text. The actual authors can be referred to, but the reference number(s) must always be given.
List: Number the references (numbers in square brackets) in the list in the order in which they appear in the text.
Examples:

Reference to a journal publication:

[1] Van der Geer J, Hanraads JAJ, Lupton RA. The art of writing a scientific article. J Sci Commun 2010;163:51–9. https://doi.org/10.1016/j.Sc.2010.00372.

Reference to a journal publication with an article number:

[2] Van der Geer J, Hanraads JAJ, Lupton RA. The art of writing a scientific article. Heliyon. 2018;19:e00205. https://doi.org/10.1016/j.heliyon.2018.e00205

Reference to a book:

[3] Strunk Jr W, White EB. The elements of style. 4th ed. New York: Longman; 2000.

Reference to a chapter in an edited book:

[4] Mettam GR, Adams LB. How to prepare an electronic version of your article. In: Jones BS, Smith RZ, editors. Introduction to the electronic age, New York: E-Publishing Inc; 2009, p. 281–304.

ANEXO III

Normas para o *Journal of Medical Micology*

Use of word processing software

It is important that the file be saved in the native format of the word processor used. The text should be in single-column format. Keep the layout of the text as simple as possible. Most formatting codes will be removed and replaced on processing the article. In particular, do not use the word processor's options to justify text or to hyphenate words. However, do use bold face, italics, subscripts, superscripts etc. When preparing tables, if you are using a table grid, use only one grid for each individual table and not a grid for each row. If no grid is used, use tabs, not spaces, to align columns.

Manuscript types and word count

Please adhere to the following maximum word and reference counts when submitting manuscripts. The word count excludes References, Tables, Figures captions and Supplementary data. Editorials. Editorials do not have abstract, but include 2 or 3 keywords. They typically should be no longer than 1500 words and include no more than 20 references.

Reviews and Minireviews Review articles typically should be fewer than 4000 words and 100 references, and should include an unstructured abstract of no more than 250 words and 6 keywords. Minireviews follow the same general instructions as those for "general" Reviews, but take an in-depth look at the recent developments in the chosen subject.

•Original articles. Research reports should not exceed 3000 words of text and 30 references. They should include a structured abstract of no more than 250 words and 6 keywords, and the following sections: Introduction, Material and methods, Results, Discussion.

•Case reports and Technical notes. These articles should not exceed 2000 words of text and 20 references, and should include an unstructured abstract of no more than 250 words and 6 keywords.

•Letters to the editor. Letters to the editor do not have abstract, but 2 or 3 keywords. They typically should be no longer than 1500 words for fewer than 10 references.

Article structure

Subdivision - unnumbered sections

Divide your article into clearly defined sections. Each subsection is given a brief heading. Each heading should appear on its own separate line. Subsections should be used as much as possible when cross-referencing text: refer to the subsection by heading as opposed to simply 'the text'.

Introduction

State the objectives of the work and provide an adequate background, avoiding a detailed literature survey or a summary of the results.

Material and methods

Provide sufficient details to allow the work to be reproduced by an independent researcher. Methods that are already published should be summarized, and indicated by a reference. If quoting directly from a previously published method, use quotation marks and also cite the source. Any modifications to existing methods should also be described.

Results
Results should be clear and concise.

Discussion
This should explore the significance of the results of the work, not repeat them. A combined Results and Discussion section is often appropriate. Avoid extensive citations and discussion of published literature.

Glossary
Please supply, as a separate list, the definitions of field-specific terms used in your article.

Appendices
If there is more than one appendix, they should be identified as A, B, etc. Formulae and equations in appendices should be given separate numbering: Eq. (A.1), Eq. (A.2), etc.; in a subsequent appendix, Eq. (B.1) and so on. Similarly for tables and figures: Table A.1; Fig. A.1, etc.

Essential title page information

• ***Title.*** Concise and informative. Titles are often used in information-retrieval systems. Avoid abbreviations and formulae where possible.

• ***Author names and affiliations.*** Please clearly indicate the given name(s) and family name(s) of each author and check that all names are accurately spelled. You can add your name between parentheses in your own script behind the English transliteration. Present the authors' affiliation addresses (where the actual work was done) below the names. Indicate all affiliations with a lower-case superscript letter immediately after the author's name and in front of the appropriate address. Provide the full postal address of each affiliation, including the country name and, if available, the e-mail address of each author.

Keywords
Immediately after the abstract, provide a maximum of 6 keywords, using British spelling and avoiding general and plural terms and multiple concepts (avoid, for example, 'and', 'of'). Be sparing with abbreviations: only abbreviations firmly established in the field may be eligible. These keywords will be used for indexing purposes.

Formatting of funding sources
List funding sources in this standard way to facilitate compliance to funder's requirements:
Funding: This work was supported by the National Institutes of Health [grant numbers xxxx, yyyy]; the Bill & Melinda Gates Foundation, Seattle, WA [grant number zzzz]; and the United States Institutes of Peace [grant number aaaa].
It is not necessary to include detailed descriptions on the program or type of grants and awards. When funding is from a block grant or other resources available to a university, college, or other research institution, submit the name of the institute or organization that provided the funding.
If no funding has been provided for the research, please include the following sentence:
This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Nomenclature for mycoses and fungi
Authors are advised to respect the norms published by the Société Internationale de Mycologie Humaine et Animale (J Med Vet Mycol 1992;30:1-10 and Clin Infect Dis 1993;16:610–1). The names of fungi (genus and species) should be underlined or in italics. The name of the genus should be given in full in the summary and the first time it is used in the text, subsequently it should be abbreviated.

References
Text: Indicate references by number(s) in square brackets in line with the text. The actual authors can be referred to, but the reference number(s) must always be given.
List: Number the references (numbers in square brackets) in the list in the order in which they appear in the text.
Examples:
Reference to a journal publication:
[1] Van der Geer J, Hanraads JAJ, Lupton RA. The art of writing a scientific article. J Sci Commun 2010;163:51–9. https://doi.org/10.1016/j.Sc.2010.00372.
Reference to a journal publication with an article number:
[2] Van der Geer J, Hanraads JAJ, Lupton RA. The art of writing a scientific article. Heliyon. 2018;19:e00205. https://doi.org/10.1016/j.heliyon.2018.e00205
Reference to a book:
[3] Strunk Jr W, White EB. The elements of style. 4th ed. New York: Longman; 2000.
Reference to a chapter in an edited book:
[4] Mettam GR, Adams LB. How to prepare an electronic version of your article. In: Jones BS, Smith RZ, editors. Introduction to the electronic age, New York: E-Publishing Inc; 2009, p. 281–304.

ANEXO IV

Normas para a revista Arquivo Brasileiro de Medicina Veterinária e Zootecnia

Os artigos devem ser redigidos em português ou inglês, na forma impessoal.

Seções
Título: Em português e em inglês. Deve contemplar a essência do artigo e não ultrapassar 50 palavras.
Autores e Filiação: Os nomes dos autores são colocados abaixo do título, com o número do ORCID e com identificação da instituição a qual pertencem. O autor e o seu e-mail para correspondência devem ser indicados com asterisco somente no “Title Page” (Step 3), em arquivo Word.

Resumo e Abstract: Deve ser o mesmo apresentado no cadastro contendo até 200 palavras em um só parágrafo. Não repetir o título e não acrescentar revisão de literatura. Incluir os principais resultados numéricos, citando-os sem explicá-los, quando for o caso. Cada frase deve conter uma informação completa.

Palavras-chave e Keywords: No máximo cinco e no mínimo duas*.

* na submissão usar somente o Keyword (Step 2) e no corpo do artigo constar tanto keyword (inglês) quanto palavra-chave (português), independente do idioma em que o artigo for submetido.

Introdução: Explanação concisa na qual os problemas serão estabelecidos , bem como a pertinência, a relevância e os objetivos do trabalho. Deve conter poucas referências, o suficiente para balizá-la.

Material e Métodos: Citar o desenho experimental, o material envolvido, a descrição dos métodos usados ou referenciar corretamente os métodos já publicados. Nos trabalhos que envolvam animais e/ou organismos geneticamente modificados **deverão constar obrigatoriamente o número do Certificado de Aprovação do CEUA**. (verificar o Item Comitê de Ética).

Resultados: Apresentar clara e objetivamente os resultados encontrados.

Tabela. Conjunto de dados alfanuméricos ordenados em linhas e colunas. Usar linhas horizontais na separação dos cabeçalhos e no final da tabela. O título da tabela recebe inicialmente a palavra Tabela, seguida pelo número de ordem em algarismo arábico e ponto (ex.: Tabela 1.)

Figura. Compreende qualquer ilustração que apresente linhas e pontos: desenho, fotografia, gráfico, fluxograma, esquema etc. A legenda recebe inicialmente a palavra Figura, seguida do número de ordem em algarismo arábico e ponto (ex.: Figura 1.) e é citada no texto como Fig seguida de ponto e do número de ordem (ex.: Fig.1), mesmo se citar mais de uma figura (ex.: Fig. 1, 2 e 3). Além de inseridas no corpo do texto, fotografias e desenhos devem também ser enviados no formato JPG com alta qualidade, em um arquivo zipado, anexado no campo próprio de submissão, na tela de registro do artigo. As figuras devem ser obrigatoriamente inseridas no corpo do texto de preferência após a sua primeira citação.

Discussão: Discutir somente os resultados obtidos no trabalho. (Obs.: As seções Resultados e Discussão poderão ser apresentadas em conjunto a juízo do autor, sem prejudicar qualquer uma das partes).

Conclusões: As conclusões devem apoiar-se nos resultados da pesquisa executada e serem apresentadas de forma objetiva, **SEM** revisão de literatura, discussão, repetição de resultados e especulações.

Agradecimentos: Não obrigatório. Devem ser concisamente expressados.

Referências: As referências devem ser relacionadas em ordem alfabética, dando-se preferência a artigos publicados em revistas nacionais e internacionais, indexadas. Livros e teses devem ser referenciados o mínimo possível, portanto, somente quando indispensáveis. São adotadas as normas gerais da ABNT, **adaptadas** para o ABMVZ, conforme exemplos:

Como referenciar:

1. Citações no texto

A indicação da fonte entre parênteses sucede à citação para evitar interrupção na sequência do texto, conforme exemplos:

- autoria única: (Silva, 1971) ou Silva (1971); (Anuário..., 1987/88) ou Anuário... (1987/88);
- dois autores: (Lopes e Moreno, 1974) ou Lopes e Moreno (1974);
- mais de dois autores: (Ferguson *et al*., 1979) ou Ferguson *et al*. (1979);
- mais de um artigo citado: Dunne (1967); Silva (1971); Ferguson *et al*. (1979) ou (Dunne, 1967; Silva, 1971; Ferguson *et al*., 1979), sempre em ordem cronológica ascendente e alfabética de autores para artigos do mesmo ano.

2. Periódicos (até quatro autores citar todos. Acima de quatro autores citar três autores *et al.*):

ANUÁRIO ESTATÍSTICO DO BRASIL. v.48, p.351, 1987-88.

FERGUSON, J.A.; REEVES, W.C.; HARDY, J.L. Studies on immunity to alphaviruses in foals. *Am. J. Vet. Res.*, v.40, p.5-10, 1979.

HOLENWEGER, J.A.; TAGLE, R.; WASERMAN, A. et al. Anestesia general del canino. *Not. Med. Vet.*, n.1, p.13-20, 1984.

3. Publicação avulsa (até quatro autores citar todos. Acima de quatro autores citar três autores *et al.*):

DUNNE, H.W. (Ed). Enfermedades del cerdo. México: UTEHA, 1967. 981p.

LOPES, C.A.M.; MORENO, G. Aspectos bacteriológicos de ostras, mariscos e mexilhões. In: CONGRESSO BRASILEIRO DE MEDICINA VETERINÁRIA, 14., 1974, São Paulo. *Anais*... São Paulo: [s.n.] 1974. p.97. (Resumo).

MORRIL, C.C. Infecciones por clostridios. In: DUNNE, H.W. (Ed). Enfermedades del cerdo. México: UTEHA, 1967. p.400-415.

4. Documentos eletrônicos (até quatro autores citar todos. Acima de quatro autores citar três autores *et al.*):

QUALITY food from animals for a global market. Washington: Association of American Veterinary Medical College, 1995. Disponível em: <http://www.org/critca16.htm>. Acessado em: 27 abr. 2000.