Pythiosis in sheep from Paraná, southern Brazil[1]

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ABSTRACT.- Bernardo F.D., Conhizak C., Ambrosini F., Jesus F.P.K., Santurio J.M., Kommers G.D., Elias F. & Franciscato C. 2015. **Pythiosis in sheep from Paraná, southern Brazil.** *Pesquisa Veterinária Brasileira 35(6):513-517.* Curso de Medicina Veterinária, Universidade Federal da Fronteira Sul, Av. Edmundo Gaievski 1000, Realeza, PR 85770-000, Brazil. E-mail: carinafranciscato@yahoo.com.br

This paper reports pythiosis in a sheep from southwestern Paraná, Brazil, confirmed by indirect ELISA (Enzime-Linked Immunosorbent Assay) and immunohistochemistry, as well as it describes the macro and microscopic injuries, in order to understand the pathogenicity. A 4-year-old ewe from a flock of 30 Santa Inês sheep, raised semi-extensively with access to a weir, showed cachexia, bilateral enlargement in nasal region, a serous and bloody secretion with a fetid odor from its nose and swollen submandibular and retropharyngeal lymph nodes. Blood collection was performed trough jugular vein puncture in order to make complete blood cell count (CBC) and to obtain serum for the subsequent serological examination. As the hematological counts were within the normal range for sheep, the animal was euthanized and submitted to necropsy. Indirect ELISA resulted positive for pythiosis. Necropsy revealed necrosis of the hard palate with a diameter of 3.5cm and extending up to the nasal cavity, forming a fistula. Submandibular and retropharyngeal lymph nodes were enlarged and edematous on section. Microscopic findings for submandibular and retropharyngeal lymph nodes consisted in moderate infiltration of eosinophils mainly in the subcapsular sinus, characterizing reactive eosinophilic lymphadenitis. The nasal cavity revealed rhinitis and oral cavity stomatitis with necro-eosinophilic and pronounced multifocal granulomatous infiltration and presence of hyphae. Hyphae found in palate and nasal cavity were positive for *Pythium insidiosum* by Grocott's method and immunohistochemistry, the last one considered to be confirmatory for the pathogen diagnostic. This report has an important epidemiological aspect, as it is the first case of pythiosis in sheep confirmed by serology in South Brazil and an alert of possible infection by the pathogen in floodplains.

INDEX TERMS: ELISA, histopathology, immunehistochemistry, sheep, *Pythium insidiosum*.

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RESUMO.- [**Pitiose em ovinos no Paraná, Sul do Brasil.**] Este trabalho tem como objetivo descrever um caso de pitiose confirmado por ELISA (*Enzime-Linked Immunosorbent Assay*) indireto e imuno-histoquímica em uma ovelha do sudoeste do Paraná, Brasil, bem como suas lesões macro e microscópicas, a fim de compreender a sua patogenicidade. Trata-se de um ovino, fêmea, de 4 anos de idade, de um rebanho de 30 animais, da raça Santa Inês, criados em sistema semiextensivo, com acesso à um açude. O animal vinha apresentando aumento de volume na região do focinho, associado a emagrecimento progressivo. No exame físico apresentou-se caquético, com aumento de volume bilateral na região nasal, e com uma secreção serosanguinolenta de odor fétido fluindo das narinas, além de possuir os linfonodos submandibulares e retrofaríngeos bilateral-

mente infartados. Foi realizada coleta de sangue por punção da veia jugular para realização do hemograma e obtenção do soro para posterior realização de exame sorológico. Sendo que todos os parâmetros hematológicos analisados estavam dentro dos valores normais para a espécie. Após a realização do exame clínico e da coleta de sangue, o animal foi eutanasiado e procedeu-se a necropsia. Através do Teste de ELISA indireto, a amostra apresentou resultado positivo para pitiose. Na ocasião da necropsia pode-se verificar uma área necrosada de aproximadamente 3,5 cm de diâmetro no palato duro, a qual se estendeu até o assoalho da cavidade nasal formando uma fístula. Os linfonodos submandibulares e retrofaríngeos estavam aumentados de volume e ao corte edematosos. Os achados microscópicos dos linfonodos submandibulares e retrofaríngeos consistiram de quantidade moderada de eosinófilos, principalmente nos seios subcapsulares caracterizando uma linfadenite eosinofílica (reativa). Na cavidade nasal e oral, observou-se, respectivamente, rinite e estomatite necro-eosinofílica e granulomatosa multifocal acentuada associada a hifas intralesionais. As hifas encontradas no palato e na cavidade nasal foram Grocott-positivas e positivas pela técnica de imuno-histoquímica para *Pythium insidiosum*, esta última técnica também foi considerada confirmatória para o diagnóstico do agente. Este relato possui impacto epidemiológico por ser o primeiro caso de pitiose sorologicamente confirmado na espécie ovina na região Sul do Brasil e também, serve de alerta para a população, principalmente do meio rural, visto a possibilidade de infecção com o agente quando em contato com áreas alagadiças.

TERMOS DE INDEXAÇÃO: ELISA, histopatologia, imuno-histoquímica, ovino, *Pythium insidiosum*.

INTRODUCTION

Pythium is a genus of parasitic oomycotes that includes more than 120 species, most of them pathogens of plants (Leal et al. 2001b). An important pathogen species in mammals is *Pythium insidiosum* (Mendoza et al. 1996). It causes fast-growing clinical conditions leading to death, and treatment with antimicrobial agents may be contradictory (Salipante et al. 2012). Normally, outbreaks occur during summers and in those animals that have closer contact with floodplains (Gaastra et al. 2010). Several species of domestic animals can be affected, mainly equines (Pal & Mahendra 2014). Moreover, pythiosis has been described in humans (Vanittanakom et al. 2004), so it is important to keep track of regions with water reservoirs, as there is the possibility of weir contamination in farms.

The last findings for ovine pythiosis occurred in the Brazilian northeast (Carrera et al. 2013), but none had been reported in the south. In sheep, the ulcerative cutaneous form (Tabosa et al. 2004) has been verified, but metastasis can develop as well, affecting lymph nodes and lungs (Souza et al. 2008). Recently, cases of granulomatous rhinitis were verified (Santurio et al. 2008; Souza et al. 2008, Portela et al. 2010, Carrera et al. 2013), with the destruction of the hard palate, and reaching the nasal vestibule, resulting in a fistula. Microscopically, it is characterized by the development of granulomatous lesions, where it is possible to observe necrotic tissue, *Splendore-Hoeppli* phenomenon and inflammatory infiltration (Carrera et al. 2013).

The differential diagnosis includes zygomycosis, habronemosis, epizootic lymphangitis, tumors such as sarcoid and squamous cell carcinoma (Pereira & Meireles 2007). The diagnosis of pythiosis should consider the clinical and histopathological aspects; however, for confirmation there exist laboratory techniques such as: immunodiffusion (Miller & Campbell 1982), immunohistochemistry (Gaastra et al. 2010), Enzyme-Linked Immunosorbent Assay (ELISA) (Mendonza et al. 1997, Santurio et al. 2006)

Due to the importance of the illness, both to animal sanitation and to public health, and the lack of reports of ovine pythiosis in the South of Brazil, this study aims to describe a case of pythiosis confirmed with indirect ELISA and immunohistochemistry in a sheep from the southwest of Paraná, Brazil, as well its macro and microscopic injuries, in order to understand its pathogenicity.

MATERIALS AND METHODS

A 4-year-old ewe from a flock of 30 Santa Inês sheep, raised semi-extensively in Francisco Beltrão, southwestern Paraná, Brazil. According to its owner, the animal showed enlargement in the region near the nose two weeks before veterinary assistance. In that period, the animal stopped eating and had progressive weight loss. It was reported that this animal had access to a grassy place with a weir where the animals could drink water. After anamnesis, the clinical examination was proceeded.

Blood was collected through a jugular vein puncture: 5ml of blood in a tube with anticoagulant (EDTA) to perform the hemogram, and 10ml of blood in a tube with no anticoagulant to obtain serum for the subsequent serological examination. After the clinical examination and blood collection, the animal was euthanized and submitted to necropsy. Fragments from the affected area (palate and nasal regions) and local lymph nodes were collected and fixed in a 10%-formaldehyde solution for future histopathology and immunohistochemistry analyses. The histological slides were prepared and stained with hematoxylin and eosin (HE) and Grocott.

The blood samples were processed in order to perform the blood analysis and to obtain serum through centrifugation (5.000rpm during 10 minutes); the serum was stored at a temperature of -20°C for posterior serological analysis, and later sent to the Mycological Research Laboratory (LAPEMI) of Universidade Federal de Santa Maria (UFSM), for indirect ELISA (Enzime-Linked Immunosorbent Assay).

Antibodies against *Pythium insidiosum* were measured by indirect ELISA for serodiagnosis of pythiosis, as previously described by Santurio et al. (2006) and used by Carrera et al. (2013).

RESULTS

During the physical evaluation the animal showed cachexia, bilateral enlargement of the nasal region and right nasal necrosis (Fig.1), from where a serous and bloody secretion with a fetid odor flowed, in addition to swollen submandibular and retropharyngeal lymph nodes, bilaterally; cardiac and respiratory parameters and rectal temperature were within the acceptable ranges.

At necropsy, it was possible to verify an accentuated crumbly necrosis (dark brown coloration) with an irregu-

Fig.1. Volume enlargement of the nasal region and nasal necrosis in sheep.

Fig.2. Severe destruction of hard palate of a sheep due to necrosis associated to serous and bloody secretion.

lar surface of around 3.5cm of diameter in the hard palate (Fig.2) and extending up to the nasal cavity floor, forming a fistula. Moreover, submandibular and retropharyngeal lymph nodes were enlarged in volume and became oedematous on the cut surface.

As for microscopic findings, it was possible to verify in the submandibular and retropharyngeal lymph nodes a moderate quantity of eosinophils, mainly in subcapsular sinus, characterizing the eosinophilic lymphadenitis (reactive). Among the different tissues from the nasal region (cartilage, muscles and goblet glands), a thick cellular film of inflammatory cells was observed, mainly eosinophils and, to a lesser extent, lymphocytes and plasmocytes. Necrosis was verified among this inflammatory cells film, consisting of cellular remains of eosinophils (eosinophilic necrosis), ranging from small to large ones. In those areas, there was a large amount of necrotic content, cellular debris and fibrin. There was a great quantity of epithelioid macrophages and some giant multinucleated cells (most of them Langhans giant cells) surrounding the eosinophilic necrosis. Among the necrosis, negative images of filamentary and circular structures were observed, with a diameter of around 7-10µm (a morphology compatible with hyphae), often surrounded by eosinophils granules (a reaction similar to *Splendore-Hoeppli* phenomenon) (Fig.3). Occasionally, those hyphae were inside the giant cells. Also observed were areas consisting of a great number of fibroblasts and small blood vessels with swollen endothelium (granulation tissue). Those findings from the nasal cavity have led to the diagnosis of necro-eosinophilic and pronounced multifocal granulomatous rhinitis associated with intralesional hyphae.

In the palate fragments, large areas of eosinophilic necrosis were verified in the tissue underlying the mucosa, as well as points of aggregated basophilic cocci (aggregated bacteria), a large quantity of vessels with lumen occluded by thrombi linked to the endothelium, hemorrhage areas and accumulation of complete and degenerated neutrophils and eosinophils. Areas of necrosis were surrounded by epithelioid macrophage and some giant, multinuclear cells. Linked to this, areas with a large amount of granulation tissue had developed. Those areas were adjacent to necrosis and inflammation regions. Structures consistent with hyphae were observed, similar to those described for nasal tissue fragments. Based on the hard palate microscopy, the sheep developed a necro-eosinophilic and pronounced multifocal granulomatous stomatitis associated with

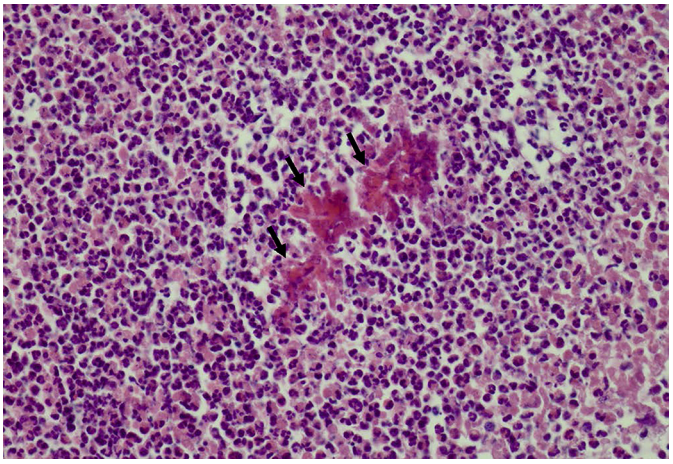

Fig.3. Nasal lesion of a sheep. Reaction similar to *Splendore-Hoeppli*, surrounded by eosinophil inflammation (arrows). H&E, obj.40x.

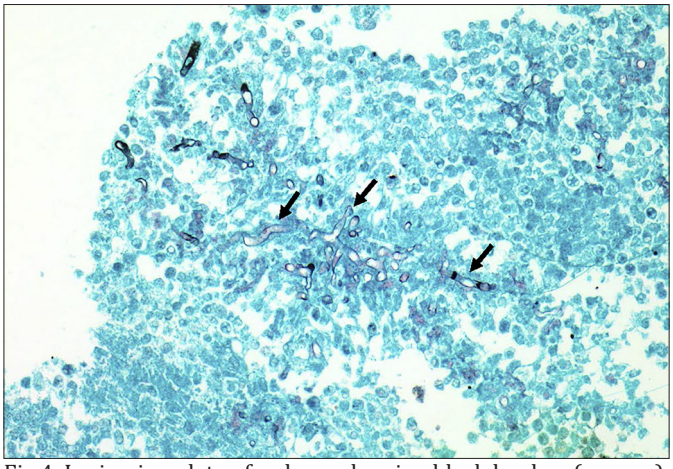

Fig.4. Lesion in palate of a sheep showing black hyphae (arrows). Grocott method, obj.40x.

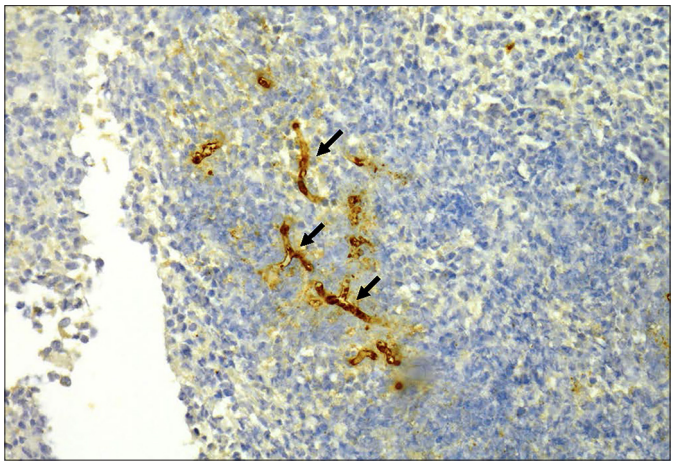

Fig.5. Immunohistochemistry technique confirming the presence of *Pythium insidiosum* (arrows). Nasal region of a sheep. Obj.40x.

intralesional hyphae. Hyphae found in the palate and nasal cavity were positive by Grocott's method (Fig. 4) and by the immunohistochemistry technique for *Pythium insidiosum* (Fig.5).

All of the hematological parameters fell within the acceptable ranges for the species. Following standardized protocol for confirmation of pythiosis by indirect ELISA, the sample tested positive for pythiosis.

DISCUSSION

According to the animal's record, it is believed that the infection source was the weir in the farm, to which animals had access to drink water. This assertion is reinforced by the fact that the evolution cycle of the pathogen occurs on floodplains (Mendoza et al. 1993), where its zoospores in the water can penetrate, causally, by chemotaxis, into the wounds of a host organism (with skin lesions generally serving as entryways), forming filaments with invasive power (Gaastra et al. 2010). It is underlined that hot weather contributes to the development of the pathogen in the environment due to its thermophilic feature (Pereira & Meireles 2007), and the free access of the animal to the floodplains was crucial to the pathogen transmission.

Macroscopic lesions showed by the sheep were similar to those described in sheep in Pernambuco (Carrera et al. 2013) and Piauí (Souza et al. 2008), which showed facial deformity with necrosis, hard palate and nasal septum fistula in the rostral region of the nasal cavity.

It is believed that the entryway for the oomycetes was a previous lesion in the oral cavity, especially in the hard palate. With the invasion of hyphae and immunological response of the host, the lesion developed to the nasal cavity, where it formed a fistula linking the two cavities. This finding was described by other authors as well, who found fistula in the hard palate linking the nose to the oral cavity (Carrera et al. 2013). Therefore, it is underlined that previous local lesions can allow the adherence of the zoospores and cause the formation of filaments with invasive power (Mendoza et al. 1993). Thus, the lesions showed by the studied animal were a consequence of the immune reaction due to hyphae migration, with the posterior development of secondary bacterial infection evolving into necrosis.

Tabosa et al. (2004) observed lesions caused by *Pythium insidiosum* in different regions, such as injuries and ulcerative lesions on legs and pre-scapular and abdominal regions of sheep after they had been on a grassy place with a weir. It is highlighted that the region where the lesions developed is related to the anatomic region with an injury and that had contact with water contaminated with the microorganisms (Pereira & Meireles 2007).

Indirect ELISA proved to be efficient for the illness diagnosis, as observed by Carrera et al. (2013), where all affected animals showed an antibody titer above the cut-off for *P. insidiosum*. The same test has demonstrated high sensibility and specificity (Santurio et al. 2006). Moreover, it makes possible the detection of early or subclinical infections (Mendoza et al. 1997), which are not always are possible to be detected by traditional diagnostic methods such as histopathological and mycological examinations (Leal et al. 2001a); but histopathology helps with the diagnosis and, in order to confirm it, the immunohistochemistry technique is necessary (Dória et al. 2014). Reis Júnior & Nogueira (2002) proved that the immunohistochemistry technique has high specificity in the diagnosis of *P. insidiosum* infection. Isolation of the pathogen shows some controversies: it is a slow process and there is a risk of secondary bacterial contamination, requiring a trained staff for zoosporogenesis induction (Mendoza et al. 1987) as well.

Other more convenient immunological techniques in relation to traditional methods of diagnosis include immune-diffusion and complement fixation tests (Miller & Campbell 1982). However, ELISA stands out as being the most efficient for diagnosing pythiosis (Mendoza et al. 1997) and allowing for an early diagnosis, because it made it possible to detect antibodies against *P. insidiosum* 14 days after inoculation in rabbits (Santurio et al. 2006).

Microscopic findings support Souza et al. (2008), who identified inflammatory infiltrate with formation of granulloma with central necrosis, in the presence of a large quantity of eosinophils, mainly, associated with hyphae surrounded by eosinophilic material - *Splendore-Hoeppli*.

Unlike other reports (Carrera et al. 2013), in this study no fungal hyphae infiltration was observed in lymph nodes; an eosinophilic lymphadenitis (reactive), however, was being the probable reason of their increase under macroscopic examination. It is highlighted that the involvement of other organs occurs through metastases, which in sheep can appear in lymph nodes and lungs (rarer), but this was not seen in the present study. In equines, metastases were verified in the intestinal tract and lungs (Tabosa et al. 2004).

Pythium insidiosum caused irreversible injury in the palate and nasal cavity floor, thus incriminating water intake as a way of disease transmission. The combination of two methods such as indirect ELISA and immunohistochemistry, considered to be highly specific, was essential for the confirmation of the case. Furthermore, this report has a crucial epidemiological impact because it is the first case of ovine pythiosis confirmed by serology in the south of Brazil

and, as well, it is an alert for the population, mainly from the countryside, to the possibility of pathogen infection when in contact with floodplains. This study is relevant to the development of additional studies to better understand the epidemiology of the disease in the Paraná State and the determining factors for the infection of the host.

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