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Cutaneous Pythiosis in calves: An epidemiologic, pathologic, serologic and molecular characterization

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ABSTRACT

This study reports the epidemiological, pathological and mycological findings of cutaneous pythiosis in cattle in southern Brazil. 23 calves, that were kept next to a river with extensive marshy regions, presented ulcerated cutaneous lesions in thoracic and pelvic limbs, sometimes extending to the ventral thoracic region. Histopathological examination revealed multifocal pyogranulomas in the superficial and deep dermis. The Grocott-Methenamine silver, immunohistochemistry anti-*Pythium insidiosum*, ELISA serology and molecular characterization demonstrated the agent *P. insidiosum* in these cases.

1. Introduction

Pythiosis is a chronic granulomatous disease caused by the oomycete *Pythium insidiosum* belonging to the Kingdom *Stramenopila* [11]. These opportunistic pathogens live in warm stagnant water and are most often reported in regions with tropical and subtropical environments [10]. In Brazil, the disease occurs predominantly in horses, however, outbreaks have been reported in sheep and cattle [16,17].

Pythiosis has three distinct forms of clinical presentation in domestic animals: cutaneous [5,6,10], gastrointestinal [15] and rhinofacial [17]. In cattle, the cutaneous form of the disease is sporadic and widely described [16] and usually occurs during the rainy season in subtropical areas [5].

The clinical diagnosis is based on epidemiological characteristics and pathological lesions [3]. However, for definitive diagnosis, cultivation and identification of the agent are crucial [10]. Techniques of immunohistochemistry, serological testing by ELISA and polymerase chain reaction (PCR) are employed to establish the definitive diagnosis of the disease [4,19]. The aim of this study is to report the epidemiological, pathological and mycological findings of cutaneous pythiosis in cattle in southern Brazil.

2. Case

During the month of February of 2016, 23 calves presented ulcerated cutaneous lesions in thoracic and pelvic limbs, sometimes extending to the ventral thoracic region. The herd consisted of 150 beef cattle (100 adults and 50 calves) in moderate body condition, belonging to a property in the city of Triunfo in southern of Brazil (29°56′08.2″S 51°27′54,2″W). The cattle were kept next to a river, in native grass areas with extensive marshy regions (Fig. 1A). The cutaneous lesions were observed after an initial period of 15 days after the animals were introduced into the area and had a clinical course of 20–30 days, with subsequent spontaneous regression in all affected calves after this period. Calves affected received supportive therapy, to prevent secondary skin infections with basic wound management techniques. Cutaneous biopsies were performed in eight calves, from which six samples were fixed in 10% neutral buffered formalin, processed routinely for histopathology, and stained with hematoxylin-eosin (HE) and Gomori methenamine silver (GMS). Two samples were kept at room temperature, and later submitted to cultivation and isolation of the agent.

Grossly, the lesions were located predominantly in the distal regions of the thoracic and pelvic limbs, and sometimes in carpal-ulnar region of forelimbs and ventral thoracic region. These consisted of multifocal ulcerated areas, with approximately 5 cm in diameter,

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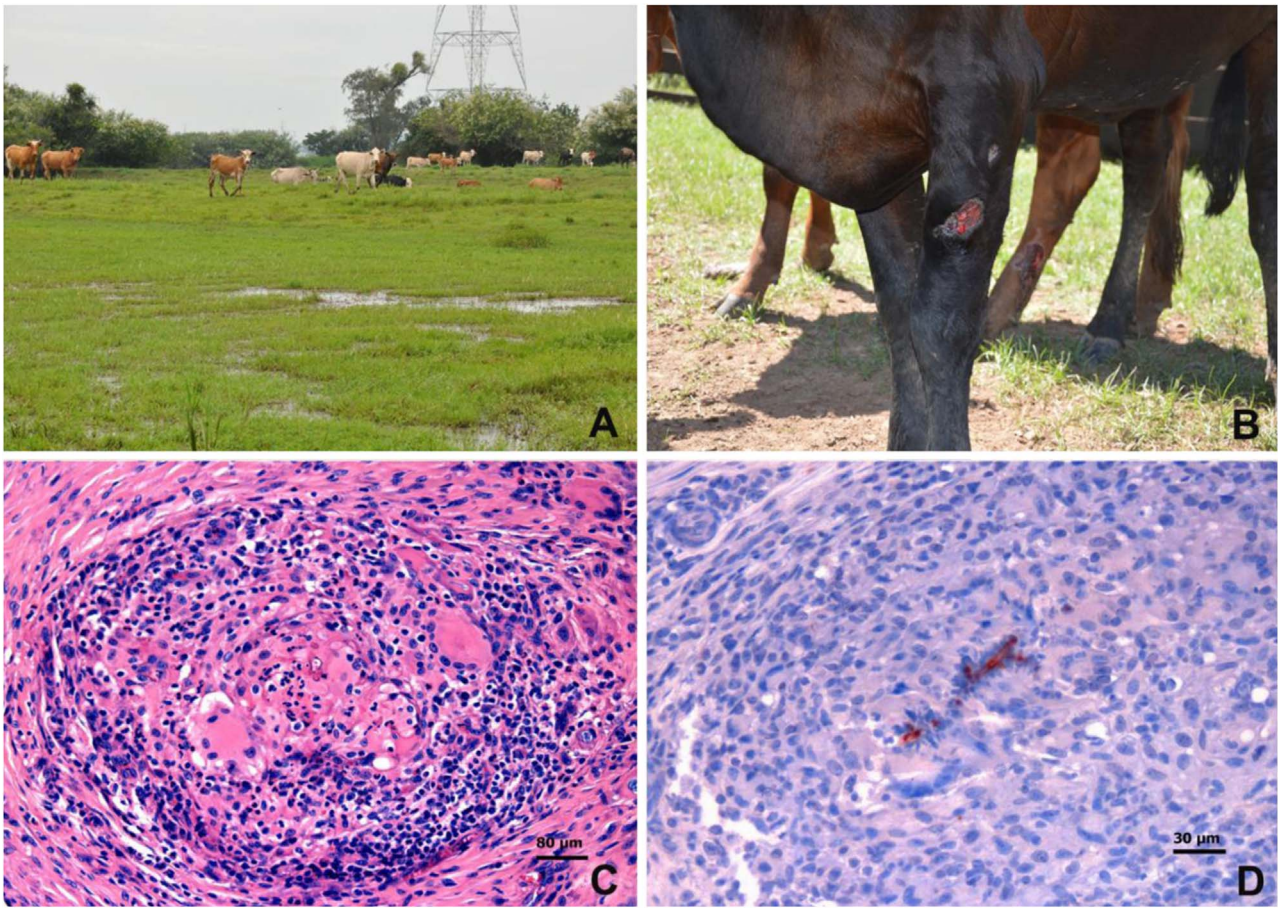

Fig. 1. Cutaneous Pythiosis in calves: an epidemiologic, pathologic, serologic and molecular characterization. (A) Cattle were kept in areas with extensive marshy regions. (B) Calf, left forelimb, an ulcerated area with approximately 3 cm in diameter reddish surface covered by crusts. (C) Skin: pyogranuloma in the dermis characterized by inflammatory infiltrate of neutrophils, eosinophils and a marked amount of epithelioid macrophages in the central region, surrounded by multinucleated giant cells, 200x. HE. (D) Skin: hyphae evidenced by immunohistochemical technique, predominantly observed in the center of the pyogranulomas, 400X. 3-amino-9-ethylcarbazole.

reddish surface, sometimes covered by crusts (Fig. 1B). Histopathological examination revealed multifocal pyogranulomas in the superficial and deep dermis characterized by inflammatory infiltrate of neutrophils, eosinophils and a marked amount of epithelioid macrophages in the central region, surrounded by rare multinucleated giant cells (Fig. 1C). In the central areas of granulomas, there were still occasional transverse and longitudinal sections of non-stained hyphae, surrounded often by eosinophilic radiated material morphologically compatible with Splendore-Hoeppli reaction. Skin sections stained with GMS showed rare centrally located hyphae with approximately 4–9 µm in diameter, ramified and rarely septate. These hyphae were also stained by immunohistochemistry, predominantly observed in the center of these pyogranulomas (Fig. 1D), employing the polyclonal antibody (non-commercial) anti-*P. insidiosum* produced in rabbits, at a 1:100 dilution in phosphate buffered saline (PBS), developed with a red chromogen, 3-amino-9-ethylcarbazole (AEC, Dako North America, Carpinteria, USA).

Serum samples were also collected for analysis by ELISA serology [19]. Plates of polystyrene of 96 wells were sensitized with the antigen diluted in PBS and incubated overnight at 4 °C. Each well received 100 µL of bovine serum albumin 0.2% and, after the incubation period, the plates were washed and stored at 4 °C until the moment of the use. The tested serums were diluted at 1:2000 in PBS in pH 7.2, distributed 100 µL in each well in the plates and incubated at 37 °C for 1 h. Then, the plates were incubated with a specific secondary antibody for the species (anti-IgG bovine conjugated with peroxidase) with dilution of 1:5000. The plates then received the chromogen buffer (*ortho-phenylene-diamine*) and the reading was carried out through a spectrophotomer of plates with 490 nm. The determination of levels of antibodies by ELISA showed positive results for the detection of anti-*Pythium*antibodies for all cattle, suggesting that these animals did develop pythiosis.

The cutaneous biopsies were cultivated on corn meal agar (CMA) at 37 °C for 48 h. A hyaline colony with a short and whitish radiating mycelium was observed. The microscopic evaluation revealed the presence of sparsely septate hyphae similar to *P. insidiosum* growth. For molecular identification, total DNA was extracted of the isolate according to the protocol described in the literature [8,13]. The PCR was performed using the primers ITS1 and ITS4 under the conditions described [1]. The sample was sequenced and data obtained were deposited in GenBank under the accession number KX369614.

3. Discussion

Pythiosis is an infectious disease that affects animals and humans [9]. In cattle, it is usually considered a sporadic disease, which occurs during the rainy season in subtropical areas [5]. The diagnosis of cutaneous pythiosis in cattle in the present outbreak was obtained through the epidemiological and the pathological findings associated with the molecular techniques, serological analyses and cultivation of *P. insidiosum*. The epidemiological findings described [12,14] are similar to those seen in this study, in which only young animals that remained in swampy regions were affected. In a previous report, the time between introduction of the animals in flooded areas and the onset of clinical signs was 15 days with a clinical course that extended over a period of two months [6], similar to that observed in this study.

High rainfall and heavy flooding in the region, influenced by El Niño [7], suggests that the precipitation factor predisposed the occurrence of this outbreak associated with the presence of the animals for long periods in this marshy environment.

The clinical and pathological findings observed in this study were similar to those described by other authors [6,12,14,16], that reported ulcerated lesions with thickening of the dermis and edema in distal regions of fore and hind limbs. One aspect observed in this outbreak and not described to date, was the presence of lesions in the proximal regions located in radio-ulnar region of forelimbs and ventral thoracic region.

The location of the lesions is therefore directly related to the parts of the body that stay in direct contact with water containing zoospores of *P. insidiosum* [14]. In bovines, histopathological lesions are characterized by multifocal pyogranulomas in superficial and deep dermis, composed of polymorphonuclear cells and a predominance of epithelioid macrophages and multinucleated giant cells [16]. A limited number of ramified septate hyphae, surrounded by Splendore-Hoeppli reactions suggest deposition of antigen-antibody complexes around the agent [18], similarly observed in this study. PCR assays, immunohistochemistry, and immunoblot analyses have been developed for identification and differentiation of *P. insidiosum* [10].

The differentiation of *P. insidiosum* from other species and organisms that cause similar clinical signs is very important to select the appropriate treatment. Pithiosis is a self-limiting condition in cattle, and spontaneous healing has been reported to occur after a few weeks to two months. Thus, it does not require specific treatment [6,16]. In horses, it is a chronic disease and effective treatment is obtained with wide surgical excision followed by the use of immunotherapy [5]. Immunotherapic treatment in horses has an effectiveness of 72% when employed alone and of 90% when combined to surgical debridement of diseased tissue [2,5,6].

The microbial culture is a traditional method to identify *P. insidiosum*; however, it requires expertise to identify this specific microorganism [20]. In this study cultured mycelium was morphologically identified as *P.insidiosum*. The PCR test followed by sequencing confirmed the previous morphological identification in the cattle samples of the present work. The molecular techniques represent an important sensitive and specific diagnostic tool for pythiosis [1]. The serological analysis by ELISA, proved to be a specific, sensitive and rapid technical in the diagnosis of bovine pythiosis [6]. The combination of pathological, serological, cultivation and molecular techniques employed in this study were crucial and allowed to characterize the outbreak of cutaneous pythiosis in cattle in Southern Brazil.

Conflict of interest

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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