INFECTIOUS DISEASE

A Comparative Study of the Histopathology and Immunohistochemistry of Pythiosis in Horses, Dogs and Cattle


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Summary

Twenty-one cases of pythiosis in horses (n = 10), dogs (n = 9) and cattle (n = 2) were investigated. The aetiology in all cases was confirmed by immunohistochemistry. Data related to the clinical course and outcome and localization of the lesions were obtained from pathology reports. The equine lesions consisted of fibrotic tissue with multiple, often coalescing, areas of immature granulation tissue encircling eosinophilic cores. Affected dogs had gastrointestinal and/or cutaneous lesions with either or both of a granulomatous/pyogranulomatous or necrotizing eosinophilic inflammatory reaction. In cattle, cutaneous lesions were characterized by multifocal to coalescing granulomas with surrounding fibrosis. The number of intralesional hyphae, the distribution of hyphae, the presence of angioinvasion and the nature of the local inflammatory reactions were associated with the different types of lesions observed.

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Introduction

Pythiosis is caused by the oomycete Pythium insidiosum. The disease affects man (Wanachiwanawin et al., 2004) and horses (Mendoza and Newton, 2005), livestock (Miller et al., 1985; Santurio et al., 1998; Tabosa et al., 2004), dogs (Miller, 1985) and cats (Rakich et al., 2005) among other domestic and wild animal species.

The clinical presentation of pythiosis varies widely between species (Santurio and Ferreiro, 2008). Although the disease is potentially fatal in all species (Mendoza et al., 2003), spontaneous healing is reported in cattle (Santurio et al., 1998; Gabriel et al., 2008; Grecco et al., 2009). It has been suggested that the nature of the immune response to the organism may determine whether the lesions resolve or persist (Mendoza et al., 2003; Mendoza and Newton, 2005).

The aim of the present study was to evaluate comparatively the histopathological and immunohistochemical aspects of pythiosis in horses, dogs and cattle.

Materials and Methods

Twenty-one cases of pythiosis in horses (n = 10), dogs (n = 9) and cattle (n = 2) were investigated. Formalin-fixed, paraffin wax-embedded samples were selected from the archives of the Laboratório de Patologia Veterinária, Universidade Federal de Santa Maria, Brazil, and from the Laboratório Regional de Diagnóstico, Universidade Federal de Pelotas, Brazil. Information related to the clinical presentation and outcome and the location of lesions were obtained from the pathology reports.

Serial sections (3 μm) were cut from the tissue blocks for histochemical staining and immunohistochemistry (IHC). The aetiology was confirmed
in all cases by IHC using a rabbit polyclonal anti-*P. insidiosum* antibody as described by Gabriel et al. (2008). Briefly, sections were dewaxed and rehydrated and endogenous peroxidase activity was blocked with H$_2$O$_2$ 3% in distilled water. Antigen retrieval was by microwaving (10 min at full power) in TRIS-EDTA (pH 9.0). Sections were incubated at 37°C for 60 min with the primary antibody diluted at 1 in 1,000. The secondary reagent was biotinylated anti-rabbit antibody (LSAB+System-AP or -HRP; Dako Corp., Carpinteria, California, USA) followed by streptavidin–alkaline phosphatase (LSAB+System-AP, Dako) or streptavidin–peroxidase (LSAB+System-HRP, Dako). Substrate development was with Liquid Permanent Red (Dako) or 3, 3′-diaminobenzidine (DAB; Sigma–Aldrich, Saint Louis, Missouri, USA), respectively. Sections were counterstained lightly with haematoxylin and coverslipped.

The specificity of the primary antibody was tested in a case of canine gastrointestinal pythiosis (dog 3, Table 1) that was confirmed by microbiological culture and zoosporogenesis (Rech et al., 2004). 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.

Serial sections were stained with haematoxylin and eosin (HE), Grocott’s methenamine silver stain (GMS), periodic acid–Schiff (PAS), sirius red (SR) and toluidine blue (TB). The SR technique, used to demonstrate eosinophil granules, was slightly modified from Wehrend et al. (2004). A combination of GMS and SR (GMS–SR) was used in selected cases to demonstrate intralesional hyphae and eosinophils.

Each technique was employed to evaluate a distinct morphological aspect and a comparative analysis of serial fields was made. HE was used to evaluate the morphology, localization and extent of the lesions and the type of inflammatory response. The intensity

### Table 1

<table>
<thead>
<tr>
<th>Species</th>
<th>Animal</th>
<th>Breed</th>
<th>Sex</th>
<th>Age (years)</th>
<th>Clinical presentation</th>
<th>Location</th>
<th>Outcome</th>
<th>Previous report</th>
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<td>NR</td>
<td>NR</td>
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<tr>
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<td>7</td>
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<td>Abdomen</td>
<td>NR</td>
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<tr>
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<td>Mixed</td>
<td>M</td>
<td>2</td>
<td>Cutaneous/SC</td>
<td>Lip</td>
<td>Humane destruction</td>
<td></td>
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<td>Mixed</td>
<td>F</td>
<td>9</td>
<td>Cutaneous/SC</td>
<td>Jaw</td>
<td>NR</td>
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<td></td>
<td>6</td>
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<td>M</td>
<td>Adult</td>
<td>Cutaneous/SC</td>
<td>Lip and SLN</td>
<td>Humane destruction</td>
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<td>NR</td>
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<tr>
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<td>Large intestine</td>
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<tr>
<td></td>
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<td>Labrador</td>
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<td>Death</td>
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<td>9</td>
<td>Boxer</td>
<td>M</td>
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<td>Cutaneous</td>
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<td>NR</td>
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<td>M</td>
<td>2</td>
<td>Cutaneous</td>
<td>Multifocal</td>
<td>Spontaneous healing</td>
<td>Greco et al., 2009</td>
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</table>

NR, not recorded; F, female; M, male; SLN, submandibular lymph node; LHL, left hindlimb; MLN, mesenteric lymph node.

*P. insidiosum* was isolated in microbiological culture.

†Tissue analysed in this study.
of the inflammatory response was graded as absent, mild (sparse, well-dispersed cells), moderate (scattered, but limited aggregates of inflammatory cells) or severe (diffuse sheets of inflammatory cells). GMS was used to analyse the quantity, distribution and morphological features of hyphae (through which hyphal viability was inferred) and angioinvasion by the agent. Eosinophils and patterns of the Splendore-Hoeppli reaction were evaluated through SR staining. PAS and TB were used to evaluate hyphae and mast cells, respectively.

Results

Clinical data are summarized in Table 1. With the exception of the cattle, the cases were isolated incidents of pythiosis. The cattle were part of two outbreaks of cutaneous pythiosis. In all cases, fungal hyphae were strongly labelled by the \textit{P. insidiosum}-specific antibody (Figs. 1a–c).

\textit{Equine Pythiosis}

Equine skin sections were extensively ulcerated with surface fibrin, neutrophils and bacterial colonies. The majority of the samples comprised of fibrous connective tissue extending from the dermis to the subcutis. There were numerous, often coalescing, foci of immature granulation tissue surrounding eosinophilic material (Fig. 2a). Grossly, sinus tracts filled by detachable tubular structures were described. These latter were yellow, firm and had an irregular surface, resembling coral. These structures are classically termed ‘kunkers’ (Miller and Campbell, 1984; Leal \textit{et al.}, 2001) and this term will be used herein to describe these structures microscopically.

Microscopically, the kunkers consisted of dense collections of eosinophils with numerous and poorly defined cell nuclei and sparse hyaline trabeculae of collagen (collagenolysis) (Fig. 2b). The kunkers were often surrounded by neutrophils and sat within a tract in the tissue, which separated them from adjacent granulation tissue. Negatively-stained hyphal profiles were present at the periphery of the kunkers. Rarely, similar smaller foci, consistent with an early stage of kunker formation, were noted (Fig. 2c). A degenerate artery (small or medium sized) was almost always observed centrally within the kunker, but there was no evidence of angioinvasion.

Rare aggregates of macrophages were present within the granulation tissue. A marked infiltration of eosinophils was present around the kunkers and occasional lymphocytes and plasma cells were present in these areas. Less frequent findings were small arterial thrombi and focal areas of dermal oedema.

With GMS staining, hyphae were seen to be located predominantly at the outer edge of the kunkers and fewer hyphal elements were scattered throughout the kunkers. The hyphae were broad
and sparsely septate and had smooth, almost parallel walls with inconspicuous dilations (Fig. 2d). Horses had a moderate (eight of 10 cases) or large (two of 10 cases) number of hyphae.

The kunkers were deeply stained by SR. The most intense staining was of clusters of intact or degranulated eosinophils surrounding hyphae at the periphery of the kunkers. This relationship was clear when the GMS and SR fields were analysed comparatively and particularly with the GMS–SR stain (Fig. 2e).

Tissue infiltration by eosinophils was also more evident following SR staining. A deeply eosinophilic, granular halo was seen around many hyphae. This finding was considered to be a reaction similar to the Splendore-Hoeppli (SH-like) reaction. Under oil immersion (×100 objective), the SH-like material consisted of multiple SR-positive granules aggregated over the surface of the hyphae (Fig. 2f).

There were few mast cells associated with the kunkers (0–8 per high power field; HPF; ×40.

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**Fig. 2.** Equine cutaneous pythiosis. (a) Dermis thickened by fibrous connective tissue encircling areas of granulation tissue with eosinophilic cores (grossly corresponding to kunkers). HE. Bar, 25 mm. (b) Kunker composed of a dense collection of eosinophils with poorly defined cell nuclei and sparse hyaline collagen trabeculae. HE. Bar, 120 μm. (c) Early stage of kunker formation, with numerous eosinophils. SR. Bar, 50 μm. (d) Hyphae confined to the periphery of a kunker. The hyphae are broad and sparsely septate with smooth and almost parallel walls, with inconspicuous dilations. GMS, Bar, 15 μm. (e) Close relationship between hyphae (dark brown) and eosinophils (red) at the outer edge of a kunker. Eosinophils converge from peripheral tissues to degranulate over the hyphae. GMS–SR, Bar, 20 μm. (f) Bright red granular halo around hyphae (SH-like reaction). The SH-like material consists of multiple SR-positive granules aggregated over the surface of the hyphae. SR, Bar, 10 μm.
and a variable number within the surrounding granulation tissue (mean 20 per HPF; range 0–50 per HPF). There was a moderate quantity of intracytoplasmic granules in these mast cells.

**Canine Pythiosis**

Two main inflammatory patterns were seen in the canine lesions, either as sole patterns or in combination. The first pattern (necro-eosinophilic) was characterized by broad zones of eosinophilic necrosis, cell...
debris, collagenolysis and variable numbers of eosinophils (Fig. 3a). Some areas of necrosis were similar to the early form of kunkers described in horses. The second pattern (granulomatous) consisted of epithelioid macrophages and Langhans giant cells (GCs) mixed in different proportions. These generally formed discrete foci, which, in some cases, were surrounded by thin capsules of connective tissue (Fig. 3b). Rarely, granulomas with necrotic cores and surrounding lymphocytes and plasma cells were present. Negatively-stained hyphal profiles were seen within the necro-eosinophilic lesions and some hyphae were weakly basophilic. Sporadically, a mild multifocal granulomatous response with vacuolated macrophages and few GCs was seen adjacent to necro-eosinophilic areas. Combined foci of both responses were considered pyogranulomatous in nature.

In the gastrointestinal tract, lesions affected one to all layers, mainly the submucosa and muscularis propria. In the cutaneous form, lesions extended from the epidermis, which was usually ulcerated, to the hypodermis. In four of nine cases, there was a predominance...

Fig. 4. Bovine cutaneous pythiosis. (a) Discrete granuloma composed of epithelioid macrophages and Langhans giant cells surrounded by a delicate connective tissue capsule. HE. Bar, 30 \( \mu \)m. (b) Discrete pyogranuloma with a moderate number of centrally located polymorphonuclear leucocytes. HE. Bar, 50 \( \mu \)m. (c) Classical SH reaction consisting of lightly eosinophilic material with irregular contours, encircling hyphae. Note the close relationship between the hyphum and the Langhans giant cell. HE. Bar, 20 \( \mu \)m. (d) Sparse silver-stained hyphae are confined to the centre of a granuloma and are largely disintegrated. GMS. Bar, 50 \( \mu \)m. (e) Strongly sirius red-stained eosinophils inside coalescing pyogranulomas. Few neutrophils are present among the eosinophils. SR. Bar, 50 \( \mu \)m. (f) SH-like reaction surrounding a hyphum. There are degranulated eosinophils immediately around the SH-like reaction. SR. Bar, 10 \( \mu \)m.
of the granulomatous pattern over the necro-eosinophilic pattern (two gastrointestinal, one cutaneous and one affecting lymph nodes). Three dogs had a predominant necro-eosinophilic pattern (two gastrointestinal and one cutaneous). Dog 7 had a predominantly granulomatous response in the gastrointestinal tract and a predominantly necro-eosinophilic pattern in the skin. In dog 5, only the granulomatous pattern was seen, and this was restricted to the gastrointestinal tract. Less frequent findings included fibrinoid necrosis of arterial walls (especially in the lymph node of dog 1) and small arterial thrombi.

In dogs, the hyphal morphology following GMS staining varied according to the type of local inflammatory response. In the necro-eosinophilic areas, hyphae were numerous, strongly GMS positive and morphologically normal (long, smooth and almost parallel walls and regular diameters) (Fig. 3c). However, hyphae confined to granulomas or inside GCs were less numerous, disintegrated (small, irregular and tortuous fragments) and, at times, less argyrophilic (Fig. 3d). Hyphae were sparse in areas where only the granulomatous pattern was observed (e.g. dog 5). Mild angioinvasion was noted rarely.

A moderate number of eosinophils were evident following SR staining and these were primarily located in the necro-eosinophilic areas. In areas of granulomatous inflammation, the eosinophils were randomly placed and intact. The comparison between eosinophils and hyphal morphology was readily established using the GMS–SR stain (Fig. 3e). The number of mast cells varied in both types of lesion and these cells were generally absent or sparse (1–5 per HPF) inside and around the lesions. Similar to horses, some hyphae were surrounded by strongly SR-positive granules that often formed the ‘red sleeve’ characteristic of an SH-like reaction (Fig. 3f).

**Bovine Pythiosis**

The lesions in both animals (each from a different herd) were indistinguishable. The main feature was the presence of multifocal to coalescing discrete granulomas surrounded by dermal collagen. Two variants of granulomas were noted. The first consisted of a centre of epithelioid macrophages and/or Langhans GCs surrounded by a thin zone of vacuolated macrophages and a delicate connective tissue ‘capsule’ as an outer layer (Fig. 4a). The second variant was similar, but in addition there was a mixture of eosinophils and neutrophils within the centre of the lesions (pyogranulomas) (Fig. 4b). Within both types of granulomas, negatively-stained hyphal profiles were seen.

Of the few hyphae in the centres of the pyogranulomas, some were surrounded by the SH-like reaction, which formed a thick-fringed halo. The classical SH presentation, consisting of lightly eosinophilic and irregular contours, was rarely seen, but always encircled hyphae inside granulomas. Interestingly, the SH-like reaction was surrounded by eosinophils, whereas the classical SH reaction was closely associated (through phagocytosis) with macrophages and GCs (Fig. 4c).

Hyphae in cattle were confined to granulomas. They were sparse and morphologically disintegrated when seen by GMS staining (Fig. 4d), except for those surrounded by the SH or SH-like reaction. Angioinvasion was not detected. Using the SR stain, it was clear that the centres of the pyogranulomas were composed almost exclusively of eosinophils, although a small number of neutrophils were also present (Fig. 4e). Under oil immersion, degranulated eosinophils were noted immediately surrounding the SH-like reaction (Fig. 4f). Conversely, the SH reaction did not stain with SR. Similar to horses and dogs, the hyphae were consistently PAS negative in cattle.

Few mast cells (2–3 per HPF) were seen and these were mostly around blood vessels surrounding the granulomas. Scattered granules were present in the cytoplasm of these cells.

**Discussion**

Although a disparate number of cases were studied from each species, which reflects the higher prevalence of equine (frequent) over canine (occasional) and bovine (rare) pythiosis in Southern Brazil, important comparative morphological aspects were identified in this study.

The lesions of equine cutaneous pythiosis are often described as areas of granulomatous inflammation (Miller and Campbell, 1984; Mendoza and Alfaro, 1986; Ginn et al., 2007); however, the cellular components characteristic of this type of inflammation were not recognized in equine lesions in this study. Therefore, the lesion was classified as an eosinophilic dermatitis and panniculitis associated with the formation of granulation tissue.

Three similar equine diseases (i.e. pythiosis, basidiobolomycosis and conidiobolomycosis) are sometimes collectively called phymycosis (Miller and Campbell, 1982). This could explain the general use of the term ‘granulomatous’, which better describes the lesions caused by zygomycetes, to characterize also the infection caused by *P. insidiosum*.

Mast cells were more often observed in the equine lesions. Mast cells and eosinophils participate in
a self-perpetuating complex cycle (Munitz and Levi-Schaffer, 2004). The higher number of mast cells within the inflammatory infiltrates, and the constant eosinophil influx located around them, may have been the cause of formation of the kunkers in the equine lesions.

Two distinct inflammatory patterns were observed in the canine lesions: granulomatous (or sometimes pyogranulomatous) and necro-eosinophilic. Similar patterns of inflammation have been described in a case study of 60 dogs with gastrointestinal phycymycosis, including pythiosis (Miller, 1985). However, in many reports of canine pythiosis, inflammation is classified only as granulomatous and/or pyogranulomatous (Fischer et al., 1994; Helman and Oliver, 1999; Hensel et al., 2003).

The inflammatory reactions observed in cattle were similar to those described in the sparse literature on bovine pythiosis. Histological presentations include pyogranulomas with a core of eosinophils and neutrophils (Santurio et al., 1998), granulomas with inconsistent central caseous necrosis (Miller et al., 1985) and multifocal areas of necrosis with eosinophils, neutrophils, plasma cells, macrophages and GCs (Pérez et al., 2005). Hyphae have been reported inside GCs and hair follicles and distributed in necrotic areas (Miller et al., 1985; Santurio et al., 1998; Pérez et al., 2005).

In this study, only the bovine lesions had two patterns of the SH phenomenon; one strongly SR positive and closely associated with the presence of eosinophils and a second that was SR negative and closely related to the presence of macrophages and GCs. The SR stain was particularly useful for quantifying and localizing the eosinophilic response in all species and it also showed the participation of eosinophil granules in the process described as SH-like (Mendoza and Alfaro, 1986).

The SH reaction is described in human conjunctival lesions associated with the presumed migration of helminth larvae. Immunohistochemically, these human SH reactions have two distinct patterns of labelling: the first reveals the presence of immunoglobulins, whereas the second reveals mainly eosinophil major basic protein (MBP) surrounding the aetiological agent. This variation is due to a number of factors (Read et al., 2005), but differences in protein composition could explain the different morphological patterns of SH noted in the bovine lesions of the present study.

Hyphae were not stained by the PAS reaction in any of the lesions studied. The PAS stain, widely utilized for identification of fungi, does not usually stain the hyphae of P. insidiosum (Grooters, 2003). This oomycete does not produce chitin as a constituent of the cell wall (Alexopoulos et al., 1996) and chitin is the main component of fungal organisms that is stained by the PAS technique (Culling et al., 1985).

Horses had the most lesional hyphae, followed by dogs and then cattle. In horses, the hyphae were confined to kunkers and morphologically appeared to be viable. It is worth noting that kunkers are generally used for culturing the agent due to the viability of the hyphae within them (Mendoza, 1987). The abundant eosinophilic response in horses, in which the disease is progressive and fatal, has been considered ineffective in combating and eliminating P. insidiosum hyphae (Mendoza et al., 2003).

In dogs, the number and morphology of hyphae varied according to the type of inflammatory response. Hyphae were more abundant and morphologically viable in necro-eosinophilic lesions and in most cases were sparse and disrupted inside granulomas. Such irregular quantitative distribution associated with the type of inflammation has already been described in canine phycymycosis (Miller, 1985); however, the author did not correlate the differences in hyphal morphology with the type of inflammatory response, as described here.

In cattle, hyphae were sparse and were limited to granulomas/pyogranulomas in which they displayed evidence of disruption. The loss of hyphal integrity was often observed in dogs and cattle in association with a granulomatous response. It is worth noting that a self-limiting clinical course was observed in three out of five reports of bovine cutaneous pythiosis (Santurio et al., 1998; Gabriel et al., 2008; Grecco et al., 2009), including all the affected cattle in the two outbreaks (n = 92) of which the two animals in the present study are representative.

One of the most intriguing aspects of pythiosis in different species is the variable frequency of vascular invasion. This feature is particularly remarkable in human pythiosis, which in some cases progresses to severe vasculitis and thrombosis and may culminate in gangrene and consequent limb amputation or radical surgical excision of affected areas (Wanachiwanawin et al., 2004).

Mild angioinvasion was seen only in some of the dogs of the present study. Nevertheless, the constant presence of a degenerate central artery in the equine kunkers, as previously reported (Miller and Campbell, 1984), could suggest that hyphae utilize vascular walls to disseminate short distances in equine cutaneous pythiosis. In the present study, hyphae were confined to kunkers and were found primarily at the periphery of these lesions and were not seen to spread between kunkers. Invasion of the vascular wall reported in a cat with gastrointestinal pythiosis did not result in distant dissemination of the lesions (Rakich et al., 2005), which may justify the hypothesis given above.
Based on the microscopical aspects of pythiosis described in the three animal species studied in this investigation, the differences in clinical course between the species may, at least in part, be attributed to the nature of the inflammatory responses made to the organism. Although it has been hypothesized that the genetic diversity of *P. insidiosum* could be implicated in causing clinical differences among affected animal species, an association of specificity among the phylogenetic groups of *P. insidiosum* and the geographical regions or hosts has not been established (Chaiprasert *et al.*, 2009).

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