Braz J Vet Pathol, 2021, 14(1), 50 – 55
DOI: 10.24070/bjvp.1983-0246.v14i1p50-55

BJVP
Brazilian Journal of Veterinary Pathology
ISSN 1983-0246

Case Report

Metastatic calcification and granulomatous grastroenteritis associated to *Pythium insidiosum* in a dog

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Submitted July, 29th 2020, Accepted December, 8th 2020

Abstract

Pythiosis is a granulomatous process of which the oomycete *Pythium insidiosum* is its etiological agent. It can affect animals and humans alike and its infection occurs when free zoospores in the water get in contact with the target tissues and encyst. The disease often occurs in tropical places with abundance of water and aquatic plants that host the fungus. Dogs infection is predominantly gastric with granuloma formations in the stomach and intestine with progressive signs of vomiting, weight loss and diarrhea. In this case report, we described clinical, surgical, necroscopic and histopathological findings of a one year and two months old, male boxer that presented clinical signs of anorexia and persistent vomiting. It was noticed on ultrasound examination an increase in stomach and intestine thickness. Laparotomy confirmed a mass affecting the gastric wall which, an incision biopsy, showed an abundant fibrous tissue associated with granulomatous reaction that was surrounded by tubuliform structures. Due to clinical complications, euthanasia was performed and in necroscopic examination a markedly increased stomach and duodenum was observed. An; histological examination of this areas it was observed that they contained granulation tissue with giant cells and epithelioids macrophages around necrosed areas associated with lymphocytes infiltrate. Also, it was possible to observe tubuliform structures by the Grocott-Gomori's Methenamine Silver (GMS) stain, this finding is compatible with the agent *Pythium insidiosum*. Therefore, this presumptive identification was confirmed by PCR analysis which amplicon had 97.83% similarity with current available genomic sequence of *P. insidiosum*.

Key words: oomycete, gastric phytiosis, granuloma, necroscopic analysis, dog.

Introduction

Pythiosis is a chronic pyogranulomatous infection that occurs in tropical, subtropical and temperate regions, it has been reported in the Americas, some European countries, Southeast Asia, Oceania and Africa. No sex, age or race predisposition have been found and its infection source is environmental zoospores which development are crucially linked with its surrounding ecosystem (Santori et al. 2006).

A retrospective study conducted by the Veterinary Pathology Laboratory of the Santa Maria Federal University (Santa Maria, RS – Brazil) described 51 cases of Pythiosis in domestic animals between 1990 and 2012. The study showed that horses were the most affected (38/51), followed by pets (12/51). Only one of the diagnosed pet cases was in a cat, the remaining others were all in dogs. Also, eight of these cases had gastrointestinal tract infection with four of them also presenting skin infection (Galiza, 2014).

The *Pythium insidiosum* oomycete is the etiological agent of pythiosis and it can affect humans and both domestic and wild animals (Martins et al. 2012), its cycle depends on the colonization of aquatic plants, in which it forms its zoosporangia. In water, free zoospores move until they find another plant (or animal), where they can encyst

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and emits a germ tube that will rise a new mycelium and then completes its cycle. The exact mechanism of which this fungus use to develop the gastroenteric form in the host remains unknown, however, it is suspected to be related with the ingestion of water containing the mobile zoospores of *P. Insidiosum*. A study (Macêdo et al. 2015) suggested that zoospores can penetrate the sublayer of the gastrointestinal tract when there is a preexisting lesions in the host.

As stated before, in Brazil, reports of pythiosis are more described in horses. This is most prominent in the Pantanal region, where animals demonstrate more frequent the cutaneous form of the disease due to their high contact with water (Santurio, 2006). Also, according to Grooters. (2003), the dogs are the second most affected species and their infections are characterized by gastrointestinal and cutaneous pyogranulomas formation (Grooters, 2003). In humans, the disease is common in Southeast Asia, mainly in Thailand, were it has a subcutaneous, systemic and ophthalmic forms, with the cutaneous and systemic forms associated with the α and β-thalassemia syndrome which are common in this region (Imwidthaya, 1994; Imwidthaya, 1995).

The gastrointestinal lesions in dogs are defined by the formation of large masses in the stomach and intestine walls, these masses are characterized by thickening of the gastric wall due to the submucosa inflammation and this is always associated with lymphadenopathy. The infection extension to pancreas, mesenteric lymph nodes and bile ducts may occur and are more frequent in immunocompetent young adults (Grooters, 2003; Berryessa, 2008). The microscopic findings of pythiosis in dogs are very similar to the ones caused by zygomycetes and Lagenidium infections (Grooters, 2003), which are part of the "phycomycosis" group and which lesions are difficult to differentiate since they have extremely similar characteristics that include areas of granulomatous to pyogranulomatous inflammation that are associated with necrosis, intense eosinophilic infiltrate and giant cells, around necrotic remains containing tubuliform structures (Guedes et al. 2016). Differential diagnosis is important due to epidemiology of each disease and choice and duration of treatment (Grooters, 2003).

The presumptive diagnosis can be made based on clinical characteristics and morphological aspect of the lesions. For the gastrointestinal form, radiography and ultrasound assay have been used to reveal segmental thickening of the gastric or intestinal wall that are associated with loss of the stratification of its layers, as well as mesenteric lymphadenopathy or abdominal mass (Grooters, 2003). Although the microbiological cultivation and isolation is considered as gold standard for identification of *P. insidiosum*, its execution is not always possible due to inadequate sampling and preservation of the suspect material (Trost et al. 2009; Galiza et al. 2014). In histopathological evaluation, the main histochemical technique used is that of Grocott's silver methenamine nitrate (GMS) that evidentiate its pseudo-hyphae (Pimentel et al. 2015). The definitive diagnosis can be obtained by serological and molecular tests as well as by immunohistochemical analysis (Grooters and Foil, 2015). Therefore, this manuscript aims to describe the clinical-pathological aspects of a case of gastroenteric pythiosis in a boxer dog, who had a severe gastroenteric condition.

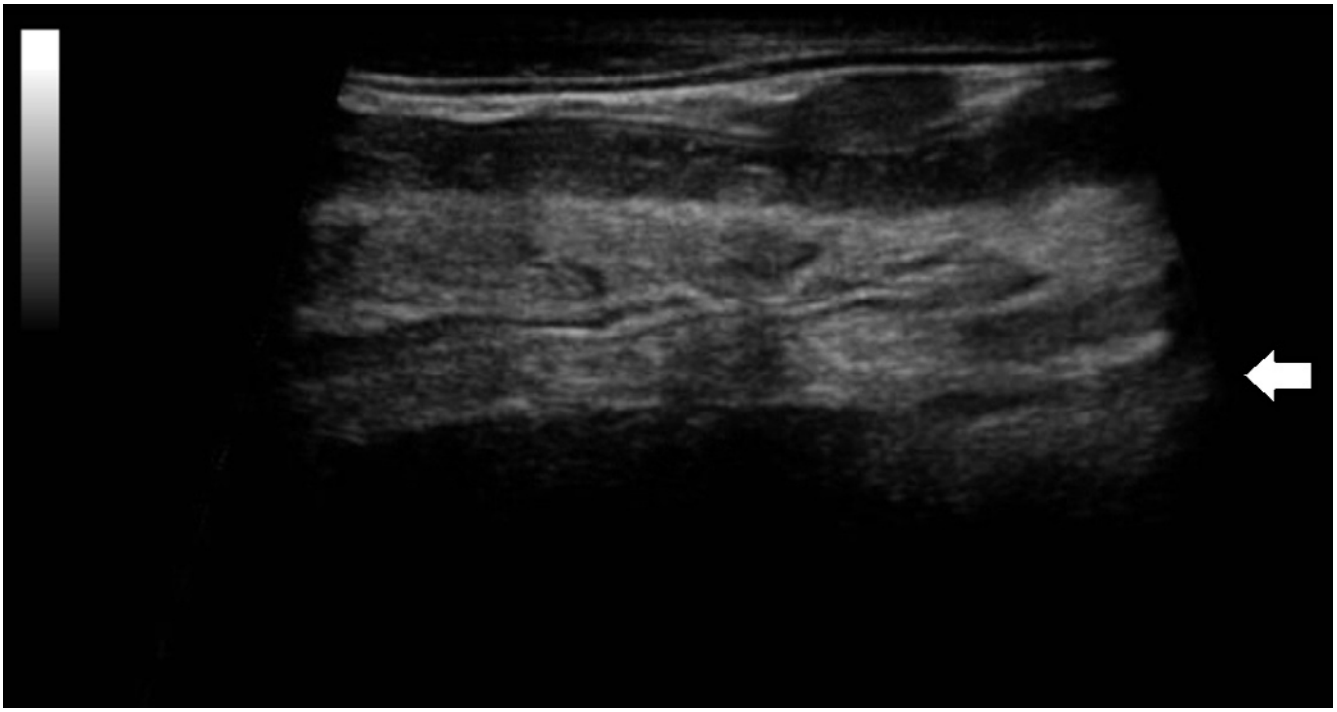

Figure 1. Ultrasonographic findings of a dog with gastric pythiosis, severe and diffuse thickening of the gastric wall is noted (arrow).

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Case report

The medical clinic service of Veterinary Hospital "Governador Laudo Natel" (HV) treated a one year and two months old male Boxer dog, with approximately 15 kg, who had history of anorexia, regurgitation, progressive weight loss and melena. The patient lived in a farm next to a lake of which it had access. During physical examination, the presence of a consistent mass was found in the epigastric area during abdominal palpation. It was possible to observe changes in the morphology of the stomach by ultrasound examination, these were characterized by walls with diffuse thickening that measured approximately 1.66 cm, with hypoechogenic and heterogeneous portions and eccentric thickening (Fig. 1). The proximal portion of descending duodenum was also thickened, with no loss of stratification, but with its muscle layer evident. Iliac, medial, hepatic and gastric lymph nodes were enlarged, hyperechogenic and homogeneous. The patient was send to HV surgical clinic service for an exploratory laparotomy.

During exploratory laparotomy, the presence of a mass affecting most of the gastric wall, with increased stiffness and yellowish color, was confirmed. An incisional biopsy was accomplished and, then, a double invaginating suture with Cushing pattern was performed on the gastric wall with non-absorbable 3-0 size nylon monofilament, followed by suture omentalization. Due to total obstruction of pylorus, that was confirmed by endoscopy and also during the surgical procedure, it was decided that a jejunostomy to be perform with a Foley tube for feeding. Routine laparorraphy was executed.

The biopsy of the gastric wall was referred to the Veterinary Pathology Service and, in the microscopic analysis of the fragment, the presence of abundant fibrous tissue associated with granulomatous inflammatory reaction surrounding tubuliform structures were observed and were evidenced by the GMS.

The patient received an itraconazole and terbinafine drug therapy and presented a worsen in its clinical sings. Therefore, the owner decided to euthanize the animal, which was referred to the Veterinary Pathology Service for necroscopic examination. In *in situ* examination of the abdominal cavity, an accentuated amount of cloudy brown intraperitoneal free fluid (Fig. 2A) was observed, as well as regions of adherence between the stomach and the right rib and an increase in stomach volume. In the detailed evaluation of the organs, the presence of focal areas with irregular, hardened and whitish surface (calcification) was noted in the epiglottis and initial portion of the trachea. The pulmonary artery, thoracic (Fig. 2E) and abdominal aorta presented an irregular, whitish and firm intimal surface (calcification). The same aspect was observed on the endocardial surface of the left atrium (Fig. 2F). Additionally, there were ulcerations at the edges of the tongue (Fig. 2D).

The stomach and the initial portion of the duodenum were markedly enlarged, with an irregular serous surface (Fig. 2B). Adherence areas were observed in the liver and epiplon. The mesenteric, gastric and hepatic lymph nodes were markedly enlarged. When cutting the gastric and duodenal walls, there was an accentuated and diffuse firm thickening,

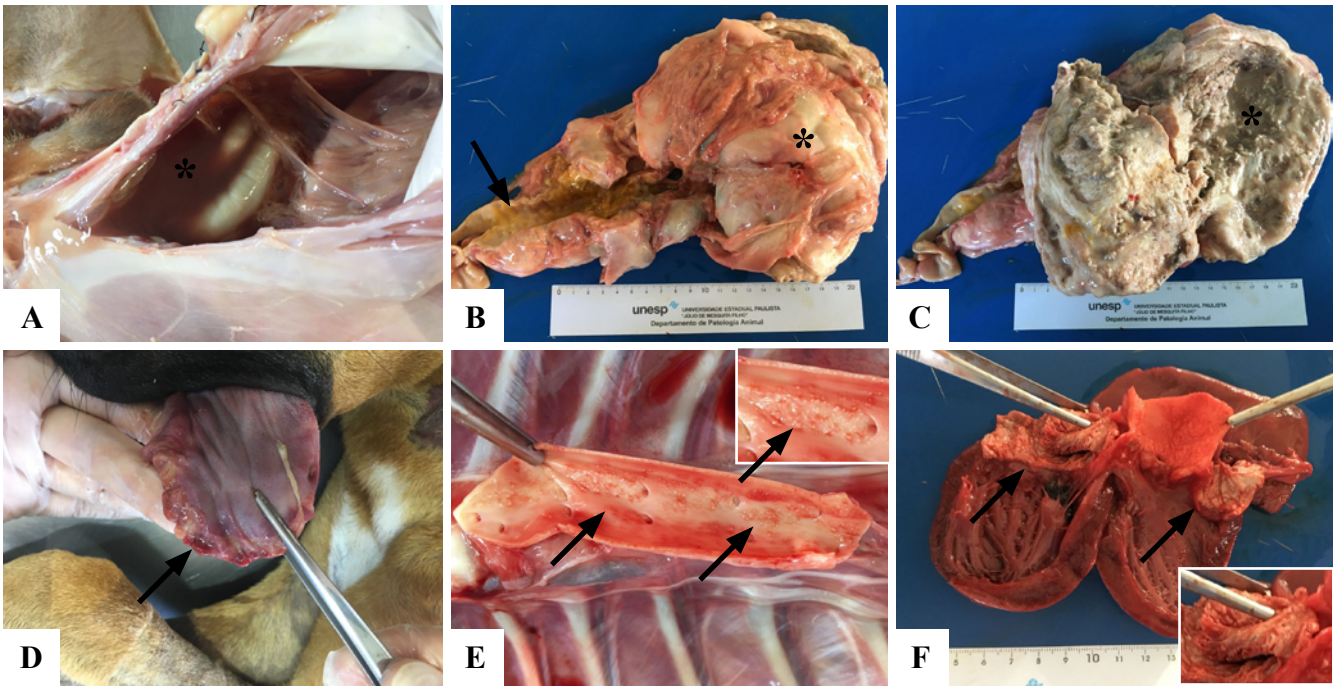

Figure 2. Main necroscopic findings in a dog with gastric pythiosis. **A.** In the abdominal cavity there is a cloudy and brownish fluid (*). **B.** Stomach with irregular serous surface (*) and thickened duodenal wall (arrow). **C.** Gastric content is grayish and caseous aspect (*). **D.** Ulcerations are noted at the edges of the tongue (arrow). **E-F.** The intimal surfaces of the aorta (**E**) and the left atrial endocardium (**F**) show rough, hardened plaques (arrows, details), which creak when cutting (calcification).

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which extended to the initial portion of the duodenum (Fig. 2C). The gastro-duodenal content was fluid to caseous and grayish, with a mild amount of food interspersed. Additionally, an accentuated pyloric stenosis was noted.

Microscopic analysis of the lungs revealed, on the alveolar wall, areas with deposition of basophilic granular content, also calcification were found distributed throughout the lung parenchyma. The same lesions were observed in the left atrial endocardium, kidneys, aorta, larynx and trachea, intercostal muscles and on the tongue. Additionally, in the kidneys, it was observed membranoproliferative glomerulonephritis, with foci of glomerulosclerosis. There were also areas of calcification and coagulative necrosis of renal tubules and areas of interstitial fibrosis associated with inflammatory debris. In the stomach, it was possible to observe extensive areas with granulation tissue surrounding giant cells and epithelioids macrophages, associated with lymphocytes, which arranged around areas of necrosis containing longitudinal branched tubular structures (Fig. 3). The identification of pseudohyphae was possible through the histochemical technique of Grocott's Methenamine Silver (GMS), which allowed the observation of foci with right-angled ramifications, suggesting they were from *P. insidiosum*. Inside the intestine there were accentuated lymphoplasmacytic inflammatory infiltrate in the serous layer, loss of the enteric architecture due to the same histological aspects observed in the gastric compartment. The conclusion of the anatomopathological findings were granulomatous gastroenteritis associated with intralesional pathogen suggestive of *P. insidiosum*.

The association of anatomopathological findings with clinical signs led to the suspicion of pythiosis in the gastrointestinal tract. To confirm the presence of the etiologic agent of pythiosis, a paraffin block was sent for molecular analysis (PCR), to search for the DNA of *Pythium insidiosum*, in the Department of Microbiology and Immunology, Biosciences Institute, UNESP, Botucatu, São Paulo State. The paraffin-embedded tissue was submitted to DNA extraction using Genomic DNA from tissue (NucleoSpin®Tissue, Macherey-Nagel) after deparaffinization with xylol. PCR with the amplification of translation elongation factor 1-alpha were specific for *Pythium insidiosum* and the amplicon was submitted to DNA sequencing. Query cover 100% presented 97,83% similarity with *P. insidiosum* sequence deposited at GenBank (accession number JRHR01003226.1).

Discussion

The patient in this report was a one year and two months old male boxer which showed signs of anorexia, regurgitation and progressive weight loss that had a palpable mass in abdomen. These were similar clinical signs as the ones described by Berryessa (2008), who reported that the majority of cases of pythiosis in young, large breeds dogs, showed chronic signs of gastrointestinal disease and in more than 50% of the cases, the dogs had a palpable abdominal mass. In the literature, the lesions described in dogs often affect the small intestine, stomach and colon, respectively; affection of only the esophagus is considered to be rare (Patton et al. 2008), in this report, the lesions involved the stomach and duodenum.

The animal in this report lived in a warm climate area and had access to a lake, the frequent exposure to water sources is considered a risk factor for pythiosis (Berryessa, 2008). Also, there are reports that suggest several forms of infection, such as *in vitro* analyzes that demonstrated attraction of zoospores for hair, animal and plant tissues, the chemotaxis is attributed to some substances present in these tissues, which suggests that horses in contact with contaminated water could attract zoospores by this process and these would germinate from a small cutaneous

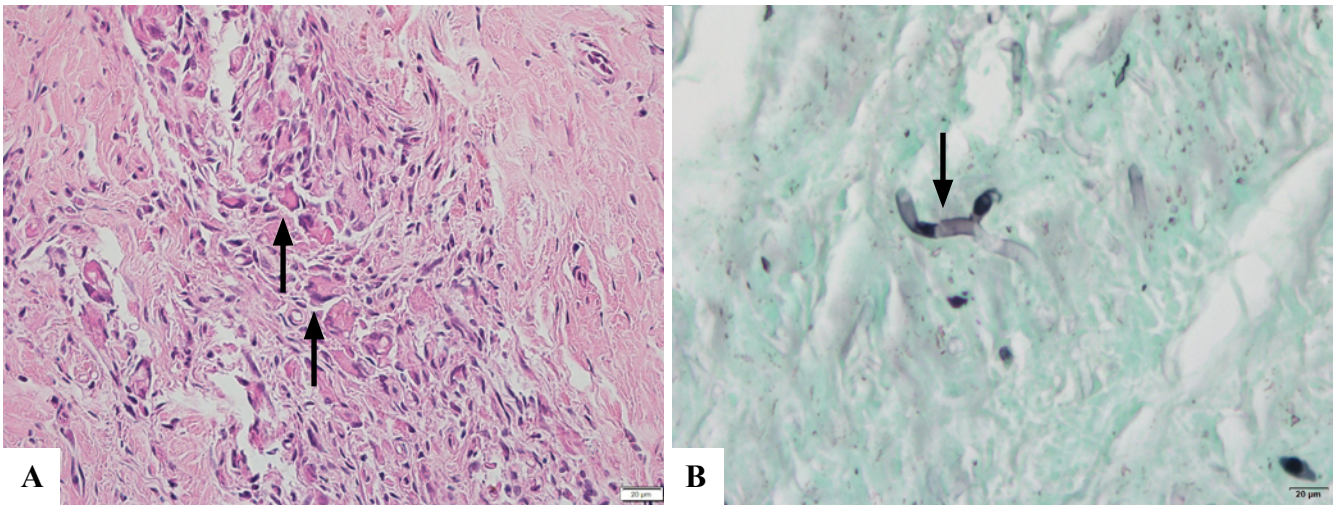

Figure 3. Photomicrographs of the dog stomach with gastric pythiosis. **A.** Presence of multinucleated macrophages associated with inflammatory infiltrate (arrows, scale bar = 20 µm, Hematoxylin and Eosin). **B.** In the necrosis areas, tubuliform structures stained in dark gray with right-angled branches (arrow, GMS, scale bar = 20 µm) were noted.

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lesion (Santurio et al. 2006). There is also the possibility of zoospores penetration through the hair follicles, this is based on detection of hyphae within the follicle of naturally infected cattle (Santurio et al. 2006). For the *P. insidiosum* to maintain its cycle, it needs water with a low ion concentration, pH close to neutrality, high temperature (between 30º and 40º C) and a specific kind of host plant (Chaffin et al. 1995, Mendoza et al. 1996).

Normal canine stomach and small intestine have ultrasound characteristic appearance, with five definable wall layers. The normal gastric wall measures up to 5 mm in thickness, and measurements above 7 mm are associated with gastric changes (Grahan et al. 2000). In the present case, the gastric wall measured 1.66 cm which is a thickening much higher than the normal values described in the literature. In this regard, a survey of gastrointestinal diseases, conducted by Grahan et al. (2000), highlighted ultrasound abnormalities such as moderate to severe thickening of the wall, loss of stratification and moderate to severe regional increase in lymph nodes are suggestive of gastrointestinal neoplasia. However, these findings are non-specific and in pythiosis endemic areas, gastrointestinal pythiosis should be considered a differential diagnosis, as its ultrasound characteristics are very similar to other pathological processes (Grahan et al. 2000). It is important to consider that a relatively small number of cases of pythiosis are reported annually, regardless of the infected species and it is very likely that this number is underestimated since the disease occurs mainly in rural areas and in developing countries (Gaastra et al. 2010). Therefore, pythiosis should be considered in the differential clinical diagnosis of chronic debilitating diseases, with weight loss, vomiting and/or diarrhea and palpable abdominal masses, particularly in young and rural dogs. It is also important that some differential diagnoses may include eosinophilic gastroenteritis, regional granulomatous enteritis, gastrointestinal foreign body, intussusception and intrabdominal neoplasia (Miller, 1985).

The granulomatous lesions in this case were also emphasized by some authors and were as similar as those observed in infections caused by other oomycetes, such as *Lagenidium spp.* And zygomycete fungi of the order Entomophthorales (*Basidiobolus ramarum* and *Conidiobolus coronatus*) (Grooters, 2003). Therefore, other diagnostic methods should be considered and includes immunohistochemical analyzes, serological tests (ELISA or immunoblot), PCR (Brown et al. 1988) and DNA extraction from paraffin blocks, followed by sequencing. In this study, last two tests were used to confirm the diagnosis of gastric pythiosis since genomic DNA is a more reliable and effective methods of pathogen identification. Other ways of observing the presence of the microorganism are histochemical techniques such as GMS. Although Periodic Acid-Schiff (PAS) is widely used to identify fungi in general, the cell wall of oomycetes does not contain chitin (Alexopoulos et al. 1996), a substance that is stained by the PAS technique (Culling et al. 1985) and therefore, this technique will not be effective to identify the tubuliform structures (pseudo-hyphae) of *P. insidiosum* (Grooters, 2003).

Metastatic calcification is defined by a precipitation of calcium salts in soft tissues due to the persistent concentration of serum calcium, with no prior tissue injury (DOERR et al., 2013; FOSTER, 2016). Its pathogenesis is derived from the increased secretions of parathyroid hormones associated with the destruction of bone tissues due to primary bone marrow tumors or metastases, disorders related to vitamin D and renal failure with the calcification damage occurring more frequently in gastric mucosa, kidneys, lungs, systemic arteries and pulmonary vessels (KUMAR et al. 2005; MAJNO & JORIS, 1996). The calcification observed in different tissues of the dog reported with pythiosis can be correlated to the granulomatous inflammation observed in the stomach and intestine. In the literature, there are descriptions of hypercalcemia associated with granulomatous diseases that affect humans such as sarcoidosis, tuberculosis and fungal diseases (JACOBS et al, 2005). Another hypothesis to be considered in the reported dog is hypercalcemia caused by hyperparathyroidism secondary to nutritional origin, since it presented severe injury to the gastric wall and part of the duodenum, which would probably result in difficulty in absorbing nutrients, including calcium and due chronicity occurred hypocalcemia that stimulated the parathyroid to produce PTH and remove calcium from bones, in the reported necropsy there was no change in bone consistency, but in the early stages of osteodystrophy this would not be evident.

In dogs, gastrointestinal pythiosis is generally a fatal disease (Mendoza et al. 2003), with low response to therapy, as in the present case report. Therapies such as wide surgical excision, antimicro-agents and immunotherapy have been used with some success in the treatment of pythiosis. However, regardless of the therapy chosen, it is extremely important to initiate it as soon as possible (Gaastra et al. 2010). In most cases, it is diagnosed when the lesions are very advanced and surgical excision becomes impractical (Hensel et al. 2003). Likewise, the success of therapy with the exclusive use of traditional antifungals are difficult, since the *P. insidiosum* is not a fungus and the ergosterol, the target substance of most antimycotic drugs, is absent in this oomycete cell wall (Grooters, 2003). Immunotherapy, which is widely used of equine pythiosis treatment, has not yet shown satisfactory results in the canine cases (Santurio et al. 2006).

Therefore, based on the current available literature and despite of it being common, the gastrointestinal pythiosis is not a frequently reported. In addition, despite the clinical history, the characteristic lesions found at necropsy and histopathological analysis, the PCR assay was extremely important for the identification of *P. insidiosum* as the etiological agent of this gastroenteric condition.

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