Treatment of canine cutaneous leishmaniasis by Leishmania (Viannia) braziliensis *in dogs with furazolidone and β-cyclodextrin: case report.*

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Summary
Euthanasia of animals is not accepted as a control for cutaneous leishmaniasis caused by *Leishmania (Viannia) braziliensis* and drugs used in humans for the treatment of leishmaniasis are not allowed for animals in Brazil. Miltefosine was authorized for dogs infected by *Leishmania infantum* with variable results for *L. braziliensis*. Thus, nine dogs infected with *Leishmania (V.) braziliensis* were treated by a combination of furazolidone and β-cyclodextrin. The nine dogs were mongrels, weighing between 4-17 kg and 3-10 years old. These dogs had ulcerous lesions in different regions such as scrotal tissue, auricular pavilion and nostrils. Serological, molecular and protozoal culture techniques were used for laboratory diagnosis. The treatment used furazolidone + β-cyclodextrin complex (1: 2) at a concentration of 60 mg/mL given orally at a dose of 15 mg/kg every 12 hours. The re-epithelialization of lesions occurred between 35 and 41 days of treatment. During fourteen months the animals were monitored and there was no reactivation of lesions or growth of the protozoan in a culture medium of the biopsies. This study demonstrated that treatment with FZD and CD is effective in reducing the cutaneous lesions caused by *L. braziliensis* in dogs.

Introduction

Cutaneous leishmaniasis (CL) is an infectious, non-contagious disease caused by 2 different species of protozoa of the genus *Leishmania*, which affects the skin and mucous 3 membranes. CL is a zoonosis, which affects animals and secondarily humans. Infection 4 invariably starts with a bite of the infected sandfly vector taking a blood meal followed 5 by sequestration of the *Leishmania* parasites in the macrophages of the host. The earliest 6 clinical symptoms, a cutaneous lesion or a fever, have no diagnostic or prognostic value 7 (De Bruijn & Barker 1992). Domestic animals are potential vectors of different types of 8 leishmaniasis, and pets play an important role as new opportunistic hosts, maintaining 9 endemic peri-urban areas. Dogs can increase the potential for transmission by virtue of 10 representing an untreated reservoir for the parasite. In human, the drug traditionally used 11 is meglumine antimoniate, but its use is not allowed in animals . Drugs like pentamidine 12 isethionate, miltefosine, amphotericin B and liposomal amphotericin B can be used 13 (PAHO 2020). In Brazil, CL occurs throughout the country and the causative agent responsible for the largest number of cases in both humans and animals is *Leishmania (Viannia) braziliensis*. Euthanasia of wild and domestic animals is not accepted as a control measure for CL and although there is treatment for infected humans, infected animals are not treated with the same drugs in order to prevent the development of parasite

resistance for human treatment (Brasil 2017). However, after 2016 the control of leishmaniasis in dogs has taken a new approach in Brazil (Brasil 2016) following the approval of a product based on miltefosine for the treatment of visceral canine leishmaniasis. This commercial product offered in the Brazilian veterinary products market has no therapeutic recommendation inserted in its package for the treatment of cutaneous leishmaniasis caused by *L. braziliensis*. A variable action of this drug on *L. braziliensis* was observed by Espada and colleagues (Espada *et al.* 2017). In humans, the drug traditionally used is meglumine antimoniate, but its use is not allowed in animals. Among several drugs used experimentally, furazolidone (FZD) has shown promising results as a leishmanicidal agent (Tempone *et al.* 2010, Passos *et al.* 2014). The pharmacological activity of FZD is due to its ability to rupture the parasitic nuclear and mitochondrial membrane, causing cell organelles to leak (Hunder 1987). Tempone and colleagues administered FZD in liposomal and free form (50 mg/kg/day) to animals infected with *Leishmania* (*Leishmania*) *chagasi* and observed that both forms of the drug were effective in reducing the number of parasites (Tempone *et al.* 2010). Passos and colleagues treated eight dogs infected with *L. (V.) braziliensis* with FZD (35 mg/kg/day) for 21 days followed by domperidone (2 mg/kg/day) for 10 days and observed that the therapy was effective for regression of the lesions, which were epithelialisation without subsequent recurrence and without changes in biochemical and haematological patterns (Passos *et al.* 2014). Also new associations of FZD complexed to β-cyclodextrin (CD) for the treatment of leishmaniasis have been proposed to a lower dosage of FZD in relation to the dosage already used in the literature for the treatment of CL (Carvalho *et al.* 2018, Carvalho *et al.* 2020). It should also be noted that the use of FZD orally is recommended by the Brazilian Society of Gastroenterology in human therapy, particularly for the treatment of *Helicobacter pylori* infection (Coelho *et al.* 2013). Thus, this report aims to describe the treatment of cutaneous leishmaniasis by *L. braziliensis* in nine dogs using a reduced dose of furazolidone complexed to β-cyclodextrin.

Case report

The Microbiology and Zoonoses laboratory – UFES (campus Alegre, ES, Brazil) has an extension project with the municipality of Íuna, state of Espírito Santo, Brazil for diagnosis and control of dogs with leishmaniasis. All experimental protocols were reviewed by a state ethics commission and have been approved by the competent authority (CEUA UFES 13/2018). Specifically in this study, nine dogs from the southern region of the state of Espírito Santo which had ulcers suggestive of CL were examined. The dogs were males between 3-10 years of age, mongrels, weighing between 4 to 17 kg, residing in rural areas of different municipalities in the region. The nine animals had ulcerative lesions in different places such as scrotal epidermis, pinna and nostrils. The laboratory diagnostic protocol was carried out in two stages, first serological diagnosis followed by molecular diagnosis. For serological diagnosis, samples of venous blood were collected to perform the indirect enzyme linked immunosorbent assay (i-ELISA) (Ribeiro *et al.* 2007) and Western Blot (Zanini *et al.* 2010). For the i-ELISA, soluble antigen was extracted from the *L. (V.) braziliensis* seed sample (MHOH/BR/1975/M2903) provided by the Oswaldo Cruz Foundation according to authentication certificate n. 034/2012.

All dogs were seropositive by the i-ELISA with a cut-off point of 3 standard deviations (3SDs) of negative controls using polyclonal antiserum IgG and soluble antigen of *L. braziliensis*. This cut-off of 3SDs allows to differentiate *L. braziliensis* from sporotrichosis (Ribeiro *et al.* 2007). In the Western Blot test, the sera of the nine dogs reacted with fractions of the protein soluble antigen distributed between 54, 66, 70, 80 and 97 kDa extracted from a seed sample of *L. (V.) braziliensis* (MHOH/BR/1975/M2903) as already observed (Zanini *et al.* 2010) using secondary polyclonal IgG antibody for symptomatic and asymptomatic dogs in a serological study for CL.

For molecular diagnosis, the animals were sedated with dissociative drugs (10% ketamine 15 mg/kg/IM and 20% xylazine 0.2 mg/kg/IM) and were anesthetized locally in the region where the procedure was performed, using lidocaine 0.2% (1:10 diluted). After sedation, tissue samples from the edge of the lesions were collected for smear, polymerase chain reaction (PCR) and protozoan culture. The following lesion samples were collected: dog 1, pinna; dog 2, pinna; dog 3, pinna; dog 4, scrotal epidermis; dog 5, pinna and nostril; dog 6, pinna and scrotal tissue; dog 7, pinna; dog 8, nostril; dog 9, pinna. The smears of the lesions were stained with Leishman stain and suggestive forms of intracellular phagocyte amastigotes were observed in the sample collected from the ear of dog 5, scrotal epidermis sample from dog 6 and the ear of dog 9. For PCR, primers B1 and B2 (De Bruijn and Barker 1992) were used since the southern region of the state of Espírito Santo is endemic only for CL, and these primers amplify a sequence of 750 base pairs of DNA from the sub-genus *Leishmania (Viannia)*. All collected samples, except for the nostril lesion of dog 5, were positive to PCR.

Plots of the nine biopsy fragments were inoculated in NNN – Novy, MacNeal, and Nicolle (modified blood agar) and LIT (Liver Infusion Tryptose) media after

treatment with penicillin-streptomycin solution for the decontamination of prokaryotes. These cultures of tissue were incubated in a BOD (biochemical oxygen demand) incubator at 24 ± 1 °C for 15 days (Passos *et al.* 2014), before examining for the presence promastigote forms of the parasite were researched. Promastigote forms were confirmed as *L. braziliensis* by PCR in four samples of auricular lesions (dogs 1, 2, 3, 5 and 9) while the other cultures were contaminated.

All animals were subjected to a standardized health treatment, wormed with a commercial product composed of the association of praziquantel, pyrantel pamoate and febantel and started to be fed with commercial food with 21% crude protein. The nine dogs were also evaluated by complete blood test with comprehensive metabolic panel-CMP (aspartate aminotransferase, alanine aminotransferase, gamma-glutamyltransferase and hepatic bilirubin) and basic metabolic panel-BMP (urea, creatinine and albumin). The sizes of the ulcers of dogs caused by *L. braziliensis* were monitored using a pachymeter (Mitutoyo, Taipei, Taiwan), and expressed in mm^2 according to Table I.

After laboratory confirmation of the infection with *L. (V.) braziliensis*, it was decided to medicate the animals with the FZD associated with CD in the proportion 1:2 manipulated according to literature (Carvalho *et al.* 2018, Carvalho *et al.* 2020). The complex of the FZD + CD (Sigma Aldrich, Darmstadt, Germany) manipulated had a concentration of 60 mg/mL. The animals which remained in their houses, were weighed and treated orally at a dosage of 15 mg/kg (5 mg/kg of active FZD) 12/12 hours x 50 days. Every 15 days, the animals were evaluated for lesion size, metabolic panel-BMP) and metabolic panel-CPM (Vidal *et al.* 2009). After 20 days of treatment, the lesions started to regress (Table I) and, at 35 days, there was a re-epithelialization of the pinna and scrotal tissues. The lesions of the mucosa of the nostrils had re-epithelialization at 41 days. After these observations, the animals were monitored for fourteen months, with no observed reactivation of lesions. A complete blood test and biopsy of the tissue for recovery of the lesions of the pinna and scrotal epidermis were then performed after regeneration (biopsy of the region of the regenerated nostril was not performed), ELISA, PCR and culture tests were repeated. All animals remained positive i-ELISA whereas four ear biopsies (dogs 1, 3, 5 and 6) and one scrotal epidermis (dog 6) resulted positive to PCR amplifying fragments of 750 bp. When culturing in NNN-LIT medium, four tissues showed secondary contamination by different microorganisms whereas the others did not show growth of promastigote forms and all cultures were negative to PCR.

Discussion

The treatment for cutaneous leishmaniasis with FZD and domperidone in dogs (Passos *et al.* 2014) at a dosage of 35 mg/kg/day has been used and regression of lesions were consistently observed after 15 days of continuous use of FZD. However, especially when dogs are in good nutritional status and have weight over 10 kg (unpublished data), nervous manifestations such as motor incoordination and vomiting were frequently observed because FZD gradually accumulates in the fatty tissue causing an overdose after the second week of treatment. The application of this experimental therapy (35 mg/kg/day) requires constant monitoring by the veterinarian to manage possible intoxication. The FZD is limited in the clinic due to its potential side effects, such as hepatotoxicity and to the incidence of observed severe side effects when the dose was increased (Deng *et al.* 2015). Thus, immunomodulation contributes to the treatment of leishmaniasis. This can be achieved by the use of domperidone, a gastric prokinetic with antiemetic activity. It is a dopamine D2 receptor antagonist and hyper pro-lactinaemic drug that causes the release of serotonin, which in turn stimulates prolactin production. Prolactin appears to have a central role in the specific cellular immune response to leishmaniasis (Sabaté *et al.* 2014). The association of FZD + CD causes an increase in the solubility of FZD and consequently

Table I. *Chronology of lesion regression (mm^2) in dogs 1 to 9 treated with furazolidone and β-cyclodextrin.*

Days of treatment	Dog										
	1	2	3	4	5		6		7	8	9
	PAN	PAN	PAN	EE	PAN	NA	PAN	EE	PAN	NA	PAN
00	576	289	630	900	328	130	414	382	271	372	402
10	505	292	598	910	340	110	410	386	265	180	367
20	100	112	182	332	92	77	224	102	192	66	162
30	00	16	00	87	00	48	38	00	88	41	27
40	00	00	00	00	00	02	00	00	00	00	00

PAN = Pinna; NA = Nostril; EE = Scrotal epidermis.

a reduction of the toxicity, particularly when using high doses of the complex. These results stimulated the production of a dense liquid formulation containing 60 mg/mL of the FZD + CD complex (1:2) where the active ingredient FZD has a concentration of 20 mg/mL. To facilitate the dog acceptance, a meat flavouring was added to the product that was administered with the aid of a disposable syringe. For humans, the literature mentions the use of FZD in the treatment of *Giardia* and *H. pylori* (Coelho *et al*. 2013) using concentrations of FZD up to 400 mg/day for persons over 12 years old (+/- 50 kg) which indicates a dosage of 8 mg/kg/day.

A dark yellowish colour was observed in the urine resulting from renal excretion of FZD but there was no change in the values of the complete blood test, metabolic panel-BMP and metabolic panel-CPM repeating the finding already observed (Passos *et al.* 2014, Vidal *et al*. 2009) for *L. braziliensis*. Despite the clinical healing and absence of reactivation or development of lesions, the PCR was positive for six of the seven lesions evaluated after fourteen months, however similar findings were observed (Schubach *et al.* 1998, Wu *et al.* 2020) when treating CL with a pentavalent antimonial. It was observed that the inactivated parasites persist on the skin for many years after treatment without reactivating the lesions. Quantitative PCR (qPCR) biopsies to assess the nucleic acid load of the parasite in the lesion were not performed since several authors reported that the presence of parasite nucleic acids in regenerated CL lesions is a constant presence and the clinical cure of CL is rarely associated with sterile healing (Schubach *et al.* 1998, Mendonça *et al.* 2004, Passos *et al.* 2014). Thus, further studies with quantitative PCR (qPCR) in CL lesions should be carried out to determine the threshold between asymptomatic and symptomatic patients, as well as the level of residual parasitic infection that can lead to a new outbreak of leishmaniasis and relapse (Wu *et al.* 2020). Culture and conventional PCR are extremely sensitive for qualitative diagnosis of CL (Mesa *et al.* 2020) while the use of quantitative PCR (qPCR) to assess parasite load in tissue biopsy would be a reference of confusion.

The use of miltefosine to treat CL has shown the need of different concentrations to inhibit the growth amastigote forms of the isolates of *L. braziliensis* from different regions of Brazil (Espada *et al.* 2019). In addition, the use of miltefosine for the treatment of canine visceral leishmaniasis has been consolidated when associated with continuous use of allopurinol (Daza González *et al.* 2019) but the allopurinol is not effective in inhibiting *L. braziliensis* (Minodier and Parola 2007). This study presents an applicable form for the treatment of canine cutaneous leishmaniasis caused by *L. braziliensis*.

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