Evaluation of the effectiveness of three therapeutic protocols used in the treatment of visceral canine leishmaniosis

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Abstract

Leishmaniasis is a tropical and subtropical disease caused by an intracellular protozoan transmitted by a bite from a vector, mainly from the genera Phlebotomus and Lutzomyia, and affects humans and other mammals, especially dogs. The main objective in controlling canine visceral leishmaniasis is to reduce the number of human cases by reducing its prevalence in dogs. In Brazil, glucantime antimoniate and Amphotericin B, utilized for treating the disease in humans, are prohibited so that only miltefosine, which is not employed for treatment of humans, is permitted for use in dogs. This work aimed to evaluate the efficacy of three different therapeutic protocols employed in the treatment of dogs naturally infected with visceral leishmaniasis. Fifty-six (56) dogs, of both sexes, were treated and evaluated utilizing three treatment protocols. The following protocols were utilized: association of several drugs; miltefosine associated with allopurinol; and immunotherapy with anti- Leishmania vaccine associated with Allopurinol. Immunotherapy was the most efficient protocol, followed by an association of drugs and miltefosine. The use of these protocols diminishes the constant relapses of the disease. Associations of therapeutic protocols produced clinical improvement of patients even with presentation of subsequent negative serology. However, the study did not include aspects related to hemoparasitoses, thus a further study is required.

Keywords: Canine leishmaniasis; Chemotherapy; Immunotherapy

1. Introduction

Leishmaniasis is a tropical and subtropical disease caused by an intracellular protozoan transmitted by a bite of a vector, principally of the genera *Phlebotomus* and *Lutzomyia*. It is present in approximately 89 countries distributed among the continents of Europe, Africa, Asia and South America. It of the seven most important tropical diseases, representing a serious global public health problem. It presents a wide spectrum of clinical manifestations (cutaneous, mucocutaneous, cutaneous-diffuse and visceral forms) with a potentially fatal result (Torres-Guerreiro et al. 2017). According to the World Health Organization (WHO), the visceral form is considered the most important and severe, infecting from 200,000 to 400,000 persons per year throughout the world, with a mortality rate between 10% and 20% (Savoia 2015).

In addition to humans, the visceral form also affects several mammalian species, including dogs, considered the main peri-domicilliary reservoir of the parasite in endemic areas. The main objective of controlling canine visceral leishmaniasis is to reduce the number of cases of the disease in humans by reducing its prevalence in dogs. Among the control methods utilized are: diagnosis and euthanasia of sick animals, diagnosis and treatment of symptomatic or asymptomatic infected dogs, vector control (use of repellents on the animal and in the environment), use of vaccines and canine population control. In recent years, studies have not proven the efficacy of elimination of infected animals and have shown that in endemic regions that have employed this method there were no reductions in the number of human cases. A large portion of the population, especially in developed countries, consider the practice of this method ethically unacceptable (Solano-Gallego et al. 2011, Vulpiani et al. 2011, Ribeiro et al. 2013).

The objectives of canine leishmaniasis treatment are to reduce the parasitic load in the animal in order to diminish its transmission capacity, to promote a clinical cure, to restore effective immune responses and to stabilize the clinical improvement to avoid relapses. Diverse drugs and protocols have been used for treatment of infected dogs (Oliva et al. 2010, Vulpiani et al. 2011).

The main drugs utilized for the treatment of leishmaniasis in humans are pentavalent antimonials (sodium stibogluconate and meglumine antimoniate), Amphotericin B, miltefosine and pentamidine (Croft & Coombs 2003). In Europe, drugs such as meglumine antimoniate, miltefosine and Amphotericin B are commonly used in the treatment of canine leishmaniasis (Solano-Gallego et al. 2009, 2011). In Brazil, glucantime antimoniate and Amphotericin B, utilized for treating the disease in humans, are prohibited (Ribeiro et al. 2013), whereas only miltefosine is permitted for use in dogs. Given this context, there is on ongoing search for other drugs or alternative therapeutic protocols for treating canine leishmaniasis.

This work aimed to evaluate the efficacy of three different therapeutic protocols (association of several drugs; miltefosine associated with Allopurinol; and immunotherapy with anti-Leishmania vaccine associated with Allopurinol) utilized in the treatment of dogs with visceral leishmaniasis, naturally infected.

2. Methodology

2.1 Animals

The study was conducted in the city of Campo Grande-MS, Brazil, a region endemic for canine

visceral leishmaniasis.

The animals were attended in a specialized veterinary clinic, where the diagnosis, treatment and accompaniment were carried out between April of 2007 and December of 2019. Data collection was performed in the period from April 24, 2017, to January 11, 2020.

Fifty-six (56) dogs were evaluated (26 males and 30 females), aged between one and 15 years; diverse breeds and individuals of undefined breed were included, naturally infected by *Leishmania infantum*.

The diagnosis was based on observation of clinical alterations associated with leishmaniasis, positive serology with detection of anti-Leishmania antibodies by the tests Enzyme-Linked Immunosorbent Assay - ELISA and Indirect Fluorescent Antibody Test - IFAT (Hermes Pardini Laboratory and Tecsa Laboratory), hematological, renal and hepatic laboratory evaluation, and observation of amastigote forms in aspirative puncture cytology of the lymph nodes and / or bone marrow. The first protocol for the first treatment was decided by evaluating the animal clinically, and also by complementary exams. A second protocol was performed if the animal was serologically positive after the end of the treatment, with any titer being considered positive and negative when the serology presented non-reactivity. The protocol instituted at that point should differ from the prior one. The same animal may continue to be reactive and be administered up to a third different treatment protocol.

The time between the application of the protocols and the result of the final diagnosis, according to serology, varied from one case to another. It should be emphasized that until 2019, the majority of the animals were alive, clinically monitored by semiannual exams and submitted to new protocols depending on post-treatment monitoring exams.

All the animals' owners that were included in this retrospective study signed terms of duly informed consent for participation.

2.2 Clinical and laboratory evaluation

First, the animals were submitted to a clinical exam for evaluation of the presence or absence of clinical signs associated with visceral leishmaniasis. Next, blood samples were collected for the serological exam (IFAT and ELISA), blood counts, biochemical exams (dosing of alanine aminotransferase-ALT, alkaline phosphatase-FA, urea, creatinine) and aspiration of lymph nodes for direct cytological examination. After confirmation of the disease, the treatment was initiated.

2.3 Therapeutic protocols

Three therapeutic protocols were utilized:

T1 – Miltefosine (Milteforan®, Virbac, Carros, France) 2mg/kg, orally once per day for 30 days associated with allopurinol (10mg/kg orally, twice per day, continuous use).

T2 - Association of the different drugs.

Various drugs were utilized in an associated form, composing 2 formulas, under the form of a manipulated medication. The treatment time of each formula comprised 60 days, totaling a cycle of 4 months of treatment. The composition of each formula depended on each clinical case in particular.

The principal substances utilized were:
- allopurinol, daily dose 20mg/kg, orally, divided into two doses.
- ketoconazole 14-20mg/kg, orally, every 12 hours, between meals (acidic stomach)
- metronidazol 20-30mg/kg, orally, every 12 hours.
- L-arginine aspartate 10-20mg/kg, orally, every 12 hours.
- zinc sulfate 10-15mg/kg, orally, every 12 hours.
- doxycycline hydrochloride, 10-20mg/kg, orally, every 12 hours.
- domperidone 0.5-1mg/kg, orally, every 12 hours.

The formulas were elaborated by a veterinary physician, after clinical and laboratory evaluation, being adjusted according to animal body weight and the necessary components.
In addition to the formula, prednisone was also prescribed at 0.1mg/kg, orally, every 12 hours.
Example of the master formula (in capsule) for one 10kg dog:
Allopurinol 100mg
Cetoconal 100mg
Metronidazol 200mg
Cloridrato de doxiciclina 200mg
L-arginine aspartate 100mg
Zinc sulfate 100mg
Domperidone 5mg

T3 - Immunotherapy with the vaccines Leishmune® (Zoetis, New Jersey, USA) (Borja-Cabrera et al. 2004) and Leish-Tec® (Ceva Santé Animale, Paris, France), with application of two doses of vaccine every 21 days, totaling three applications associated with allopurinol 10 mg/kg orally, two times per day, continuous use.

At the end of each treatment, new clinical and laboratory exams were performed to verify the efficacy of the protocol utilized. If the disease was not controlled, the animal was submitted to a new cycle of treatment employing the same protocol or a different one. Thus, the protocols were classified in the following manner: T1- animals that were submitted only to treatment with miltefosine and Allopurinol; T2 – treatment only with an association of drugs; T3 – treatment only with immunotherapy and Allopurinol. The order and time interval in which the treatments were carried out in the associated protocols varied on a case-by-case basis. Some of the animals were counted more than once since they had been submitted to more than one treatment. In the post-treatment evaluation, if the animal presented persistence of serological positivity, another treatment protocol was instituted according to the clinical and exam results.

2.3 Post-treatment evaluation

Immediately after the end of treatment, a new clinical evaluation and collection of blood samples were carried out for the serological exam (IFAT and ELISA), blood count, biochemical exams (dosage of alanine aminotransferase-ALT, alkaline phosphatase-FA, urea, creatinine) and lymph-node aspiration for direct cytological examination. The treatment efficacy was evaluated through the presence or absence of clinical signs, negative serology, hematological status (remission of anemia, leukopenia, thrombocytopenia) and

biochemical (kidney and liver function). Subsequently, these exams were repeated every 4 months in the first year; in the second year every 6 months and afterwards one time per year to control the infection and monitor the animal.

3. Results

3.1 Clinical signs

All the animals from the study presented one or more clinical symptoms associated with canine visceral leishmaniasis. The main alterations observed were: weight loss, dermatopathies (ulcerated skin lesions, crusts, alopecia, seborrhea, etc.), ocular injuries, epistaxis, pallid mucosae, lymphoadenomegaly, hepatomegaly, splenomegaly and onycogryphosis. In 49 of the 56 animals two dermatopathies, namely crusts and seborrhea, were observed. After the treatments, independent of the protocol utilized, there was total or partial remission of the clinical signs, including the dermatopathies (Table 1).

Table 1. Evaluation of the presence of seborrhea and crust lesions before and after treatment according to clinical observation

Clinical Observation of Lesion	Crusts		Seborrhea	
	Before Treatment	After Treatment	Before Treatment	After Treatment
Absence	36 (73%)	48 (98%)	33 (67%)	42 (86%)
Presence	13 (27%)	1 (2%)	16 (33%)	7 (14%)
TOTAL	49 (100%)	49 (100%)	49 (100%)	49 (100%)

As shown in Table 1, 13 animals presented lesions and crusts, whereas after the treatment there was improvement in 93%, that is, only one animal continued to present lesions. Seborrhea lesion was presented by 14 animals, but after the treatment, only 7 animals continued presenting the symptom, characterizing an improvement in 50%. The appearance of new lesions was not detected after the treatments.

3.2 Cytology

Table 2. Comparison of results of the exams conducted before and after conducting treatment protocols

Animal Identification	Protocol	Serology		Cytology	
		Before	After	Before	After
3	T1+T2+T3	POS	POS	-	POS
4	T1+T2	POS	NEG	NEG	-
5	T1	POS	POS	POS	-
11	T3	POS	POS	POS	-
15	T1+T2	POS	POS	-	POS
16	T1+T2+T3	POS	POS	NEG	-
22	T1	POS	POS	-	NEG
29	T3	POS	NEG	POS	-

30	T2	POS	POS	-	NEG
33	T11+T2	POS	POS	-	POS
36	T3	POS	NEG	POS	NEG
52	T3	POS	NEG	POS	NEG
53	T2	POS	POS	-	NEG

Cytology was carried out in 13 animals, including five animals only before the treatment, six only afterwards and two both before and after the treatment. As to the two animals tested before and after the treatment, both presented positive results before and negative results after undergoing the protocol.

Of the five animals on which cytology was performed only before the protocol, only two obtained a negative result.

Five of the six animals subjected to cytology only after the protocol obtained a negative result; of these, three still presented a positive result in serology.

It should be emphasized that when the cytology exam has a positive result, it is considered confirmation of the disease, but in the case of a negative result other exams must be performed to obtain a conclusive diagnosis.

3.3 Serology

Before the treatment, the animals were considered serologically positive when antibodies were detected in ELISA with an optical density above the cutoff and presented titer starting from 1:40 in IFAT. All the animals presented positive serology in ELISA and IFAT.

Table 3. Comparison of serological results after conducting post-treatment exams

PROTOCOL	POS	NEG	TOTAL	% NEG
T1	8	3	11	27%
T2	12	5	17	29%
T3	16	8	24	33%
T1+T2	6	1	7	14%
T1+T3	3	0	3	0%
T2+T3	5	3	8	38%
T1+T2+T3	5	1	6	17%
TOTAL	**55**	**21**	**76**	

Table 3 shows the percentage of seronegative and seropositive animals after the treatments. Among the animals submitted to only one treatment protocol, T3 (immunotherapy with vaccines) was the most efficacious, followed by T2 (association of drugs) and T1 (miltefosine). The animals that presented relapses were submitted to new treatment cycles utilizing protocols different in each cycle, with the best response being observed in the animals treated with an association of drugs and immunotherapy, protocols T2+T3 with a negative percentage of 38%, as displayed in Table 3.

To evaluate the treatment efficacy, the animals were expected to present negative serology in the

ELISA and IFAT tests after the treatments.

3.4 Hematology

For the animals submitted to different protocols, the median values of blood counts were found near or within the normal range of values. The seropositive animals were those that presented a higher percentage of hematimetry outside the normality limit (60%) while 43% of negative animals were outside of normality.

Table 4. Number and percentage of animals with blood count outside normal limits after treatment according to diagnosis after the last serology performed

Final Diag.	Protocol	Total Number of Animals	Erythrocytes	Hgb	Hct	Platelets	Leuk	Lymph	Seg	Eosin
POS	T1	7	43%	71%	57%	86%	29%	43%	29%	29%
POS	T2	13	69%	77%	69%	54%	23%	46%	23%	15%
POS	T3	13	46%	54%	46%	62%	23%	38%	15%	15%
POS	T1+T2	6	50%	67%	67%	67%	50%	67%	50%	0%
POS	T1+T3	2	50%	50%	100 %	100%	50%	50%	100%	50%
POS	T2+T3	3	33%	33%	0%	33%	0%	33%	33%	0%
POS	T1+T2+T3	5	40%	80%	40%	60%	40%	60%	20%	0%
POS		**49**	**51%**	**65%**	**55%**	**63%**	**29%**	**47%**	**29%**	**14%**
NEG	T1	2	50%	50%	100 %	0%	50%	0%	0%	0%
NEG	T2	3	33%	67%	67%	67%	67%	33%	67%	0%
NEG	T3	7	14%	14%	14%	57%	29%	43%	29%	14%
NEG	T1+T2	1	0%	0%	0%	0%	100 %	100%	0%	.
NEG	T1+T3	0	.	.	.	.	.	.	.	.
NEG	T2+T3	3	0%	0%	0%	33%	0%	0%	0%	67%
NEG	T1+T2+T3	0	.	.	.	.	.	.	.	.
NEG		**16**	**19%**	**25%**	**31%**	**44%**	**31%**	**25%**	**25%**	**19%**

As shown in Table 4, 100% of the seropositive animals submitted to the protocol T1+T3 presented a platelet score outside of normality, while in the animals submitted to the protocol T1+T2 this value was 67%. Of the seronegative animals submitted to the protocols T3 and T2+T3, 67% presented platelet count outside of normality and of the seronegative total, 57% presented an abnormal platelet count.

3.5 Biochemical exams

The median values of FA, ALT, urea and creatinine, with the different protocols demonstrated that the majority of animals with final result serologically positive or serologically negative remained within normality, as expressed by reference values, as displayed in Table 5.

Table 5. Number of animals that presented biochemical values outside normal limits after treatment, according to the diagnosis after the last serology performed

FINAL DIAG.	PROTOCOL	TOTAL NUMBER OF ANIMALS	FA	ALT	UREA	CREATININE
POS	T1	6	0%	33%	17%	17%
POS	T2	11	27%	9%	0%	0%
POS	T3	10	40%	0%	20%	30%
POS	T1+T2	5	60%	40%	20%	40%
POS	T1+T3	4	50%	50%	0%	0%
POS	T2+T3	3	0%	0%	33%	0%
POS	T1+T2+T3	5	20%	0%	60%	40%
POS		**44**	**30%**	**16%**	**18%**	**18%**
NEG	T1	3	33%	0%	67%	0%
NEG	T2	4	0%	50%	0%	0%
NEG	T3	8	13%	25%	50%	13%
NEG	T1+T2	1	0%	0%	100%	100%
NEG	T1+T3	3	33%	33%	67%	33%
NEG	T2+T3	0	.	.	.	.
NEG	T1+T2+T3	19	**16%**	**26%**	**47%**	**16%**
NEG		**3**	0%	33%	17%	17%

It should be emphasized that two animals, despite final results with negative serology, presented urea levels above the reference values, one of which showed at the beginning of treatment urea at 143mg/dL with protocol T1, and the other one during the treatments T1+T2+T3. In addition to the three protocols, these two animals were treated with renal diet and homeopathy and are clinically well through the present moment.

4. Discussion

It was observed that the different protocols did not interfere in relation to dermatopathies, given that there were no manifestations of lesions after the treatments and that all were equally effective at reducing skin lesions. The more severe the disease stage the more severe the dermatopathies (Solano-Gallego 2011).

Cytology revealed that the animals serologically positive after treatment may present negative cytology. In this manner, cytology is an exam in which a positive result indicates 100% confirmation of

leishmaniasis, but in the case of a negative result requires other exams for diagnosis.

Since it was a retrospective study and not a controlled experiment, there was no exact order in the choice of protocols of treatments. We found that, among the individual treatments, the most effective was immunotherapy with vaccines followed by an association of drugs. The absence of evolution of the disease after the use of two vaccine doses may indicate an immunomodulatory protective response against progression of the infection, provided by immunotherapeutic treatment with antigen A_2 associated with one milligram of saponin, which induces a Th1 response (Ribeiro et al. 2014). The immunotherapy associated with allopurinol promoted a reduction of clinical signs while some animals remained serologically negative in post-treatment control exams. The owners were instructed to use collars and repellant continuously since they are in endemic areas.

When the protocols T2+T3 were associated, independent of the order, the greatest number of serologically negative animals was obtained. As to the use of the formulas, the owner was oriented to administer the medication between meals to optimize the effect of ketoconazole against gastric acidity. Few cases of emesis were observed after the administration of the formulas, being well tolerated. Utilization of the drugs presents efficacy due to the different actions of the medications utilized in the formula. Allopurinol when administered orally acts on the enzyme xanthine oxidase, and inhibits metabolism of Leishmania (Reguera et al. 2016). Orally administered ketoconazole is leishmaniostatic (Noli & Auxilia 2005, Favrot & Saridomichelakis 2010). Metronidazole administered orally is a weak leishmaniostatic with few side effects and provides a satisfactory synergistic effect when given concomitantly with cetoconazol (Noli & Auxilia 2005, Favrot & Saridomichelakis 2010). L-arginine aspartate is an immunostimulant amino acid (Durante et al. 2007). Zinc sulfate has an immunostimulant effect (Afshari et al. 2016). Domperidone, utilized to avoid the nausea or vomiting induced by the medications taken, elevates the Th1 immune response, inhibits the Th2 response, and stimulates a neutrophilic response with memory effect of up to one month after suspension of the medication (Reguera et al. 2016). Prednisone was utilized in order to act as an immunomodulator, controlling the deleterious autoimmune effects of the disease (Afshari et al. 2016).

After 30 days of formula administration, it was possible to observe a considerable reduction in the clinical signs, whereas at the end of the second formula, at about 120 days after initiating the treatment, there was complete diminution of the clinical signs. With the use of different medications, the majority of these animals presented negative serology after four months of treatment. For the animals that remained serologically positive, but with low titers of antibodies, the greatest number of serologically negative animals post-treatment was obtained by the chosen protocol of immunotherapy with vaccines.

The association of miltefosine with allopurinol was the treatment that presented more relapses of the disease, given that miltefosine only reduces the replication of *Leishmania* and does not remove the parasite from the lymph nodes (Laura Manna et al. 2015).

After accompaniment with annual negative serology, it is believed that these animals presented a clinical cure.

Before initiating any of the protocols, the exams performed included blood count, biochemical tests and cytology. Two of the animals showed very high urea levels, but were clinically well, without apparent symptoms. Nevertheless, treatment was chosen. The two animals continue to be monitored, serologically negative, but still showing kidney alteration, and are being treated adequately. It is believed that this kidney

alteration is a probable sequela of the disease.

It was observed that even the animals serologically negative after treatment presented anemia, in some cases severe, requiring blood transfusion; in some cases, associated diseases such as *Erlicha* sp. and *Babesia* sp. were observed concomitantly with leishmaniasis, which hampered the treatment and accounted for the non-regenerative anemia. In general, the alterations in the initial diagnostic blood count were only mild anemia and slight reduction of platelets, without clinical importance.

5. Conclusion

Leishmaniasis is a disease that is difficult to treat, presents constant relapses and can be associated with other diseases such as hemoparasitoses. However, associations of therapeutic protocols produced clinical improvement of patients even with presentation of subsequent negative serology, a clinical picture that if maintained would require lifelong monitoring. It is also noteworthy that this study evaluated only the aspects related to the disease leishmaniasis, and did not include those related to hemoparasitoses, thus requiring a further study.

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