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A COMPARISON OF THE IMMUNE PARAMETERS OF DOGS INFECTED WITH VISCERAL LEISHMANIASIS USING WESTERN BLOT AND NEUTRALIZATION TECHNIQUES

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SUMMARY

The *Western blot* technique was used to demonstrate the presence of antibodies in the blood of dogs that presented canine visceral leishmaniasis. This technique was used against some specific molecules present in the lysate of the promastigote form of *Leshmania chagasi.*Through the association of the results of the *Western blot* technique with the morphological alterations seen as a result of the serum neutralization technique performed in McCoy cells (which mimetizes the macrophage) it was possible to observe the role of some molecules of great relevance in determining the disease in symptomatic dogs as well as that of some other molecules associated with asymptomatic infected dogs that may become transmitters as well as differentiating them as asymptomatic resistant dogs. In the sera analyses carried out during the immunobloting a variation of 9 to 27 immunoreacting bands was observed, which were then compared using Dice's similarity coefficient. In the dendrogram constructed on the basis of the coefficient, 50% similarity was observed among the total number of reagent bands with the promastigote lysate, thus creating five groups. The main difference observed related to the clinical condition of the dogs: symptomatic and asymptomatic dogs were found in separate groups. The asymptomatic group of dogs was distributed in two different places in the dendrogram because they presented two different behavior patterns regarding the cellular morphology in the serum neutralization reaction: the presence or absence of cellular lysis. According to this analysis it is possible to evaluate the immune status and associate it with specific markers observed in the reaction found in the *Western blot strips.*

KEYWORDS: Canine visceral leishmaniasis; Western blot*;* Serum neutralization; Immunity status; McCoy Cell lineage.

INTRODUCTION

Several studies were undertaken using the Western blot technique to demonstrate the presence of antibodies to some specific molecules in the lysate of the promastigote forms in dogs that presented canine visceral leishmaniasis transmitted by *Leishmania donovani infantum* in the Mediterranean region[6,11,12,13,17].

On the other hand, other studies using the same technique, but working with the lysate of the amastigote form, showed that different molecules were characteristic of the infection and of the illness itself[13].

Further, in studies of infection using animal or *in vitro* models (using macrophage cell cultures) it was possible to identify the important role of such molecules as heat shock proteins (Hsp) of various molecular weights as hsps70-80-90 and hsp100[22,27]. These molecules are related to the stress that the promastigote suffers on being phagocytized by the macrophages and converted into amastigotes[24].

There are still other aspects of the interactions of the cytokines, gamma interferon, tumoral necrosis factor (TNF-alpha)[3], which are also involved in the cell immune response, as in the immune response mediated by the lymphocytes CD4 or CD8; there are, further the immunoglobulins responsible for the humoral response due to the first contact with promastigotes transmitted by the vector *Lutzomia longipalpis* as in the case of the visceral leishmaniasis which occurs in Brazil.

This complex interaction of the immunological response does not succeed in avoiding the escape mechanism that these protozoa present on protecting themselves from the various kinds of antibodies, cytokines and chaperones (Hsps). This mechanism is responsible for the perpetration of the species which will characterize those dogs that are classified as potential transmitters[4,10,23].

However, not all the asymptomatic infected dogs are transmitters and those which are not, are considered resistant to the disease. In this case the mechanism of protection that even promotes self-cure is triggered off by the immune system, which implies an effective cell immune response[21]. Using serological techniques, immune histochemistry, and the reaction of late sensitivity, it is possible to evaluate the characteristics and the kind of immune response that is involved and thus to define the immune status of the dogs.

However, these techniques that use animal models or even macrophages coming from infected animals make their use in the laboratory routine for the control of the illness by Epidemiologic Surveillance impossible.

This study proposes the utilization of the Western blot technique to identify the molecular markers of the symptomatology of canine visceral leishmaniasis and associate it with another *in vitro* technique, serum-neutralization[10,24], which may indicate the presence of immunoglobulins of isotypes IgG1 and IgG2, the production of which is induced by the cell response of types Th1 and Th2.

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In this study, a McCoy cell[19], a cell model that mimics the macrophages, was used to evaluate the cell lysis caused by the opsonization of the parasite by the isotypes IgG1 and IgG2, which the Fc fragments interacts as receptors on the surface of the cell membrane and promote phagocytosis and cell lysis.

MATERIAL AND METHODS

Sera: a 10% sample selection of the 240 sera employed in the serum epidemiological inquires which took place in an endemic area with ELISA testing[20] was used. The 24 sera were selected from dogs that presented either positive or negative serology, which means that they had a relative absorbance value superior or inferior to that of the cut-off. The Western blot technique was performed with the lysate of the promastigotes. Samples of the leukocyte cream from the same dogs were used for analysis by the use of the *polymerase chain reaction* (PCR).

ELISA: the ELISA test, produced in house (in Laboratório de Patologia de Molestias Infecciosas - Departamento de Patologia, Faculdade de Medicina da Universidade São Paulo), with a soluble antigen of promastigotes of *L.(L.) chagasi* was used for this research into antileishmania IgG. The sera were diluted (1:400) and the immune reaction undertaken for 30 minutes at a temperature of 37 ºC. The revelation was undertaken with anti-dog conjugate linked to alkaline phosphatase (BETHYL-USA), the plate was again incubated for 45 minutes at 37 ºC and the substratum and the chromogene (p-nitro phenyl phosphate) was added in carbonate-bicarbonate buffer, the reaction was interrupted with NaOH after 30 minutes to room temperature and reading undertaken with 405 nm filter.

The cut-off point was defined for the reading of the relative optical density (O.D.) = 1.

Polymerase chain reaction (PCR): this reaction was undertaken with leukocyte cream obtained after the centrifugation of the blood samples collected from the dogs.

The criterion of choice of the 24 samples was based on the O.D. of the ELISA test with results varying from negative values (< 1,000), with borderline values with O.D. up to 1,500 and with blood samples considered positive with values superior to 2,000.

The procedure for the extraction of DNA from the leukocyte cream was carried out in accordance with the description of ODORIZZI[20] and FICHOUX *et al.*[9].

The characterization of the species *L.(L.) chagasi* was defined by the pair of primers RV1/RV2[9], which amplifies a sequence of 140 base pairs from the specific region of the mini-circle of KDNA of *L.(L.) donovani* with an annealing temperature of 60 ºC. To identify the gender the primers LT1 (Leish 150) and LT2 (Leish 152), which amplify 120 base-pairs of the mini-circle of the KDNA in the conserved region at an annealing temperature of 55 ºC in accordance with the description[9], were used.

Western blot: the procedures described by ANTOINE *et al.*[1] were used to prepare the strips of nitrocellulose that were incorporated into the lysate of promastigotes of *Leishmania chagasi*.

Dendrogram: the band analysis observed in the nitrocellulose strips after the serologic reaction had been carried out was undertaken with Gel Compar software, using Dice's similarity coefficient[7], employed in populational studies, to compare the similarity between bands.

Serum-neutralization: the test was performed in duplicate using 24 well- plates. A cover slip and a solution of 0.5 mL of the promastigotes grown in completely conditioned medium were added to each well. After the growth of the McCoy[18] cells with $2x10^4$ promastigotes/mL, 0.5 mL of each serum diluted 1:40 was added and underwent a 30-minute incubation period at 37 °C. Thereafter 10^4 cells/mL of McCoy cells grown in supplemented Eagle medium with fetal calf serum were added. The plates were incubated at 37 °C for 24 hours.

The final dilution of the sera (1:40) was used because this is the standard dilution used in the indirect immunofluorescence reaction employed in the commercial kits for determination of reactive sera for Epidemiological Surveillance.

Morphological analysis: after serum-neutralization, each well was photomicrographed before the fixation of the cells with a solution of 0.2% glacial acetic acid. The cells were then processed for the immunofluorescence reaction using anti-dog serum marked with flourescein produced by the Biomanguinhos unit (Fundação Oswaldo Cruz, RJ). This was done after the reaction and the complete drying of the cover slips. Each cover slip was mounted on a microscope slide and observed by epifluorescence microscopy and then photomicrographed. A photo digital system was used in association with Image-pro Plus 5.1 software. Afterwards the morphological profile of each associated serum was mounted together with the analysis of the Western blot strips in the dendrogram.

RESULTS

The clinical variables are given in Table 1: the ages of the dogs, the PCR results provided by a previous study[20], the most important molecular markers and the presence of cellular lysis observed in the serum-neutralization reaction were added.

Figure 1 shows the serum–neutralization reaction following the indirect immunofluorescence reaction of serum # 191 in which the phagocytosis in the presence of parasites from the interior of a cell was observed and serum #169 where the potential process disintegration of the cell with the parasites inside it may be observed, while with the serum # 37 the total disintegration of the cell and the cellular control without the presence of the serum was observed.

The serum reaction analysis showed a variation of 9 to 27 immunoreactive bands which were compared using Dice's similarity coefficient. In the dendrogram constructed on the basis of the coefficient 50% of similarity was observed among the reactive bands with the lysate of promastigotes, forming five groups. Each group evidenced the kind of clinical condition that separated the symptomatic and asymptomatic dogs into separate groups, the asymptomatic dogs being divided into subgroups, the last of them for negative control.

It may be observed from the dendrogram that the positive and negative controls, respectively, present 90% of the positive sera and

Table 1

Results of the immune reactive bands most identified with the clinical conditions compared with results of PCR (gender and species), also the values of ELISA testing (O.D.), serum neutralization and dogs ages.

Sera	ELISA O. D.	Disease	Symptoms	PCR gender *L. chagasi*	PCR species *L. chagasi*	The most important bands analyzed in Western blot strips									cellular lysis	age/ years
						63kDa	50-55kDa	46kDa	42kDa	36kDa	26kDA	23kDa	14kDa	12kDa		
	Negatives															
83	0.788	no	no	neg	neg	x	-	-	-	-	-	-	-	-	-	4
116	0.302	no	no	neg	neg	-	-	-	-	-	-	-	-	-	-	1
118	0.779	no	no	neg	neg	-	-	-	-	-	-	-	-	-	-	7
120	0.55	no	no	neg	neg	-	-	-	-	-	-	-	-	-	-	3
	Boderlines		no													
11	1.047	no	no	neg	neg	-	-	-	-	-	-	-	-	-	-	8
151	1.047	no	no	neg	neg	-	-	-	-	-	-	-	-	-	-	3
192	1.421	no	no	neg	neg	-	-	-	-	-	-	-	-	-	-	1
	Positives															
103	2.836	no	no	neg	neg	x	-	x	-	-	-	-	-	-	-	6
43	1.825	no	no	neg	neg	x	-	-	-	-	-	-	-	-	-	1
55	4.718	no	no	neg	neg	x	-	x	-	-	-	-	-	-	-	3
	Symptomatics															
63	2.655	yes	yes	neg	neg	x	x	-	x	-	x	x	-	-	-	2
77	2.707	yes	yes	**pos**	**pos**	x	x	-		-	x	x	-	-	-	4
10	3.453	yes	yes	neg	neg	x	-	-		-	x	x	x	-	-	6
100	4.846	yes	yes	neg	neg	x	x	x		-	x	x	-	-	-	2
19	4.449	yes	yes	**pos**	**pos**	x	-	x	x	-	x	x	-	-	-	3
163	6.469	yes	yes	neg	neg	x	-	x	x	-	x	x	-	-	-	3
164	6.306	yes	yes	**pos**	**pos**	x	-	x	x	-	x	x	x	x	-	1
196	3.289	yes	yes	neg	neg	x	-	-		-		-	-	-	2+	20
	Asymptomatics															
95	4.019	no	no	**pos**	**pos**	x	x	x	-	-	x	x	x	-	-	2
56	1.597	no	no	neg	neg	x	-	x	x	-	x	x	-	-	-	0.6
139	1.106	no	no	neg	neg	-	-	-	-	-	-	x	-	-	-	0.3
169	1.421	no	no	neg	neg	x	x	-	x	x	x	-	-	-	1+	2
191	7.196	no	no	neg	neg	x	x	x	x	x	x	-	-	-	1+	1
37	2.403	no	no	neg	neg	-	-	-	-	-	x	-	-	-	4+	5

Nd+ not determined Cut-off = D.O. = 1; Disease = dogs showing three or more symptoms: lost weight, alopecia, growth nails, wounds and conjuctivitis.

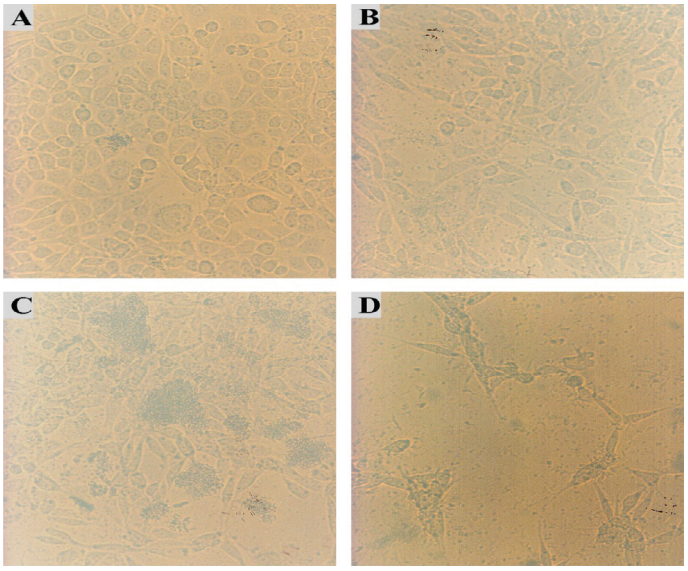

Fig. 1-A - Shows the serum-neutralization and cell lysis: (A) the cellular control; (B) serum #191 1+; (C) serum #169 2+ and (D) serum #37 4+.

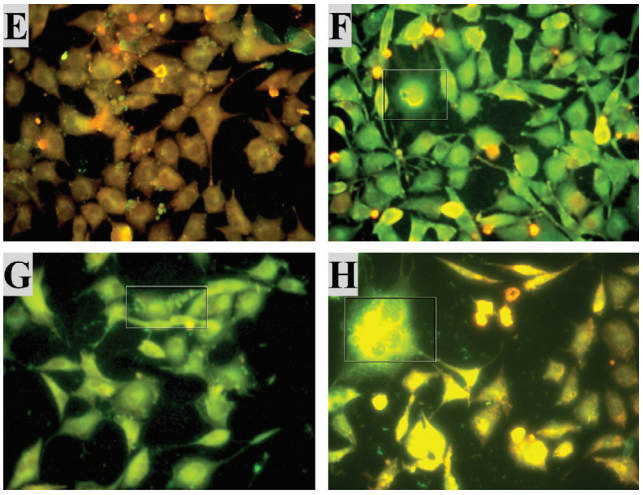

Fig. 1-B - Shows the serum–neutralization reaction following the indirect immunofluorescence reaction of (E) the cellular control without the presence of the serum; (F) serum #191 in which the phagocytosis in the presence of parasites from the interior of a cell; (G) serum #169 where the potential process disintegration of the cell with the parasites inside and (H) serum #37 the total disintegration of the cell.

80% of similarity with serum #118, considered negative in the ELISA test. Generally the similarities among the bands in the five groups varied between 50 and 90%.

Sera #11, #151 and #192 presented positive values of O.D., though very close to the cut-off point, but none of them presented antibodies to any of the bands analyzed. The optical density of serum #137, which presented antibodies to only one band 23 kDa was 1.647; because this dog was only three months old it may be supposed that this antibody had been acquired by vertical transmission (from mother to pup).

Thus, in the symptomatic dog group (sera #95, #100, #63, #77, #10, #19, #163 and #164) the antibodies to the proteins with molecular weights 63kDa, 26kDa and 23kDa are present as also are the antibodies to 42kDa or 46kDa. Among these sera only those numbered #19, #77 and #164 presented a positive PCR, representing 43% of the symptomatic dogs.

However, though serum # 95 presented positive PCR, it was clinically classified as asymptomatic. But in the dendrogram this serum belongs to the groups of symptomatic dogs, thus reinforcing the agreement of PCR, with the Western blot technique, in which antibodies to the proteins 63 kDa, 46 kDa, 26 kDa and 23 kDa are observed. As for serum #56, also clinically asymptomatic, it is in the dendrogram also to be found in the symptomatic group, presenting antibodies to proteins 63 kDa, 46 kDa, 42 kDa, 26 kDa and 23 kDa. These two sera

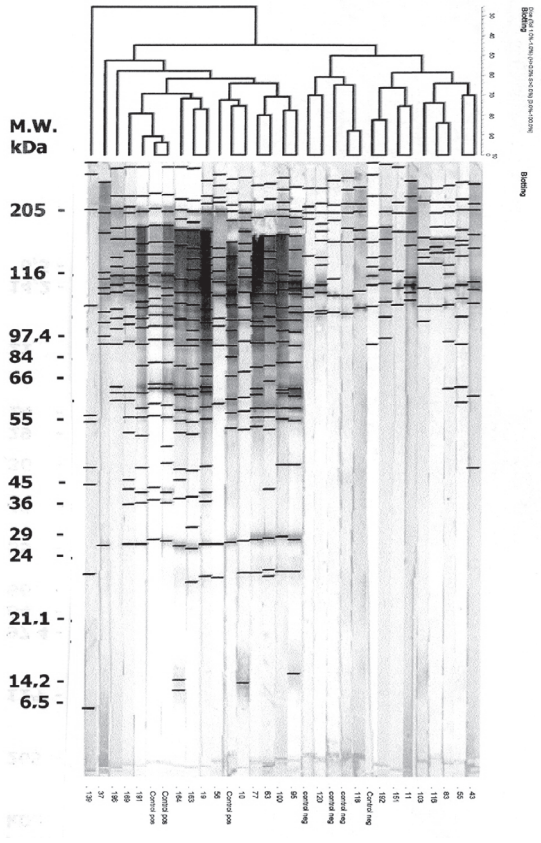

Fig. 2 - Dendrogram of immunoblotting performed with the 24 dog's sera and positive and negative controls.

suggest that these are pre-clinical cases.

Serum # 10 which presented as clinically ill with symptoms, also presented antibodies to proteins 63, 26 and 23 kDa, in accordance with the observations of all symptomatic dogs, though disagreeing with PCR.

The asymptomatic dog sera are distributed in two different groups in the dendrogram, each group presenting a different pattern of behavior related to the morphology in the serum-neutralization reaction, indicating the presence or absence of cellular lysis.

Thus the group considered as clinically asymptomatic showed antibodies to the proteins of molecular weights 63kDa, 55kDa, 36kDa and 26kDa, as also to the proteins of 46kDa and 42kDa; sera #191 and #169 they also presented cellular lysis. Serum #37 presented cellular lysis but only presented antibodies to the protein 26kDa. Besides that, serum #139 did not presented lysis but neither did it present antibodies to the proteins 42kDa and 23kDa. Under these circumstances, in order for a dog to show symptoms, the presence of antibodies to at least three proteins is necessary, one of which must be to the protein of molecular weight 23kDa.

In these cases these dogs may be considered asymptomatic, because they came into contact with leishmania and produced antibodies though without clinical symptoms.

The other group of sera numbered #43, #55, #83, #116, #11, #103, #151 and #192 did not show cellular lysis in the serum-neutralization, only the first three presenting antibodies to the protein 63kDa. All of these were considered clinically asymptomatic.

The sera #118 and #120 did not present antibodies to any of the above mentioned bands and were negative by both the ELISA and PCR, presenting a reactive pattern similar to that of the negative control sera. Serum #118 did, however, present cell lysis.

DISCUSSION

Proteins with molecular weight varying among 14kDa, 16kDa, 18kDa, 44kDa and 21kDa, 23kDa and 31kDa have already been described in sick dogs with canine visceral leishmaniasis[12,13,14,17]. COUVREUR *et al.*[5] characterized the antigen 24kDa as corresponding to a multi-antigen family of six to nine members varying from 20kDa to 31kDa, all of them presenting in some measure in dogs with visceral leishmaniasis, though these proteins were not identified in the negative sera of endemic areas[14]. Asymptomatic dogs presented antibodies, associated with humoral response[12,13], to antigens of 14kDa and 16kDa. Those authors suggested, in the 90's, that the Western blot would be a very useful method for diagnosing sick dogs.

In 2006 TALMI-FRANK[25] *et al*. used a computerized system which enable them to undertake a quantitative Western blot, thus assessing the intensity of the bands in the responses of antibodies during experimental infection by *Leishmania infantum* and after treatment with alloprurinol. This system permitted discrimination among immunodominant reactive bands in symptomatic dogs. These dogs presented antibodies to the following bands: 12, 14, 24, 29, 48 and 68 kDa and showed a significant increase in the intensity of reaction as compared to the non-immunodominant bands. The authors also observed that the bands 14,

48 and 68 kDa were associated with early infection (the first contact) while the increase in reactivity of bands 14, 24 and 29 kDa were associated with an unfavorable treatment prognosis.

However, due to the association of the Western blot technique with the morphological alterations observed with the serum-neutralization technique, the McCoy cell made it possible to observe the role of certain molecules which were of greater relevance in determining illness in symptomatic dogs and another important ability to predict the condition of infected but asymptomatic dogs that are potential transmitters and also distinguish these latter from asymptomatic dogs that present greater immunological responses that allow them to be considered as either healthy asymptomatic or as having the immunological protection that guarantees the neutralization of the parasites[21].

The active mechanism involved in the formation of these antibodies lies beyond the scope of this present study.

MYUNG *et al.*[16] showed that the glycoprotein GP63, present in the surface of the leishmania as a metalloproteasis gives resistance to the lysis mediated by the complement of the host cell.

As for GP46, also a surface glycoprotein of unknown function, it is also present together with GP63 in the promastigotes of several species of leishmania and other trypanosomatides[11]. The liberation of active proteases is associated with the density of the promastigotes in samples of SDS-PAGE gels; these have molecular weights varying from 43kDa to 100kDa. It has been demonstrated that the activity of these proteins may be inhibited by zinc metalloproteasis and by antibodies to GP63.

JAFFE & DWYER[11] showed that a protein of 66kDa liberated into the extracellular medium is recognized by the antibody anti-GP63, the main protein of the promastigotes' membrane; its function has not been clearly defined but is associated with the virulence and resistance to complement[5].

MYUNG *et al*.[16] observed that the metallo sulfo-proteasis (MSP), another name for GP63, besides creating resistance also allows the internalization of the promastigotes in the macrophage´s interior and increases the intracellular survival of the amastigotes in the macrophage phagolisossomes. Besides the authors´ evaluating the virulence factor as the MSP increases in the stationary phase when it is considered as having greater virulence, this factor increases the GP63 by about 30 times more than in the phase of logarithmic growth when the promastigote is less infectious.

The expression of the surface proteins (PSA) or GP46 in promastigotes of *Leishmania chagasi* is also differentiated and it increases more in the less infectious logarithmic phase than in the highly infectious stationary phase. The promastigote in the stationary phase presents the same proportion of PSA and MSP[2].

This dynamic may explain the situation of the sera that presented antibodies to GP63 and GP46 and yet did not present cellular lysis in the serum-neutralization as in this phase and immediately after, when the promastigotes entered the host, they present the less infectious logarithmic growth; this would be the first contact of the infectious agent with the host and it would induce a humoral response that could eventually result in the re-infection with neutralizing antibodies, thus creating a secondary response that would prevent the entrance of the promastigote into the macrophage.

FERNANDEZ-PEREZ *et al*.[8] demonstrated that asymptomatic dogs which presented cellular response with IgG2 in the serum were capable of recognizing bands ~67 and 45 kDa, while those dogs which only presented IgG1 did not recognize these antigens.

In the sera in which cellular lysis was observed in the serum-neutralization the immunologic responses would be greater and would involve a previous contact of the promastigote stimulating the lymphocytes CD4 and CD8 in which the host would be forced to develop the cellular response Th1 and Th2; on that occasion other mediators such as cytokines and interferon-gamma play a part in the action, as well as the induction of IgG2 and IgG1 that would promote the opsonization and the increase of the receptors of the fragment Fc of these immunoglobulins resulting in the cellular lysis mediated by the complement[10,15,23].

As was observed with sera #169, #191 and #37, cellular lysis in these cases may indicate that antibodies to leishmania present in dog sera would react to the leishmanias of the serum-neutralization reaction and be phagocytized and destroyed, thus promoting resistance factors in these sera. Although serum #118 was considered a standard negative serum, it also presented cellular lysis, which may indicate not the production of specifically anti-leishmania antibodies, but it may identify in the serum other factors involved in macrophage activation, possibly indicating a co-infection.

Then the presence of the cell lysis activate factors as: cytokine, as interferon-gamma, IgG1, IgG2[26], which associated with the specific antibodies in the strips of Western blot may provide clues as to the immune status of the dog, in which case these factors may help to elucidate aspects of the resistance in the canine population. This knowledge is of great value in population dynamics which might influence decisions to eliminate all the serum-positive dogs in programs for the control of canine visceral leishmaniasis.

This study offers some clarification as to the possibility of having more knowledge about the immunity of a small number of dogs from the endemic area, the Western blot that may be useful and provide elements as to the moment at which the clinical condition occurs with the manifestation of the disease, though a populational study is necessary to evaluate the consistency of the observations presented.

RESUMO

Comparação dos parâmetros imunológicos de cães infectados com leishmaniose visceral usando as técnicas de Western blot e neutralização

A técnica de *Western blot* foi utilizada para demonstrar a presença de anticorpos do soro de cães, que apresentavam leishmaniose visceral canina, contra algumas moléculas específicas no lisado da forma promastigota de *Leshmania chagasi.* Através da associação da técnica de *Western blot* com as alterações morfológicas observadas como resultado da técnica de soro-neutralização em células McCoy (que mimetizam o macrófago) foi possível observar o papel de algumas moléculas de maior relevância para a determinação da doença em cães sintomáticos bem como o papel de outras

moléculas na predição de cães infectados assintomáticos com o potencial de serem transmissores e ainda diferenciá-los como cães assintomáticos resistentes. Na análise dos soros durante a reação de *immunoblotting* observou-se uma variação de 9 a 27 bandas imunorreagentes, que foram comparadas utilizando-se o coeficiente de similaridade de Dice. No dendrograma construído com base no coeficiente, observou-se 50% de similaridade entre as bandas totais reagentes com o lisado de promastigota formando cinco agrupamentos. A principal diferença foi observada com respeito à condição clínica, ou seja, cães sintomáticos e assintomáticos ficaram em grupos separados. Os soros dos cães assintomáticos distribuídos em dois grupos diferentes do dendrograma apresentaram padrões de comportamento diferentes, quanto à morfologia celular na reação de soro-neutralização, ou seja, a presença ou ausência de lise celular. De acordo com esta análise foi possível avaliar o *status* imunitário e associá-lo com determinados marcadores específicos observados na reação encontrada nas fitas de *Western blot.*

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