Performance of DPP™ immunochromathographic rapid test (IRT) for canine visceral leishmaniasis:
comparison with other serological methods in suspected dogs from Cuiabá, Mato Grosso State, Brazil

Desempenho do teste rápido imunocromatográfico (TRI) para o diagnóstico da leishmaniose visceral canina: comparação com outros métodos sorológicos em cães suspeitos de Cuiabá, Mato Grosso, Brasil

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Abstract

The immunochromathographic rapid test (IRT) for canine visceral leishmaniasis (CVL) was tested in suspected dogs from the urban area of Cuiabá. The performance of IRT was compared with IFT and EIE and the direct parasitological test (DPT) as the gold standard. The sample, comprising 45 dogs, was selected by the Zoonosis Control Center. Twenty (51%) were male and thirty-four (75.5%) were both mongrel and had an estimated age of less than three years old. According to clinical data and lab tests: 10 (26.3%) dogs have been classified as exposed or infected and 18 (47.4%) as sick. IRT has achieved the best result for sensibility, 62%, high specificity, 87% and for positive and negative predictive values: 83.3% and negative 81.48%, respectively, reinforced by a *k* coefficient equal to 0.50, representing substantial agreement.

Keywords: Canine Visceral Leishmaniasis. Rapid test. Diagnosis.

Resumo

O teste rápido imunocromatográfico (TRI) para leishmaniose visceral canina (LVC) foi testado em cães suspeitos da área urbana de Cuiabá. O desempenho do teste foi comparado com RIFI e ELISA e, como padrão ouro, o teste parasitológico direto (TPD). A amostra com 45 cães foi selecionada pelo Centro de Controle de Zoonoses, sendo 23 (51,1%) machos, 34 (75,5%) sem raça definida e com idade estimada inferior a três anos. De acordo com os dados clínicos e os exames laboratoriais, 10 (26,3%) cães foram classificados como expostos, outros 10 (26,3%) como infectados e 18 (47,4%) como doentes. O TRI alcançou os melhores resultados quanto à sensibilidade, 62%, elevada especificidade, 87% e para os valores preditivos positivo e negativo: 83,30% e 81,48%, respectivamente, consolidado pelo coeficiente Kappa igual a 0,50, de concordância moderada. Os resultados confirmaram TRI como um bom preditor de doença e infecção para LVC.

Palavras-chave: Leishmaniose visceral canina. Teste rápido. Diagnóstico.

Introduction

In Brazil, visceral leishamaniasis (VL) is distributed in 21 states, and records show an annual average of 3,600 human cases and 230 deaths (BRASIL, 2009). The disease is caused by *Leishmania (Leishmania) chagasis* (MAURICIO; STOHARD; MILES, 2000) and it is transmitted by Lutzomyia longipalpis to a

great variety of mammals, including animals and human beings. Over the past thirty years, VL has spread towards the centers of cities in the Northeast, Southeast and Middle West Regions, characterizing an urbanization process of the disease (BRASIL, 2006) and dogs *(Canis familiaris)* have been referred to as the main carriers responsible for the persistence of VL in these endemic areas (FRANCA-SILVA et al., 2003; WORLD HEALTH ORGANIZATION, 2008). Meanwhile Almeida et al. (2009) pointed out the permanence of dogs in the peridomicile and the proximity of the residence to the forest are the main risk factors for canine infection. All outbreaks reported in the scientific literature are associated with the presence of seropositive dogs (MOURA et al., 1999; FRANCA-SILVA et al., 2003), that precedes the human infection (ALENCAR, 1961).

Nevertheless the strategy used by Brazil's Ministry of Health (BMH) (2006) for the control of VL includes the disposal of seropositives dogs. However it should be reconsidered due to the social problems caused by dog culling in addition to the replacement of new puppies by their owners (COSTA, 2011). Moreover, the diagnosis of canine visceral leishmaniasis (CVL) is complex, due to broad and nonspecific clinical signs and the presence of asymptomatic dogs. Therefore the diagnosis of CVL should be conducted using an integrated approach considering local residence, travels, habits, careful clinical examination and accurate laboratory analysis.

Even with the development of new laboratory techniques, the diagnosis of CVL remains a challenge for veterinarians and authorities responsible for the formulation of effective public policies for zoonosis control. The BMH performs the following serological tests: the indirect fluorescent test (IFT) and the enzyme-linked immunosorbent assay (EIE), both being reference techniques and manufactured by Bio-Manguinhos (BRASIL, 2006).

IFT and EIE are the techniques most commonly used for detection of antileishmanial canine antibodies

(LIRA et al., 2006; BISUGO et al., 2007; SOLANO-GALLEGO et al., 2009). IFT is highly sensitive and specific for detecting dogs with clinical signs of CVL, in addition to those infected but seemingly healthy (MOURA et al., 1999; TOLEZANO et al., 2007; ALMEIDA et al., 2009) and the test allows quantification of specific antibodies. Although it has disadvantages that may harm its performance, such as the subjective interpretation and the cross-reacting caused by metabolic discords and against *Trypanosoma cruzi* that have long been recognized (CAMARGO; REBONATO, 1969), there is still a lack of consensus regarding the threshold titer (DESJEUX, 1996).

EIE is a test with a medium-high sensibility and specificity that increase, depending on the antigens employed (SOLANO-GALLEGO et al., 2009). The best results were obtained when recombinant antigens were used (MIRÓ et al., 2008; MARCONDES et al., 2011), as K39 (CARVALHO et al., 2003; PORROZZI et al., 2007). Recombinant K39 antigen is a marker of active infection in human visceral leishmaniasis (CARVALHO et al., 2003) and canine disease (OTRANTO et al., 2005; MAIA et al., 2012).

Immunochromathographic tests combine particular characteristics that are important during screenings. Otranto et al. (2005) have found this test to be a useful tool for the serological diagnosis of clinical and sub-clinical canine leishmaniasis. The authors have pointed out the speed, sensitivity, specificity and usefulness of the test both under field conditions and for surveys, in addition to its importance in routine veterinary practices requiring a minimum of equipment and training of the handler.

Canine visceral leishmaniasis immunocromathographic test (TR DPP™ Leishmaniose-visceral-canina-Bio-Manguinhos/FIOCRUZ kits) is a new assay manufactured by Bio-Manguinhos/FIOCRUZ/Brazil and Chembio Laboratories – USA. The device uses technology with dual path platform (DPP™), insuring a high-sensitivity for the test and offering the result in about 15 minutes. The aim of this study was to

compare the performance of IRT with IFT, EIE assays (both manufactured by Bio-Manguinhos/FIOCRUZ) and DPT, as gold standard, in dogs suspected of being infected by *L. (L.)* chagasi (DE SANTIS et al., 2011) from Cuiabá, Mato Grosso state.

Material and Methods

Study design: The present study has conducted with dogs domiciled in Cuiabá (Mato Grosso state) and captured by the Zoonosis Center Control (ZCC - Cuiabá), from July to November 2009.

Sample selection: The selection adopted by ZCC included one or more of the following criteria: (i) animals with positive serology, by indirect fluorescent test (IFT) and/or enzyme-linked immunosorbent assay (EIE), previously held in the ZCC; (ii) dogs from neighboring areas where others were found to be infected and (iii) animals coming from homes or trapped in its vicinity where the ZCC had been collected phlebotomine. Dogs not meeting any of these criteria were not included in this study, resulting in the selection of 45 dogs.

Animals attributed data: Variables such as gender, breed and age were evaluated.

Clinical examination: All animals were examined by veterinarians from ZCC for clinical signs compatible with CVL. Next, based on the results of the laboratory tests, the dogs were grouped according to the classification proposed by Paltrinieri et al. (2010), in following categories: (i) exposed, for dogs without evidence of parasite in tissues but with antibodies for *Leishmania* spp but clinically normal or having clinical signs associated with other diseases; (ii) infected, when the presence of *Leishmania* organisms are confirmed by parasitological or molecular methods and low-titer specific antibodies are detected. Some of these animals can be healthy or not; (iii) sick, including dogs with positive results regardless of the tests and signs indicative of VL disease and (iv) severely sick, comprising dogs with severe clinical conditions and cytological findings. Dogs with none of the clinical or laboratory signs described above were considered negative.

Laboratory tests: Kits of Canine Visceral Leishmaniasis immunocromathographic rapid test (IRT) (TR DPPTM Leishmaniose-visceral-canina-Bio-Manguinhos/FIOCRUZ kits) (Bio-Manguinhos/FIOCRUZ and Chembio Laboratories – USA), indirect fluorescent test (IFT) (IFI Canine Visceral Leishmaniasis Bio-Manguinhos/FIOCRUZ) and enzyme- linked immunosorbent assay (EIE Canine Visceral Leishmaniasis Bio-Manguinhos/FIOCRUZ). Serological techniques and immunochromathographic assay were performed according to the manufacturer's instructions. The following criteria were adopted to classify the results as positive: IFT titers equal to or higher than 1:40, EIE readings over the cut-off and, for IRT, the emergence of two lines 15 minutes after buffer placement.

The direct parasitological test (DPT) was carried out by bone marrow aspiration to detect amastigotes in stained cytological smears (OLSSON et al., 2008) and was used as the gold standard. When necessary the animals were pre-anesthetized with Acepram® 1% (acepromazine 0.1 mg/kg IM, 30 to 40 minutes before the assay) (MIRÓ et al., 2008; MÜLLER et al., 2009). However, others, still agitated, required general anesthesia, with use of 25 mg/kg of sodic thiopental in order to perform the procedure. All of the laboratorial procedures were executed according to the Principles of Good Laboratory Practices (BRASIL, 2006) for the protection of the manipulator, sample and environment.

Kappa Coefficient: Agreement *(kappa coefficient)* among the laboratory tests and the evaluation of positive and negative predictors were calculated. In order to identify the agreement between each test and the DPT gold standard the authors employed the set of Landis and Koch (1977) guidelines: values *(k)* < 0 as indicating no agreement; 0 – 0.20 as slight; 0.21 – 0.40 as fair; 0.41 – 0.60 as moderate; 0.61 – 0.80 as substantial, and 0.81 – 1 as almost perfect agreement.

Ethics Committee: The project was submitted to the Use of Animals Committee (CEUA - FIOCRUZ) and duly approved (nº 0300/2006).

Results

Distribution of variables, gender, age, breed and clinical findings: The results on gender, breed, age and clinical signs of the 45 selected dogs by ZCC-Cuiabá showed that 23 (51.1%) were male, 34 (75.5%) were mongrel and they had an estimated age under three years old, and in 20 (44.4%) clinical findings were related to the skin region, localized or generalized desquamative dermatitis (53.3%), alopecia (44.4%) and ulcerative dermatitis (40%).

Laboratory assays: Among the 45 studied animals, 38 (84.4%) were positive for one of the four laboratory analyses carried out. Direct parasitological test (DPT) was used as gold standard and was prepared for all 45 animals and 16 (42.1%) were positive. When commercial kits for serological methods have been evaluated, IFT has proved to present higher frequency, 38 (100%) serum samples have been achieved titers equal to or above 1:40, as recommended by the kit's instructions. IRT and EIE test generated positive responses: 21 (55.3%) and 19 (50.0%) samples, respectively (Table 1).

According to the dogs infected by *L. (L.) chagasi* classification, proposed by Paltrinieri et al. (2010), among the 38 (100%) positive animals, 10 (26.3%) have been accounted as exposed or infected and 18 (47.4%) as sick; none was classified as a severely sick dog. Among the regarded accounted as sick and exposed animals, all of them were detected by IFT (Table 1).

Analysis of agreement among the serological and parasitological tests: Benchmarks were then between each serological technique and the DPT gold standard (Table 2). All IFT positive sera have been also DPT positive, total of 16 (100%), followed by IRT and EIE, 13 (81.3%) and 10 (62.5%), respectively. Sensibility and specificity were calculated for each test. The results showed high sensitivity of 81.3% and 100% for IRT and IFT, respectively and moreover 65.5% obtained in EIE However, the specificity was lower, respectively, 72.4%, 69.0% and 24.1% for IRT, EIE and IFT.

Our results have confirmed all negatives DPT were also in IFT. Indeed the negative predictive values were 100% between the gold standard and IFT and the positive predictive value detected was low, 42.1% (Table 2).

Kappa index: Kappa coefficient (k) was determined (LANDIS; KOCH, 1977) among each test and the DPT. The best agreement was IRT: k equal

Table 1 - Clinical classification matched with positive and negative results of laboratory assays in 38 samples from dogs screened by ZCC-Cuiabá, from July to November of 2009

Clinical Approach (N)	Laboratory assays*			
	DPT (%)	IRT (%)	IFT** (%)	EIE (%)
EXPOSED (10)	00	00	10 (26.3)	01 (5.3)'
INFECTED (10)	02 (12.5)	05 (23.8)	10 (26.3)	04 (21.1)
SICK (18)	14 (87.5)	16 (76.2)	18 (47.4)	14 (73.7)
TOTAL 38	16 (42.1)	21 (55.3)	38 (100)	19 (50.0)

* Seven (15.56%) were negative in all laboratory assays
** Positive results for titers equal or above 1:40

DPT – Direct Parasitological Test
IRT - Immunocromathographic Rapid Test (TR DPPTM Leishmaniose Visceral Canina - Manguinhos/FIOCRUZ and Chembio Laboratories/USA)
IFT – Indirect Fluorescent Test (IFI Canine Visceral Leishmaniasis - Bio-Manguinhos/FIOCRUZ)
EIE– – Enzyme-linked Immunosorbent Assay (EIE Canine Visceral Leishmaniasis – BioManguinhos/FIOCRUZ)

Table 2 - Agreement estimated among the results obtained from DPT, the golden standard and serological tests, as well the respective rate of sensibility and specificity, positive and negative predictive values and the kappa index with data interpretation from 38 studied dogs screened by ZCC-Cuiabá, between July and November of 2009

ASSAYS		DPT (+)	DPT (-)	Sensibility (%)	Specificity (%)	PPV (%)	NPV (%)	Kappa Value (interpretation)
IRT	(+)	13	08					
	(−)	03	21	81.3	72.4	61.9	87.5	0.50
Total		16	29					(substantial)
EIE	(+)	10	09					
	(−)	06	20	62.5	69.0	52.6	76.9	0.13
Total		16	29					(slight)
IFT**	(+)	16	22					
	(−)	00	07	100.0	24.1	42.1	100.0	0.18
Total		16	29					(slight)

** Positive results for titers equal or above 1:40
CI - 95%

DPT – Direct Parasitological Test
IRT - Immunochromatographic Rapid Test (TR DPPTM Leishmaniose Visceral Canina - Manguinhos/FIOCRUZ and Chembio Laboratories/USA)
IFT – Indirect Fluorescent Test (IFI Canine Visceral Leishmaniasis - Bio-Manguinhos/FIOCRUZ)
EIE– – Enzyme-linked Immunosorbent Assay (EIE Canine Visceral Leishmaniasis – Bio-Manguinhos/FIOCRUZ)
PPV – Positive Predictive Value
NPV – Negative Predictive Value

to 0.50 (substantial) and 0.18 and 0.1.3 (slight) for IFT and EIE, respectively (Table 2).

Discussion and Conclusions

The dogs studied were from Cuiabá and they were collected or taken to the Zoonosis Control Center due to the possibility of being harboring the etiological agent. CVL is a systemic disease, but it is not clear yet which factors may influence resistance or susceptibility. Gender (TRAVI; OSORIO; MELBY, 2002), breed (FERROGLIO; VITALE, 2006) and age (CARDOSO et al., 2004) are some of the possibilities that may suggest an infection prognosis. In our study, no gender differences were observed (48.9% and 51.1% for females and males, respectively); about the breed of the studied dogs, 75% were mongrels. The disease prevalence was higher in groups of up to three years old, 34.4% were considered sick, perhaps due to the low immune response, usually seen in this age group; despite the small numbers of dogs examined, data were similar to Cardoso et al. (2004) findings.

Canine disease is also marked by clinical signs, which are common to several other pathologies inspiring an accurate diagnosis. Among the main clinical signs described by Baneth et al. (2008) for CVL, our study has detected desquamation (53.3%), followed by alopecia (44.4%) and ulcers (40%).

The Brazil's Ministry of Health (2006) advocates the IFT and EIE kits (Bio-Manguinhos/FIOCRUZ) for use not only in individual diagnosis but also in field surveys. Moreover, at new transmission areas, the parasitological confirmation has also been required, by means of bone marrow aspirative punction in order to prepare smears and further species identification (BRASIL, 2006). In our study, DPT was used as the reference method, due to its ability to identify the *Leishmania* spp parasite. However, it seems to be an invasive assay that requires skilled laboratory technicians, besides it does not show good sensitivity (TAFURI et al., 2001). Our findings have showed of 16 (42.1%) dogs DPT positive, 14 (87.5%) were classified as sick. Indeed the likelihood of these animals have high parasite load permitted its recognition on bone marrow smears.

The high sensitivity of IFT was also confirmed in the 45 samples studied, all 38 animals classified as exposed, infected or sick being positive to IFAT with titers between 1:40 and 1:1280. None of the other tests used in the study showed a similar performance. Probably due to low cut off recommended by the kit's instructions. Boarino et al. (2008) considered 1:40 cut off as doubtful. However, the IFAT positive predictive value was lower, 42.1%, when compared with DPT. Indeed, IFAT is not able to distinguish between CVL progression states and it seems that may not be useful as a marker of disease. The group of dogs classified as exposed displayed clinical signs consistent with other diseases (FERROGLIO et al., 2007; FIGUEIREDO et al., 2010).

Furthermore, it might be more prudent to include differential diagnoses before a conclusive diagnosis that may follow the domestic dog elimination. This procedure has not been able to produce elimination or even the reduction in the human cases incidence satisfactory results, which highlights the absence of strong evidence for a significant impact (ROMERO; BOELAERT, 2010).

The positive result obtained by EIE (Bio-Manguinhos/FIOCRUZ) assay has shown a weak performance. The sensibility and specificity were the lowest ones, 62.5% and 69.0%, respectively. Actually this kit was also tested by other authors (LIRA et al., 2006; BISUGO et al., 2007) with different results, emphasizing the inconsistent results produced by EIE (PORROZZI et al., 2007). However, the serological and parasitological methods implementation requires special conditions, such as laboratory infrastructure and trained staff. In addition, it takes a long time to deliver the results. Thus, given the complexity of CVL diagnosis, laboratory methods easier to perform, interpret and rapid results are increasingly required.

Immunochromathographic rapid test (IRT) (TR DPP™ Leishmaniose-visceral-canina-Bio-Manguinhos/FIOCRUZ kits) was developed by Bio-Man-guinhos/FIOCRUZ in partnership with Chembio (USA) for the diagnosis of CVL. The results have showed a higher sensibility (81.3%) and intermediate specificity (72.4%). IRT has allowed the identification of 5 (50.0%) infected and 16 (88.9%) sick dogs, but failed, as expected, to spot the exposed animals. Still, the new assay has presented the best result for the positive and negative predictive values, 61.9% and 87.5%, respectively, with a substantial degree of agreement ($k = 0.50$), different from the two others assays as slight for both IFT ($k = 0.18$) and EIE ($k = 0.13$). IRT has been ensured better results than IFT and EIE both recommended by the Brazilian Health Ministry and guide the selection, sometimes wrongly, of animals for disposal.

In this study, the results showed that the combination of the dual path platform, with selected joining recombinant antigens of *L. (L.) chagasi*, has allowed to the device IRT to be a good infection and disease predictor for CVL, besides being a simple procedure, easy to handle, rapid and adapted for use in both the field and the laboratory.

Acknowledgements

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