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Reliability of techniques used in the diagnosis of canine visceral leishmaniasis by the national control program in Brazil: A survey in an area of recent transmission

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ABSTRACT

One of the key components of the Brazilian Program for the Control of Visceral Leishmaniasis (PCLV) is the euthanasia of *Leishmania*-infected canine reservoirs, the detection of which depends on a screening procedure involving a Dual Path Platform® (DPP) immunoassay and a confirmatory *enzyme*-linked immunosorbent assay (ELISA). The aims of the present study were to evaluate the reliability of these techniques in a region of recent transmission of canine VL, to follow up the seroconversion 3–4 months after the initial diagnosis of DPP reactive but ELISA indeterminate or non-reactive dogs, and to identify the species of *Leishmania* in circulation in the area. Each animal was submitted to DPP under field conditions, performed by municipal health workers using peripheral blood (DPP-field), to DPP under laboratory conditions using serum (DPP-lab) and to ELISA using serum. The agreements between the tests were determined using McNemar's χ^2 test, Cohen's kappa coefficient (k) at the 95% confidence interval and *prevalence*-adjusted bias-adjusted kappa (PABAK). Of the 1130 dogs examined, 74.2% were non-reactive in all three tests applied. Based on the PCLV positive-infection criterion, seroprevalence was 8.9% (101/1130) with 83.2% (84/101) of infected animals showing reactivity in all three tests while 7.8% (8/101) were reactive in DPP-field and ELISA and 8.9% (9/101) in DPP-lab and ELISA. The proportions of disagreements were substantial in all comparisons. Inter-rater reliability between DPP-field and ELISA (k = 0.55; PABAK = 0.78) and DPP-lab and ELISA (k = 0.59; PABAK = 0.81) were considered moderate, while that between DPP-field and DPP-lab (k = 0.61; PABAK = 0.79) was classified as marginally good. The proportion of seroconversions in DPP reactive animals that were initially ELISA indeterminate was significantly higher than in those that were DPP reactive but initially ELISA non-reactive. Restriction fragment length polymorphism analysis revealed the presence of *Leishmania infantum*, the etiologic agent of VL, in bone marrow samples from VL-infected animals. Our data showed that the techniques and protocols currently employed in the PCLV screening approach are not entirely reliable. Further consideration should be given to monitoring dogs with undetermined results in ELISA and a better training should be provided for health workers responsible for performing DPP tests applied under field conditions.

1. Introduction

Visceral leishmaniasis (VL) is the most serious form of leishmaniasis by virtue of its high incidence and lethality rates in various parts of the world. On the American continent, most of the reported cases of VL (90%) occur in Brazil (Alvar et al., 2012) where the disease is distributed throughout the country. According to the Brazilian Ministry of Health (Brasil, 2014), the incidence of VL is particularly high in urban areas and the trend is for geographic expansion of the disease.

Recent studies in regions of Brazil that are endemic for VL have established a firm association between human and canine VL (Belo et al., 2013a; Teixeira-Neto et al., 2014). Indeed, from an epidemiological standpoint, canine VL is so important as human VL because

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dogs represent the main reservoirs of *Leishmania infantum* in urban environments (Gontijo and Melo, 2004). On this basis, the Brazilian Program for the Control of Visceral Leishmaniasis [*Programa de Controle da Leishmaniose Visceral* (PCLV)] calls for the rapid diagnosis and treatment of human cases, the control of vectors, and the identification and eradication of *Leishmania*-infected canine reservoirs in endemic areas.

The need for rapid and reliable diagnoses is more than justified considering the high prevalence of canine VL and the presence of asymptomatic animals that could infect the vector in the same way as symptomatic ones (Alvar et al., 2004). Swift and accurate diagnosis eliminates the unnecessary euthanasia of false-positive dogs and the unintentional maintenance of false-negative animals that could serve as sources of vectorial contamination (Leandro-Júnior, 2014).

Up until 2011, the diagnosis of canine VL in Brazil involved preliminary screening by *enzyme*-linked immunosorbent assay (ELISA) followed by an *indirect fluorescent-antibody test (IFAT)* to confirm the result (Brasil, 2014). This approach was not only inaccurate (Fraga et al., 2016) but also incurred a protracted period (of around 60 days) between sampling and release of the results, thereby maintaining infected animals in circulation longer than necessary. Due to these limitations, towards the end of 2011, a rapid Dual Path Platform® (DPP) immunoassay developed by the Instituto de Tecnologia em Imunobiológicos (*Bio-Manguinhos, Fiocruz, Rio de Janeiro, RJ, Brazil*), began to be used in the screening procedure with ELISA retained for confirmation (Coura-Vital et al., 2014; Leandro-Júnior, 2014). DPP test produce faster results in field conditions with samples of peripheral blood and can be performed in lab conditions with serum or plasma samples (Brasil, 2011).

A number of studies have documented the specificity and sensitivity of the DPP test (Arruda et al., 2016; Grimaldi et al., 2012; Ribeiro et al., 2015; Santis et al., 2013), while Fraga et al. (2016) demonstrated that the DPP/ELISA procedure offers higher specificity and greater positive predictive power than the earlier screening approach, although the sensitivity is substantially the same. However, there are few reports concerning the agreement between the two adopted techniques (i.e. DPP *versus* ELISA) or the effect of experimental conditions under which the DPP test is performed (i.e. field *versus* laboratory) (Schubach et al., 2014). Although Santis et al. (2013) claimed that the DPP test is as effective in the field as in the laboratory, as far as we are aware, no studies have evaluated the agreement between the DPP test performed using peripheral blood in the field and using serum under laboratory conditions, with both tests employing visual readout.

In order to test the hypothesis that the screening strategy involving a combination of DPP-field or DPP-laboratory tests and ELISA is reliable, we aimed to: (i) apply the PCLV procedure in a region of recent transmission of canine VL; (ii) establish the level of agreement between DPP-field, DPP-laboratory and ELISA tests; (iii) to follow up the seroconversion of animals presenting DPP-reactive but ELISA indeterminate or non-reactive tests; and (iv) to identify the species of *Leishmania* in circulation in this area. The results reported herein will contribute to our understanding of the issues involved in the diagnosis of canine VL and will be of value in establishing benchmarks that could be useful in the development of screening procedures.

2. Materials and methods

2.1. Ethical statement

Details of the study were submitted to and approved by the Ethics Committee on Animal Research of the Universidade Federal de São João del Rei (protocol no. 015/2014), and all procedures followed the guiding principles established by the Council for the International Organizations of Medical Sciences.

2.2. Study area

The study was performed in the Recanto da Lagoa district (19° 51.606′ S, 44° 36.462′ W; 791 m altitude) of the municipality of Pará de Minas, State of Minas Gerais, Brazil (Fig. 1). The municipality encompasses an area of 551 km^2 and according to data gathered in 2014, has a population of 90,306 individuals of which 6496 were domiciled in the district under study (Instituto Brasileiro de Geografia e Estatística, 2013). Recanto da Lagoa was chosen as the sampling area because this district had the highest incidence of human VL in the municipality (four cases notified since 2009) together with a high prevalence of canine VL (12.3% of seropositive dogs in both ELISA and RIFI, in 2013) as indicated by a survey carried out by the municipal health authorities.

2.3. Design of study

The survey took place during the first half of 2014 and included all dogs in the households of Recanto da Lagoa district except for animals less than six months old and those for which permission to participate in the research program could not be obtained from the owner. Each dog was submitted to three assessments as follows: (i) DPP-field: the DPP test was performed *in situ* using total peripheral blood, (ii) DPP-lab: DPP test was performed in the Laboratory of Parasitic Diseases at Fundação Ezequiel Dias (FUNED, Belo Horizonte, Brazil) using serum, and (iii) ELISA: the test was performed in the same laboratory using serum. Dogs that tested positive for DPP-field and/or DPP-lab and also in ELISA were considered *Leishmania*-infected as defined by the Diretoria de Vigilância Ambiental/Superintendência de Vigilância Epidemiológica, Ambiental e Saúde do Trabalhador/Sub Secretaria de Vigilância e Proteção à Saude (technical note number 140/2012).

2.4. Blood sampling and serological analysis

In the DPP-field test, sampling of blood and collection of data were performed by two municipal health workers following the instructions issued by the manufacturer and using a routine procedure adopted in the municipality. Briefly, a sterile disposable blade was used to make a small cut in the ear tip of the animal and peripheral blood was collected using the sampling loop supplied with the test kit which, when filled with blood in the correct manner, delivered a sample aliquot of exactly 5 μL. The blood sample was applied to the DPP strip provided and the result obtained by visual inspection after 10–15 min reaction between sample and *Leishmania* antigens.

For the DPP-lab and ELISA tests, two trained professionals collected samples (~5 mL) of peripheral blood by jugular or cephalic vein puncture using sterile and disposable syringes and needles. Blood samples were transferred to vials without anticoagulants and refrigerated overnight at 4–8 °C. Samples were then centrifuged at 2000 rpm for 10 min and the sera separated and stored at −20 °C until required for analysis. For the DPP-lab test, each sample was thawed, mixed thoroughly and a 5 μL aliquot applied to a DPP strip with the aid of a micropipette. The results were assessed by visual inspection in the same manner as the DPP-field test. ELISA was performed using the *EIE*-LVC (*Ensaio Imunoenzimático para Diagnóstico da Leishmaniose Visceral Canina*) kit produced by *Bio-Manguinhos*according the recommendations of the manufacturer.

Blood samples for the field and laboratory tests were collected on the same day. However, in order to avoid possible bias, the professionals responsible for the DPP-lab and ELISA tests had no access