Performance of an indigenous β-mercaptoethanol-modified antigen in comparison with a commercial reference in direct agglutination test for detection of canine visceral leishmaniasis

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We compared the performance of a locally produced β-mercaptoethanol-modified promastigote antigen (β-ME-Ag) of an indigenous Leishmania infantum strain against that of a trypsized Leishmania donovani reference (REF-Ag) in the direct agglutination test (DAT) for detection of canine visceral leishmaniasis (CVL). One hundred and fifty-one serum samples collected from dogs belonging to four groups with different conditions were included. At a DAT titre of 1:320, statistically determined as optimal cut-off value for β-ME-Ag, and 1:160 for REF-Ag, a sensitivity and a specificity of 100% were estimated for β-ME-Ag in comparison with 96.6% and 100%, respectively, for REF-Ag. Overall, levels of agglutination titres recorded for the two antigens were highly concordant (Cohen’s k = 0.879) in both the CVL and non-CVL groups. Based on current results, and ease experienced in processing the antigen and reading the test outcome, we recommend incorporation of β-ME-Ag in DAT for confirmation or exclusion of suspected CVL in dogs.

INTRODUCTION

In the Mediterranean region, visceral leishmaniasis (VL) is a zoonotic disease caused by Leishmania infantum. As in most northern European countries, many people in this region own dogs which, due to optimal habitat, attract phlebotomine flies, increasing the chance of disease transmission to humans and other animals (Gramiccia, 2011). Based on available data, it is assumed that among the existing 15 million dogs residing in southern Europe 2.5 million (16.7%) are infected with Leishmania (Moreno & Alvar, 2002). Canine visceral leishmaniasis (CVL) seroprevalence rates varying from 1.7% in southern Cyprus to more than 40% in southern Italy have been reported (Deplazes et al., 1998; Oliva et al., 2006). More than half of these dogs present asymptomatic Leishmania infection and are thus considered as eligible for transmitting the disease (Molina et al., 1994; Michalsky et al., 2007). Unfortunately, none of the available serological tests has the required efficiency for detecting CVL at the early phase. This shortcoming has urged public health and veterinary authorities to seriously search for tools that are sensitive and reliable for detection of the disease at this phase (Rajasekariah et al., 2008). Establishing diagnostic methods...
that are, in addition to being sensitive, also easily applicable and simple to interpret, such as the direct agglutination test (DAT), is therefore highly recommended (WHO, 2010).

Following its development and evaluation for VL diagnosis at both laboratory and field levels, a similar version of the DAT was established for the disease in the canine reservoir (el Harith et al., 1986, 1989). Since then, the DAT has been used as reference diagnostic procedure for the disease in both human and canine hosts worldwide. Among further improvements introduced to DAT procedures were the incorporation of a homologous indigenous or autochthonous *Leishmania donovani* strain and substitution of trypsin by β-mercaptoethanol (β-ME) as cleaving agent in antigen processing, which have significantly improved test sensitivity (el Harith et al., 1995). These two improvements significantly motivated evaluation of the test for detection of VL in human patients who presented with negative parasitological outcome but demonstrated favourable response to the administration of specific anti-leishmanial therapy (Chowdhury et al., 1993).

In this study, we have compared the performance of a locally produced β-ME-modified antigen of an indigenous *L. infantum* (β-ME-Ag) with a reference *L. donovani* antigen conventionally prepared by trypsin treatment (REF-Ag) in the DAT for CVL detection.

**METHODS**

**Promastigote mass cultivation.** *L. infantum* strain MHOM/PT/01/IMT387, isolated from an adult Portuguese VL patient and kindly supplied by the Institute of Hygiene and Tropical Medicine in Lisbon, was maintained as described in more detail previously (el Harith et al., 1995). Mass cultivation of the promastigotes for preparation of the antigen was performed in liver infusion tryptose/haemin medium (Sadigursky & Brodskyn, 1986), supplemented with 20 % heat-inactivated (30 min at 56 °C) and penicillin/streptomycin, with continuous slow orbital shaking at constant temperature (26–27 °C) for a minimum period of 7 days.

**β-ME-modified DAT antigen.** Promastigotes at the exponential phase (mostly elongated forms) were harvested by cold (4 °C) centrifugation (4000 g). The parasite suspension was further washed by centrifugation in cold Locke’s solution (pH 7.2) for 10 min. After a third wash, the promastigote pellet was resuspended in Locke’s solution supplemented with 1.6 % (v/v) β-ME. The parasite suspension was then incubated at 37 °C for 45 min. Following β-ME treatment, promastigotes were fixed in 1 % formaldehyde/Locke’s solution for at least 20 h at 4 °C. After washing in citrate saline (0.15 M sodium chloride, 0.056 M sodium citrate, pH 7.4), the promastigotes were stained with 0.1 % Coomassie brilliant blue/citrate saline under constant shaking for 90 min. The stained parasites were then washed in citrate saline and resuspended in 1 % formaldehyde/citrate saline. The suspension was finally filtered through 40 μm mesh size nylon gauze and adjusted to a promastigote concentration of 5 x 10^6 ml^-1. The ready-for-use antigen was stored at 4 °C until required.

**DAT reference antigen.** The reference antigen in the DAT was prepared as originally described by el Harith et al. (1986). Shortly before use the lyophilized antigen was reconstituted by addition of 5 ml physiological saline (0.9 % NaCl) followed by gentle mixing to ensure thorough distribution of promastigotes in the suspension medium as instructed.

**Test sera.** A total of 151 serum samples from male and female dogs of different breeds, ages and functions were studied. Group 1 (G1) consisted of 31 dogs with confirmed CVL (lymph node aspirates positive for *Leishmania*). Group 2 (G2) consisted of 38 dogs with clinical signs typifying CVL but that had negative lymph node aspirates for *Leishmania*. Group 3 (G3) consisted of 45 dogs with diseases other than CVL: 26 collected during an annual anti-rabies vaccination campaign in 2010 carried out in the municipality of Évora, Portugal; 16 from dogs diagnosed at the Veterinary Hospital of the University of Évora; 3 from Brazilian dogs (municipality of Patos, state of Paraíba, Brazil). Final diagnosis for those 45 dogs was as follows: neoplastic disorders (*n* = 12), dirofilariasis (*n* = 8), babesiosis (*n* = 4), ehrlichiosis (*n* = 3), trypanosomiasis (*n* = 3), distemper (*n* = 3), haemorrhagic gastroenteritis (*n* = 2), skin disorders (*n* = 2), autoimmune disorder (*n* = 1), leptospirosis (*n* = 1), leukaemia (*n* = 1), conjunctivitis (*n* = 1), epilepsy (*n* = 1), acaetocholeinemia (*n* = 1), respiratory disorder (*n* = 1) and toxocariasis (*n* = 1). Group 4 (G4) consisted of 37 healthy dogs with no history of CVL from the municipality of Évora that had negative lymph node smears. With the exception of three sera from the trypanosomiasis cases, all the remaining 148 were collected either during an anti-rabies campaign (March–July 2010) or from dogs presented at the veterinary clinic of Évora since 2009. All 151 sera were continuously kept at −20 °C until the start of this study in March of this year.

**DAT execution.** The DAT using either β-ME-Ag or REF-Ag was executed according to the improved protocol described for diagnosis of CVL by el Harith et al. (1989). Serum dilutions from 1 : 10 up to 1 : 10 240 with a diluent containing normal saline supplemented with foetal bovine serum (1 % v/v) and β-ME (1.56 % v/v) were performed in V-shaped microtitre plates. After incubation for a period of 1 h, the antigen was added. The test was read after a second incubation lasting 18 h at room temperature following similar criteria to those previously described by el Harith et al. (1986); To determine the ideal cut-off titre value for either the locally produced or reference antigen, a receiver-operating characteristics (ROC) curve was constructed with the MedCalc software program for Windows (version 12.7, 2013); accordingly, titres of 1 : 320 for the β-ME-Ag and 1 : 160 for the REF-Ag were determined as optimal.

**Study design and data analysis.** All 151 canine sera comprising groups G1, G2, G3 and G4 were tested against both β-ME-Ag and REF-Ag antigens in the DAT. The results were compared for sensitivity and specificity. Analysis of data was performed using MedCalc software, version 12.7 (2013). The sensitivity and specificity of the locally produced β-ME-Ag and the REF-Ag were estimated as percentages of the true (parasitologically confirmed) positives (G1, n = 31) and negatives (free of *Leishmania* amastigotes or having had final diagnosis of conditions other than CVL (G3 + G4, n = 82) with exact binomial 95 % confidence limits. Agglutination titres were transformed into simple categorical data (number of the twofold serial dilution showing the highest agglutination titre). The mean titre values obtained with each antigen were then compared using Student’s t-test at critical x-level of 0.05; all tests were two-tailed and any P-value ≤ 0.05 was considered to be significant. Concordance between titre values obtained with each antigen was determined using Cohen’s kappa coefficient (κ), where values <0.6 were considered as poor, 0.60–0.80 good and 0.81–0.99 optimal.

**RESULTS**

Unlike the REF-Ag antigen, which gave negative DAT titre readings of 1 : 80 in one sample, the indigenous β-ME-Ag scored positive results in all 31 serum samples belonging to group G1 (confirmed CVL) (Table 1). All sera collected
from the dog group with suspected CVL (G2) tested positive against β-ME-Ag, showing titres in the range 1:320 to $\geq 1:10240$. In this same group, seven sera revealed negative titres (1:20–1:80) with REF-Ag. In the group consisting of dogs diagnosed with diseases other than CVL (G3), all sera tested negative against both antigens. One sample in this group, from a dog with dirofilariasis, tested negative at a relatively higher titre (1:160) against β-ME-Ag. In this group, all titres obtained with REF-Ag were below 1:160. Without exception, all samples collected from the group that had a negative parasitological outcome (G4) tested negative against both antigens at a titre of $\leq 1:80$. Therefore, a sensitivity and specificity of 100% were estimated for β-ME-Ag and of 96.7% for REF-Ag. At their respective optimal cut-off values (1:320 and 1:160) both antigens showed 100% specificity.

With the exception of group G2, similar mean titre values were obtained with the two antigens for the other three groups. In this group with suspected CVL, β-ME-Ag demonstrated a higher mean titre value than REF-Ag due to the higher positive titre levels encountered in the individual samples tested (Fig. 1). Overall, the levels of titre obtained with the two antigens against sera collected from all four groups were highly concordant ($\kappa$=0.879).

**DISCUSSION**

This is, we believe, the first study in which performance of the recently improved aqueous β-ME-treated and reference trypsinized and lyophilized (REF-Ag) antigens has been compared for diagnosis of CVL. The 100% sensitivity obtained with the β-ME-treated antigen in this study is in very good agreement with that reported by el Harith et al. (1995) in human patients using the same test version.

The sensitivity of 96.7% obtained here with REF-Ag, although slightly lower than that obtained with β-ME-Ag, is still within the range reported by others. Sensitivity and specificity levels of 95.5% and 100%, 100% and 95.0%, and 95.1% and 96.5% for aqueous DAT trypsinized antigen were also published for southern Europe (Neogy et al., 1992; Vercammen et al., 1997) and northern Africa (Aoun et al., 2000). Using a freeze-dried version of the trypsinized antigen in the DAT and three times the cut-off value employed here for REF-Ag, Oskam et al. (1996) and Schallig et al. (2001) reported higher sensitivity and specificity levels of 100% and 97.8% or 98.8% in areas known to be endemic for *L. infantum* and *Leishmania chagasi*, respectively.

Less sensitive by comparison with the indigenous (*L. infantum*) β-ME-modified antigen, the trypsinized reference of *L. donovani* failed to detect one dog with genuine

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**Table 1.** Relative efficiency of β-ME-treated locally produced (β-ME-Ag) antigen in comparison with a commercial reference (REF-Ag) in DAT for diagnosis of CVL

<table>
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<tr>
<th>DAT cut-off</th>
<th>Sensitivity* (%) [95% CI]</th>
<th>Specificity† (%) [95% CI]</th>
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<tbody>
<tr>
<td></td>
<td>β-ME-Ag</td>
<td>REF-Ag</td>
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<tr>
<td>1:10</td>
<td>31 (100) [86.2–100]</td>
<td>31 (100) [86.2–100]</td>
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<td>1:20</td>
<td>31 (100) [86.2–100]</td>
<td>31 (100) [86.2–100]</td>
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<td>1:40</td>
<td>31 (100) [86.2–100]</td>
<td>31 (100) [86.2–100]</td>
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<td>1:80</td>
<td>31 (100) [86.2–100]</td>
<td>31 (100) [86.2–100]</td>
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<tr>
<td>1:160</td>
<td>31 (100) [86.2–100]</td>
<td>30 (96.7) [88.4–99.8]</td>
</tr>
<tr>
<td>1:320</td>
<td>31 (100) [86.2–100]</td>
<td>29 (93.5) [77.1–98.8]</td>
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*Sensitivity is defined as the number (percentage) of DAT-positive cases from 31 cases positive by lymph node aspiration.

†Specificity is defined as the number (percentage) of DAT-positive cases negative for CVL either by lymph node aspiration ($n=37$) or by final diagnosis of clinical conditions other than CVL ($n=45$).
CVL. Whether this slightly relatively low sensitivity is to be attributed to use of the heterologous *Leishmania* subspecies as antigen in the DAT is difficult to conclude based on this limited number of sera. Using an aqueous trypsinized antigen of *L. donovani*, Garecz *et al.* (1996) reported much lower sensitivity (71.4%) in Brazilian dogs with confirmed CVL. Terán-Angel *et al.* (2007) suggested that the lower sensitivity encountered with the DAT in identifying dogs with genuine CVL is most likely associated with the use of a different *Leishmania* species lacking particular antigen characteristics unique to the strain that is endemic in the area wherein the study was carried out. The favourable effect of using an indigenous or autochthonous strain of *Leishmania* as antigen in the DAT was unambiguously demonstrated by el Harith *et al.* (1995) in human VL patients residing in three different endemic areas. In that study, the authors reported a threefold increase in titre for VL sera that were tested against antigens processed from the indigenous *L. donovani* subspecies.

The β-ME-treated antigen showed titres indicative of CVL in all samples belonging to the group that had high suspicion of CVL (G2). Taking into consideration the clinical signs and symptoms typifying CVL presented by those dogs at the time of clinical examination and the level of titre (≥1:320) subsequently obtained in their sera we concluded that those animals had genuine CVL. Based on these observations and findings previously published by others concerning the favourable effect of using homologous antigens of *L. donovani* subspecies in addition to incorporating β-ME treatment in antigen processing on sensitivity of the DAT for diagnosing the disease in humans, we strongly believe that using similar antigens will also enhance CVL diagnosis. We also think that by both using homologous antigen and incorporating β-ME in antigen processing, DAT sensitivity can be significantly enhanced even for the detection of CVL at the early and subclinical phases.

No cross-reaction with any of the two antigens was observed against the 43 sera comprising group G3, implying a high level of specificity. It is, however, worth mentioning that unexpectedly low titres (1:10-1:160) were obtained with both β-ME-Ag and REF-Ag (1:20–1:80) against sera collected from dogs with *Trypanosoma cruzi* infection. Cross-reactions were, however, reported for the DAT trypsinized antigen against sera of dogs infected with *Dirofilaria* spp. or *Ehrlichia canis* (Ferreira *et al.*, 2007).

We believe that the observed improvement in the specificity of the locally produced antigen is most likely due to the favourable effect of β-ME treatment in unselectively cleaving promastigote surface epitopes, thereby increasing the ability of the parasite to react specifically with antibodies. Trypsin treatment, on the other hand, is known to be limited to cleaving peptides at the arginine-lysine bond.

It is important to mention in this connection that the group of dogs with confirmed diseases did not undergo lymph node aspiration to exclude the possibility of infection with *Leishmania*. Concomitant infections in *Leishmania*-infected dogs are plausible and this could affect the accuracy of the new test. However, considering the highly concordant results (κ=0.88) obtained here with the two antigens, we can conclude that their diagnostic performance was generally comparable for detection of clinically manifest CVL.

As recommended by other researchers, we also believe that detection of CVL at an early stage is of paramount importance as it permits prompt administration of therapy, therefore overcoming development of drug resistance and preventing onset of serious disease manifestation. Subsequently, the chances of further transmission to the human and canine population will also be reduced (Abranches *et al.*, 1991).

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