Serodiagnosis of canine visceral leishmaniasis in Portugal comparison of three methods.

Nurit Rachamim[1], P. Abranches[2], M.C.D. Silva-Pereira[2], Lionel F. Schnur[3], Raymond L. Jacobson[3] and Charles L. Jaffe[1*].

[1]Department of Biophysics and MacArthur Center for Molecular Biology of Tropical Diseases, Weizmann Institute of Science, Rehovot, Israel, [2]Disciplina de Protozoologia, Instituto de Higiene e Medicina Tropical Lisbon, Portugal, and [3]Department of Parasitology, Kuvin Center, Hebrew University-Hadassah Medical School, Jerusalem, Israel.

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Please send correspondence regarding manuscript to: Dr. Charles L. Jaffe, Dept. of Biophysics, Weizmann Institute of Science, Rehovot 76100 Israel. Tel. 972-8-343103 FAX. 972-8-468256.

*Address for reprint requests.

Abstract

Sera collected in Portugal from 43 dogs were screened for specific antibodies to Leishmania donovani antigens. Three different techniques were compared: an indirect immunofluorescence assay (IFA), a direct enzyme-linked immunosorbent assay (ELISA) and a competitive-ELISA (C-ELISA) using two species-specific monoclonal antibodies, D2 and D13. By IFA, 22 of the sera examined showed positive reactions, compared to 26 by ELISA or 27 by C-ELISA. There was no direct correlation ~~was observed~~ between the serum titre by IFA and the strength of reaction in ELISA or inhibition in C-ELISA. However, a good correlation was observed between sera identified as positive (95.5%) by all three techniques. Western blotting on leishmanial membranes showed that common antigens with M_r of 26,000 and 70 - 94,000 were recognized by all infected dog sera, regardless of the serum titre. In large scale studies, ELISAs are preferred to IFA for the rapid diagnosis of canine visceral leishmaniasis because of their greater simplicity.

INTRODUCTION

Leishmania donovani sensu lato is prevalent in many regions of the Old and New Worlds, where it causes fatal human visceral leishmaniasis (VL) (Peters and Killick-Kendrick, 1987). In man, the disease is typified by irregular fevers, immunosuppression, hepatosplenomegaly, hypergammaglobulinemia and anaemia. With the exception of a few well studied foci, relatively little is known about the epidemiology of this disease (Laison and Shaw, 1987). Dogs and wild canids have been considered important reservoir hosts ever since Nicolle and Comte (1908) first isolated L. d. infantum from dogs. The prevalence of canine VL in the Mediterranean countries varies from 1.1% to 37% (Bettini and Gradoni, 1986), with typical levels of infection ranging from 1 - 10%. Dogs play an important role in the ecology of human VL because they are in close contact with people, though there is no apparent direct correlation between the incidence of canine and human VL (Bettini and Gradoni, 1986; Ashford and Bettini, 1987).

Due to the long incubation time of this disease (Ashford and Bettini, 1987), parasites are found in tissue biopsies from only 40 - 50% of dogs with positive immunofluorescence titres (Lanotte et al., 1979; Pozio et al., 1981). The other seropositive dogs are a pool of asymtomatic carriers, of which 30 - 40% become active infections and 10% or more resolve spontaneously. Asymtomatic cases represent part of the disease reservoir.

Canine VL has been diagnosed by three methods: clinical examination, microscopical examination of tissue biopsy preparations and parasite cultures, and indirect immunofluorescence (IFA). Frequently, it proves difficult to compare the prevalence of infection by these methods (Ashford and Bettini, 1987). IFA, though widely used, involves titration of each serum and microscopical examination. This is tedious and tiring when numerous samples have to be examined in extensive epidemiological studies.

The metropolitan area of Lisbon (Portugal) comprises urban and rural regions of

human and canine VL (Abranches et al., 1983). The epidemiology in the rural region has been extensively studied (Abranches et al., 1987). All L. d. infantum isolates obtained from humans, dogs and foxes have belonged to zymodeme MON-1 (LON-49). Dog sera from this region were used to compare the efficacy of diagnosing canine VL by three different serodiagnostic methods: an indirect immunofluorescence assay (IFA), a direct enzyme-linked immunosorbent assay (ELISA) and a competitive-ELISA (C-ELISA, see Jaffe and McMahon-Pratt, 1987). The two ELISAs require only single dilution of serum samples that are analyzed directly in a microplate reader.

MATERIAL AND METHODS

Sera

Forty-three dog sera were collected in the Setubal Peninsula south of Lisbon (Abranches et al., 1983; Abranches et al., 1987). This is a rural area with an 8.8% incidence of canine VL (Abranches et al., 1987).

Serodiagnostic assays

IFA was carried out (Lanotte et al., 1975; Abranches, 1984), using promastigotes of a local L.d. infantum strain (MCAN/PT/81/IMT 82) as antigen. Serial dilutions of each dog serum were examined until a visual end point was reached. Direct ELISA and C-ELISA were carried out exactly as described by JAFFE et al. (1988), using antigen prepared from a virulent Sudanese L.donovani strain (MHOM/SN/00/Khartoum). A single dilution was used for the C-ELISA and ELISA, 1/100 and 1/1000, respectively.

Western blotting

Membranes isolated from L.donovani (Khartoum, 200 µg/gel) were separated by electrophoresis on 10% sodium dodecylsulfate polyacrylamide gels and transfered to nitrocellulose paper (0.45µm) (Jaffe and Zalis, 1988a). After blocking with 0.3% Tween-20 in phosphate buffered saline (T-PBS) for 1h, separate strips were incubated with each dog serum at 1/10,000 dilution in 0.05% T-PBS for 2h, then washed several times with 0.05% T-PBS and probed with horseradish peroxidase -

conjugated to Protein A at 1/2000 dilution in 0.5% T-PBS for 30 min. The reactions were developed with 3,3'-diaminobenzidine tetrahydrochloride at 0.5mg/ml in PBS.

RESULTS

The Table and Fig. 1 summarize and compare the results. The number of seropositive dogs found with each assay is very similar: 22 by IFA, 26 by ELISA and 27 by C-ELISA. Positive reactions are, by definition, titres $\geq$ 1/128 in IFA (Abranches, et al., 1983); ratio absorbance at 405nm positive / negative sample (P/N ratio) $\geq$ 3 for ELISA and inhibition of test serum by either L. donovani monoclonal antibody (mAb) binding to crude parasite antigen $\geq$ 30% for C-ELISA. No correlation was observed between the magnitude of serum reactions with antigen, when measured by the IFA and ELISA, or the percent inhibition of mAb binding to crude antigen in the C-ELISA. A strong positive in one assay was often a weak positive in either or both of the other assays. However, when sera were scored as positive or negative, a significant consensus (95% confidence level by the Fisher exact test) was found between all three assays. All of the 21 seropositive samples in both the IFA and ELISA were positive by the C-ELISA. This is clearly seen in Fig. 1 upper right quadrant (o is positive for C-ELISA). Of 16 seronegative samples by both the IFA and ELISA, 13 were also negative by C-ELISA (Fig.1 lower left quadrant, o is negative in C-ELISA).

Immune blotting on L.donovani membrane antigen was also carried out for 15 of the 43 sera. These sera were selected according to their IFA titres and are arranged in Fig. 2 according to increasing titre from left to right. None of the 5 sera with IFA titres $\leq$ 1/32 (lanes a - e) reacted. All the sera with titres $\geq$ 1/128 (lanes h - n) showed extensive reactivity with the promastigote membranes. Sera with IFA titres of 1/64 (lanes f and g) showed disparity in reactions (lane g was positive by ELISA and C-ELISA). Multiple membrane antigens from $\leq$ 26 kDa to $\geq$ 150 kDa were recognized by all the positive sera. However, antigens in two molecular

weight fractions, between 70,000 - 84,000 and a distinct band at 26,000, were recognized by the positive dog sera examined. These antigens appear to be highly antigenic, eliciting strong humoral responses even when the IFA titres were relatively low.

DISCUSSION

Cross-reactivity between leishmanial antigen and sera from infections caused by other trypanosomatids and organisms occurs with IFA and ELISA (Jahn and Diesfeld, 1983; Badaro et al., 1983; Jaffe and Zalis, 1988b; Gottstein et al., 1988), the C-ELISA was developed to specifically serodiagnoses human VL (Jaffe and McMahon-Pratt, 1987). In the C-ELISA, 90% of the human VL cases were correctly identified and no false positives were found among sera from patients with Chagas disease, leprosy, schistosomiasis, malaria, cutaneous and mucocutaneous leishmaniasis. The mAbs used in the C-ELISA react with epitopes on 70 kDa antigens of L. donovani. Bands with similar M_r are recognized by western blotting, using dog sera positive for VL (Fig. 2 and Gottstein et al., 1988). These antigens seem to be highly immunogenic and are recognized by positive sera from dogs that were parasitologically positive but with low IFA titres (1/64). In addition, antibodies to the pure protein antigens (dp72 and gp70-2) appear early following experimental infection with either promastigotes or amastigotes (Abranches et al., unpublished results). Reactions to either pure protein can be detected at least 1 month prior to the appearance of seropositivity by direct ELISA or IFA (Abranches et al., unpublished results). All of the samples showing as positive by both ELISA and IFA were also positive by C-ELISA, but 3 "false positives" were found by C-ELISA, compared to the other two tests. Unfortunately, these dogs were not biopsied for parasites and it is not possible to concluded that these "false positives" represent an early diagnosis of canine VL missed by IFA or ELISA. Three sera were also found to be 'positive' and one 'negative' by ELISA that were not confirmed by

the other two tests. These 'false positive' results from both types of ELISA test might indicate increased specificity with reduced sensitivity.

All the infected dog sera reacted with a 26,000 M_r band. This antigen, in addition to the pure proteins dp72 and gp70-2 recognized by mAb D13 and D2, could be used in developing a rapid assay for canine VL. The pure 70,000 M_r proteins have already been successfully used in the specific serodiagnosis of human VL (Jaffe and Zalis, 1988b).

The reason for the lack of correlation between serum IFA titres and strengths of ELISA reaction (P/N ratio) remains unclear. Studies on human VL have shown that differences between strains of L. donovani can affect the accuracy of serodiagnosis (Badaro, et al., 1986). Two different L. donovani antigens were use in the IFA and ELISA, a local Portuguese strain and Sudanese strain respectively, however a lack of correlation between IFA and ELISA has been noticed even when the same antigens were used (Gottstein et al., 1988).

Earlier studies (Abranches et al., 1983) in the Sebutal region described a bimodal distribution of anti-leishmanial IFA titres with 1/64 at the nadir between the positive and negative peaks. Titers $\geq$ 1/128 were arbitrarily chosen as signifying a positive response. Results obtained by both types of ELISAs clearly suggest that sera with IFA titers of 1/64 are not negative, but represent borderline values. Indeed 2/4 dogs were also found to be parasitologically positive following tissue biopsy and culturing (Abranches, et al., unpublished data). These sera should be re-examined by another method able to expose borderline positives that would be negative by the IFA.

Serodiagnosis of canine VL is valuable in epidemiological studies, especially for determining the latent frequency of disease in a population (Pozio et al., 1981; Bettini and Gradoni, 1986; Ashford and Bettini, 1987). IFA has been used more widely than any other technique to diagnose VL, however it is not efficient for analysing large numbers of samples. ELISAs are simple, rapid and have been adapted

for the diagnosis of many infectious diseases, including human VL (JAHN and DIESFELD, 1983). We have shown that an ELISA can be reliably used to diagnose canine VL in place of IFA. The ELISA gives the same qualitative diagnosis as the IFA without necessitating the titration of serum antibodies to the reactions on leishmanial antigen. Since the ELISA requires a single dilution and can be evaluated by a microplate reader, many samples can be simultaneously assayed. This makes the ELISA more rapid and simpler than IFA. In addition, the ELISA can be read qualitatively by eye.

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Legends to Figures.

Fig. 1. Correlation between three serodiagnostic assays for canine visceral leishmaniasis. Results from indirect fluorescence assay (IFA), direct enzyme-linked immune assay (ELISA) and competitive - ELISA are compared for 43 dog sera from Portugal. IFA - final fluorescent serum titre, titre > 1/64 is positive; ELISA - absorbance at 405 nm, P/N ratio > 3 is positive; C-ELISA - positive o or negative o, inhibition of monoclonal antibody binding > 30% is positive.

Fi. 2. Reaction by immune blotting of canine visceral leishmaniasis sera with Leishmania donovani membrane antigen. Membranes from a Sudanese L.donovani isolate (MHOM/SN/00/Khartoum) were separated by gel electrophoresis and transfered to nitrocellulose paper. Selected dog sera with increasing immunofluorescence titres (0 to 1/8192, left to right) were incubated with the paper strips. Dog serum samples (DS, reciprocal titres): lane a - DS1, 0; lane b - DS5, 0; lane c - DS10, 16; lane d - DS13, 16; lane e - DS16, 32; lane f - DS18, 64; lane g - DS21, 64; lane h - DS24, 128; lane i - DS29, 256; lane j - DS31, 512; lane k - DS34, 512; lane l - DS36, 1024; lane m - DS38, 2048; lane n - DS41, 4096 and lane o - DS43, 8192.

TABLE

DIAGNOSIS OF CANINE VISCERAL LEISHMANIASIS IN PORTUGAL

	ELISA	IFA	C-ELISA
Positive	26	22	27
Negative	18	21	16
False Positive*	3	0	3
False Negative	1	2	0

* If the individual serum test result does not concur with those found for the same serum in the remaining two assays it is considered either a false positive or false negative, respectively.

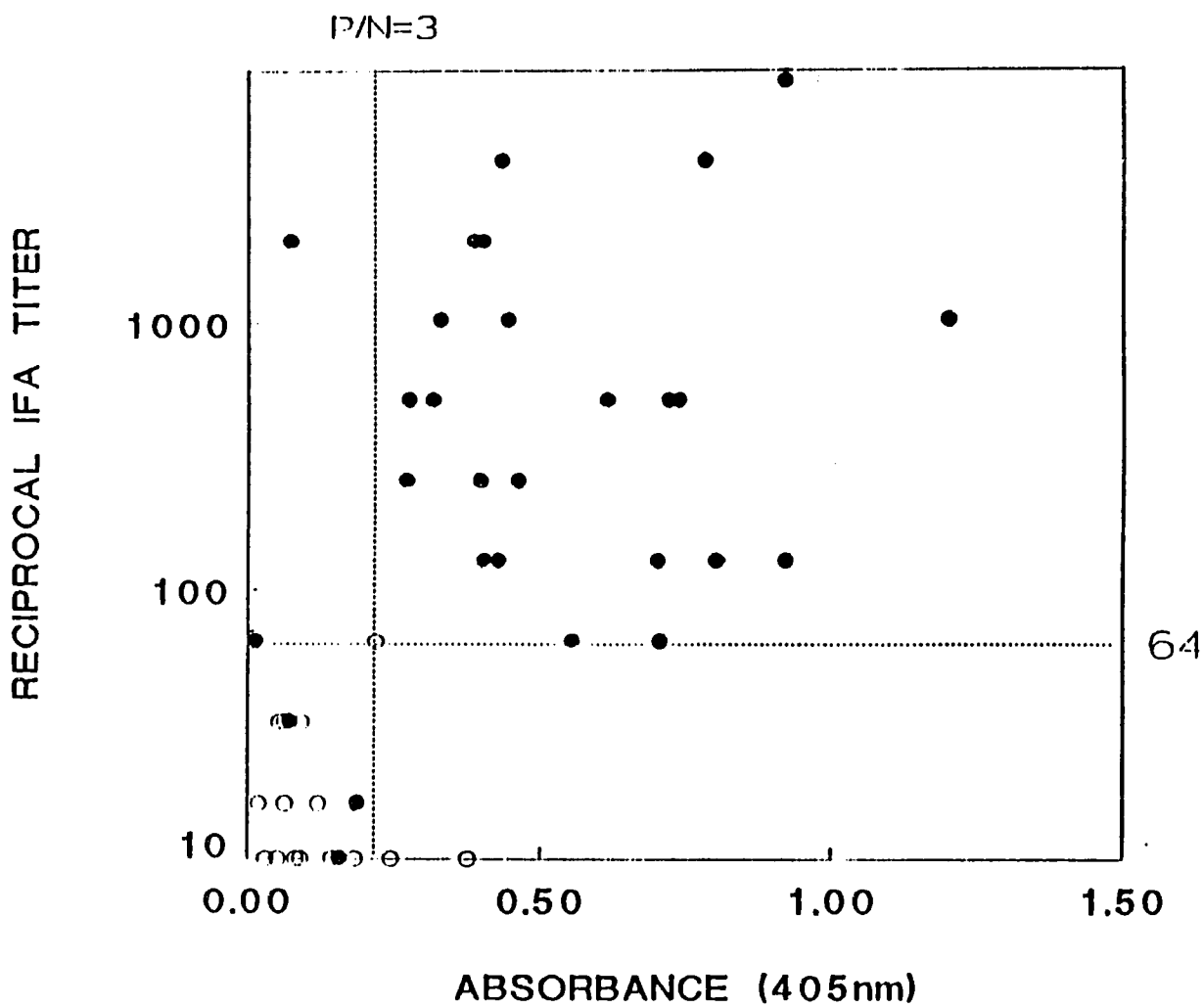
P/N=3
RECIPROCAL IFA TITER
1000
100
10
64
0.00
0.50
1.00
1.50
ABSORBANCE (405nm)

Fig. 1.

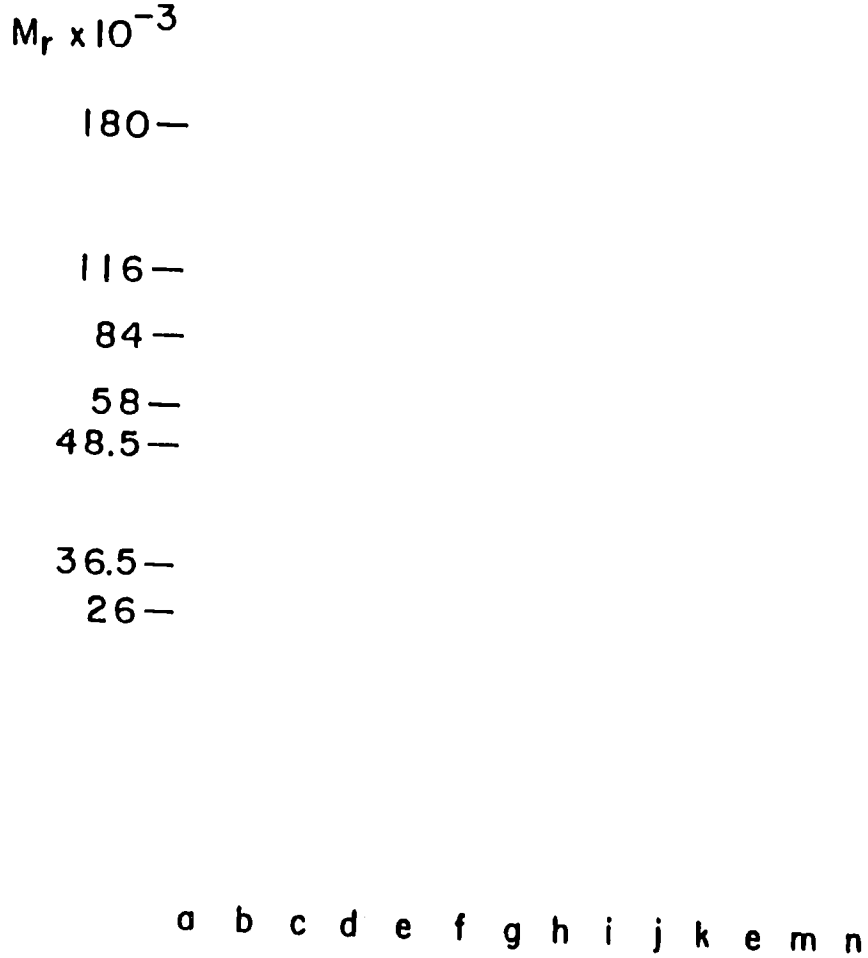

Fig 2