

Application of a Direct Agglutination Test for Detection of Specific Anti-*Leishmania* Antibodies in the Canine Reservoir

ABDALLAH EL HARITH,^{1*} ROB J. SLAPPENDEL,² INGRID REITER,³ FRANS VAN KNAPEN,⁴
PAULA DE KORTE,⁵ ELIZABETH HUIGEN,¹ AND AREND H. J. KOLK¹

N. H. Swellengrebel Laboratory of Tropical Hygiene, Royal Tropical Institute, Amsterdam,¹ Utrecht University Small Animal Clinic, Utrecht,² National Institute of Health and Environmental Protection, Bilthoven,⁴ and Laboratory of Parasitology, Institute for Tropical Medicine, University of Leiden, Leiden,⁵ The Netherlands, and Institute of Comparative Tropical Medicine and Parasitology, University of Munich, Munich, Federal Republic of Germany³

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A direct agglutination test (DAT) for detection of visceral leishmaniasis in humans has been developed. In this study, it was evaluated for applicability to detection of infections in dogs, a reservoir species. The reliability of the test was improved by treating the test sera with 0.2 M 2-mercaptoethanol and incubating them at 37°C. Sensitivity was 100% and specificity was 98.9% when the test was used on serum samples from 220 dogs, including 26 with parasitologically confirmed canine leishmaniasis, 12 with suspected but unconfirmed leishmaniasis, and 182 with other conditions. The DAT detected specific antibodies in 10 dogs with canine leishmaniasis diagnosed by case history, clinical signs of leishmaniasis, and seropositivity in an immunofluorescence test using either promastigotes or amastigotes, as well as in 2 dogs suspected of having leishmaniasis. The performance of an antigen prepared from a homologous isolate of *Leishmania infantum* in the DAT was compared with that of an antigen from a laboratory-adapted strain of *L. donovani* (sensu lato). The homologous antigen compared favorably with the standard antigen, and the results provided further evidence of the potential of the DAT for detection of *Leishmania* infection in the canine reservoir host. The results of this study, together with those of our previous studies in human visceral leishmaniasis, demonstrate that the DAT is highly suitable for wide-scale epidemiological and ecological field work. This technique could also facilitate diagnosis of leishmaniasis in dogs in veterinary health services.

A variety of vertebrate species, both wild and domestic, play a major role in the epidemiology of visceral leishmaniasis (VL) in many parts of the world (5, 7, 20, 21). Because of its cryptic form in canid and rodent reservoirs and the low sensitivity of the techniques used to detect infections in them (1, 24), the actual prevalence of VL in the Mediterranean littoral, Central Asia, Brazil, and East Africa may have been underestimated (2, 8, 13, 15, 24, 25). In canine hosts, extensive dermal infections and lack of a spontaneous cure provide optimal conditions for sandfly transmission (8, 20).

The occurrence of leishmaniasis in dogs indicates the possibility that human infections may have been overlooked. Sudden flare-ups of VL, or its appearance in a subclinical form, have sometimes been attributed to the presence of infected reservoir host species (13, 27). Perhaps because of conditions unfavorable to transmission and the lesser susceptibility of the final host, a high incidence of canine leishmaniasis has not been found to be correlated with that of VL in humans (18, 20, 24). In nonendemic areas, such as The Netherlands, the United Kingdom, and the Federal Republic of Germany, leishmaniasis has been imported by dogs which have been in Mediterranean areas and Portugal (14, 19, 24, 26).

For effective control of VL, simple, sensitive, and specific techniques for identification of *Leishmania* reservoir hosts are required. The clinical signs of leishmaniasis in dogs can easily be confused with those of other diseases, such as systemic lupus erythematosus, malignancies, lymphoproliferative diseases, and endocrine disorders. The conventional parasitological methods of diagnosis suffer from low sensitivity; their usefulness is limited by the possible scarcity of

parasites in the target organs, by contamination of cultures, and by postmortem decay of material collected under field conditions (13).

Among the serological techniques used to detect canine leishmaniasis, the immunofluorescence (IF) test has been recommended (2, 23, 24), but the anti-complement effect of canine sera renders the complement fixation test less reliable (24). Specific antibodies to the immunoglobulins of the various reservoir hosts are required for the enzyme-linked immunosorbent assay and the IF test. The simplicity and reliability of our previously reported direct agglutination test (DAT) for detection of specific antibodies in final hosts (10, 11) encouraged us to evaluate it for detection of *Leishmania* infections in canine reservoir hosts.

MATERIALS AND METHODS

DAT. Antigens of a laboratory-adapted *Leishmania donovani* sensu lato strain and of *L. infantum* LRC-L47 were prepared by improved DAT procedures (11). Following trypsin treatment and five washes in Locke solution (154 mM sodium chloride, 6 mM potassium chloride, 2 mM calcium chloride, 2 mM sodium bicarbonate), promastigotes were fixed with 2% (wt/vol) formaldehyde in Locke solution for 20 h. Excess formaldehyde was removed by two washes in cold (4°C) citrate-saline (0.15 M sodium chloride + 0.05 M sodium citrate, pH 7.4). Promastigotes were stained with Coomassie brilliant blue (0.1% [wt/vol]), and after two washes in citrate-saline they were resuspended at a final concentration of 7.5×10^7 /ml in 0.43% (wt/vol) formaldehyde in the same citrate-saline solution. The ready-for-use antigens were kept at +4°C until needed.

The DAT with canine sera was first performed as described for detection of human VL. It was observed that

* Corresponding author.

nonspecific agglutinating antibodies in canine sera were much less sensitive to 2-mercaptoethanol (2-ME) than were those in human sera under the same conditions (11) (addition of 0.1 M 2-ME before dilution and incubation at 21°C). To achieve satisfactory reduction of nonspecific agglutination, the canine sera were treated with 2-ME at 37°C for 1 h. The sera were tested in twofold serial dilutions (50 µl per well), starting at 1:10, in gelatin diluent (0.15 M sodium chloride and 0.2% [wt/vol] gelatin in distilled water) supplemented with 0.1 or 0.2 M 2-ME. After incubation, the antigen was added (50 µl per well); after further incubation at room temperature for 18 h, the DAT was read. Since the antigen suspension was supplemented with 0.43% (wt/vol) formaldehyde, there was no problem with bacterial proliferation in the antigen-serum mixture during the 18 h of incubation. The test is read by locating the first sharp button in the row which is identical with the one in the control well; the preceding dilution is then considered to be the highest positive dilution or the titer of the test serum. Because treatment with 0.2 M 2-ME resulted in higher specificity and greater ease in locating the endpoint negative button, this concentration was used in all further studies.

Promastigote IF. The promastigote IF test was performed by standard procedures routinely used at the National Institute of Health and Environmental Protection, Bilthoven, The Netherlands (26). Three human *Leishmania* isolates, one visceral (*L. donovani* sp.) isolate, and two cutaneous (*L. tropica* and *L. braziliensis*) isolates, in addition to a fourth isolate (probably *L. infantum*) from the bone marrow of a naturally infected dog, were used as antigens.

Promastigotes cultured in NNN medium (22) were washed three times in PBS (0.07 M phosphate buffer in 0.15 M NaCl, pH 7.2) and dispensed in demarcated circles (diameter, 4 to 5 mm) on glass slides at a concentration of 5×10^4 per circle. The antigen-coated slides were then air dried and fixed with absolute methanol for 10 min. Test sera (25 µl per circle) were added in twofold serial dilutions in PBS, from 1:5 to 1:1,280, and the slides were incubated for 1 h at 37°C. After three washes in PBS, bound immunoglobulin G antibodies were stained with fluorescein isothiocyanate-labeled anti-dog immunoglobulin G (heavy and light chains) conjugate (Nordic, Tilburg, The Netherlands), diluted 1:20 in PBS (25 µl per circle). Unbound conjugate was removed by three washes in PBS, and the preparation was counterstained with a 1:40 dilution of eriochrome black solution (Difco Laboratories, Detroit, Mich.) in PBS (25 µl per circle) for about 20 s. The slides were examined under a Leitz Orthoplan fluorescence microscope with epi-illumination and a filter combination for fluorescein isothiocyanate fluorescence at a magnification of $\times 250$. The IF titers were defined as the highest dilution in serum still displaying fluorescence detectable to the observer with reference to a negative control serum. The promastigotes showed body and flagellar fluorescence.

The cutoff titer in the above-described promastigote IF test was determined by earlier tests with four *Leishmania* antigens on 92 sera collected from 52 dogs with parasitologically confirmed leishmaniasis and 40 dogs with conditions other than leishmaniasis representing 35 breeds (26). On the basis of the results obtained, promastigote IF titers of $\geq 1:10$ could be considered indicative of canine leishmaniasis.

Amastigote IF. The amastigote IF technique is routinely performed for diagnosis of leishmaniasis at the Institute of Comparative Tropical Medicine and Parasitology, Munich, Federal Republic of Germany (23). Preparations of *L. donovani* Calcutta strain amastigotes were obtained by experi-

mental intraperitoneal infection of 8- to 10-week-old Syrian hamsters. The animals were killed 6 to 8 weeks postinfection, and their heavily parasitized spleens were removed; the parasite density was approximately 6+ on a World Health Organization scale (100 amastigotes per microscope field; magnification, $\times 40$ with an $\times 8$ objective in glycerin). Dab preparations of infected spleen tissue were prepared on IF slides (Hözel, Dorfen, Federal Republic of Germany). The slides were air dried overnight at approximately 20°C, fixed with absolute methanol for 5 min, and stored desiccated at -20°C until use.

Twofold serial dilutions of serum in PBS from 1:16 to 1:8,192 were transferred (25 µl per circle) to the antigen-coated slides and incubated in a moist chamber for 45 min at 37°C. After two washes in PBS, fluorescein isothiocyanate-labeled anti-dog immunoglobulin G (heavy and light chains) conjugate (Organon Teknika, Malvern, Pa.) diluted 1:20 in PBS was added (25 µl per circle). After removal of unbound conjugate by three washes in PBS, the air-dried slides were examined under a Leitz Orthoplan fluorescence microscope with epi-illumination and a filter combination for fluorescein isothiocyanate fluorescence at a magnification of $\times 250$. Dilutions in serum giving fluorescence on at least two-thirds of the amastigote population per view were considered to be positive. The amastigotes showed fluorescence covering the whole parasite.

The cutoff titer in this amastigote IF test was determined in previous studies by experimental infection (23). Seventeen beagle dogs (eight males and nine females) were infected with the *L. donovani* Calcutta strain and sacrificed 145 to 400 days postinfection. The dogs were examined for parasites intra vitam and postmortem, and development of anti-*Leishmania* antibodies was studied. In all infected dogs, *Leishmania* parasites were detected either by direct microscopical examination or by in vitro culturing of isolates. Of the 17, 16 had IF titers of $\geq 1:128$. Serum samples taken before infection or from control dogs had titers of $\leq 1:16$. In 200 sera collected from dogs with conditions other than leishmaniasis, including anemia, skin alterations, coughing, leucopenia, babisiosis, and fever, referred to the Veterinary Faculty in Munich, titers were never above 1:64. Accordingly, 1:64 was taken as the cutoff for canine leishmaniasis in this test.

Dog serum samples. Two hundred and twenty male and female dogs of various breeds and ages (5 months to 13 years) were tested. On the basis of case histories and clinical and parasitological findings, the sera from these dogs were grouped as follows. (i) There were 26 samples from dogs with parasitologically confirmed leishmaniasis. *Leishmania* parasites were identified either by direct microscopical examination of bone marrow and/or lymph node aspirates or by culturing of the isolates in NNN or Schneider medium (20, 22). Eighteen were dogs that had contracted leishmaniasis during a stay in one or more of the Mediterranean countries or Portugal, and eight were imported from the same endemic areas. The animals in this group presented one or more of the classical signs of leishmaniasis, including pronounced weight loss, lymphadenopathy, splenomegaly, renal failure, generalized skin lesions, especially around the eyes and on the dorsal aspect of the nose, excessive scaling of the epidermis, and diffuse hair loss. (ii) There were 12 samples from dogs with unconfirmed leishmaniasis. Of these 12 dogs imported from the Mediterranean region, 8 eventually manifested dermal and/or visceral symptoms resembling those of leishmaniasis. All eight dogs were amastigote IF positive, and three of them also had positive indirect hemagglutination

titers. Of the four dogs treated with sodium antimony gluconate (Pentostam) or meglumine antimonate (Glucantime), two showed visible improvement, but in the other two the lesions persisted. Instructions for treatment were issued to the veterinary clinics for the other four animals. Additional samples were collected from 4 of 12 dogs that had returned from a stay in the same endemic areas. One of these had a positive titer in the promastigote IF test, and its condition improved markedly after treatment; a second died shortly after treatment. Both dogs had clinical signs characteristic of canine leishmaniasis, although no *Leishmania* parasites could be demonstrated in their lymph node or bone marrow aspirates. The remaining two animals were not presented for a second clinical examination. (iii) There were 182 samples from other dogs, including six sera from healthy experimental dogs housed at the Department of Experimental Surgery, Faculty of Medicine, University of Amsterdam, Amsterdam, The Netherlands, and 176 samples from dogs of different sex, breed, and age presented at the Utrecht University Clinic for Small Animals, Utrecht, The Netherlands, because of various internal diseases. None of them had a history or clinical signs suggesting leishmaniasis. Most had never been in areas endemic for leishmaniasis.

With the exception of 22 samples, all 198 others were tested blindly with the DAT against the standard *L. donovani* sensu lato antigen. One hundred and twenty-seven samples, including 26 with canine leishmaniasis, were used to evaluate the performance of *L. infantum* antigen in comparison with the standard DAT antigen from *L. donovani* sensu lato. In addition, some of the positive sera from the confirmed and unconfirmed leishmaniasis cases were tested not only in the DAT but also for comparison in the promastigote (27 serum specimens) and amastigote (16 serum specimens) IF tests.

RESULTS

We first performed the DAT with the canine sera by using the improved procedure already described for detection of human VL (11). In contrast to results obtained with sera from VL-free people, the cross-reacting agglutinating antibodies in canine sera were not eliminated by treatment with 2-ME (data not shown). Addition of 0.2 M 2-ME to the canine sera and incubation of treated sera at 37°C for 1 h, however, resulted in a remarkable improvement in DAT specificity. Sensitivity and the titers of positive sera were unaffected (Fig. 1). If only parasitologically confirmed canine leishmaniasis cases were considered the optimal cutoff point would be a titer of 1:320; this would give 100% sensitivity and 98.9% specificity (Table 1).

The results obtained with homologous *L. infantum* antigen matched those of the standard *L. donovani* sensu lato. In all 26 confirmed cases, DAT titers were >1:320 with either antigen (Fig. 2). Of 89 dogs from the control group, 87 had titers of <1:160. All of the animals diagnosed on clinical grounds ($n = 10$) or suspected of having leishmaniasis ($n = 2$) were positive in the DAT with both antigens. Imported dogs ($n = 16$) with confirmed or unconfirmed leishmaniasis that were found to be amastigote IF positive were also positive in the DAT against *L. donovani* or *L. infantum* antigen (Table 2).

Titers obtained in the promastigote IF test with canine leishmaniasis sera against an *L. donovani* antigen were generally higher than those obtained with *L. tropica* or *L. braziliensis* (Table 3). While all 25 dogs with confirmed canine leishmaniasis and 2 dogs with unconfirmed canine

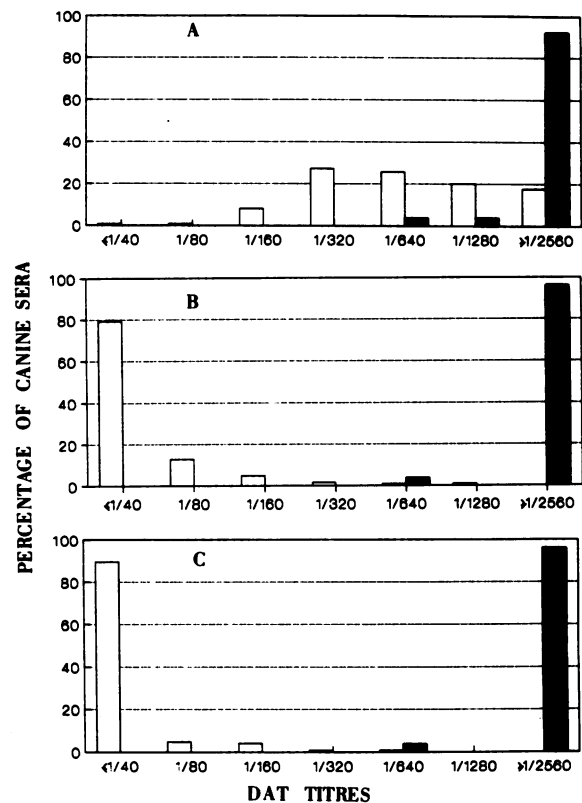


FIG. 1. Effect of 2-ME on DAT titers. One hundred and fifty-one serum specimens from 26 dogs with canine leishmaniasis (■) and 125 dogs with other conditions (□) were tested before (A) or after treatment with 2-ME at a concentration of 0.1 (B) or 0.2 (C) M.

leishmaniasis had positive titers ($\geq 1:320$) in the DAT, 13 of them gave results at or below the cutoff for the promastigote IF test (1:10), depending on the antigen used.

DISCUSSION

Epidemiological studies on the reservoir hosts for VL have been discouraged by the lack of sensitive and practical methods to detect infections in the various species. Negative parasitological results do not rule out *Leishmania* infection in dogs. Not uncommonly, a combination of clinical, parasitological, serological, and therapeutical tests has had to be done to confirm *Leishmania* infection in this reservoir host (1, 3, 13, 24, 26).

In the present study, the DAT was evaluated for detection of specific anti-*Leishmania* antibodies in natural canine leishmaniasis. With a recently modified DAT, a sensitivity of 100% and a specificity of 98.8% were obtained in a population which included not only patients with VL but also those with the closely related infections *Trypanosoma gambiense* and *T. cruzi* (4, 11, 12). This high reliability was reproduced in the findings reported here, with dogs with evident *Leishmania* infection (Table 1). The elevated titers ($>1:320$) obtained in 10 cases which had been diagnosed only on clinical grounds can be attributed with great certainty to *Leishmania* infection. All 10 dogs had been in endemic Mediterranean areas and manifested clinical signs typical of leishmaniasis. Their serological results in two different tests using three antigens of *L. donovani* (Tables 2 and 3) were indistinguishable from those of dogs with known *Leishmania*

TABLE 1. DAT results for sera of dogs with parasitologically confirmed or unconfirmed leishmaniasis and dogs with other conditions

Diagnosis	No. of dogs	No. with a DAT titer of:						
		≤1:40	1:80	1:160	1:320	1:640	1:1,280	≥1:2,560
Confirmed leishmaniasis	26	0	0	0	0	1	0	25
Clinically diagnosed (n = 10) or suspected leishmaniasis (n = 2)	12 ^a	0	0	0	0	3	0	9
Other conditions	182 ^b	169	6	5	1	1 ^c	0	0

^a Twelve dogs were imported from or had stayed in Mediterranean areas; nine had clinical signs of leishmaniasis and a positive amastigote IF test (n = 8) or promastigote IF test (n = 1).

^b One hundred and seventy-six serum samples were from dogs with various internal diseases, and six serum samples were from healthy experimental dogs (see Materials and Methods).

^c One dog had an ovarian cyst and ascites (permanently resided in The Netherlands).

infection. Moreover, three of six dogs put on specific anti-*Leishmania* chemotherapy showed pronounced improvement. On the basis of clinical history and positive DAT titers, *Leishmania* infection in the remaining two animals with suspected infections cannot be excluded.

The reports of failure to demonstrate parasites in canine leishmaniasis (2, 16, 17) are evidence of the considerable shortcomings of the available parasitological detection methods. Although lymph node aspiration is currently practiced for the diagnosis of VL in human patients because of its

relative safety, it is considered to be less sensitive than either bone marrow or spleen aspiration (R. A. Kager and P. H. Rees, Ph.D. thesis, University of Amsterdam, Amsterdam, The Netherlands, 1983). As a confirmatory procedure, bone marrow puncture is performed on dogs suspected of leishmaniasis but having negative lymph node aspirates (26). In the leishmaniasis cases examined here, parasites could not be detected in lymph node or bone marrow aspirates of two of the dogs, although clinical history, signs, and DAT results indicated infection. Similar findings have been reported in dog populations with prepatent leishmaniasis with positive IF test results (16, 17). Since organ aspiration was not performed on any of the other eight dogs with only a clinical diagnosis, no conclusion can be drawn as to whether those dogs had early leishmaniasis. However, application of chemotherapy was justified by the amastigote IF test results, which were later confirmed by positive titers in the DAT. The reliability of this IF technique has been demonstrated by comparison with the enzyme-linked immunosorbent assay and indirect hemagglutination assay (23). All 200 sera collected from dogs with conditions other than leishmaniasis had titers of <1:64, and the only cross-reaction found in the

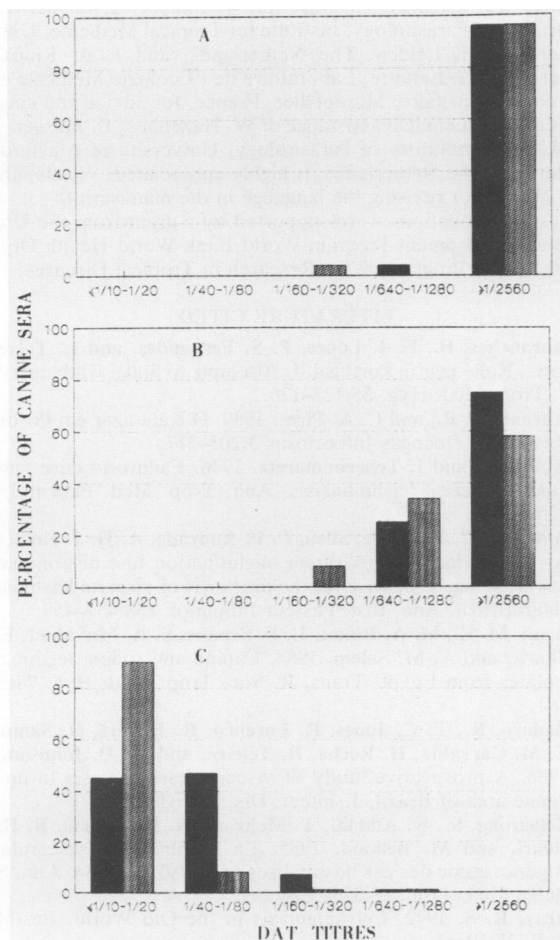


FIG. 2. DAT titers in 127 serum specimens from 26 dogs with parasitologically confirmed canine leishmaniasis (A), 12 dogs with unconfirmed canine leishmaniasis (B), and 89 dogs with other conditions (C) tested against antigens of *L. donovani* sensu lato (■) and *L. infantum* LRC-L47 (▨).

TABLE 2. Comparison of DAT and amastigote IF test results for confirmed and unconfirmed canine leishmaniasis

Diagnosis and dog no.	Reciprocal of antigen titer by DAT		Amastigote IF test reciprocal of antigen titer by (<i>L. donovani</i> Calcutta strain)
	<i>L. donovani</i> sensu lato	<i>L. infantum</i> LRC-L47	
Confirmed leishmaniasis^a			
16	>10,240	2,560	128
23	640	320	1,024
117	>10,240	>10,240	512
295	>10,240	5,120	1,024
426	>10,240	2,560	1,024
512	>10,240	5,120	512
522	>10,240	5,120	2,048
560	>10,240	>10,240	4,096
Unconfirmed leishmaniasis^b			
35	>10,240	>10,240	2,048
41	>10,240	>10,240	1,024
128	640	320	1,024
300	>10,240	>10,240	2,048
355	10,240	1,280	2,048
375	>10,240	>10,240	8,192
506	5,120	1,280	512
521	>10,240	>10,240	256

^a Parasitologically confirmed leishmaniasis.

^b Diagnosed by clinical signs and positive amastigote IF tests.

TABLE 3. Comparison of DAT and promastigote IF results for confirmed and unconfirmed canine leishmaniasis

Dog no.	Reciprocal of titer to <i>L. infantum</i> LRC-L47 antigen by DAT ^{a,b}	Reciprocal of antigen titer by promastigote IF ^b			Dog isolate ^c
		<i>L. donovani</i> sp.	<i>L. tropica</i>	<i>L. braziliensis</i>	
16	2,560	20	20	20	10
27 ^d	>10,240	80	20	80	80
136	5,120	40	20	40	20
146	>10,240	160	20	40	80
199	>10,240	40	10	10	80
200	>10,240	20	10	<10	40
204	>10,240	40	20	20	10
206	>10,240	40	10	<10	10
211	>10,240	40	20	10	20
212	>10,240	20	<10	<10	10
214 ^d	>10,240	20	10	<10	20
215	>10,240	80	20	20	40
522	5,120	40	40	40	40
560	>10,240	80	10	20	40

^a All dogs tested had reciprocal titers of >10,240 in response to *L. donovani* sensu lato antigen.

^b Thirteen dogs had DAT titers of 1:320 to >1:10,240 against the *L. donovani* sensu lato or *L. infantum* LRC-L47 antigen or IF titers of ≤1:10 against the *L. donovani* sp., *L. tropica*, or *L. braziliensis* antigen or the dog isolate antigen.

^c Probably *L. infantum*.

^d Dog with clinical history and signs of leishmaniasis.

amastigote IF was with sera from dogs infected with *T. congolense*.

In a previous study (9), a similar promastigote IF provided better sensitivity in human VL than that obtained here with canine leishmaniasis. For diagnosis of South American VL with an IF test, an antigen prepared from *L. chagasi* promastigotes was reported to give higher sensitivity and specificity than the amastigote antigen of the same species. However, for detection of the low humoral response in South American (muco)cutaneous leishmaniasis, the IF results were much better with an amastigote antigen of *L. braziliensis* than with a promastigote antigen of the same species (28).

Even if it were true that IF techniques in general are more reliable than other serological techniques, their application requires a high level of skill and experience. This fact and the need for complex equipment and expensive antisera to immunoglobulins of the various potential reservoir species constitute limitations to its use in epidemiological studies of leishmaniasis.

Because of easy access to sandfly microhabitats and close contact with both potential wild reservoir species and humans, dogs can be an ideal biological monitor for assessing the real endemicity of VL. To some extent, the intensive VL transmission in Southeast Asia, indicated by the exposed lesions in post-kala-azar dermal leishmaniasis, is comparable to that associated with the canine form in the Mediterranean littoral and Brazil. Zoonotic transmission of VL in the latter two areas is clearly reflected in the percentages of infected children and dogs (2, 6, 7). Measures for VL control in these endemic areas could include eradication of *Leishmania*-infected dogs (29). However, this approach would meet emotional and ethical resistance. On the other hand, the drugs currently in use for human VL are not effective for treatment of evident canine leishmaniasis (3); resistance and relapses are common, and dogs harboring infections for more than about 2 years cannot be cured (3, 20, 26).

Therefore, early detection of leishmaniasis in dogs, followed by prompt therapy, would be most appropriate. The very high sensitivity observed here, as well as that in our previous studies on sera of ex-VL patients collected 2 to 3 years after parasitological cure (9, 11, 12), demonstrate the potential of the DAT for detection of low antibody levels in early or cryptic canine leishmaniasis. The practicability and ease of performance permit wide-scale application in epidemiological and ecological studies of VL, including both reservoir and final hosts. Among the merits of the DAT are the stability of the antigen under tropical temperatures and the extremely low costs involved (11). It will be essential, however, to evaluate this DAT for applicability and usefulness in endemic areas as a practical field tool for detection of leishmaniasis in reservoir animals. Furthermore, modification of the DAT for rapid reading and the use of simple devices other than regular volumetric pipettes seem necessary to cope with the situation in the field and in moderately equipped laboratories.

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