Reliability of Serological Methods for Detection of Leishmaniasis in Portuguese Domestic and Wild Reservoirs

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A direct agglutination test (DAT) and an immunofluorescence technique (IFAT) were compared for detection of Leishmania infantum *infection in 43 dogs and five foxes from Alto-Douro and Arrábida, two known endemic areas in Portugal. In four dogs with proved canine leishmaniasis, both DAT and IFAT showed positive readings (titres ³1:320 and ³1:128). Of 34 samples collected from apparently healthy dogs, ten were positive by both serological tests and eight were serologically positive by one test or the other. Three foxes out of five captured in this area, scored titres indicative of leishmaniasis in both DAT and IFAT. The concordance between DAT and IFAT in all collected samples (48) was 81.25%. Considering these and previous studies in the adjacent Mediterranean areas, the seroprevalence of* L. infantum *infection in the canine and vulpine populations appear to be of high magnitude.*

Key words: canine - vulpine - leishmaniasis - direct agglutination test - immunofluorescence technique

The zoonotic nature of leishmaniasis in the south of Europe has initiated intensive epidemiological and ecological studies aiming towards control of the disease in this area (Lanotte et al. 1974, Reiter et al. 1985). In Portugal, besides the domestic dog, the fox was identified as an important wild reservoir for *Leishmania infantum* (Abranches et al. 1984). The cryptic nature of infections caused by this *Leishmania* species made it rather difficult to determine the exact level of endemicity (Macnemar 1979). Estimations based on parasitological findings only, lead to underemphasis of *L. infantum* infections in the canine and vulpine populations (Rioux et al. 1971). Seroprevalence surveys using the immunofluorescence test, showed higher degree of transmission in the south of France (Lanotte et al. 1979). Being readily detectable after onset of infection, antibodies to *L. infantum* therefore, seem to be more sensitive parameters for monitoring of infections (Lanotte et al. 1974, Abranches et al. 1987). Epidemiological studies using other serological techniques including the counter-immunoelectrophoresis (CIEP) (Dieng 1985), latex agglutination (Dereure 1986), the enzyme-linked immunosorbent assay (ELISA) (Binhazim et al. 1993) and the direct agglutination test (DAT) (De Korte et al. 1990, Semião-Santos et al. 1995) revealed comparable high levels of endemicity.

In this study the performance of DAT is further evaluated on the canine and vulpine hosts against the parasitological and clinical findings and results obtained by the immunofluorescence technique.

MATERIALS AND METHODS

Forty eight serum samples were analyzed in this study: 43 collected from dogs examined during a field visit to Alto-Douro and Arrábida and five from foxes captured in/or around the second area.

Immunofluorescence technique (IFAT) was performed according to the original method reported by Quilici et al. (1968) and modifications introduced by Lanotte et al. (1974) and Abranches et al. (1984). Promastigotes of *L. infantum* [zymodeme MON-1 (Lon.-49)] isolated from a dog were employed for antigen preparation. Parasites were fixed with acetone on IFAT-slides and dog or fox sera were tested at start dilution of 1:8 using FITC labelled anti-Dog IgG (H+L) (Immuno Biological ICN, Lisle, Israel). Positive IFAT titres were determined as the highest serum dilutions show-

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ing whole fluorescence detectable to the microscopist. On ground of previous results (Abranches et al. 1984), a titre of 1:128 was considered indicative of *Leishmania* infection in the canine or vulpine hosts.

Antigen for DAT performance was prepared at the Department of Medical Microbiology, University of Amsterdam, The Netherlands, according to the standard procedures later improved (Harith et al. 1988). The test was performed following the modified version for the canine reservoir (Harith et al. 1989). According to previous studies on evaluation of DAT for the canine reservoir (Harith et al. 1989, De Korte et al. 1990, Semião-Santos et al. 1995), 1:320 titre was taken as a cut-off for *L. infantum* infection in the canine and vulpine hosts.

Clinical examination of the studied population was carried out whenever possible. Absence of signs or symptoms resembling *Leishmania* infection were considered clinically negative. Dogs presenting all or some signs or symptoms characteristic of the disease (ex lymph node enlargement, skin involvement, abnormal locomotion) were considered clinically positive.

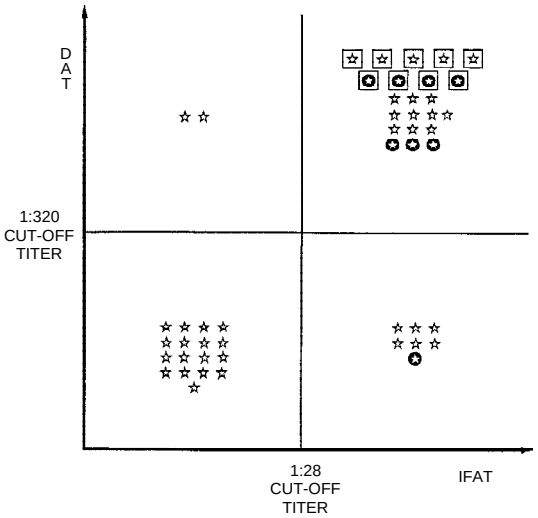
All dogs showing clinical signs or symptoms referable to *Leishmania* infection were submitted to parasitological examination; aspirated tissue from popliteal lymph nodes was obtained by injecting a small quantity of sterile Locke's solution into the nodule. After aspiration the content of the syringe was smeared on to a glass slide, fixed with methanol and stained with Giemsa for microscopical examination according to the technique described by Lanotte et al. (1974) and Abranches et al. (1991). Foxes were subjected to histopathological examination. In one of the foxes, bone-marrow aspiration was performed and aspirate immediately inoculated in NNN in order to observe whether later, *Leishmania* promastigotes could be demonstrated.

A Mcnemar's statistical test (Macnemar 1979) was applied to compare the results of matched sera in the IFAT and DAT tests.

RESULTS

Of the canine (43) and vulpine (5) sera tested, 22 (46%) scored positive results in DAT (≥ 1:320) and IFAT (≥1:128) (Fig). Negative DAT (≤ 1:160) and IFAT (≤ 1:64) titres, were obtained in 17 (35%) canine samples. In 6 of the canine samples tested, weak positive titres were obtained with IFAT (1:128) and negative ones with DAT (≤ 1:160) and in 2 others, the DAT was positive and IFAT negative.

Out of 19 dogs found positive in both tests, 9 had symptoms suggestive of leishmaniasis. In 4 of those 9 dogs *Leishmania* amastigotes were found in lymph node aspirates. The other 5 were parasitologically negative. The remaining 10, were parasitologically and clinically negative. Of the 5 foxes tested, 3 were positive in both serological techniques and in these *Leishmania* amastigotes were found despite absence of clinical symptoms. One fox was negative in all tests and another was negative in DAT but positive in IFAT. Histopathological examination of the liver section in this fox showed no amastigote but a granuloma forming. After inoculation of bone-marrow aspirate from this fox in NNN, *Leishmania* promastigotes were demonstrated. The concordance between the two tests in all canine and vulpine samples studied was significant being 81.25% in both negative and positive samples. The direction of change in the signal according to the Mcnemar's statistical test was the same in both tests (p = 0.41).



Results obtained by direct agglutination test (DAT) and immunofluorescence test (IFAT) in ☆ 43 dogs and 5 foxes, ✪ parasitologically confirmed and □ diagnosed on clinical grounds.

DISCUSSION

Previous results obtained in a Kenyan population including active and treated VL cases and endemic controls, showed good concordance (80%) of DAT and IFAT (Harith et al. 1987). Following further modifications in DAT procedures, sensitivity of DAT was significantly improved to monitor low antibody levels and prepatent infections in the human and the reservoir host (Harith et al. 1988, 1989).

Although the population studied here was rather small, the results obtained indicated a concordance

of 81.25% between DAT and IFAT. Recent studies in Central Tunisia (Ben Said et al. 1992) and in the south of Portugal (Semião-Santos et al. 1995) revealed even higher concordance between DAT and IFAT, of respectively 94.04% and 99.62% when evaluated in seroepidemiological studies aimed at local dog populations. In the study reported here, all four parasitologically confirmed dogs with leishmaniasis were positive in both tests. The same holds for the other five dogs with typical clinical symptoms of the disease. The positive readings obtained by both tests in ten apparently healthy dogs may indicate subclinical or prepatent *Leishmania* infection. This phenomena was earlier pointed out by other scientists (Lanotte et al. 1979, Abranches et al. 1991). A comparatively high prevalence rate has been reported in a dog population in the south of France (De Korte et al. 1990). In another study, 12 out of 38 dogs with negative parasitological findings showed a positive response to anti-*Leishmania* chemotherapy (Harith et al. 1989). IFAT positivity in six of the cases was of a minimum value (titre 1:128) where DAT showed a borderline titre (1:40-1:160) in the vicinity of the cut-off.

The demonstration of *Leishmania* parasites in one of the foxes with negative DAT titres could possibly be due to the use of *L. donovani* as antigen instead of homologous or authochtonous *L. infantum*. A recent study (Harith et al. 1995) showed that by incorporating the reducing agent 2-mercaptoethanol (2-ME) instead of trypsin when processing DAT antigen and when simultaneously combined with the use of an indigenous strain in the process, both sensitivity and specificity levels are increased in human as well as in canine sera. Nevertheless, one should not eliminate the possibility of deteoration of serum or the fact of being in presence of one of the few "false positives" as demonstrated in an earlier study (Zijlstra et al. 1991).

Although presence of granulomas was observed in *Leishmania* infection (Abranches personnal communication) its presence in this study, in one fox, does not exclude infection due to other pathogens. To differentiate between *Leishmania* and other pathogens such as *Hepatozoon canis*, which is also prevalent in domestic and wild canids in this area (Conceição-Silva et al. 1988), more specific techniques are required. As reported in previous studies using DAT and IFAT, significant positive results were obtained in 16 dogs having early or prepatent leishmaniasis (Harith et al. 1989, De Korte et al. 1990, Semião-Santos et al. 1995). This again emphasizes the need to incorporate reliable serological techniques in routine surveillance and control of leishmaniasis in the south of Europe. However, the need for anti-immunoglobulins specific to incriminated reservoirs constitute difficulties in applying IFAT and similar techniques for regular epidemiological surveys.

The importance of the fox as wild reservoir for *Leishmania* in the Mediterranean region has not yet been throughly evaluated. Both the domestic and wild reservoir should be considered for proper control of leishmaniasis in the Mediterranean areas. The steadily growing tourist traffic in this area and the increase in the number of acquired immunodeficiency sindrome (AIDS) cases necessitate broader approach towards transmission of infections due to *L. infantum*.

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