*Epidemiol. Infect.* (1993), **103**, 647–656



# Serological diagnosis of leishmaniasis: on detecting infection as well as disease

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(*Accepted 13 January 1993*)

## SUMMARY

Serological tests are very frequently used in epidemiological surveys of leishmaniasis and other parasitoses. Their sensitivity and specificity are generally defined with respect to parasitism and disease, rather than infection. The reason is that known positives are those individuals most likely to yield parasites, or who have distinctive clinical signs, and concomitantly high antibody titres. This paper investigates the performance of one serological method, the indirect fluorescent antibody test (IFAT), in detecting *Leishmania infantum* infection during an intensive 2-year cohort study of dogs in southern France. The results show that sensitivity and specificity with respect to infection can be simultaneously high, but maximum sensitivity is probably $< 80\%$, and lasts for a relatively short period of 2–3 months after a lengthy incubation period. The IFAT gave the incidence of infection as 18–65% in the first year, whereas the best estimate of incidence based on parasite isolation and clinical observation was 72%. But data from the second year suggest that the 72% was itself an underestimate. We argue that, during epidemiological surveys, the IFAT in particular, and serological tests for leishmania in general, will underestimate prevalence, incidence and hence the scale of the control problem. However, there is evidence that tests for canine leishmaniasis employing high threshold titres will identify the most infectious animals, allowing selective treatment or culling of those which contribute disproportionately to transmission.

## INTRODUCTION

Incidence, prevalence and basic case reproduction number, $R_0$, are fundamental measures of the scale of any infectious disease control problem. We have recently attempted to obtain all three from data collected during a cross-sectional survey of canine leishmaniasis in the Maltese islands [1, 2]. The greatest obstacle to doing so was the interpretation of serological data.

The use of serological tests in epidemiological surveys of parasitic infections is often rightly preceded by a study of sensitivity (proportion of true positives

detected) and specificity (proportion of true negatives detected). The usual study design compares the distribution of antibody titres in known positives, from whom parasites have been isolated or who have distinctive clinical signs, with titres found in a sample of non-endemic or endemic controls. The former are certainly uninfected; the latter are individuals who have been found parasitologically or clinically negative, usually after a single examination. Some examples of this approach applied to both canine and human visceral leishmaniasis, among the very many which have been published, are in the references [3–14]. It has often been concluded, particularly when using non-endemic controls, that it is possible to choose a cut-off point (threshold titre) giving sensitivity and specificity close to 100%.

Sensitivity and specificity obtained by this method are, broadly speaking, defined with respect to disease, and not with respect to infection. The distinction is underlined by the fact that antibody titres obtained from populations of known positives and non-endemic controls are often bimodally distributed [e.g. 11], whereas titre distributions obtained from natural populations in which disease is endemic are commonly unimodal [1, 4, 8, 12]. Where bimodal distributions of titres are found in field samples, the peaks corresponding to positives and negatives are never completely separated [15–17]. The shallow trough between peaks has been interpreted as the highest titre given by true negatives [15, 17], but could in fact contain a substantial number of infected individuals.

There are two ways in which all these observations can be consistent. First, titre takes a significant and/or variable period of time to rise following the establishment of infection (parasitism which leads sooner or later to seroconversion is the definition of 'infection' here), but becomes distinctively large by the time clinical illness has developed, or by the time parasitological isolation is possible. Second, a substantial fraction of individuals in the population never develop high titres in response to infection, and never become clinically ill, or never yield parasites. (The rest, of course, do.) Either way, cut-off points chosen to separate positives from negatives by the method described above will lead to a test which has good specificity but poor sensitivity with respect to infection.

In this paper, we focus on one serological method, the indirect fluorescent antibody test (IFAT). To find out how good the test is we would ideally compare its performance in two groups of animals, one known to have established infections, and the other known not to be infected. The perfect experiment would be carried out with a laboratory dog population, some individuals of which are infected by the bite of the appropriate species of phlebotomine sandfly. Laboratory cohort studies are, however, constrained by small sample sizes. This applies primarily to dogs, though rearing and infecting large numbers of sandflies is not a trivial technical problem.

Larger sample sizes are possible in field experiments with naturally infected dogs. However, because the act of infection cannot be observed, such experiments cannot guarantee detecting all instances of it. It will be possible to argue that few infections are missed only by virtue of the sheer intensity and variety of observations made. Here we attempt to make that argument with reference to the recent 2-year, longitudinal study by Vidor and colleagues [18], carried out with a cohort of 50 dogs in the Cévennes region of France. Because sera were collected

monthly for 2 years from animals under detailed clinical observation, and efforts were made to isolate parasites from lymph nodes, bone marrow and skin (including cutaneous lesions), we believe this field study comes closer than any previous one to providing a 'gold standard' for infection.

We use the data to answer the following particular questions: are sensitivity and specificity ever simultaneously high? If so, how high and at what threshold titre? What period is required for them to become so, and for how long do they remain so? Is antibody titre associated with the success of parasite isolations, and with the presence of cutaneous lesions? How consistent are these data with other published results, and what is their relevance for visceral leishmaniasis control?

## METHODS

Details can be found in Vidor and co-workers [18]. Briefly, 50 uninfected male beagles aged 6 months were introduced in May 1989 to kennels situated at Taleyrac in the heart of the Cévennes focus of visceral leishmaniasis. The parasite here is *Leishmania infantum*, and transmission by the principal vector, *Phlebotomus ariasi*, occurs from late May to early September, but is usually concentrated in July [19–21].

Each month until May 1991 (except September 1989, and August and October 1990), each dog was examined for cutaneous chancres, and for the other well-known clinical signs associated with leishmaniasis – enlarged lymph nodes, onychogryphosis, depilation, amyotrophy, keratoconjunctivitis, eczema, furfuraceous dermatitis and weight loss. Attempts were made to isolate parasites in culture (NNN or HOSMEM medium) from samples taken more or less randomly on 162 occasions beginning in February 1990, an average of 3·24 (range 0–9) per dog. Of these samples, 48 were aspirates from popliteal lymph nodes, 87 were aspirates from bone marrow, and 27 were biopsies of chancres. Dogs found positive by any of these methods constituted the reference, infected population.

IFAT on monthly serum samples were performed against *L. infantum* promastigotes (zymodeme MON1, ref. MCAN/FR/73/LEM1425), with FITC-conjugated anti-dog IgG. Serial twofold dilutions were made starting at either 1:20 or at 1:25. Thresholds titres for negatives were defined as $< 40$ or $< 80$. When considering the frequency distribution of titre in the entire population we have combined together titres of 20/25, 40/50 and 80/100, and indicated titre with the higher value of each pair. The sensitivity of the IFAT is defined as the fraction of all infected animals found to be seropositive. Specificity is the fraction of all uninfected animals found to be seronegative.

## RESULTS

Thirty-six of 50 dogs apparently acquired infection during the first transmission season. Thirty-four of these were animals which yielded parasites in culture (22 dogs) and/or developed leishmanial chancres (30 dogs). One of the remaining two died of leishmaniasis in January 1990, with severe amyotrophy and onychogryphosis. The other dog died in September 1991, also with typical signs of leishmaniasis; having first become ill in June 1990, it is possible, but unlikely, that

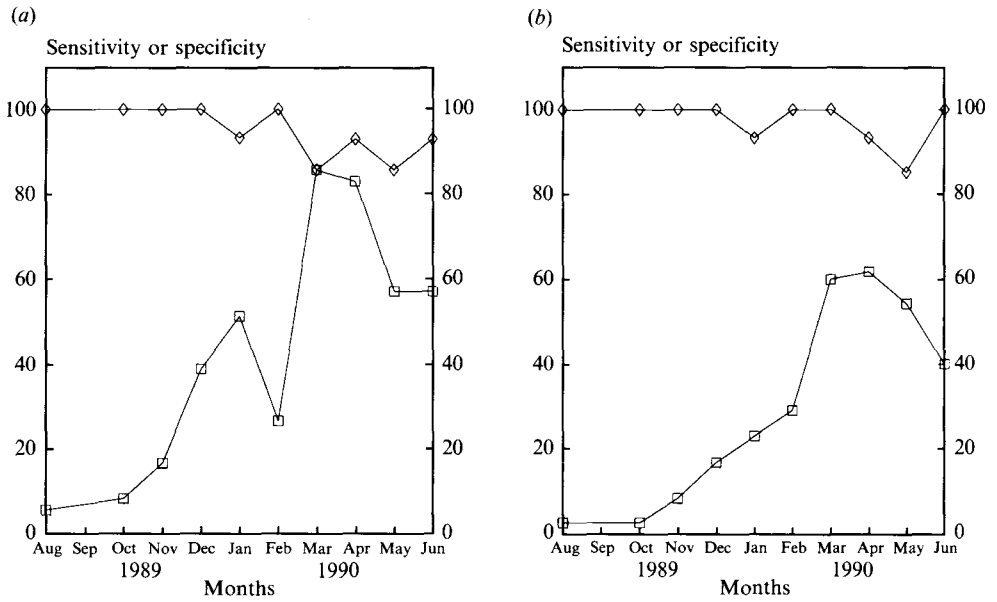


Fig. 1. Sensitivity (squares) and specificity (diamonds) of the IFAT from the end of the transmission season in August 1989, to the beginning of the transmission season in June 1990. Uninfected dogs were assumed to be those with titres of (*a*) $< 40$ and (*b*) $< 80$.

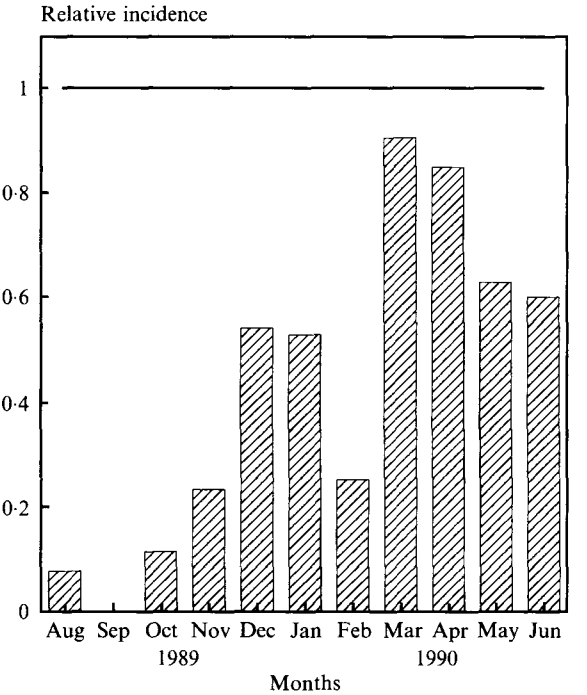


Fig. 2. The ability of the IFAT to estimate true incidence after the first transmission season. Relative incidence is incidence estimated from the lower of the two threshold titres divided by incidence estimated from clinical condition and parasitological isolation.

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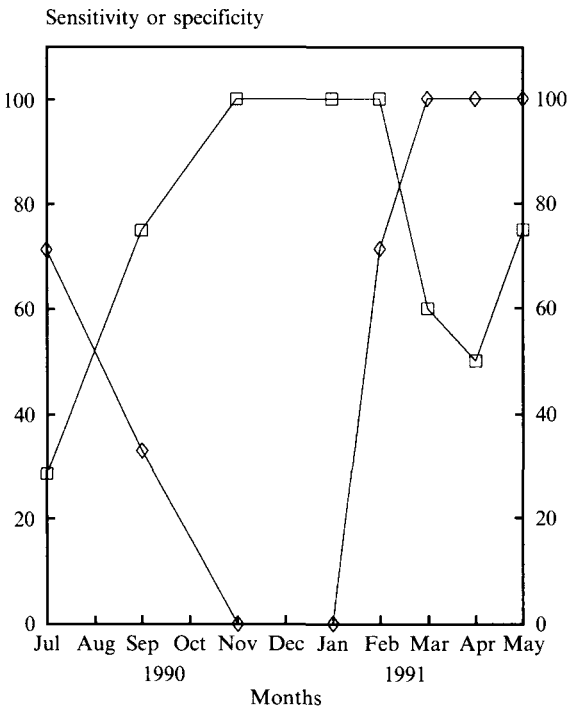


Fig. 3. As Fig. 1, but for those dogs which were supposedly uninfected during the first transmission season.

it became infected during the second transmission season. We assume that it acquired infection during the first season.

Monthly changes in sensitivity and specificity from August 1989 to June 1990 are shown in Figure 1. In Figure 1*a*, all those with titres $< 40$ are assumed negative, and in Figure 1*b* all those with titres $< 80$ are assumed negative. Both parts of Figure 1 reveal that, whilst specificity was always high with these threshold titres, sensitivity rose slowly after the end of the 1989 transmission season, taking 8–9 months to reach a peak in March and April 1990. That peak marks a high sensitivity only for the lower threshold titre (86% and 83% in March and April respectively, Fig. 1*a*), and only for that brief period of 2 months.

In Figure 2 we show how well, taking the lower threshold titre, the IFAT estimated the 'true' incidence of infection. Here 'relative incidence' is calculated as the number of seropositives divided by the number presumed infected. As expected, it provided close estimates in March and April 1990, but not at any other time. Our estimate of the true incidence during the first transmission season was 72% per year, and our best estimate obtained by IFAT in March 1990 was 65% per year.

Figure 3 plots sensitivity and specificity for the 14 dogs which supposedly escaped infection in 1989. Seven of these were identified as reference positives because they yielded parasites (4), developed chancres (4), or had other clinical signs (5). The pattern is quite different from that shown in Figure 1. Sensitivity increases significantly earlier, and sensitivity and specificity are never simultaneously high.

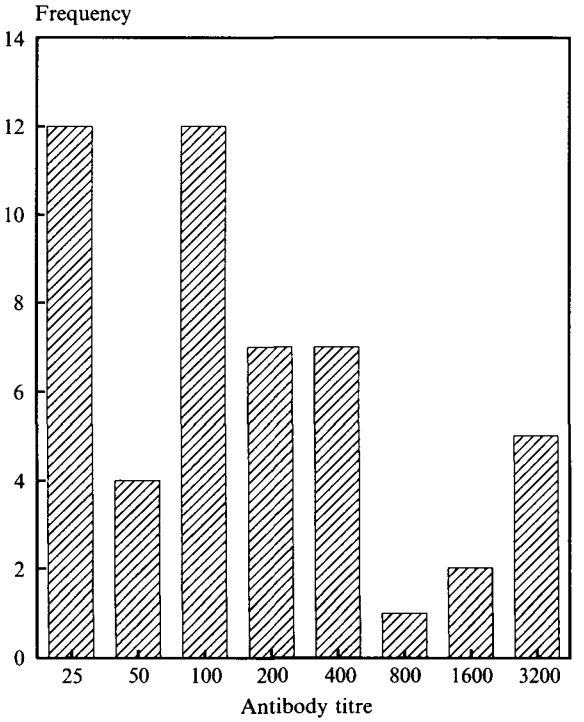


Fig. 4. Frequency distribution of antibody titres obtained using, for each dog, the highest ever titre recorded between the two transmission seasons. Limiting dilutions are given on the $x$-axis.

Treating the data collected in any one month as the results of a cross-sectional survey, we also examined the frequency distributions of antibody titres. On no occasion did we obtain a bimodal distribution of titres which could help to determine an appropriate cut-off point. The only way to obtain such a distribution with these data was to use the highest titre ever recorded for each dog between August 1989 and May 1990 (Fig. 4).

Finally, we examined the way in which success of parasite isolation was related to antibody titre and to the presence of leishmanial chancres. There is a strikingly good association between parasite isolation and titre (Fig. 5): use of logistic regression [22], and Efron's [23] entropy method for calculating the equivalent of $r^2$ with binomial data, revealed that 65·3% of the variation in proportion of samples positive, by all methods of isolation, could be explained by reciprocal log antibody titre at the time of isolation ($\chi^2 = 46{\cdot}1$, D.F. $= 1$, $P < 0{\cdot}001$). There was also a significant but weaker association between parasite isolation and the presence of a chancre ($\chi^2 = 4{\cdot}66$, D.F. $= 1$, $P = 0{\cdot}031$).

## DISCUSSION

We began by acknowledging that there can be no true 'gold standard' for infection in field experiments of this kind. We conclude that, even in a cohort of dogs under relatively intensive observation, a significant number of infections were missed.

Whilst sensitivity and specificity of the IFAT appears high (> 80%) during

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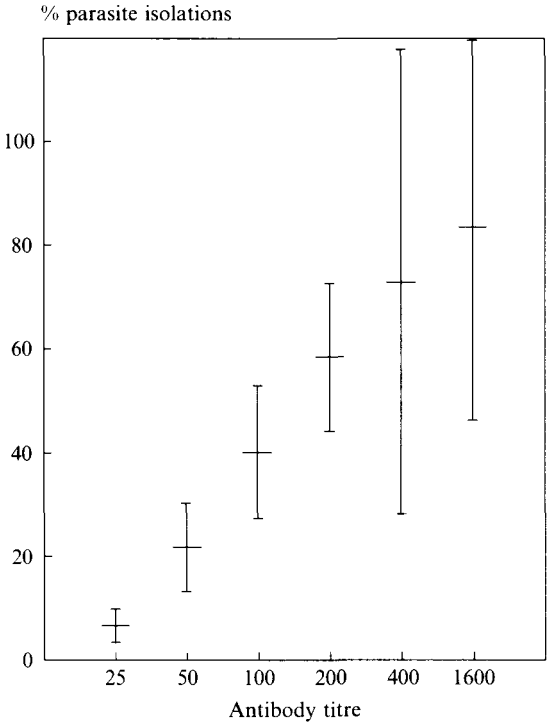


Fig. 5. Association between the percentage of successful parasite isolations and log antibody titre. Binomial standard errors become larger at higher titres because sample sizes are smaller. Notice that the $x$-axis is not a continuous logarithmic series. Statistics are given in the text.

March and April 1990 (Fig. 1*a*), the identical analysis carried out for the second transmission season (Fig. 3) indicates that our criteria for identifying infection in the first season are unsatisfactory. More attempts at parasite isolation would presumably have found more infected animals. We suggest that the more rapid serological response to infection seen late in 1990 is attributable to previous exposure. Specificity fell to zero when sensitivity was high because the titres of presumed negatives rose at the same time, a sign of earlier exposure of these animals too. It is worth recording that 6 of these 7 presumed negatives developed antibody titres $\geqslant 50$ on at least one occasion between August 1989 and May 1990.

All of the seven dogs found parasitologically or clinically positive after the second transmission season but not after the first were seropositive by November 1990 but none was serologically positive in March or April of that year. So the maximum sensitivity seen in Figure 1*a* is probably an overestimate. Note that a yearly incidence of 72% implies a force of infection of $\lambda = -ln(1-0{\cdot}72) = 1{\cdot}27\ \text{yr}^{-1}$, but if the true incidence is, say 11% higher at 0·8, $\lambda = 1{\cdot}61\ \text{yr}^{-1}$, which is 27% higher. In any event, that peak in sensitivity lasted only 2–3 months (Figs 1*a* and 3). following a significant incubation period. The question of maximum sensitivity may be particular to the way the IFAT was handled in this study, but the existence of a lengthy incubation period is likely to be a general problem with serological tests for leishmania infections.

The implication is that, in environments like the French Cévennes [20, 21] and


https://doi.org/10.1017/S0950268800051074 Published online by Cambridge University Press

the Brazilian north-east [4, 24] where transmission is highly seasonal, sero-epidemiological studies attempting to estimate the true prevalence of infection would have to be very carefully timed. Even then, sensitivity could not be guaranteed with this IFAT. In areas where transmission occurs all year round, as in Pará state in the Brazilian Amazon [25], cross-sectional surveys of asynchronous infections in dogs will inevitably lead to still larger underestimates of the prevalence of infection.

Figure 4 may tempt those who have observed bimodal distributions of antibody titre in random samples collected in endemic areas [15, 17] to take dogs with titres $\geqslant 100$ as positive, as indeed did Vidor [18]. Our analysis suggests that such a high threshold would give a test which is even more insensitive to infection.

The decline in sensitivity after April 1990 (Fig. 1) is probably explained by a proportion of dogs resolving their infections [15, 26]. These may be dogs that we have earlier termed type-B [1, 2] – animals which become transiently seropositive, but which never become infectious, or do so only briefly. The decline may be apparent rather than real, since we cannot know whether those animals becoming seronegative have also been able to clear their infections. They are in any case individuals which survived leishmania infection, in the short term at least. Having become seropositive, the 16 which died of leishmaniasis before the end of the study became seronegative ($< 40$) again on only 12/114 occasions.

In Brazil, China and the Mediterranean basin, seropositive dogs are often destroyed or treated as part of visceral leishmaniasis control programmes [8, 27, 28]. Our analysis suggests that any population of seropositives will not include all infected animals. However, if there is a good correlation between antibody titre and infectiousness, then culling or selective treatment would effectively remove those animals most responsible for transmission.

A certain amount of evidence for this correlation now exists, and data collected during this study add to it. From results in Pozio and co-workers [26] we can calculate by logistic regression that 89·2% of the variation in proportion of dogs parasite positive was explained by log reciprocal antibody titre (IFAT: $\chi^2 = 22{\cdot}8$, D.F. $= 1$, $P < 0{\cdot}001$). The same calculation performed on data in Abranches and colleagues [17], gives 52% variation explained (IFAT: $\chi^2 = 16{\cdot}02$, D.F. $= 1$, $P < 0{\cdot}001$). These strong correlations are consistent with our own (Fig. 5). This argument is almost completed by Adler and Theodor's [29] observation that where more infected macrophages were seen during histological study of the skin, infectiousness to sandflies was greater.

There are, however, proven connections between antibody titre and infectiousness. Pozio's data also show a strong association between clinical condition and titre (patent vs asymptomatic or oligosymptomatic cases, compared with titres $< 160$ or $\geqslant 160$, $\chi^2 = 16{\cdot}92$, D.F. $= 1$, $P < 0{\cdot}001$). (Although Abranches and colleagues [17] found no significant association, their minimum titres, 128, were already moderately high.) And clinical condition has repeatedly been associated with increased infectiousness to sandflies, albeit from studies on just a few dogs [27, 29, 30].

In sum, the rarely-stated (but see [27]) aim of serology carried out during continuous control programmes should be, logically, to detect infectiousness rather than infection. The selective treatment or destruction of those dogs which

contribute most to transmission ought, if carried out regularly and thoroughly, be very effective. As for the timing of control, Gradoni and colleagues [27] proposed that the decision about which dogs to treat or cull should be made just before the beginning of the Mediterranean sandfly season. Our data support this view: if there is a good correlation between titre and infectiousness then animals which have already reconverted to seronegative by the start of next season will play little part in transmission. These are animals who would not need to be treated, or whose lives could be spared. Remarkably, though, our results from the Cévennes indicate that about 45% of dogs surviving to May 1990 would have to have been dealt with one way or the other.

There is a final irony attached to the relatively quantitative analysis of which this work is part. Where treatment or culling programmes based on serology do succeed, they may be seen as victories for an empirical approach over a theoretical one. The reason is that the survey data would at no stage have allowed accurate calculation of the magnitude of the control problem – expressed as incidence or prevalence of infection, or as the basic case reproduction number, $R_0$. The dangers in this view are, of course, those which are always associated with being right, but for not quite the right reason.

ACKNOWLEDGEMENTS

We particularly thank R. W. Ashford for bringing us together in the first place. N. Dubreuil, G. Bissuel and Y. Moreau played an important part in the practical work, and R. W. Ashford, M. A. Miles and R. J. Quinnell made helpful comments on the manuscript. The project was supported by Rhône Mérieux as a component of research towards a vaccine for canine leishmaniasis.

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