The epidemiology of canine leishmaniasis: transmission rates estimated from a cohort study in Amazonian Brazil

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SUMMARY

We estimate the incidence rate, serological conversion rate and basic case reproduction number (R_0) of *Leishmania infantum* from a cohort study of 126 domestic dogs exposed to natural infection rates over 2 years on Marajó Island, Pará State, Brazil. The analysis includes new methods for (1) determining the number of seropositives in cross-sectional serological data, (2) identifying seroconversions in longitudinal studies, based on both the number of antibody units and their rate of change through time, (3) estimating incidence and serological pre-patent periods and (4) calculating R_0 for a potentially fatal, vector-borne disease under seasonal transmission. Longitudinal and cross-sectional serological (ELISA) analyses gave similar estimates of the proportion of dogs positive. However, longitudinal analysis allowed the calculation of pre-patent periods, and hence the more accurate estimation of incidence: an infection–conversion model fitted by maximum likelihood to serological data yielded seasonally varying *per capita* incidence rates with a mean of $8{\cdot}66 \times 10^{-3}$/day (mean time to infection 115 days, 95% C.L. 107–126 days), and a median pre-patent period of 94 (95% C.L. 82–111) days. These results were used in conjunction with theory and dog demographic data to estimate the basic reproduction number, R_0, as 5·9 (95% C.L. 4·4–7·4). R_0 is a determinant of the scale of the leishmaniasis control problem, and we comment on the options for control.

Key words: canine leishmaniasis, *Leishmania infantum*, serology, domestic dog, incidence rate, basic reproduction number.

INTRODUCTION

Leishmania infantum (= *Leishmania chagasi*, Rioux *et al*. 1990), the agent of human and canine visceral leishmaniasis in Europe and Latin America, is probably maintained largely in domestic dog populations. In order to control disease by reducing the number of infected dogs, we should first have quantitative estimates of those epidemiological variables which describe the transmission rate between individuals in a population – the incidence, prevalence and basic case reproduction number of infection (R_0). These quantities define the magnitude of the control problem.

To measure the incidence of infection we need to distinguish infected from uninfected dogs. An obvious method would be to use the presence of parasites to detect infection. Unfortunately, existing methods of detecting *Leishmania* parasites in dogs (microscopical examination, culture or hamster inoculation of biopsy material) are known to be variable and insensitive (Schnur & Jacobson, 1987). DNA-based techniques, such as PCR, may have greater sensitivity, but are expensive to perform, and have yet to be tested in large-scale field surveys. A recent study in Brazil confirmed that PCR is more sensitive than parasitological diagnosis; however, 23% of seropositives were PCR-negative, suggesting that further evaluation is needed (Ashford *et al*. 1995).

Alternatively, the presence of anti-parasite antibodies can be used as a marker of infection, with production of antibody as the definition of infection or challenge. In previous attempts to calculate incidence rates in Europe (Dye *et al*. 1992; Hasibeder, Dye & Carpenter, 1992) and Latin America (Courtenay *et al*. 1994), we have made use of cross-sectional serological data describing the change in prevalence with age. There are 2 main drawbacks with such data. First, not all infected dogs are expected to be seropositive: there is known to be a significant pre-patent period before seroconversion (Abranches *et al*. 1991; Dye, Vidor & Dereure, 1993), a fraction of infected dogs may never convert (Dye *et al*. 1993; Killick-Kendrick *et al*. 1995), and dogs may revert to seronegative but remain parasite positive. So the relationship between infection and serology changes during the course of infection. Secondly, it is difficult to discriminate between seropositive and seronegative dogs: bimodal distributions of antibody titre, identifying a distinct population of seropositive animals, are rarely seen (see Lanotte *et al*. (1979) for an exception), and different tests rarely agree on the proportion of positives in a sample (Evans *et al*. 1990).

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It should be easier to interpret serological data from longitudinal studies which monitor conversion in initially uninfected dogs. We have therefore carried out a cohort study which followed a population of sentinel dogs exposed to natural rates of infection in an endemic area of Brazil. This allows us to separate out the time-dependent processes of parasite acquisition, and serological conversion and reversion, and to establish more reliably a cut-off point for diagnosis. Our particular aims were (1) to develop a procedure for estimating the number of seropositives in cross-sectional data from underlying frequency distributions, (2) to compare methods for estimating the numbers of serological conversions and reversions in longitudinal data, (3) to compare the sensitivity and specificity of parasitology and serology through time, (4) to develop a model for estimating seasonally varying incidence rates and the pre-patent period from seroconversion data, (5) to calculate R_0 from these incidence rates using the appropriate epidemiological theory, and (6) to consider the implications of our results for the control of canine leishmaniasis.

MATERIALS AND METHODS

Study design

We worked in 24 villages in the municipality of Salvaterra, Marajó Island, Pará State, Brazil (48° 03′ W, 00° 46′ S), which has been described elsewhere (Quinnell, Dye & Shaw, 1992; Courtenay *et al*. 1994; Quinnell & Dye, 1994).

Uninfected dogs enrolled in the study came from 2 sources: 99 were young adults (generally 6–18 months old) obtained in the nearby city of Belém, where there is no leishmaniasis, and 27 were young (< 6 months old), serologically (IFAT) negative animals born in the study area. All these dogs were given to households to be kept as pets. The study ran from April 1993 to July 1995, during which time we took samples at approximately 10-week intervals from each animal (mean interval 67·3 days, S.E. 0·854, range 58–80 days). The first cohort contained 30 dogs, and additional animals were enrolled into the study at most sampling dates, giving a total of 126 dogs (Table 1). Permission was obtained from all householders to use their dogs, and dogs were given, without payment, only to those households which were willing to receive them. In some instances, permission to use a dog was withdrawn during the course of the study. Where a dog was obviously being mistreated or neglected, we sought agreement to transfer it to another household.

Sampling

At each sampling round dogs were brought to our laboratory in Salvaterra town. Each dog was anaesthetized with a mixture of Medetomidine hydrochloride (Domitor®) and Ketamine (Vetelar®) at dosages recommended for minor surgery. Twenty ml of blood were taken by venepuncture (jugular) and defibrinated in a sterile 50 ml vol. polypropylene tube with 30–40 glass beads. Triplicate serum samples were taken after centrifugation and stored at −20 °C.

Bone marrow was aspirated from the iliac crest with a 16 × 25 mm Klima needle (Veterinary Instruments, Newcastle) into a 20 ml syringe containing 0·5 % EDTA. The sample was inoculated onto 2 sterile Difco blood-agar slopes (rounds 1–9; Walton, Shaw & Lainson (1977)) or into 2 golden hamsters (rounds 10–13) for parasite isolation, and used to make 1–4 smears.

Serology

Controls. We used 3 groups of uninfected dogs as serological controls: (1) 127 young adults (6–18 months old) from Belém, (2) 26 IFAT-negative puppies (< 6 months old) from Marajó, (3) 85 Dutch dogs of various ages and breeds which had attended a veterinary clinic in Utrecht, The Netherlands. The first 2 groups included all dogs from the cohort study.

IFAT. Immunofluorescent antibody-tests (IFAT) were performed by standard techniques using FITC-conjugated anti-dog IgG (Sigma) and amastigote antigen prepared from dabs of hamster spleen infected with *L. infantum* (MCER/BR/81/6445). Sera were tested at 2-fold dilutions from 1/20 to 1/320. IFAT titres were used solely as an entry criterion to the study for Marajó puppies (see above). To be conservative, only dogs with titres of < 20 were included in the study, whereas titres of < 40 are generally considered negative (Courtenay *et al*. 1994).

ELISA. *L. infantum* (MHOM/BR/74/PP75) promastigotes were cultured in RPMI 1640 supplemented with 10 % foetal calf serum, 100 units/ml penicillin–streptomycin, 2 mM glutamine and 1 mM pyruvic acid. Mixed logarithmic/stationary-phase cultures were harvested, washed × 3 in PBS, counted and sonicated.

Antigen was added to 96-well plates (Linbro, ICN Flow) in 100 µl/well carbonate coating buffer (pH 9·6) at 5×10^5 parasite equivalents/ml and incubated overnight at 4 °C. Coated plates were blocked for 1 h at 37 °C with 200 µl/well 3 % bovine serum albumin (BSA) in phosphate-buffered saline + 0·05 % Tween 20 (PBS-T). Plates were then washed × 3 in 0·9 % NaCl + 0·05 % Tween 20, sera were plated in 100 µl incubation buffer (PBS-T + 0·5 % BSA) and plates incubated for 2 h at 37 °C. After washing, rabbit anti-dog IgG peroxidase conjugate (Sigma) was added at a dilution of

Table 1. Numbers of dogs originating from Belém and Marajó Island which were enrolled in the study between April 1993 and July 1995

Sampling round	Date	Day	Belém dogs	Marajó dogs
1	11 April 93	0	19	11
2	30 June 93	80	20	1
3	28 Aug. 93	139	30	7
4	5 Nov. 93	208	10	6
5	13 Jan. 94	277	6	0
6	23 March 94	346	2	0
7	30 May 94	414	7	2
8	6 Aug. 94	482	0	0
9	12 Oct. 94	549	0	0
10	10 Dec. 94	608	5	0
11	19 Feb. 95	683	0	0
12	24 April 95	746	0	0
13	6 July 95	818	0	0
Total			99	27

1/1000 in incubation buffer and incubated for 1 h at 37 °C. Substrate, ABTS in phosphate–citrate buffer (pH 4·5) + 0·1 % H_2O_2 was added after washing. Plates were read in a Dynatech plate reader at 410 nm. Optimum concentrations of antigen and conjugate were determined by chequerboard titration.

Standardization. A positive control serum was titrated 2-fold on every plate from 1/50 to 1/3 276 800. This positive serum was assigned an arbitrary number of units, 409 600/ml, defined as the reciprocal of the highest dilution at which absorbance was greater than the mean + 3 S.D. of background (no antibody) wells. Test sera were titrated from 1/50 to 1/800, with all sera from a single dog being tested on a single plate: when space allowed, sera were tested in duplicate. Absorbance values were calculated as observed absorbance minus the mean background absorbance.

A standard line was fitted to the positive control serum absorbance values using a log-logit transformation, over the range 1/100 to 1/409 600 (Peterman & Butler, 1989). Thus, the slope and intercept of the equation $\log(B/(1-B)) = a + b\log(\text{units})$ were calculated by linear regression, where $B =$ observed/maximum absorbance, and the latter was estimated by least squares. Absorbances of test sera at 4 dilutions from 1/100 to 1/800 were then expressed in antibody units using the standard line, from which we calculated the geometric mean number of units in a sample. Where the dilution curve for any test serum was noticeably non-parallel to the standard, tests were repeated at dilutions of 1/800 to 1/12 800. The geometric mean number of units was then estimated using the dilution range over which the new dilution curve was parallel to the standard.

Parasitology

Blood-agar cultures were examined at 7, 14 and 28 days by microscopy. If both culture tubes of a sample were contaminated at day 7 or 14, that sample was excluded from the analysis. Smears were Giemsa-stained and examined by microscopy. Hamsters were killed 6–12 months after inoculation: impression smears were made of spleen and liver, and a small piece of each was triturated and cultured on blood-agar slopes. The 34 successful parasite cultures were examined by monoclonal antibodies (J. J. Shaw, personal communication): all were identified as *L. infantum*.

Incidence, pre-patent period and R_0

For a dog to become serologically positive as a result of infection, it must first be exposed to infection, and then produce detectable antibody after some pre-patent period. The analytical problem is to separate these 2 processes using data which specify the proportion of dogs seroconverting between sampling occasions. We define p_i and s_i, respectively, as the proportion of dogs acquiring infection and the proportion of infected dogs seroconverting in the interval between rounds $i-1$ and i, beginning with round zero. Thus, in the first interval a proportion p_1 dogs are infected, of which $s_1 p_1$ seroconvert during that interval, $s_2 p_1$ in the second interval, and so on. In the second interval $p_2(1-p_1)$ dogs acquire infection, so the proportion of dogs seroconverting in the second interval is $[s_1 p_2(1-p_1) + s_2 p_1]/(1-s_1 p_1)$. In general, the expected number of converts, C_t, out of N_t negatives, over a chosen time interval is:

$$C_t = \sum_{i=1}^{t} \left\{ s_i p_{t-i+1} \prod_{j=i+1}^{t} (1-p_{t-j+1}) \right\} N_t, \qquad (1)$$

where t is the number of intervals a cohort of dogs has been exposed to infection, and $N_t = (N_{t-1} - C_{t-1}) \times$ proportion surviving between sampling intervals. Values of s_i and p_i are estimated by maximum likelihood following the procedure of Williams & Dye (1994), and making use of the forward differencing, quasi-Newton iterative method available in Microsoft Excel®. We checked the reliability of this procedure by applying it to 5 sets of simulated data with known incidence and conversion rates. The mean incidence rate and the median time to conversion were estimated, respectively, to within 7 and 14 % on average. Having estimated time-varying incidence rates, we can calculate R_0 using the methods laid out in Appendices 1 and 2.

RESULTS

Serological analysis

Uninfected controls. The geometric mean number of units of antibody activity (95 % C.L., mean + 3 S.D.)

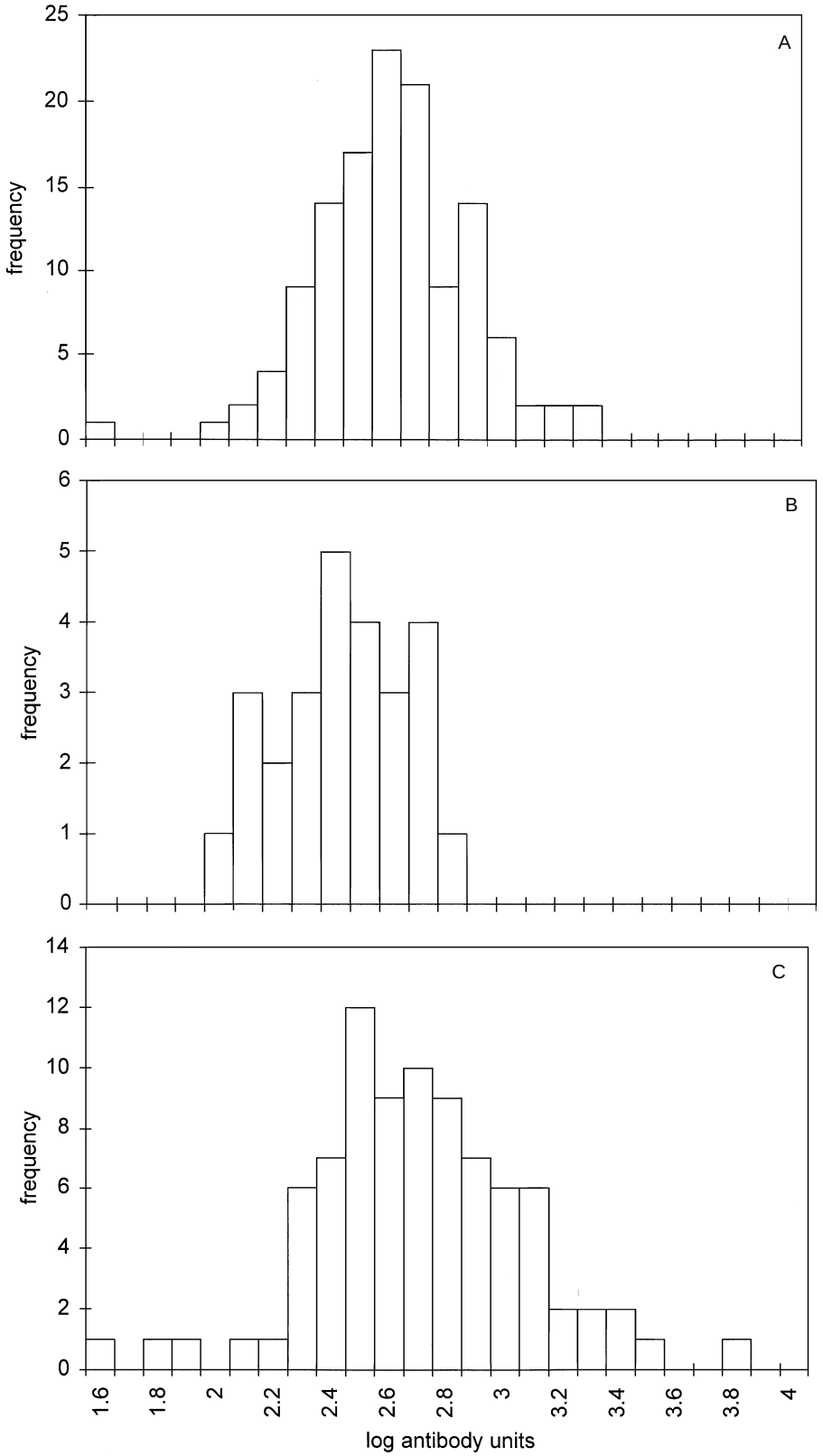

Fig. 1. Frequency distributions of log units of anti-*Leishmania* antibody in groups of uninfected dogs from (A) Belém, (B) Marajó and (C) Utrecht, Netherlands.

in the 3 groups of control dogs were: Belém 473 (424–526, 3054, Fig. 1A), Marajó 374 (308–455, 1605, Fig. 1B), and Dutch 569 (475–681, 6941, Fig. 1C). The mean log antibody units of Belém and Marajó dogs did not differ significantly ($F_{1,152} = 3{\cdot}28$, $P = 0{\cdot}072$). Marajó dogs, but not Belém dogs, had significantly less antibody activity than Dutch dogs (Mann–Whitney $U = 1477$, $P < 0{\cdot}01$ and $U = 6156$, $P = 0{\cdot}08$). The variation in log antibody units among Dutch dogs was significantly greater than

Table 2. Numbers of Belém and Marajó dogs sampled with time since enrolment

Sampling round	Belém dogs	Marajó dogs	Total
1	99	26	125
2	97	27	124
3	76	22	98
4	62	16	78
5	59	12	71
6	48	11	59
7	40	11	51
8	30	9	39
9	23	10	33
10	22	6	28
11	17	6	23
12	12	5	17
13	6	4	10

among Belém or Marajó dogs (variance ratio test: $F_{84,126} = 1{\cdot}84$ and $F_{84,25} = 2{\cdot}94$ respectively, $P < 0{\cdot}01$), but the variation among Belém and Marajó dogs did not differ significantly ($F_{126,25} = 1{\cdot}60$, $P > 0{\cdot}10$). The distributions of antibody units of both Belém and Dutch dogs did not differ significantly from a log-normal distribution (Kolmogorov–Smirnov test, $D = 0{\cdot}048$ and $0{\cdot}064$ respectively; Fig. 1).

Cohort serology : cross-sectional analysis. A total of 756 serum samples was obtained from 126 dogs (Table 2). Levels of anti-*Leishmania* antibodies in these sera were expressed as units of antibody activity measured by ELISA. The frequency distribution of log units is skewed to the right, but not obviously bimodal (Fig. 2). The graph does not show 2 distinct distributions, and we have no independent standard with which to assess seropositivity; parasitology, for example, is not expected to relate directly to serology. However, since the frequency distributions of antibody units among known negatives are lognormal (Fig. 1), we expect the distribution of negatives among all sera also to be lognormal. We can therefore estimate the proportion of animals in a sample which are seronegative by fitting a lognormal distribution to the left-hand tail of the distribution in Fig. 2. We did this by maximum likelihood, fitting distributions from $-\infty$ up to a truncation point c. The means and standard deviations of a series of distributions were calculated, with c varying from the mode upwards, and the best-fit distribution was identified with the modified Kolmogorov–Smirnov test (Lilliefors, 1967). The goodness of fit to the lognormal distribution varies with c as shown in Fig. 3. There is an optimum at $c = 3{\cdot}08$, and the fit is almost as good at $c = 3{\cdot}2$. The means and standard deviations of the 2 corresponding lognormal distributions are indistinguishable, 2·76 (S.D. 0·327) log units, or 575 units. These procedures were also tried with antibody levels expressed as scaled units (the number of units at round i divided by the number of units at time zero). However, the left-hand tail of the distribution of scaled units was not well described by a lognormal ($D = 0{\cdot}0484$, $n = 261$, $0{\cdot}15 > P > 0{\cdot}10$), so this measure was discarded.

The expected distribution of positive sera was then obtained by subtracting the lognormal distribution of negatives from the observed data (Fig. 2). The lower boundary of the positive distribution is by definition c; that is, at 3·08 log units (1208 units). We take this method of subtracting distributions to be the best way of classifying sera by cross-sectional analysis; it gives 362 (48 %) positives.

The procedure above estimates the number of seropositive dogs in a sample, but not whether any individual dog is positive. The simplest technique, and effectively the maximum likelihood discriminator, for separating individual sera is to choose the cut-off as the point of intersection (found by linear interpolation) of negative and positive distributions. In this case, the cut-off is 3·25 log units (= 2253 units); 3·5 % of the negative distribution lies above it, and 3·3 % of the positive distribution lies below it; 26/756 sera (3·4 %) are misclassified, and the specificity and sensitivity are 96·5 % and 96·7 %. A total of 365 sera are classed as positive (> 2253 units), close to the best estimate of 362.

Cohort serology : longitudinal analysis. In order to estimate incidence rates we need to identify conversions in longitudinal serological data, and below we consider 3 possible methods for defining conversions. Since the true number of conversions is unknown we again have no independent standard or reference against which each method can be assessed. However, we can compare longitudinal methods with each other, and against the results of cross-sectional analysis.

We first use the cut-off from cross-sectional analysis (2253 units) and look at the distribution of positive and negative sera through time (method A). Of 80 dogs which become positive by this criterion, 75 remain positive thereafter. The 5 remaining dogs, which have positive titres followed by negatives, may be true reversions, or false positives. We arbitrarily define a false positive as a single positive followed by a negative (2 dogs), and a reversion as 2 or more positives followed by a negative (3 dogs).

We can also define conversion as a distinct change in antibody titre through time (method B). Following the same procedure as the cross-sectional analysis, we ask whether we can reliably define a change in antibody titre between consecutive samples. The frequency distribution of changes in titre (titre at round i divided by titre at round $i-1$) is given in Fig. 4. The distribution is again skewed to the right, but the left tail is approximately lognormal. The best-fit lognormal distribution has a mean of 0·086

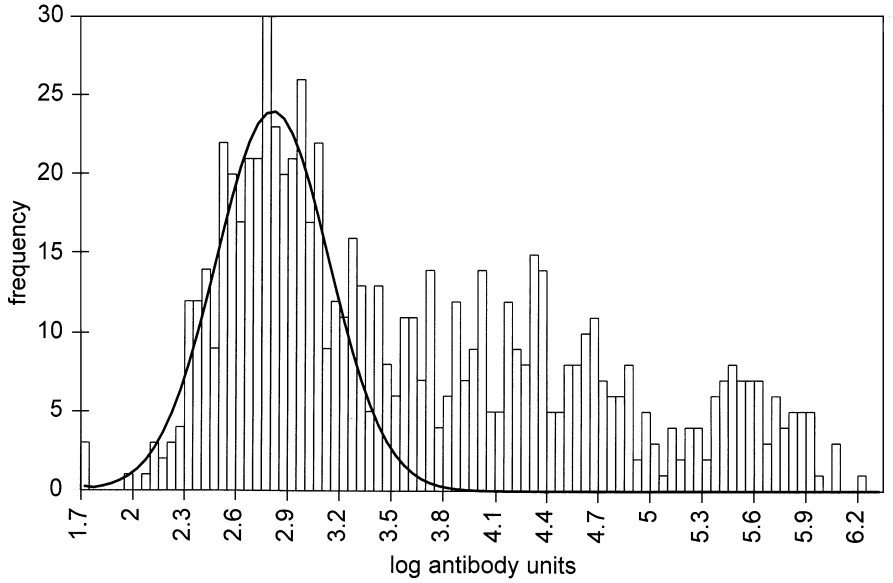

Fig. 2. Frequency distribution of log units of anti-*Leishmania* antibody in the cohort study dogs. Solid line is the fitted lognormal distribution (see text).

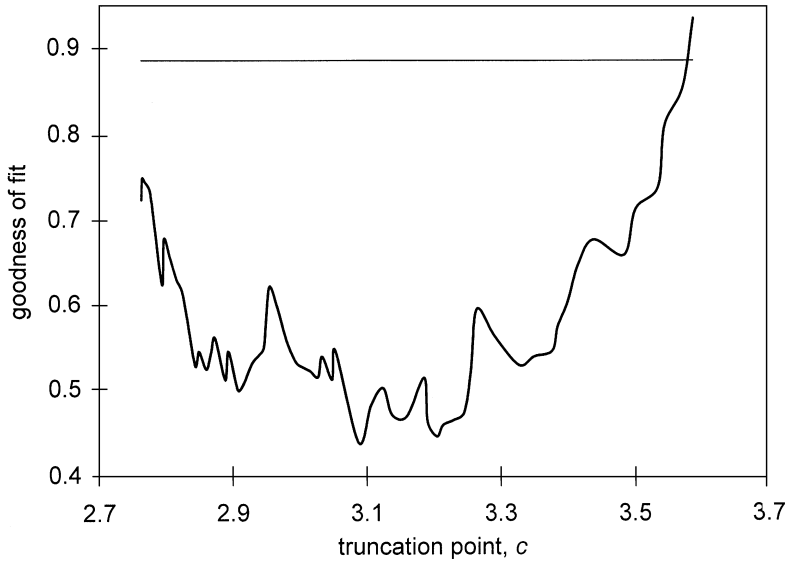

Fig. 3. Goodness of fit of the lognormal distribution against the truncation point, c (heavy line), for the distribution of antibody units. Goodness of fit is measured with the Kolmogorov–Smirnov statistic, D, multiplied by the square root of the sample size. A smaller value indicates a better fit. There are significant ($P < 0{\cdot}05$) departures from lognormal only above the horizontal line. The best fit in this example for antibody units is at $c = 3{\cdot}08$. The corresponding mean and standard deviation were used to draw the fitted distribution in Fig. 2.

(S.D. 0·246), that is, a 1·2-fold change between samples on average. Repeating the above procedure for establishing a cut-off, we arrive at a threshold of 0·6; in other words, a 4-fold ($> \times 3{\cdot}98$) change in titre is considered to be a positive change. This gives 114 positive changes, with $13/622 = 2{\cdot}1\,\%$ of observations misclassified.

Finally, we can in principle improve the algorithm by combining information on the number of units of antibody activity and their rates of change (method C). One such 'mixed method' uses the following rules: (1) conversion occurs at the first change in units greater than $\times 4$, provided units exceed 1208; otherwise conversion occurs at the second $\times 4$ change; (2) if a dog becomes clearly seropositive ($>$ mean + 3 S.D. of the fitted negative distribution, i.e. $>$ 5500 units) without a $\times 4$ change, then conversion occurs when units first exceed 2253.

The 3 methods actually give similar results: 79, 75 and 75 seroconversions respectively, and totals of 360, 345 and 355 seropositives, allowing for seroreversion as defined above. As C is the best method in principle, we use it from now on.

Serological reversions. Only 3 dogs which converted according to method C showed titres which subsequently fell below the threshold 2253 units. Sera

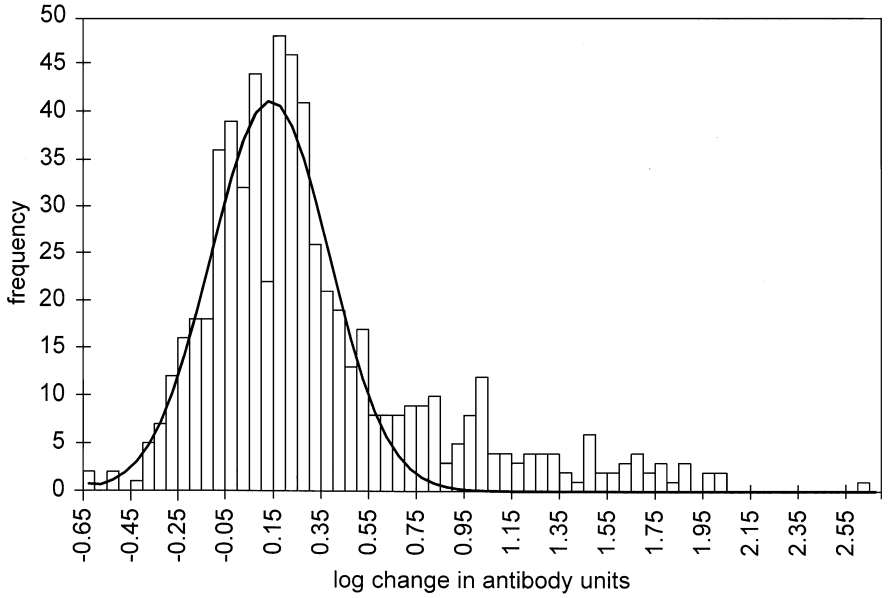

Fig. 4. Frequency distribution of log changes in units of anti-*Leishmania* antibody in the cohort dogs. The solid line is the fitted lognormal distribution (see text).

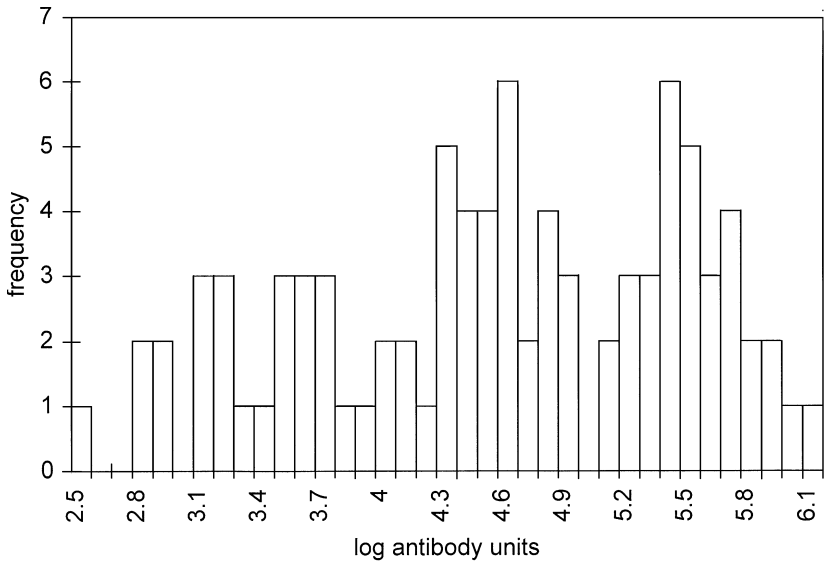

Fig. 5. Frequency distribution of log units of anti-*Leishmania* antibody among cohort dogs from which parasites were isolated.

from 1 of these dogs showed units < 2253 on the final observation only, 1 on the final 3 occasions, and 1 on 6 of the final 8 occasions. These dogs reverted after 2, 3 or 5 consecutive positive readings. The overall reversion rate was 3/221 per interval, or $2{\cdot}01 \times 10^{-4}$/day.

Relationship between parasitology and serology

A total of 89 bone-marrow samples was positive by parasite culture, direct examination or inoculation into hamsters. Fig. 5 shows the distribution of antibody units for these samples (cf. Fig. 2). The sensitivity of serology to detect parasite-positive dogs was low, only 76/89 (85·4%). However, of the 13 seronegative dogs from which parasites were isolated, all 8 dogs which survived did seroconvert, 7 at the next round. The other 5 died or disappeared at the next round. All parasite-positive seronegatives were thus in the pre-patent phase; no infected dogs failed to seroconvert.

The sensitivity of parasite isolation was low, as expected. Only 49/224 (22%) of bone-marrow samples from seropositive dogs were positive by parasite culture and 23/65 (35%) were positive by hamster inoculation. Overall, sensitivity was greatest 2 months after seroconversion, declining thereafter (Fig. 6). Bone-marrow smears were the least sensitive method of detecting infection: parasites were found in smears from just 9/145 (6·2%) samples from

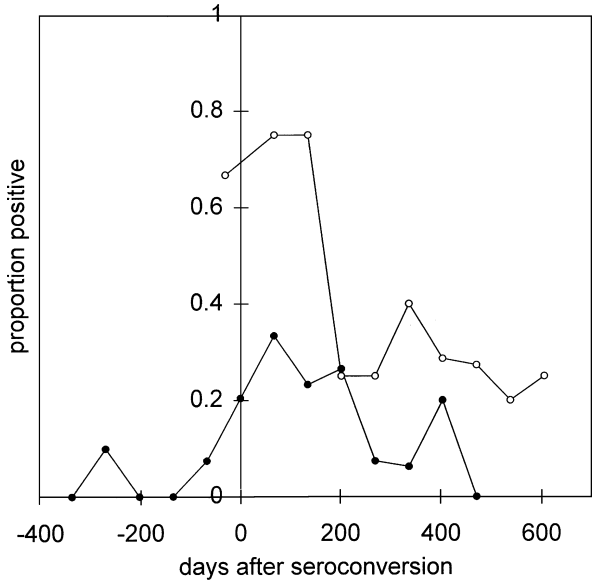

Fig. 6. The proportion of dogs from which parasites were isolated by culture (●) and hamster inoculation (○) before and after seroconversion.

seropositive dogs. Comparing smears and cultures made from the same bone-marrow samples, the numbers found with parasites were significantly different (7 and 30 out of 207 respectively; $z = 4{\cdot}4$, $P < 0{\cdot}001$).

Incidence rates, pre-patent period and R_0

Table 3 contains the number of serologically negative dogs in 35 groups at the start of each 10-week interval and the number which converted during that interval. The Table combines the data for Belém and Marajó dogs: although the latter had been exposed to infection for up to 6 months longer, the number and timing of seroconversions in the 2 groups were not significantly different ($F_{1,17} = 2{\cdot}44$, $P > 0{\cdot}05$). Dogs experienced 11 different incidence rates during the 11 sampling rounds, and analysis was restricted to data from the first 5 intervals each cohort was in the field, beyond which few animals survived (Table 3). We thus fitted eqn (1) to these data with 16 ($= 11p + 5s$) parameters, subject to the obvious constraints $0 < p_i < 1, 0 < s_i < 1$ and $\Sigma_{i=1}^{5} s_i \leqslant 1$. The fitted incidence and conversion rates are given in Table 3. Note that the final incidence rate (p_{11}) could not be estimated, since $p_{10} = 1$, so was calculated as the mean incidence rate for the same period in the previous year. The expected total number of seroconversions obtained with this model was 67·6, which compares well with the 68 observed. The 11 seasonally varying rates are given in column 2 of Table 3. They show that transmission was low between January and March/April (wet season), intermediate between March/April and August (dry/wet transition), and high between October and December (dry season). The last row of Table 3 shows the proportion of dogs converting in each interval after infection: most dogs seroconverted during weeks 10–20. The *per capita* conversion rate increased with time: conversion rates were 0·112, 0·464, 0·490, 0·440 and 1·0/dog/sampling interval over intervals 1–5. Note that, although the best estimates of the incidence and conversion rates come from this full model, the number of parameters can be reduced to 3 different incidence rates and 2 conversion rates without significantly reducing the goodness of fit (likelihood ratio test).

We can calculate the mean incidence rate over 11 rounds (approximately 2 years) from the seasonal rates in Table 3, which gave a mean instantaneous *per capita* incidence rate of $8{\cdot}93 \times 10^{-3}$/day, i.e. a mean time to infection of 112 days. Assuming that the average dog acquired infection at the mid-point of any 10-week interval, the pre-patent period was 101 days.

The complexity of the fitting procedure and of subsequent averaging suggests that there is no simple method to calculate variances at point estimates, so we used a jackknife approach (Selvin, 1991). The mean incidence rate was recalculated, ignoring data from each of the 8 cohorts of dogs in turn. In each case the starting values for the iteration were those derived from the full data set. When ignoring data from one cohort meant that a particular seasonal incidence rate could not be calculated, we used the value from the full data. These 8 separate estimates of incidence rate gave a jackknife mean of $8{\cdot}66 \times 10^{-3}$/day (S.E. $0{\cdot}30 \times 10^{-3}$/day), and a mean time to infection of 115 days (95% C.L. 107–126 days). Similarly, the jackknife median pre-patent period was 94 days (95% C.L. 82–111 days). We use these values as the best estimates of incidence rate and pre-patent period.

The mean incidence rate and pre-patent period can be used to reconstruct, roughly, the change in prevalence of dogs through time (solid line, Fig. 7). We can also apply to this graph of prevalence (P) against time (T) the same cross-sectional analysis used by Courtenay *et al.* (1994) to estimate incidence (λ) and reversion (ρ) rates, in which $P(T) = [\lambda/(\lambda+\rho)]\,[1 - \exp(-(\lambda+\rho)\,T)]$. This analysis ignores the pre-patent period, and the dotted line in Fig. 7 shows clearly that the penalty is a relatively poor fit to the data. The most important consequence is an underestimate of incidence: the maximum likelihood fit of the incidence–recovery model gives $\lambda = 3{\cdot}65 \times 10^{-3}$/day, which is less than half that above. The reversion rate is $\rho = 3{\cdot}4 \times 10^{-4}$/day, which is somewhat larger than the estimate of $2{\cdot}0 \times 10^{-4}$/day from longitudinal data.

Finally, we calculate the basic case reproduction number from $R_0 = 1 + L/A$ (Appendix 1). The average life-expectancy of serologically positive, Marajó dogs measured longitudinally over 4 intervals

Table 3. Observed, O, and fitted (F) numbers of serological conversions among groups of negative dogs of different sizes, N, by sampling round (down) and duration of exposure (10-week periods, across)

(The expected numbers of conversions are calculated from the p_i and s_i given in the second column and the last row, as described in the text. *The final incidence rate was estimated from the previous year's data (see text).)

Sampling interval	p_i	0–1 O (F) N	1–2	2–3	3–4	4–5	Totals
April–June 93	0·478	1 (1·61) 30	—	—	—	—	1 (1·61) 30
June–Aug. 93	0·000	0 (0·00) 21	4 (5·24) 25	—	—	—	4 (5·24) 46
Aug.–Nov. 93	0·903	4 (3·65) 36	1 (1·93) 19	6 (3·94) 18	—	—	11 (9·52) 73
Nov. 93–Jan. 94	0·693	1 (1·24) 16	12 (12·35) 29	6 (7·24) 17	5 (4·71) 11	—	24 (25·54) 73
Jan.–March 94	0·000	0 (0·00) 6	3 (1·87) 6	5 (4·14) 9	3 (4·14) 9	4 (3·39) 6	15 (13·55) 36
March–May 94	0·393	0 (0·09) 2	0 (0·18) 4	0 (0·27) 1	2 (1·21) 3	1 (2·01) 5	3 (3·76) 15
May–Aug. 94	0·353	0 (0·36) 9	0 (0·39) 2	2 (0·78) 4	0 (0·28) 1	1 (0·81) 1	3 (2·62) 17
Aug.–Oct. 94	0·000	—	2 (0·92) 6	0 (0·23) 1	0 (0·47) 2	0 (0·45) 1	2 (2·07) 10
Oct.–Dec. 94	1·000	—	—	0 (0·76) 4	1 (0·23) 1	0 (0·23) 1	1 (1·22) 6
Dec. 94–Feb. 95	1·000	2 (0·56) 5	—	—	2 (1·39) 3	—	4 (1·95) 8
Feb.–April 95	0·147*	—	—	—	—	0 (0·56) 1	0 (0·56) 1
Totals		8 (7·50) 125	22 (22·87) 91	19 (17·37) 54	13 (12·43) 30	6 (7·45) 15	68 (67·63) 315
s_i		0·112	0·415	0·232	0·106	0·135	—

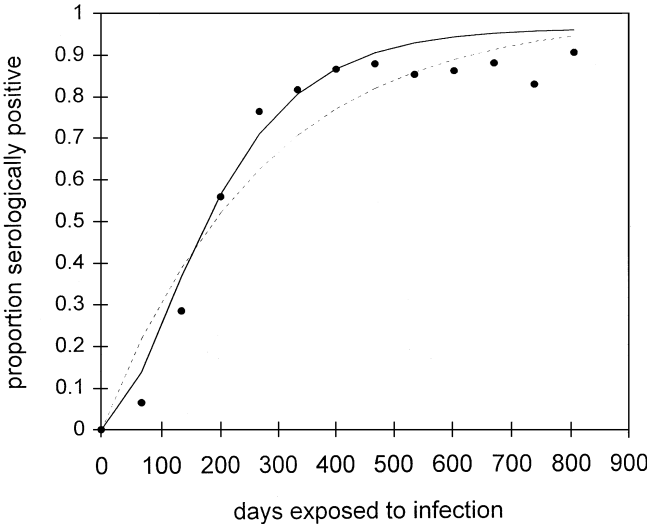

Fig. 7. The relationship between seroprevalence and time in the field for cohort dogs (●). (——) Numerical reconstruction of the change in prevalence using estimates of the mean incidence and reversion rates, and the pre-patent period. (– – – –) The maximum likelihood fit of an incidence–seroreversion model (see text), which markedly underestimates incidence.

(10–18 months) between 1989 and 1994 was $L = 563$ days (95% C.L. 468–708 days; Courtenay *et al.* 1994; O. Courtenay, R. J. Quinnell & C. Dye, unpublished data). Given $A = 115$ (95% C.L. 107–126) days, we arrive at $R_0 = 5{\cdot}9$ (95% C.L. 4·4–7·4).

DISCUSSION

In a previous, cross-sectional analysis of *L. infantum* transmission rates in Marajó dogs (Courtenay *et al.* 1994), we found that *per capita* incidence varied seasonally from $1{\cdot}86$ to $6{\cdot}79 \times 10^{-3}$/day. That analysis was based on the more difficult appraisal of cross-sectional serological (IFAT) data only, and seasonal variations in transmission were coarsely classified, *a priori*, as high or low. Cross-sectional analysis of ELISA data from the present study gave a similar estimate of the average incidence of $3{\cdot}65 \times 10^{-3}$/day. However, in a more flexible analysis based on longitudinal data, we have obtained a higher mean incidence rate of $8{\cdot}66 \times 10^{-3}$/day. This estimate is higher than those derived from cross-sectional analysis because it accounts for the serological pre-patent period. A time-delay between infection and conversion lowers the rate of increase of sero-positivity with age, and it is this rate of increase which determines incidence in cross-sectional analysis. Significantly, that time-delay for *L. infantum* on Marajó Island (94 days) is about as long as the mean time to infection (115 days). Our model also makes no prior assumptions about seasonality, but the

fitted pattern of seasonal variation does coincide with previous experience (Courtenay *et al*. 1994; Kelly, Mustafa & Dye, 1996): transmission was lowest in the wettest months (January–March) and increased during the dry season (up to December). In the present study we have used ELISA in preference to IFAT, since ELISA is more easily standardized (e.g. all samples from 1 dog can be tested on 1 plate), thus aiding the interpretation of longitudinal data.

A general problem in *Leishmania* serology is that the frequency distributions of antibody titres for infected and uninfected animals show considerable overlap, regardless of the serological test used. There are broadly 2 approaches to determining a cut-off from such data: use the distribution of titres from reference negative and positive control animals, or calculate an intrinsic cut-off using the observed frequency distribution of the sample. Here we have done the second, fitting a lognormal distribution to assumed negatives in the left-hand tail of the observed distribution of titres. The distribution of positives is then found by subtracting the fitted distribution of negatives from the total distribution. The most discriminating cut-off is the point of intersection between the distributions of negatives and positives. A similar approach was taken by Greiner *et al*. (1994), who fitted a mixture of log-normal distributions to observed titres. Our analysis is both simpler, in fitting only 1 distribution, and avoids making assumptions about the form of the distribution of positives, which in our data was clearly not lognormal.

By calculating an intrinsic cut-off, we avoid problems associated with the selection of controls. Negative reference populations are typically animals from a non-endemic area, which may not be representative. For example, antibody levels in our best-matched negative reference population (Belém dogs) were somewhat lower than those of the fitted distribution of negative cohort study dogs (mean + 3 S.D. 3054 and 5500 respectively). Moreover, a cut-off based purely on a negative population, typically mean + 3 S.D., will have a very low sensitivity if positive and negative distributions overlap. For example, the mean + 3 S.D. of our fitted negative distribution would detect only 292/362 seropositives, a sensitivity of 80·6%. Positive controls are typically dogs with proven infection – those from which parasites have been isolated. But not all parasitized dogs are expected to be seropositive, so parasitism is not a gold standard for serology. In our study, only 87% of samples yielding parasites came from dogs which were simultaneously seropositive.

We conclude that, with the assumption that negative titres are distributed lognormally, the method of subtracting a fitted negative distribution will give the most accurate estimate of the proportion positive in a sample, and that is often what is required in epidemiological studies. An intrinsic cut-off will probably give a similar estimate of the number of positives, as the number of false positives and false negatives tend to cancel out. Cut-off points do perform less efficiently when used to determine whether a given individual is infected, especially when the overlap between the distributions of negative and positive titres is large. However, although we commonly want to know whether a particular individual is infected or not, this is often for differential diagnosis. Leishmaniasis patients usually have high antibody titres, lying well above the zone of ambiguity (Dye, 1994).

Compared with cross-sectional analysis, a cohort study ought to provide a more accurate estimate of the number of seropositives because we know the sequence of positive and negative titres and the rate of change of antibody titres. In fact, we found that the extra information made little difference in this study, since most dogs were consistently positive after the first positive sample. However, the great advantage of longitudinal data is that they allow direct observation of the number of seroconversions. We explored 3 possible methods of estimating the number of seroconversions, based on the number of antibody units, the rate of change of antibody units, and a mixed method combining information of both kinds. All give reassuringly similar results.

Longitudinal data also allow for a more accurate assessment of the rate at which hosts lose detectable antibody. Our estimate of the *per capita* reversion rate was very low, only 0·0002/day, whereas Courtenay *et al*. (1994) recorded reversion rates of 0·0003–0·005/day. It is possible that reversion is time dependent, although this did not appear to be the case here. Alternatively, the difference may lie in the fact that most of our dogs were imported from Belém. They appeared to be more susceptible to diseases including leishmaniasis, and had higher death rates than Marajó dogs (O. Courtenay, R. J. Quinnell & C. Dye, unpublished data).

The separation of infection from serological conversion has not previously been done in longitudinal studies of canine leishmaniasis (Corredor *et al*. 1989; Vidor *et al*. 1991). Our data clearly show that parasites can be isolated from dogs up to 8 months before seroconversion and, as we have already remarked, the pre-patent period is a significant fraction of the mean time to infection. Previous estimates of pre-patent period from field or laboratory data vary from < 30 days to 120–150 days; the shorter periods are associated with greater inoculating doses in experimental infections (Lanotte *et al*. 1979; Abranches *et al*. 1991; Dye *et al*. 1993). Our estimates of conversion rates suggest that all infected dogs did eventually seroconvert, and this is again confirmed by the parasitological data. In contrast, other studies suggest that a fraction of exposed dogs do not seroconvert, at least after a single exposure (Dye *et al*. 1993; Killick-Kendrick *et*

al. 1995). However, these studies used healthy European dogs, which may be able to mount a stronger and swifter cellular immune response.

The incidence estimates can be used, together with data on dog life-expectancy, to calculate the basic case reproduction number from the formula $R_0 = 1 + L/A$ (Appendices 1 and 2). This calculation of R_0 makes the assumption that the incidence rate in dogs which become infectious is the same in those which do not. If so, we can ignore the uninfectious dogs: we do not need to know whether seroconversion in these dogs results from successful infection or unsuccessful parasite challenge, nor the fraction of dogs which become infectious. Use of this formula also assumes that the death rate of infectious dogs (by contrast with susceptibles and latents) is not markedly elevated by leishmaniasis (Appendix 1). In fact, the death rate of infectious dogs probably *is* higher. Our estimate of R_0 may therefore be too large, but we cannot presently say by how much.

R_0 is perhaps the best single indicator of the magnitude of the disease control problem. Since our estimate of $R_0 = 5{\cdot}9$ is calculated from mean incidence, it indicates the mean effort required, over each yearly transmission cycle, to ensure that *L. infantum* infection dies out in the long term. Thus, if it were possible to immunize dogs, we would have to maintain a coverage of more than $100(1-1/R_0) =$ 77–86% for elimination (though infection could perhaps be maintained in the wild fox population). R_0 here does not measure the maximum seasonal potential for an outbreak, nor can we obtain that quantity from our seasonal incidence rates. So we cannot determine from these data (or from the deterministic theory in Appendix 2) how to ensure that a dog population never suffers an epidemic of *L. infantum* infection.

Neither does our calculation of R_0 account for spatial heterogeneity in transmission, although we know from entomological studies that this is likely to be large (Quinnell & Dye, 1994). One approach, used by Woolhouse *et al*. (1997), is to examine heterogeneity in the distribution of vectors/host across the endemic area. Using data on the abundance of sandflies and dogs in different villages on this part of Marajó Island, they estimate that R_0 could be 3·4 times greater than assumed under homogeneous mixing. The precise magnitude of this factor is questionable because the data describe the distribution of sandflies among animal pens, rather than on dogs, but the calculation usefully cautions that R_0 might be as high as 20.

Elsewhere we have shown that, for $R_0 \approx 10$, the most effective methods of canine and human visceral leishmaniasis control should in principle be insecticide application, vaccination (dog or human), nutritional improvement (mainly children), drug treatment (dogs) and culling, in roughly that order (Dye, 1996). This conclusion is not particularly sensitive to the precise magnitude of R_0 and is actually more robust for $R_0 < 10$. These predictions come from epidemiological theory and data; the need now is to test them in carefully designed field trials. Surprisingly, the definitive insecticide trial remains to be done (Kelly, Mustafa & Dye, 1997), but is especially desirable in endemic areas where the vector lives peridomestically and can be attacked in houses and animal pens, as with *Lutzomyia longipalpis* in Amazon Brazil. As no vaccine is yet available, the relation between nutrition and susceptibility is poorly understood, and treatment or culling of dogs is expensive and of limited effectiveness, insecticide trials are a priority on grounds of both feasibility and expected relative efficacy.

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APPENDIX 1

Estimation of R_0 *for canine leishmaniasis in a stable endemic area*

Here, following Dietz (1975) on measles, we derive an expression which can be used to estimate the basic case reproduction number from data collected in areas where canine leishmaniasis is stably endemic. We begin with a set of coupled, non-linear, partial differential equations which describe the change in numbers of susceptible (S), latent (L) and infective (I) dogs ($N = S+L+I$) with time (t) and age (a):

$$\frac{\partial S(t,a)}{\partial t} + \frac{\partial S(t,a)}{\partial a} = \partial N + \alpha I(t,a) - (\lambda+\delta)\, S(t,a) \qquad \text{(A 1)}$$

$$\frac{\partial L(t,a)}{\partial t} + \frac{\partial L(t,a)}{\partial a} = \lambda S(t,a) - (\sigma+\delta)\, L(t,a) \qquad \text{(A 2)}$$

$$\frac{\partial I(t,a)}{\partial t} + \frac{\partial I(t,a)}{\partial a} = \sigma L(t,a) - (\alpha+\delta)\, I(t,a) \qquad \text{(A 3)}$$

$$\text{and } S(t,0) = \delta N + \alpha \int_0^\infty I(t,a)\, \partial a \qquad \text{(A 4)}$$

Notice that this set of equations deals only with dogs which can potentially become infectious, and not with dogs which are for some reason refractory (e.g. because they mount an effective cellular immune response); λ is the *per capita* rate at which dogs acquire infection from sandflies and σ is the rate at which they move from the latent to the infectious class; α is the mortality rate imposed on infective dogs by leishmaniasis, over and above the disease-independent rate δ.

It is well-known for vector-borne diseases that the basic case reproduction number of infection, R_0, takes the form:

$$R_0 = \frac{C\sigma}{(\sigma+\delta)(\alpha+\delta)}, \quad \text{(A 5)}$$

which is the product of the vectorial capacity (C), the proportion of dogs which survive the latent period ($s_l = \sigma/(\sigma+\delta)$), and a dog's expectation of infective life ($L_i = 1/(\alpha+\delta)$). Because the vectorial capacity is very difficult to measure (Dye, 1992), we want to replace C in eqn (A 4) by parameters from eqns (A 1–A 3), which are more tractable. We begin by identifying the incidence rate in dogs as the infective biting rate of sandflies:

$$\lambda = \frac{abI_v}{N} \quad \text{(A 6)}$$

in which I_v is the size of the infective vector (sandfly) population, a is the daily biting rate of a female sandfly on dogs, and b is the proportion of infective sandflies which actually transmit infection when taking a bloodmeal. Equation (A 5) demands an expression for I_v, and we have previously shown (Dye & Williams (1995) following others) that

$$I_v = \frac{Vab'I\exp(-\mu\tau)}{\mu N + ab'I}, \quad \text{(A 7)}$$

where μ is the female sandfly mortality rate, τ is the duration of the latent period in sandflies and b', in symmetry with b, is the proportion of uninfected sandflies which acquire infection when taking a bloodmeal from an infective dog (I). The vectorial capacity is

$$C = \frac{Va^2bb'\exp(-\mu\tau)}{\mu N}. \quad \text{(A 8)}$$

Using eqns (A 6) and (A 7) together,

$$I_v = \frac{C}{ab[(N/1)+(ab'/\mu)]}. \quad \text{(A 9)}$$

We now evaluate I in eqn (A 8) by assuming that infection is stably endemic in the dog population. So we can drop the time-dependency in eqns A 1–A 3, and find solutions for S, L and I with respect to age only. By standard methods,

$$S(a) = N'\exp(-(\lambda+\delta)a) \quad \text{(A 10)}$$

$$L(a) = \frac{\lambda N'}{\sigma-\alpha}[\exp(-(\lambda+\delta)a) - \exp(-(\sigma+\delta)a)] \quad \text{(A 11)}$$

$$I(a) = \frac{\lambda\sigma N'\exp(-(\alpha+\delta)a)}{\sigma-\lambda}\left[\frac{\exp((\alpha-\lambda)a)-1}{\alpha-\lambda} + \frac{\exp((\alpha-\sigma)a)-1}{\alpha-\sigma}\right]. \quad \text{(A 12)}$$

Here, $N' = \delta N + \alpha I^*$, and I^* is the total number of infective dogs of all ages at equilibrium. This total is found by integrating eqn (A 11):

$$I^* = \int_0^\infty I(a)\,da = \frac{\delta\lambda\sigma N}{(\alpha+\delta)(\lambda+\delta)(\sigma+\delta) - \alpha\lambda\sigma}. \quad \text{(A 13)}$$

Substituting for I in eqn (A 8), then for I_v in eqn (A 5), and finally for C in eqn (A 4), we get

$$R_0 = 1 + \frac{\lambda}{(\sigma+\delta)} + \frac{\lambda\sigma(1+ab'/\mu)}{(\alpha+\delta)(\sigma+\delta)}. \quad \text{(A 14)}$$

A simpler interpretation of eqn (A 13) emerges from the following observations, to add to those above: $1/\lambda = A =$ the average time taken for a dog to acquire infection; $1/(\sigma+\delta) = L_l =$ the latent period of infection in dogs; $1/ab' = A_v =$ the average time taken for a vector to acquire infection as a result of biting infective dogs (I); and $1/\mu = L_v =$ the life-expectancy of an infective sandfly. Then, more transparently,

$$R_0 = 1 + \frac{L_l + s_l L_i(1 + L_v/A_v)}{A}. \quad \text{(A 15)}$$

Notice how the bracketed vector term $1 + L_v/A_v$, trivial though it is (because usually $L_v \ll A_v$), nestles neatly in the numerator, reflecting in miniature the structure of the more important terms for host infection. Neglecting the vector term, eqn (A 14) becomes:

$$R_0 = 1 + \frac{L_l + s_l L_i}{A}. \quad \text{(A 16)}$$

When $\alpha \ll \delta$, eqn (A 15) converges to Dietz's (1975) now-classic $R_0 = 1 + L/A$, in which L stands for life-expectancy. This is true even though Dietz first derived this expression for an infection which induces lifelong immunity, whereas an infective dog in our model remains infective for the rest of its life. The two approaches suggest different intuitive interpretations of the condition for persistence: Dietz's formula says that, for infection to persist, the average individual must live long enough to get infected; for canine leishmaniasis, eqns (A 14) and (A 15) say, as expected, that R_0 depends on how much the rate of gain of infected dogs exceeds the rate of loss.

APPENDIX 2

Estimation of R_0 *when transmission varies seasonally*

The abundance of the sandfly vectors of leishmaniasis changes seasonally and so, therefore, do R_0 (as $R_0(t)$) and the incidence and prevalence rates in the dog population. How do we use eqn (A 14) under these circumstances?

Various approaches have been taken to the problem of infectious and vector-borne disease persistence in periodic environments (e.g. Heesterbeek & Roberts, 1995; Lord *et al.* 1996). Here, specifically, we want to know whether the net value of the basic case reproduction number, across the typical 1-year cycle (call it $\bar{R}_0$), is greater than unity. First, it is possible to show formally (Williams & Dye, 1997), for a deterministic system in which transmission rate varies periodically, that infection will persist when $\bar{R}_0 > 1$, provided $\bar{R}_0$ is the arithmetic mean (rather than the geometric mean) value of $R_0(t)$. In practice, we measure seasonal variation in the incidence rate, not $C(t)$, and the question then arises of how well the average value of the incidence rate ($1/\bar{A}$) estimates $\bar{R}_0$ via eqns (A 14) or (A 15).

One answer comes from numerical simulation of the model described by eqns (A 1–A 3). We produced stable cycles of incidence and prevalence with $C(t)$ fluctuating sinusoidally around a mean of 0·145/week, and constants $\delta = 0{\cdot}01$/week, $\sigma = 0{\cdot}1$/week, $\alpha = 0$, and $N = 963$. The amplitude of the variation in $C(t)$ is expressed in column 1 of Table A 1 as $(C(t)_{\max} - \bar{C})/\bar{C}$. With these parameter values, eqn (A 4) gives $\bar{R}_0 = 13{\cdot}132$ for an infection which is stably endemic, and this is the value we want to estimate by measuring the changes in incidence.

Table A 1. Estimates of R_0 obtained from a simulation model of canine leishmaniasis

Amplitude of $C(t)$	$R_0 = N/\bar{S}$ Arithmetic mean	$R_0 = 1 + L/\bar{A}$ Arithmetic mean	$R_0 = 1 + L/\bar{A}$ Geometric mean
0	13·132	13·132	13·132
0·25	12·870	13·105	12·906
0·5	12·222	13·043	12·211
0·75	11·246	12·939	10·859
0·999	10·031	12·775	6·990

Table A 1 shows that $\bar{R}_0$ is estimated quite efficiently by eqn (A 15) in the face of seasonal variation in the transmission rate, so long as we use the arithmetic mean value of incidence as in column 3 (cf. geometric mean in column 4). For comparison, column 2 of Table A 1 estimates $\bar{R}_0$ from the related formula $N/\bar{S}$, where $\bar{S}$ is the arithmetic mean of $S(t)$. The results compare poorly with those in column 3. The reason for the difference is that incidence, and hence $A(t)$, is very closely correlated with $C(t)$ (typically $r > 0{\cdot}99$ in these simulations). So oscillations in $C(t)$ and $A(t)$ have the same form and frequency; we can expect $\bar{A}$ to reflect $\bar{C}$, and it is $\bar{C}$ which determines long-term persistence. By contrast, $S(t)$ responds to $C(t)$ with a marked time delay: the number of susceptibles starts to rise as soon as $C(t)$ falls below its maximum, and continues to rise after $C(t)$ has reached its minimum. Consequently, there are too many susceptibles during the average cycle.

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