Development of immunodiagnostic tests for leprosy: from biomarker discovery to application in endemic areas

Hooij, A. van

8 Chapter

The anti-PGL-I antibody response in naturally *M. leprae* infected squirrels, a free-roaming animal model

Adapted from:

Detection of humoral immunity to mycobacteria causing leprosy in Eurasian red squirrels (Sciurus vulgaris) using a quantitative rapid test[§]

&

Clinical Progression of leprosy in Eurasian red squirrels (Sciurus vulgaris) in A Naturally infected wild population¶

Anna-Katarina Schilling[1*], Anouk van Hooij[2,*], Paul L.A.M. Corstjens[3], Peter W.W. Lurz[1], Jorge DelPozo[1], Darren Shaw[1], Karen Stevenson[2], Anna Meredith[1,4] and Annemieke Geluk[2]

[1]The Royal (Dick) School of Veterinary Studies and The Roslin Institute, University of Edinburgh, Easter Bush Campus, Midlothian, Edinburgh;
[2]Dept. of Infectious Diseases, Leiden University Medical Center, The Netherlands;
[3]Dept. Cell and Chemical Biology, Leiden University Medical Center, The Netherlands;
[4]Melbourne Veterinary School, Faculty of Veterinary and Agricultural Sciences, The University of Melbourne Parkville Campus, Melbourne.

* these authors contributed equally

[§]Eur J Wildl Res 65, 49 (2019)
.J Zoo Wildl Med. *In press*

Abstract

Eurasian red squirrels (Sciurus vulgaris, ERS) in the British Isles are a recently discovered natural host for *Mycobacterium leprae* and *Mycobacterium lepromatosis*. Infected squirrels can develop skin lesions or carry the bacteria without showing clinical signs. Until now the clinical diagnosis of leprosy could only be confirmed in squirrels by isolating DNA of leprosy bacilli from carcasses or by establishing the presence of acid-fast bacilli in skin sections of carcasses with clinical signs. In this study, we assessed the performance of a field-friendly diagnostic test for detection of *M. leprae*/*M. lepromatosis* infection in ERS. This up-converting phosphor lateral flow assay (UCP-LFA) is well established for detection of *M. leprae* specific anti-phenolic glycolipid-I antibodies (αPGL-I) IgM antibodies in humans and associated with bacterial load. Assessment was performed on serum and blood drops from live squirrels. Clinically diseased squirrels showed significantly higher αPGL-I antibody levels than healthy animals or subclinically infected animals ($p < 0.0001$), both in serum and whole blood drop samples. Subclinically infected animals were identified using molecular methods to detect the presence of leprosy bacilli DNA in punch biopsy tissue samples. This study shows that the αPGL-I UCP-LFAs presented here allows a field-friendly serological confirmation of *M. leprae* infection in clinically diseased live ERS. For surveillance purposes, the combination of clinical assessment, αPGL-I UCP-LFAs, and molecular methods allow the identification of both diseased animals and subclinically infected animals.

Introduction

Leprosy was first described in Eurasian red squirrels (*Sciurus vulgaris*; ERS) in 2014 (1). Since then, DNA of the causative mycobacteria (*Mycobacterium leprae* and *Mycobacterium lepromatosis*) has been detected in ERS populations throughout the British Isles (2-5), but not on the European continent (6, 7). Individual squirrels may carry leprosy bacteria without showing clinical signs (2) or present pathognomonic clinical lesions with individual variation (1). The most clinically similar disease in ERS is atypical histiocytosis, which has been described only in a few animals in Scotland (8). Differential diagnosis using histological and molecular methods is possible using carcasses but for live ERS accurate diagnosis is more challenging. Minimally invasive rapid tests would provide a field-friendly and humane method to confirm clinical diagnosis of leprosy.

In humans levels of antibodies against the *M. leprae*-specific phenolic glycolipid I (αPGL-I) closely correlate with bacterial load and higher risk of developing leprosy. αPGL-I serology is used to detect infections with leprosy bacilli in humans (9) and nine-banded armadillos (Dasypus novemcinctus) (10). Previously, a qualitative lateral flow test was used to detect αPGL-I in body fluid samples of ERS (2). However, this test lacked a quantified read-out, was subject to operator bias, and test results could not be correlated with disease severity. In humans, lateral flow assays (LFAs) combined with up-converting phosphor (UCP) reporter particles as a quantitative label to assess αPGL-I antibody levels (αPGL-I UCP-LFA) are highly sensitive, field friendly, low-complexity diagnostic tools in leprosy endemic areas (11). We investigated the applicability of an αPGL-I UCP-LFA in ERS for diagnostic purposes using different sample types; serum and blood drops from live squirrels.

Materials and methods

Sampling and ethical approval

Samples were obtained from two squirrel populations (Isle of Arran, Ayrshire; Brownsea Island, Dorset) in which leprosy had been confirmed previously (2). They were collected between 2016 and 2018 from 90 different ERS (87 adult, 3 sub-adult). Field-based clinical assessment and sampling took place under general isoflurane anesthesia, following a previously published protocol (12). Once fully recovered from anesthesia, ERS were released at the trapping site. A microchip was placed subcutaneously between the shoulder blades as permanent identification to document repeated assessments of individual ERS. Since the population was free-living, return and re-assessment of a previously trapped individual could not be guaranteed. Scanning for the presence of a microchip was always performed at the end of an assessment, to avoid subconscious bias in lesion assessment

and categorization. To be able to compare information from individual ERS seen for the first time at different sessions, results were noted as a timeline of 0 to a maximum of 24 months (up to 5 time points).All procedures took place under Home Office license authority (Project license 70/9023), Natural England License 2016–24517-SCI-SCI and with ethical approval from the University of Edinburgh's Animal Welfare and Ethical Review Body.

Diagnostic methods to establish the leprosy status of ERS

At the time of sampling, all animals were clinically assessed by a veterinarian and grouped according to the absence or presence of clinical pathognomonic leprosy lesions (areas of alopecia, shininess, and firm rubbery swelling of the skin). Those with lesions were classified into four severity categories (1 = mild, 2 = mild-moderate, 3 = moderate, 4 = severe) derived from an additive numerical (2–96) score assigned after assessing the number of affected body areas, size, shape, and ulceration of lesions (Supplementary Table S1). For 64 live ERS without clinical lesions, a small tissue punch sample was taken from the left ear under general anesthesia. Tissue punch samples from clinically diseased ERS were not collected to avoid altering the progression of lesions in the first four sampling sessions (autumn 2016 to spring 2018), but were collected from all ERS assessed in the final session (autumn 2018). The presence of leprosy bacilli DNA in these tissues was assessed via PCR as described by Avanzi et al. (2016).

Blood samples were taken from the femoral vein under general anesthesia. Serum samples (n = 132) were prepared at room temperature by centrifugation (10 min/2000g) and were either used immediately or stored at − 20 °C until required. Blood drop samples (n = 65) were obtained either using remaining blood in the syringe after ejection of the whole blood sample (n = 26) or via a skin prick using disposable 20-µL Minivette® collection tubes (heparin coated; Sarstedt) (n = 39). Where the blood flow from the prick site was insufficient to fill the minivette, filling was completed from the whole blood sample. Full details of samples are provided in Online Resource 1 (https://link.springer.com/article/10.1007/s10344-019-1287-1#Sec5). The information obtained from clinical assessment, serological and molecular diagnostics were combined to establish each ERS' leprosy status (Supplementary Table S2).

αPGL-I UCP-LFA

The αPGL-I UCP-LFA were produced as described previously (13). Briefly, the nitrocellulose of the LF strips was provided with a test line (T) of 100 ng synthetic PGL-I and a 50-ng rabbit anti-goat IgG (G4018, Sigma-Aldrich) flow control line (FC). The reporter, 85-nm-

sized NaYF4:Yb3+, Er3+ up-converting reporter particles (UCP; Intelligent Material Solutions Inc., Princeton, NJ, US) was covalently coated with 125 µg goat anti-human IgM (I0759, Sigma-Aldrich) per mg UCP (14) and 200 ng dried on the conjugate-release pad of the LF strip. LF strips were stored at ambient temperature in plastic containers with silica dry pad. Irrespective of type (serum, blood drop) samples were diluted 50-fold in LF assay buffer (100 mM Tris pH 7.2, 270 mM NaCl, 1% (v/v) Triton X-100, 1% (w/v) BSA). The diluted sample (50 µl) was applied to the PGL-I strips and immunochromatography continued until strips were dry.

Data analysis

LF strips were scanned in a Packard FluoroCount microtiter plate reader adapted for measurement of the UCP label (980 nm IR excitation, 550 nm emission) (15). Test results were displayed as ratio (R) between T and FC signals (550 nm emission) measured upon IR excitation.

Graphpad Prism version 7.00 for Windows (GraphPad Software, San Diego CA, USA) was used to perform Mann-Whitney U tests, one-way ANOVA for non-parametric distribution (Kruskall-Wallis), and Dunn's correction for multiple testing and to plot ROC curves and calculate the area under curve (AUC). Cut-offs were calculated using Youden's index (16).

Results

αPGL-I antibody levels indicate clinical leprosy and correlate to disease severity

Only adult animals showed clinical signs of leprosy or tested positive for the presence of *M. leprae* DNA. *M. lepromatosis* DNA was not detected in this study. Detailed information on all ERS including clinical category and lesion score is presented in Online Resource 1 (https://link.springer.com/article/10.1007/s10344-019-1287-1#Sec5).

Of the serum samples analyzed, 25 were from ERS with pathognomonic leprosy lesions, 11 from individual ERS with no clinical signs but detectable *M. leprae* DNA, 53 from ERS with no lesions or detectable *M. leprae* DNA, and 43 samples from ERS with no lesions and from which no tissue sample was available. Elevated αPGL-I antibody levels clearly discriminated clinically positive ERS from clinically negative/PCR positive ($p < 0.0001$; AUC 0.94) and clinically negative/PCR negative ERS ($p < 0.0001$; AUC 0.96; Figure 1a). However, they did not significantly differ between clinically negative/PCR positive ERS and clinically negative/PCR negative ERS ($p > 0.9999$). The UCP-LFA has a sensitivity of 88% and a specificity of 96% in sera for detection of *M. leprae* infection in clinically diseased animals (cut-off ratio > 0.1).

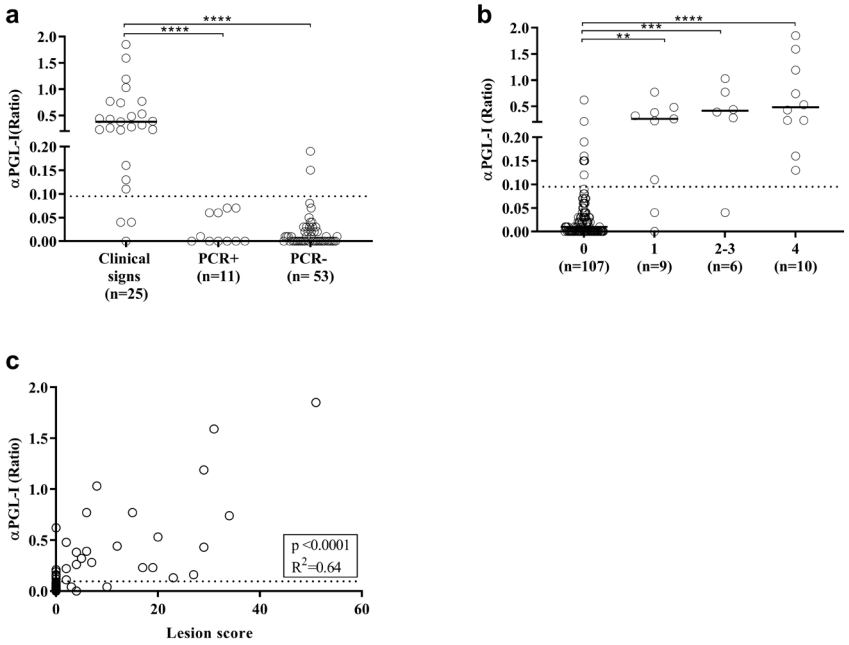


Figure 1: αPGL-I antibody levels correlate with clinical presentation of leprosy in squirrels. αPGL-I antibody levels were determined by UCP-LFA and ratio values are displayed on the y-axis. The cut-off for positivity (R = 0.1) is indicated by the dotted line. Comparisons were made using Kruskal-Wallis tests with multiple Dunn's correction. **a** Comparison of serum αPGL-I antibody levels from ERS with clinical lesions with ERS PCR+ without lesions or ERS negative for both PCR and lesions (only animals with clinical disease or tissue sampled for PCR included). **b** Comparison of serum αPGL-I antibody levels from ERS classified into different lesion categories ranging from negative (0) to severe (4). **c** Comparison of serum αPGL-I antibody levels from ERS classified according to severity of lesions expressed by a continuous numerical score (Pearson's correlation). **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001

The αPGL-I antibody levels showed to correspond with disease severity; for ERS in category 1 elevated levels of αPGL-I antibodies could be detected (p = 0.0012; AUC 0.88; Figure 1b) compared with ERS lacking lesions, but this difference became more significant for animals with lesions of a higher category (2–3: p = 0.0005; AUC 0.96; 4: p < 0.0001; AUC: 0.99. Figure 1b). By representing the clinical signs as a continuous numerical score a significant correlation (p < 0.0001; $R^2 = 0.64$) between αPGL-I antibody levels and severity of the lesions confirmed this observation (Figure 1c).

Longitudinal αPGL-I antibody levels correlate to disease progression

A total of 31 ERS were trapped in multiple assessment sessions. Two ERS were assessed the maximum of five times (i.e. at 0, 6, 12, 18 and 24 months), two were assessed at four time points, 12 at three time points and 15 at two time points. Eight (25.8%) ERS were

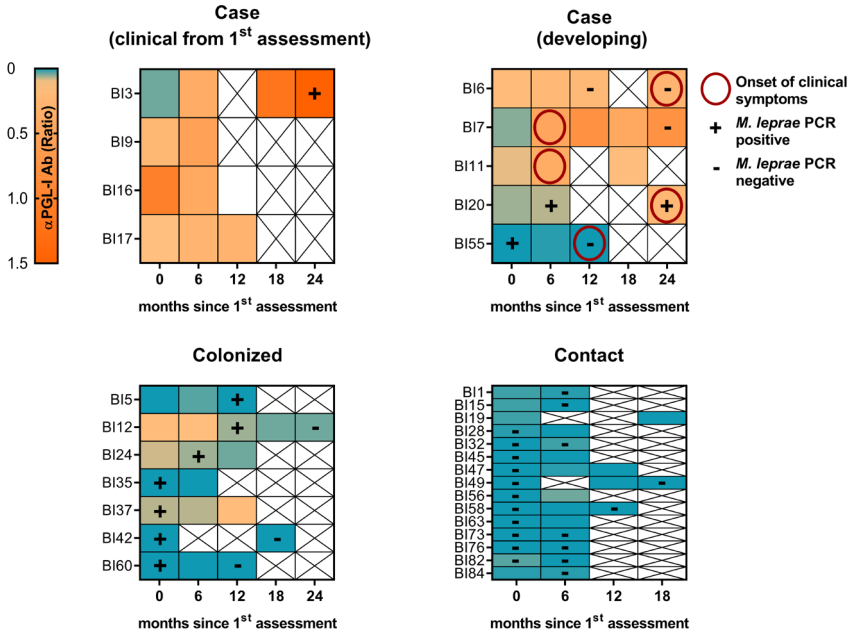


Figure 2 Longitudinal anti-phenolic glycolipid-I (PGL-I) antibody levels and presence of *M. leprae* DNA in 31 free-roaming Eurasian red squirrels (Sciurus vulgaris). Squirrels were assessed for αPGL-I antibody levels at all time points of assessment, with a maximum of 5 timepoints (Supplementary Figure S1). The color indication at each timepoint corresponds to the Ratio value observed in the αPGL-I antibody lateral flow assay. Blue indicates Ratio values below the cut-off for positivity determined for this study (0.1) and orange represents Ratio values above this cut-off. PCR was performed to determine the presence of *M. leprae* DNA for the time points where either a plus (+, PCR positive) or a minus (-, PCR negative) is indicated. Squirrels were divided in 4 groups; Clinical leprosy, squirrels that showed clinical signs at initial assessment (top left panel). Developing leprosy, squirrels that developed leprosy during the study, timepoint of leprosy diagnosis is indicated with a red circle (top right panel). Colonized squirrels, colonized with *M. leprae* at a certain time point during the study showing no signs of leprosy (bottom left panel). Contact ERS, squirrels without symptoms of leprosy and no presence of M. leprae DNA (bottom right panel).

identified at some point during the study as leprosy cases, one as leprosy suspect (3.2%, included with leprosy cases in figures, BI55), seven as colonized (22.6%) and 15 (48.4%) as contacts (Figure 2). Three out of four squirrels that already presented with symptoms at the first clinical assessment showed αPGL-I antibody levels above the cut-off for positivity during the entire study and one from the six month timepoint until the end of the study. This animal (BI3) could be followed for two years, displaying an increase in the αPGL-I antibody levels over time (Supplementary Figure S1). Longitudinal αPGL-I antibody levels in the squirrels that developed leprosy symptoms during the study showed a clear association with the appearance of clinical symptoms. All subjects with symptoms except the suspect (BI55) showed positive αPGL-I antibody titers in the 2-year study period. Only

in one animal (Bl6), the antibodies were detected before the onset of clinical symptoms, in the other 3 animals the antibodies were detected at the appearance of clinical symptoms

In the colonized animals αPGL-I antibodies were hardly detected, two out of seven had αPGL-I antibody levels above the cut-off for positivity (Figure 2). However, the R-values in these two ERS were not as high as observed in the confirmed clinical cases (Supplementary Figure S1). Of the contacts, in which no *M. leprae* DNA was detected, the αPGL-I antibody levels remained below the cut-off for positivity at all timepoints. It is apparent from these data that αPGL-I antibody levels correspond to the appearance of clinical symptoms, rather than *M. leprae* infection.

αPGL-I antibody levels in blood drops

We assessed the performance of αPGL-I UCP-LFA on blood drop samples by comparing results for 65 sample pairs for which both serum and blood drop samples were collected from the same ERS at the same time point. Eight of these sample pairs were from ERS with lesions and 57 from ERS without lesions, in five of the latter, *M. leprae* DNA was detected. αPGL-I levels showed a significant correlation ($p < 0.0001$; $R^2 = 0.9$), indicating the compatibility of αPGL-I UCP-LFA with blood drops (whole blood) as well as serum (Figure 3).

This offers the potential to reduce the impact of sampling on the animal. However, we found it difficult to reliably get sufficient blood drop formation in the prick sites that were evaluated (ear, front and hind foot, tail; selected on the basis of accessibility without risk of injury to ERS and handler in a handling cone). Prick sites either did not bleed enough or the blood drop dispersed along the fur, even if it was clipped very short. Limited success was achieved on the underside of the last third of the tail by clipping the fur very short, disinfecting the site with ethanol and warming the tail on a heat pad prior to pricking.

Discussion

The ability to study squirrels developing leprosy is an unique opportunity to gain insight in the antibody response to *M. leprae* before the onset of clinical symptoms. Using the αPGL-I UCP-LFA previously developed for humans (11, 17) antibody levels could be easily monitored in both serum and blood drops. These levels showed to correlate to the severity of disease and corresponded with the appearance of clinical symptoms. *M. leprae* infection without clinical symptoms is not detected using the UCP-LFA, requiring PCR. On the other hand, the PCR result was not positive for all squirrels that showed clinical symptoms and the unavailability of PCR data for all time-points does not enable a direct comparison of PCR and αPGL-I data in all samples. The αPGL-I UCP-LFA thus offers a useful

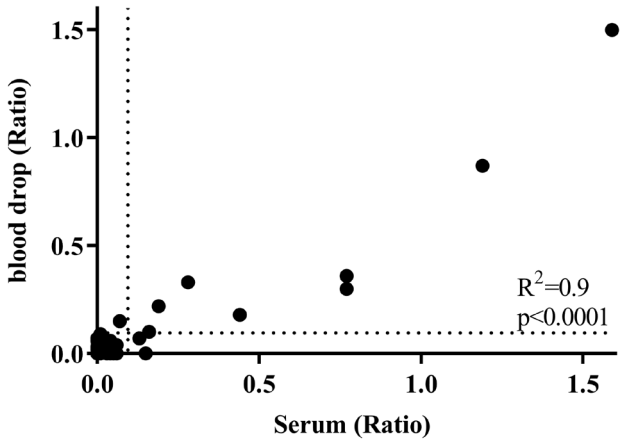


Figure 3: Significant correlation between αPGL-I levels in serum and blood drop samples. αPGL-I levels were detected by UCP-LFA in serum and blood drops of the same squirrels and Pearson's correlation indicated a significant correlation between both sample types.

rapid test to confirm clinical leprosy in ERS in the field.

For the identification of subclinical carriers, tissue sampling and molecular assessment for the presence of leprosy bacilli DNA was more sensitive. For three squirrels, *M. leprae* DNA was no longer detected at a later timepoint, suggesting that these animals were able to efficiently clear the mycobacterium without developing disease symptoms. In humans, αPGL-I antibodies are predominantly detected in leprosy patients with high bacterial loads and are difficult to detect in paucibacillary patients (11, 17). The observation that the highest αPGL-I antibody levels were observed in the animals with the most severe disease symptoms suggests that this correlation is also present in squirrels. qPCR enables the quantification of the *M. leprae* bacilli, which was not performed in this study, but would be of interest to confirm that the high αPGL-I antibody levels indeed correlate to a high bacterial load (18).

In line with observations in humans (19), αPGL-I antibody levels are poor predictors of the development of leprosy. Only in one squirrel αPGL-I antibody levels preceded the development of symptoms, but in general corresponded with the appearance of clinical symptoms. In the squirrels that did not develop clinical symptoms, both colonized and contacts, high αPGL-I antibody levels were not observed, again confirming the correlation with clinical disease.

Since none of the samples included in this study were derived from animals infected with *M. lepromatosis*, we cannot confirm that the UCP-LFA could be used to detect

infection with this bacterium in ERS. However, for humans, it is shown that αPGL-I-based immunodiagnostics are able to detect infections with *M. lepromatosis* as well (20). It will be important to verify this assumption particularly for surveillance efforts in ERS populations in which *M. lepromatosis* infections have been described to occur in more locations than *M. leprae* infections (2). Future efforts should investigate whether additional cellular immune markers can augment the identification of subclinically infected squirrels, something that has been done successfully in humans (17).

In summary, we present a field- and animal-friendly serological test to detect specific αPGL-I antibodies and confirm clinical leprosy in ERS. While it will be necessary to add other tools and/or additional biomarkers in animals that do not show visible signs of disease to estimate the prevalence of leprosy bacilli in this species, the αPGL-I UCP-LFA is a valuable tool to exclude or confirm clinical leprosy or severe infection in a captured squirrel with lesions.

Acknowledgements

We thank the local staff of the National Trust(Brownsea Island) and Dorset Wildlife Trust, especially Angela Cott, Timothy Hartley, John Lamming and Adam Poole, Luke Johns, Nicki Tutton, and Chris Thain (Brownsea Island), the National Trust for Scotland and Forestry Commission Scotland, especially Kate Sampson, Corinna Goeckeritz, Jackie Kemp, Amy Bennett, Andy Walker, Robert Logan, and Robin (Isle of Arran) for their invaluable help in preparing and executing our field research. We also thank Mizuho Suzuki, Katy Barrett, Anna George, Rebecca Amos, Julia Coats, and Amy Fish for their assistance in the field.

Funding information

This study was supported by the Q.M. Gastmann- Wichers Foundation (to AG), the Principal's Career Development PhD Scholarship by the University of Edinburgh (to AKS), and the Scottish Government Rural and Environment Science and Analytical Services Division.

Conflict of interest

The authors declare that they have no conflict of interest.

References

1. Meredith A, Del Pozo J, Smith S, Milne E, Stevenson K, McLuckie J. Leprosy in red squirrels in Scotland. Vet Rec. 2014;175(11):285-6.
2. Avanzi C, Del-Pozo J, Benjak A, Stevenson K, Simpson VR, Busso P, et al. Red squirrels in the British Isles are infected with leprosy bacilli. Science. 2016;354(6313):744-7.
3. Butler HM, Stevenson K, McLuckie J, Simpson. V. Further evidence of leprosy in Isle of Wight red squirrels. Vet Rec. 2017;180(16):407.
4. Schilling AK, Del-Pozo J, Lurz PWW, Stevenson K, Avanzi C, Shuttleworth CM, et al. Leprosy in red squirrels in the UK. Vet Rec. 2019;184(13):416.
5. Simpson V, Hargreaves J, Butler H, Blackett T, Stevenson K, McLuckie J. Leprosy in red squirrels on the Isle of Wight and Brownsea Island. Vet Rec. 2015;177(8):206-7.
6. Schilling AK, Avanzi C, Ulrich RG, Busso P, Pisanu B, Ferrari N, et al. British Red Squirrels Remain the Only Known Wild Rodent Host for Leprosy Bacilli. Front Vet Sci. 2019;6:8.
7. Tió-Coma M, Sprong H, Kik M, van Dissel JT, Han XY, Pieters T, et al. Lack of evidence for the presence of leprosy bacilli in red squirrels from North-West Europe. Transbound Emerg Dis. 2020;67(2):1032-4.
8. Smith SH, Stevenson K, Del-Pozo J, Moss S, Meredith A. Atypical Histiocytosis in Red Squirrels (Sciurus vulgaris). J Comp Pathol. 2017;156(4):446-50.
9. Spencer JS, Duthie MS, Geluk A, Balagon MF, Kim HJ, Wheat WH, et al. Identification of serological biomarkers of infection, disease progression and treatment efficacy for leprosy. Mem Inst Oswaldo Cruz. 2012;107 Suppl 1:79-89.
10. Truman RW, Shannon EJ, Hagstad HV, Hugh-Jones ME, Wolff A, Hastings RC. Evaluation of the origin of Mycobacterium leprae infections in the wild armadillo, Dasypus novemcinctus. Am J Trop Med Hyg. 1986;35(3):588-93.
11. van Hooij A, Tjon Kon Fat EM, van den Eeden SJF, Wilson L, Batista da Silva M, Salgado CG, et al. Field-friendly serological tests for determination of M. leprae-specific antibodies. Sci Rep. 2017;7(1):8868.
12. Schilling AK, Lurz PW, Meredith AL. Field anaesthesia of Eurasian red squirrels (Sciurus vulgaris). Vet Anaesth Analg. 2020;47(5):736-8.
13. van Hooij A, Tjon Kon Fat EM, Batista da Silva M, Carvalho Bouth R, Cunha Messias AC, Gobbo AR, et al. Evaluation of Immunodiagnostic Tests for Leprosy in Brazil, China and Ethiopia. Sci Rep. 2018;8(1):17920.
14. Bobosha K, Tjon Kon Fat EM, van den Eeden SJ, Bekele Y, van der Ploeg-van Schip JJ, de Dood CJ, et al. Field-evaluation of a new lateral flow assay for detection of cellular and humoral immunity against Mycobacterium leprae. PLoS Negl Trop Dis. 2014;8(5):e2845.
15. Corstjens P, Zuiderwijk M, Brink A, Li S, Feindt H, Niedbala RS, et al. Use of up-converting phosphor reporters in lateral-flow assays to detect specific nucleic acid sequences: a rapid, sensitive DNA test to identify human papillomavirus type 16 infection. Clin Chem. 2001;47(10):1885-93.
16. Fluss R, Faraggi D, Reiser B. Estimation of the Youden Index and its associated cutoff point. Biom J. 2005;47(4):458-72.
17. van Hooij A, Tjon Kon Fat EM, Richardus R, van den Eeden SJ, Wilson L, de Dood CJ, et al. Quantitative lateral flow strip assays as User-Friendly Tools To Detect Biomarker Profiles For Leprosy. Sci Rep. 2016;6:34260.
18. Tió-Coma M, Avanzi C, Verhard EM, Pierneef L, van Hooij A, Benjak A, et al. Genomic Characterization of Mycobacterium leprae to Explore Transmission Patterns Identifies New Subtype in Bangladesh. Front Microbiol. 2020;11:1220.
19. Penna ML, Penna GO, Iglesias PC, Natal S, Rodrigues LC. Anti-PGL-1 Positivity as a Risk Marker for the Development of Leprosy among Contacts of Leprosy Cases: Systematic Review and Meta-analysis. PLoS Negl Trop Dis. 2016;10(5):e0004703.
20. Singh P, Benjak A, Schuenemann VJ, Herbig A, Avanzi C, Busso P, et al. Insight into the evolution and origin of leprosy bacilli from the genome sequence of Mycobacterium lepromatosis. Proc Natl Acad Sci U S A. 2015;112(14):4459-64.

Supplementary material

Tables

Supplementary Table S1: Scoring of lesions

Points per body section*					
Points	**0**	**1**	**2**	**3**	**4**
Lesion size (mm)	None	< 2	< 5	< 10	> 10
Lesion description	None	A	B	C	D
Ulceration	None/N	T	–	–	Y
Ulcus description	None	–	Dry	Bleeding	Purulent

*To calculate the score four characteristics of lesion's present in each of the 6 body sections are assessed:

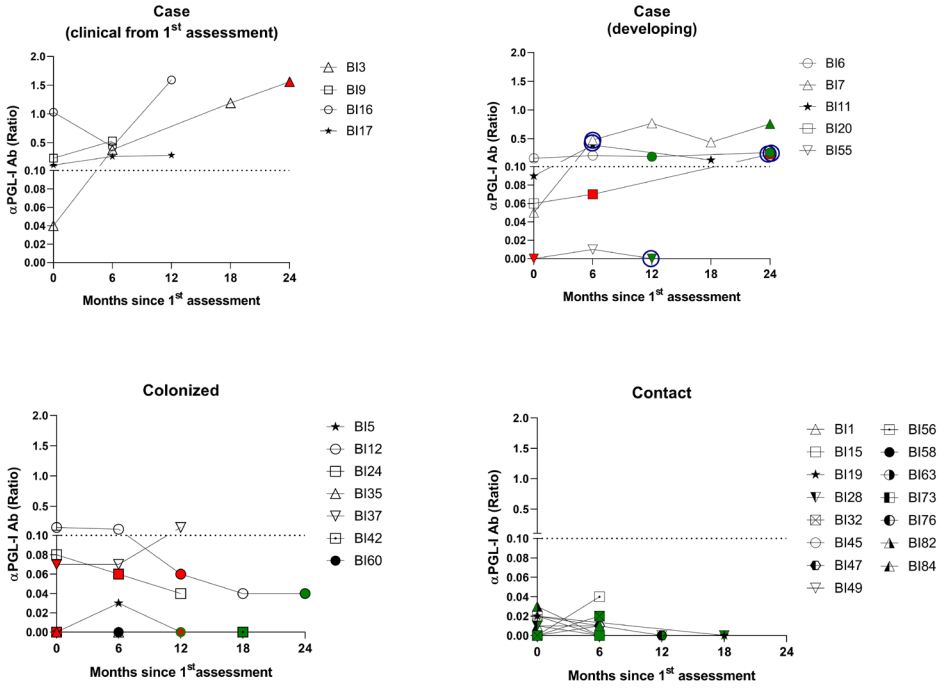
1. Lesion size (< 2, < 5, <10, > 10 mm)
2. Lesion description (*A*, one lesion with a clearly defined rim, or just alopecia; *B*, several lesions, separate with clearly defined rim; *C*, several lesions, rim not always clear/merging; *D*, cauliflower appearance due to excessive merging of several lesions)
3. Ulceration (traumatic injury or ulceration of the lesion are present or absent)
4. Ulcus description (dry, bleeding, or purulent)

The sum of the scores of all six body areas is used to calculate the total score per squirrel

Supplementary Table S2. Leprosy status definitions for Eurasian red squirrels based on clinical assessment and two diagnostic tests (anti-phenolic glycolipid-I antibody detection and PCR detection of *M.leprae* DNA)

Leprosy status	1. Clinical signs of leprosy	2. αPGL-I levels above positivity cut-off	3. *M. leprae* DNA detected in tissue
Leprosy case	Yes	Yes	Yes
	Yes	No	Yes
	Yes	Yes	No
Colonized squirrel	No	No	Yes
Leprosy contact (endemic area)	No	No/Yes	No
Leprosy suspect, further tests necessary	Yes	No	No

Figures



Supplementary Figure S1: Longitudinal anti-phenolic glycolipid-I (αPGL-I) antibody levels and presence of *M. leprae* DNA in 31 free-roaming Eurasian red squirrels (Sciurus vulgaris). Squirrels were assessed for αPGL-I levels at all time points of assessment, with a maximum of 5 timepoints. PCR was performed to determine the presence of *M. leprae* DNA for the time points indicated in red (PCR positive) or green (PCR negative) is indicated. Squirrels were divided in 4 groups; Clinical leprosy, squirrels that showed clinical signs at initial assessment (top left panel). Developing leprosy, squirrels that developed leprosy during the study, timepoint of leprosy diagnosis is indicated with a blue circle (top right panel). Colonized squirrels, colonized with M. leprae at a certain time point during the study showing no signs of leprosy (bottom left panel). Contact ERS, squirrels without symptoms of leprosy and no presence of M. leprae DNA (bottom right panel).