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METHODS PAPER

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

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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 and body cavity fluid samples from dead squirrels. Clinically diseased squirrels showed significantly higher αPGL-I levels than healthy animals or subclinically infected individuals ($p < 0.0001$), 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. In body cavity fluids, αPGL-I levels antibody levels were lower than in serum or blood drops. 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.

Keywords *Sciurus vulgaris* · *M. leprae* · *M. lepromatosis* · PGL-I · POC diagnosis · Leprosy

Anna-Katarina Schilling and Anouk van Hooij contributed equally to this work.

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Introduction

Eurasian red squirrels (*Sciurus vulgaris*; abbreviated to ERS) in the British Isles are the most recently discovered natural host for *Mycobacterium leprae* and *M. lepromatosis* (Avanzi et al. 2016). While ERS are widespread throughout Eurasia and classed as "least concern" in the IUCN Red List of Threatened Species, their numbers are declining in the British Isles where they are legally protected (Shar et al. 2016).

Individual squirrels may carry leprosy bacteria without showing clinical signs (Avanzi et al. 2016) or present pathognomonic clinical lesions with individual variation (Meredith et al. 2014). The most clinically similar disease in ERS is atypical histiocytosis, which has been described only in a few animals in Scotland (Smith et al. 2017). 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

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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 (Spencer et al. 2015) and nine-banded armadillos (*Dasypus novemcinctus*) (Truman et al. 1986). Previously, a qualitative lateral flow test was used to detect αPGL-I in body fluid samples of ERS (Avanzi et al. 2016). 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 levels (αPGL-I UCP-LFA) are highly sensitive, field friendly, low-complexity diagnostic tools in leprosy endemic areas (van Hooij et al. 2017). We investigated the applicability of an αPGL-I UCP-LFA in ERS for diagnostic purposes using three different sample types; serum and blood drops from live squirrels and body fluid collected from carcasses.

Materials and methods

Samples were obtained from two squirrel populations (Isle of Arran, Ayrshire; Brownsea Island, Dorset) in which leprosy had been confirmed previously (Avanzi et al. 2016). They were collected between 2016 and 2018 from 90 different ERS (87 adult, 3 sub-adult). Consecutive samples from the same animal were assessed separately for this study.

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 (Table 1 and details available upon request). For 64 live ERS without clinical lesions, a small tissue punch sample was taken from the left ear under general anesthesia. Tissue biopsies were not taken from live squirrels with clinical lesions ($n = 25$) or without lesions but showing skin peculiarities that could be physiological or turn into lesions ($n = 24$) to be able to follow their progress in a separate study. Ear tissue sections were taken from 24 carcasses, following a pilot study using 11 carcasses (unpublished data) identifying the ear as the best tissue for detection of *M. leprae* DNA in ERS. The presence of leprosy bacilli DNA in these tissues was assessed via PCR as described by Avanzi et al. (2016).

Table 1 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

Blood samples were taken from the femoral vein under general anesthesia. Serum samples ($n = 132$) were prepared at room temperature by centrifugation (10 min/2000*g*) 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. Body cavity fluid was collected during post mortem (PM) assessment from the thorax from 24 ERS carcasses (20 adult, 1 sub-adult, 2 juveniles, 1 age unknown), four of which were frozen immediately after sudden death or humane euthanasia and the remainder having been found dead in the wild and stored at − 20 °C until PM. Full details of samples are provided in Online Resource 1.

The αPGL-I UCP-LFA test strip materials were produced as described previously (van Hooij et al. 2018). 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 $NaYF_4$:Yb^{3+}, Er^{3+} 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 as described by Bobosha et al. (2014) 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, body cavity fluid), 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. 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) (Corstjens et al. 2001). Test results were displayed as ratio (R) between T and FC signals (550 nm emission) measured upon IR excitation.

Statistical analyses

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 (Fluss et al. 2005).

Results and discussion

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.

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 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; Fig. 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).

This suggests that the αPGL-I UCP-LFA offers a useful rapid test to confirm clinical leprosy in ERS in the field. However, for the identification of subclinical carriers, its tissue sampling and molecular assessment for the presence of leprosy bacilli DNA was more sensitive. This may be due to the fact that the test was optimized for detection of human antibodies.

Considering disease severity, while for ERS in category 1, elevated levels of αPGL-I could be detected ($p = 0.0012$; AUC 0.88; Fig. 1b) compared with ERS lacking lesions, 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. Fig. 1b). By representing the clinical signs as a continuous numerical score a significant correlation ($p < 0.0001$; $R^2 = 0.64$) between αPGL-I levels and severity of the lesions confirmed this observation (Fig. 1c).

Next, 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 (Fig. 2).

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.

As access to live animals is often limited in wildlife research, we assessed the performance of the UCP-LFA using body cavity fluid from seven carcasses with leprosy lesions, three carcasses without lesions but positive for *M. leprae* DNA, and 14 carcasses in which no *M. leprae* DNA was detected. Unfortunately, the correlation between clinical signs and positive serology was lower using body cavity fluid than blood samples. Only four of seven ERS (57%) with pathognomonic clinical signs of leprosy and PCR confirmed infection with *M. leprae* had test results above the positivity threshold. Three of these were frozen immediately after death, indicating that carcass decomposition time at room temperature prior to sampling may negatively impact the detectability of leprosy specific antibodies. Even in these three ERS, αPGL-I levels decreased from a ratio of 0.74, 1.85, and 0.16 ante mortem to 0.29, 0.28, and 0.11 PM, respectively. Furthermore, we had a positive result from the body fluid of an animal that had a negative serology prior to death, showed no clinical signs of leprosy, and from which no leprosy bacilli DNA could be isolated. Therefore, in ERS carcasses where welfare concerns no longer apply, we would currently recommend molecular methods for the diagnosis of leprosy above serological methods in ERS. However, when only few bacilli are present in an individual, even these methods may not identify all infected animals (Scollard et al. 2006).

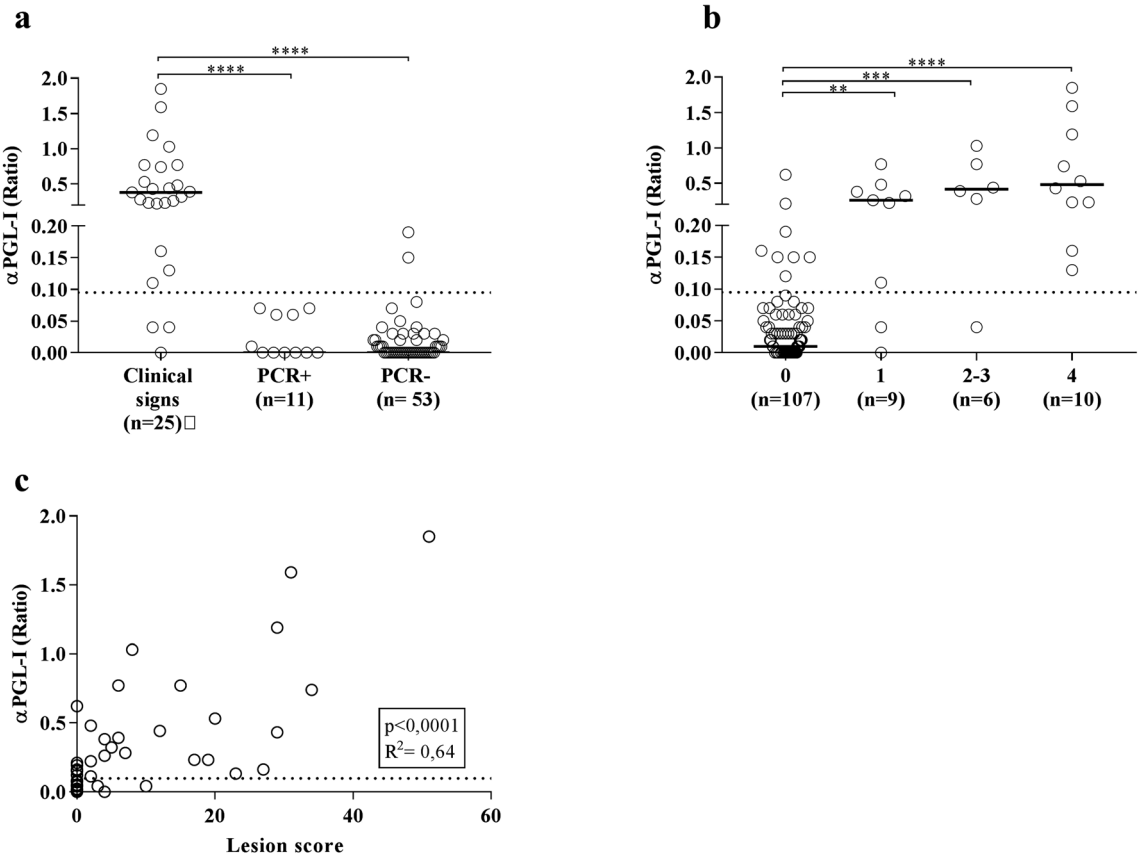

Fig. 1 αPGL-I levels correlate with clinical presentation of leprosy in squirrels. αPGL-I levels were determined by UCP-LFA and ratio values displayed on the *y* axis. The cut-off for positivity ($R = 0.1$) is indicated by the dotted line. Comparisons were made using Kruskal-Wallis tests with multiple Dunn's correction. **a** Comparison of serum αPGL-I 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 levels from ERS classified into different lesion categories ranging from negative (0) to severe (4). **c** Comparison of serum αPGL-I levels from ERS classified according to severity of lesions expressed by a continuous numerical score (Pearson's correlation). $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, $^{****}p \leq 0.0001$

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 (Singh et al. 2015). 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 (Avanzi et al. 2016). 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 (van Hooij et al. 2016).

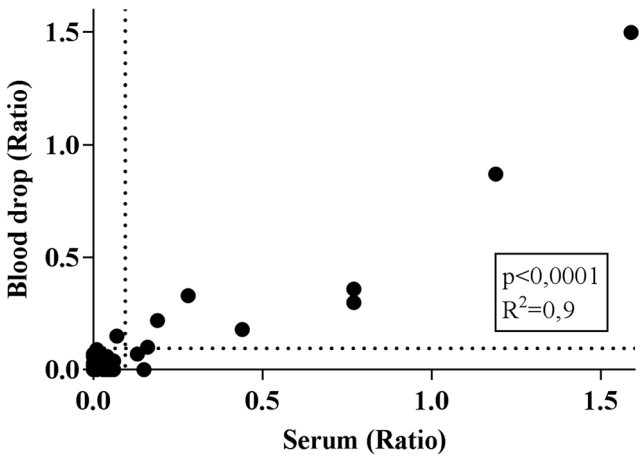

Fig. 2 Significant correlation between αPGL-I levels in serum and blood drop samples. αPGL-I levels were detected by UCP-LFA in serum and blood drops of the same squirrels and Pearson's correlation indicated a significant correlation between both sample types

In summary, we present a field- and animal-friendly serological test to detect specific αPGL-I and confirm clinical leprosy in ERS. While it will be necessary to add other tools (tissue sampling) 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.

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Compliance with ethical standards

Statement on the welfare of animals All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in this study were in accordance with the ethical standards of the Home Office (United Kingdom) and approved under Home Office Project Licence (PPL) 70/9023, Natural England Licence 2016–24,517-SCI-SCI, and Scottish Natural Heritage Licence 90,896.

Conflict of interest The authors declare that they have no conflict of interest.

References

Avanzi C, del-Pozo J, Benjak A, Stevenson K, Simpson VR, Busso P, McLuckie J, Loiseau C, Lawton C, Schoening J, Shaw DJ, Piton J, Vera-Cabrera L, Velarde-Felix JS, McDermott F, Gordon SV, Cole ST, Meredith AL (2016) Red squirrels in the British Isles are infected with leprosy bacilli. Science 354(6313):744–748

Bobosha K, Tjon Kon Fat EM, van den Eeden SJF, Bekele Y, van der Ploeg-van Schip JJ, de Dood CJ, Dijkman K, Franken KLMC, Wilson L, Aseffa A, Spencer JS, Ottenhoff THM, Corstjens PLAM, Geluk A (2014) Field-evaluation of a new lateral flow assay for detection of cellular and humoral immunity against Mycobacterium leprae. PLoS Negl Trop Dis 8(5):e2845

Corstjens P, Zuiderwijk M, Brink A, Li S, Feindt H, Niedbala RS, Tanke H (2001) 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 47(10):1885–1893

Fluss R, Faraggi D, Reiser B (2005) Estimation of the Youden index and its associated cutoff point. Biom J 47(4):458–472

Meredith A, Pozo JD, Smith S, Milne E, Stevenson K, McLuckie J (2014) Leprosy in red squirrels in Scotland. Vet Rec 175(11):285–286 Available at: http://www.ncbi.nlm.nih.gov/pubmed/25234460

Scollard DM, Adams LB, Gillis TP, Krahenbuhl JL, Truman RW, Williams DL (2006) The continuing challenges of leprosy. Clin Microbiol Rev 19(2):338–381

Shar S et al (2016) *Sciurus vulgaris*. The IUCN Red List of Threatened Species. Available at: https://doi.org/10.2305/IUCN.UK.2016-3.RLTS.T20025A22245887.en. Accessed August 20, 2018

Singh P, Benjak A, Schuenemann VJ, Herbig A, Avanzi C, Busso P, Nieselt K, Krause J, Vera-Cabrera L, Cole ST (2015) Insight into the evolution and origin of leprosy bacilli from the genome sequence of Mycobacterium lepromatosis. Proc Natl Acad Sci U S A 112(14):4459–4464 Available at: http://www.pnas.org/cgi/content/long/112/14/4459. Accessed April 26, 2018

Smith SH, Stevenson K, del-Pozo J, Moss S, Meredith A (2017) Atypical Histiocytosis in red squirrels (Sciurus vulgaris). J Comp Pathol 156(4):446–450 Available at: http://linkinghub.elsevier.com/retrieve/pii/S0021997517300683

Spencer JS et al (2015) Identification of serological biomarkers of infection , disease progression and treatment efficacy for leprosy. Mem Inst Oswaldo Cruz 107:79–89

Truman RW et al (1986) Evaluation of the origin of Mycobacterium leprae infections in the wild armadillo, Dasypus novemcinctus. Am J Trop Med Hyg 35(3):588–593

van Hooij A, Tjon Kon Fat EM, Richardus R, van den Eeden SJF, Wilson L, de Dood CJ, Faber R, Alam K, Richardus JH, Corstjens PLAM, Geluk A (2016) Quantitative lateral flow strip assays as user-friendly tools to detect biomarker profiles for leprosy. Sci Rep 6:34260

van Hooij A, Tjon Kon Fat EM, van den Eeden SJF, Wilson L, Batista da Silva M, Salgado CG, Spencer JS, Corstjens PLAM, Geluk A (2017) Field-friendly serological tests for determination of M. leprae-specific antibodies. Sci Rep 7(1):8868

van Hooij A, Tjon Kon Fat EM, Batista da Silva M, Carvalho Bouth R, Cunha Messias AC, Gobbo AR, Lema T, Bobosha K, Li J, Weng X, Salgado CG, Spencer JS, Corstjens PLAM, Geluk A (2018) Evaluation of immunodiagnostic tests for leprosy in Brazil, China and Ethiopia. Sci Rep 8(1):17920