Exploring host and pathogen biomarkers for leprosy

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Lack of evidence for the presence of leprosy bacilli in red squirrels from North-West Europe

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Abstract

Leprosy is a human infectious disease caused by *Mycobacterium leprae* or *Mycobacterium lepromatosis* that can also occur in animals and even manifest as zoonosis. Recently, both mycobacteria were detected in red squirrels (*Sciurus vulgaris*) from the British Isles.

To further explore the presence of leprosy-bacilli in North-West Europe, we screened Belgian and Dutch squirrels. Tissue samples from 115 animals tested by qPCR were negative for both pathogens. No molecular or pathological evidence was found of the presence of these zoonotic pathogens in North-West Europe.

Introduction

Leprosy, caused by *Mycobacterium leprae* or *Mycobacterium lepromatosis*, is a debilitating disease occurring in several low- and middle-income countries. Transmission is unabated as shown by stable numbers of new cases worldwide (1). Close contact to multibacillary patients (2) and, to a lesser extent, infected animals and environmental sources are presumed to play a role in transmission (3, 4).

M. leprae and *M. lepromatosis* can cause leprosy-like disease in several animal hosts, including nine-banded armadillos (4), red squirrels (5) and nonhuman primates (6).

Previously, *M. leprae* and *M. lepromatosis* have been detected in Eurasian red squirrels (*Sciurus vulgaris*) from the British Isles (5, 7), where human leprosy has not occurred for centuries. Squirrels of other European countries and Mexico, however, were not positive for DNA of these pathogens when screened by PCR (8).

To further explore the presence of *M. leprae* or *M. lepromatosis* in continental squirrels from North-West Europe we screened squirrels from the Netherlands and Belgium.

Materials and methods

Sample collection

Sixty-one wild red squirrels (*Sciurus vulgaris*) and one Japanese squirrel (*Sciurus lis*) were found dead in the Netherlands and submitted to the Dutch Wildlife Health Centre (Figure S1). Animals died due to infection with *Toxoplasma gondii* (n=21), traumatic injuries (n=23), or other pathologies (n=18). Skin lesions consistent with leprosy were not detected. Red squirrels (n=53) victims of road traffic were collected between 2010 to 2014 in Flanders (Table S1), Belgium (9).

Necropsy included macroscopic examination, cytological analysis of liver, spleen, lungs, and intestinal contents stained with HemacolorR (Hemacolor quick stain, Merck, Darmstadt, Germany), and histological examination.

Small biopsies from liver and spleen were collected in 2 ml tubes and stored at -20 °C until further processing.

DNA extraction

Biopsies from spleen [Dutch squirrels (n=62); Belgian squirrels (n=53)] and liver [Belgian squirrels only (n=53)] were used for DNA extraction.

DNA extraction was performed on 20 mg tissue using the Qiagen DNeasy Blood and Tissue Kit (Qiagen, Venlo, the Netherlands), according to manufacturer's protocol.

RLEP and 202 qPCR

A qPCR amplifying an *M. leprae*-specific repetitive sequence (RLEP) was performed (10). For *M. lepromatosis* primers and probe (202-qPCR) were designed based on criteria for

TaqMan PCR reactions to amplify a 168bp fragment in contig 202 which has two copies in the genome and is specific of *M. lepromatosis*. Amplification was carried in a final volume of 25 µl by addition of GoTaq™ Probe qPCR Master Mix (Promega, Madison, WI), 22.5 µM primers (Table 1), 6.25 µM TaqMan probe (Table 1) and 5.0 µl sample using the following profile: 2 min at 95ºC, 40 cycles of 15 s at 95ºC and 1 min at 60ºC. Nuclease-free water was used as negative control and *M. leprae* or *M. lepromatosis* DNA as positive controls. DNA of *M. leprae* Br4923 and Thai53, *M. bovis* BCG P3 and *M. tuberculosis* H37DNA were used to assess 202qPCR specificity. A Ct of 38.5 was taken as the limit for positivity.

Statistical analysis was performed in R (version 3.4.1).

Table 1. ***Primers and probes for RLEP and 202 qPCR.***

Primer/probe	Sequence 5‘ - 3‘
RLEP qPCR F	GCAGCAGTATCGTGTTAGTGAA
RLEP qPCR R	CGCTAGAAGGTTGCCGTAT
RLEP qPCR Probe FAM	CGCCGACGGCCGGATCATCGA
qPCR 202 F	CTGATCGCACACCTTGATGAGAG
qPCR 202 R	GTTAGGTTGATCGACATCTTCGGTGC
qPCR 202 Probe VIC	CACCACTAGCGCACCACGTCAGACAGGC

Forward (F) primers, reverse (R) primers and probes with dyes used to detect presence of *M. leprae* (RLEP) and *M. lepromatosis* (202) by TaqMan qPCR.

Results

To study whether leprosy-bacilli are present in Dutch and Belgian squirrels, we performed qPCR analysis on 115 red squirrels none of which showed clinical signs of leprosy. DNA samples were negative for both RLEP-qPCR (*M. leprae*) and 202-qPCR (*M. lepromatosis*). Thus, *M. leprae*- and *M. lepromatosis*-specific DNA was not detected in the Dutch or Belgian red squirrel populations with a 95% confidence interval (CI) of 0.0 to 4.1%. Part of these samples have been tested successfully for the presence of tick-borne pathogens by qPCR (9), and 45% were positive for the presence of Bartonella DNA (11).

Discussion

Since the discovery of Eurasian red squirrels suffering from leprosy-like disease in the British Isles (5) there has been an increased interest to screen other squirrels for *M. leprae* and *M. lepromatosis*. We examined 114 animals from the red squirrel population in the Netherlands and Belgium.

In Brownsea Island *M. leprae* DNA was found in 25 out of 25 red squirrels (8 with leprosy-like lesions) (5). In Scotland and Ireland *M. leprae* was not detected, but *M. lepromatosis*

was identified. The authors calculated that 21% of the squirrel population without clinical signs and all squirrels with clinical signs from the British Isles carried either *M. leprae* or *M. lepromatosis*.

As recently described (8), in a squirrel population showing no disease manifestations and with the same prevalence of leprosy as the British Isles (21%), a sample size of 19 should suffice to identify minimally one case with a 95% CI. Since our sample size (53 Belgian squirrels, 62 Dutch squirrels) was 2-3-fold larger, we should have identified leprosy-bacilli in the Dutch and Belgian squirrel population, if present, in at least the same prevalence as in the British Isles. When taking into account also previously tested continental European Eurasian red squirrels (n=96, (8)), the prevalence in mainland Europe is less than 2.2% with a 95% CI.

Our findings are consistent with previous observation in other European countries (France, Italy, United Kingdom, Germany and Switzerland) and Mexico (8) where *M. leprae*- or *M. lepromatosis*-specific DNA were not detected.

Pinnae samples have been reported to be the optimal tissue for molecular screening. Spleen and liver have also been successfully used to detect *M. leprae* and *M. lepromatosis* DNA in squirrels (5), however, the sensitivity might differ from pinnae samples. The lack of positive PCR results could be due to a lower prevalence of the leprosy-bacilli in the Dutch and Belgian red squirrel population compared to the reference UK population. It is conceivable that, red squirrels in UK could be more susceptible due to reduced immunity as a consequence of squirrelpox transmitted by grey squirrels (12). Alternatively, the animals could carry a bacillary load below the detection limit, or our tissue sampling might have been suboptimal. However, animals with leprosy-related lesions have not been observed in these populations suggesting that the absence of the pathogen is a more-plausible scenario. As we did not find evidence of the presence of *M. leprae* or *M. lepromatosis*, our results endorse the recent hypothesis (8) that Eurasian red squirrels in the British Isles are the only known wild rodent up to date carrying the leprosy-bacilli.

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Ethics Statement

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to. No ethical approval was required as wild animals were found dead and offered for necropsy.

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Conflicts of Interest

The authors declare that they have no conflict of interest.

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Supplementary Material

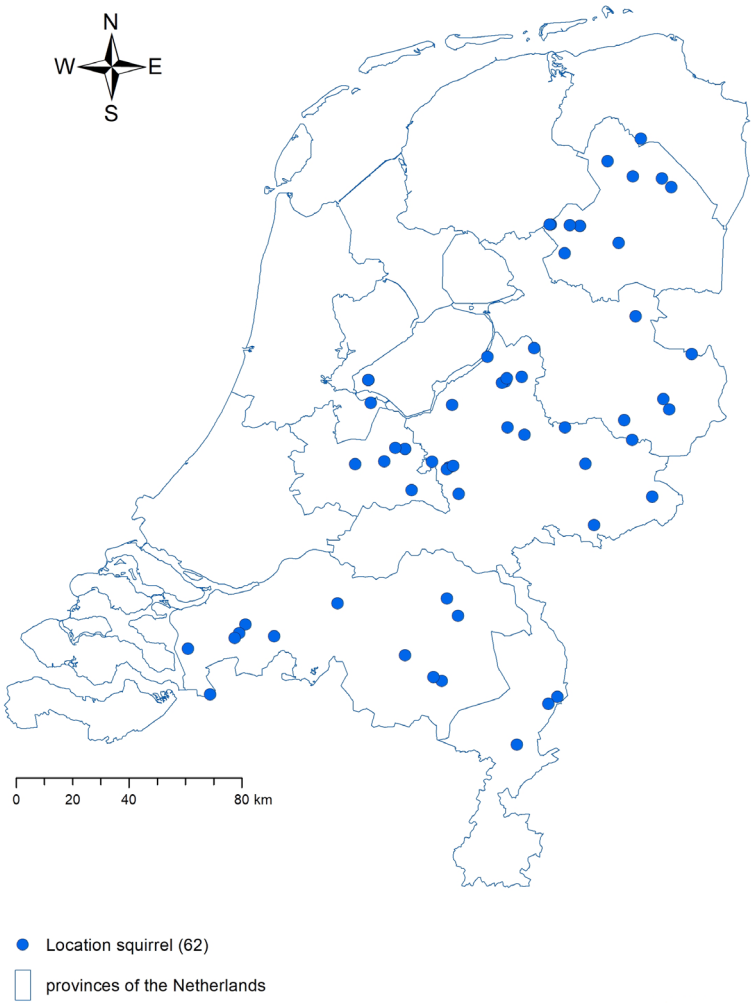

Figure S1. Distribution of necropsied and tested squirrels in the Netherlands (2015 – 2017). Locations of squirrel carcasses found in the Netherlands are indicated in blue circles. Figure designed by Ir. M.G.E. Montizaan.