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Molecular Screening of Wild Rodents for *Leishmania infantum* in Germany

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Abstract: A total of 1200 samples from eight rodent species from South Germany were analysed by real-time PCR. All tested negative for *Leishmania infantum*. There is currently no supportive evidence for wild cycles nor peri-domestic cycles of transmission of *L. infantum* involving rodents in Germany.

Key words: Rodentia, *Leishmania infantum*, leishmaniosis, real-time PCR, Germany

Introduction

Leishmanioses are widespread in the Old World and in the New World (Ashford 2000, Desjeux 2001). In Europe, visceral and cutaneous leishmaniosis due to *Leishmania infantum* is endemic around the Mediterranean basin and surrounding areas (Dujardin *et al.* 2008, Gómez-Saladín *et al*. 2005, Mary *et al*. 2004, Solano-Galego *et al*. 2007). It is common for dogs (Mettler *et al.* 2005) and to a lesser extent for Humans (Harms *et al*. 2003, Weitzel *et al*. 2005) visiting endemic areas to become infected. In many cases, infection remains asymptomatic (Campino et al. 2000, Manna *et al*. 2006, Mary *et al*. 2004). Cases of canine leishmaniosis diagnosed in Germany are believed to be linked to importation from and travel to endemic areas (Mettler *et al.* 2005). However, a few cases could not be traced back to a known endemic area. This point, combined with the existence of capture reports of *Phlebotomus mascittii* (Naucke, Pesson 2000) and *P. perniciosus* (Naucke 2007) in South Germany, fuelled speculations about hypothetical autochthonous parasite transmission foci. In contrast to phlebotomine vectors which are characterised by a short period of activity (Rossi *et al*. 2008), mammalian hosts can be surveyed over much longer periods of time. Leishmanioses are zoonotic diseases where wild and domestic canids play a prime part as reservoirs (Ashford 1996, 2000, Desjeux 2001, Dujardin *et al.* 2008, Moreno, Alvar 2002, Quinnell *et al*. 2001, Reale *et al*. 1999). Biological samples from symptomatic dogs and Humans are routinely analysed by diagnostic laboratories. This is not the case of wild rodents which most often remain unsurveyed. However, the latter ones constitute a very large biomass of potential reservoirs, and several species were already reported as carriers of *Leishmania* parasites in both the Old World and the New World (Ashford 1996, 2000, Desjeux 2001, Di Bella *et al*. 2003, Mohebali *et al*. 2004, Oliveira

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et al. 2005, Svobodová *et al.* 2003). In Europe, *L. infantum* was found in *Rattus rattus* in Italy (Di Bella *et al*. 2003) and in Spain (Martin-Sanchez *et al*. 2004). Furthermore, rodents are known to remain asymptomatic carriers for a very long time (Pozio *et al*. 1985, Svobodová, Votypka 2003, Svobodová *et al.* 2003). This makes them most suitable study species as they may thus be surveyed all year round. The present study therefore aimed to screen wild rodents from South Germany for *L. infantum* by means of real-time PCR, which is currently the most sensitive and most specific detection method.

Materials and Methods

Rodents were either (a) live-trapped as part of an epidemiological study of tick-borne diseases, or (b) snap-trapped as part of rodent control campaigns in organic orchards and forestry plantations, or (c) brought in by cats. Sampling areas are shown in Fig. 1. Live rodents were individually marked by ear-notching, and released at the point of capture. The tissue sample collected as part of the marking procedure was preserved in 80% ethanol for future molecular analyses. A similar sample was removed from the ear-flap of each dead rodent. In all cases (live and dead rodents), species, sex, body mass, visible pathologies and parasites were recorded. DNA from ear samples was extracted using a QIAamp® DNA Mini Kit (QIAGEN, Hilden Germany) following the manufacturer's instructions. For each sample, the presence and quality of rRNA as well as the absence of PCR inhibitors were assessed by amplifying two molecular markers targeting the vertebrate 18S rDNA gene (modified after Pichon *et al*. 2003). Molecular screening for *Leishmania infantum* was done by real-time PCR (modified after Francino *et al*. 2006, cf. Table 1), with a limit of detection of 0.001 parasite per PCR reaction. Fig. 2 shows calibration curves obtained with serial dilutions of the positive control. Positive and negative controls were systematically included in each PCR run.

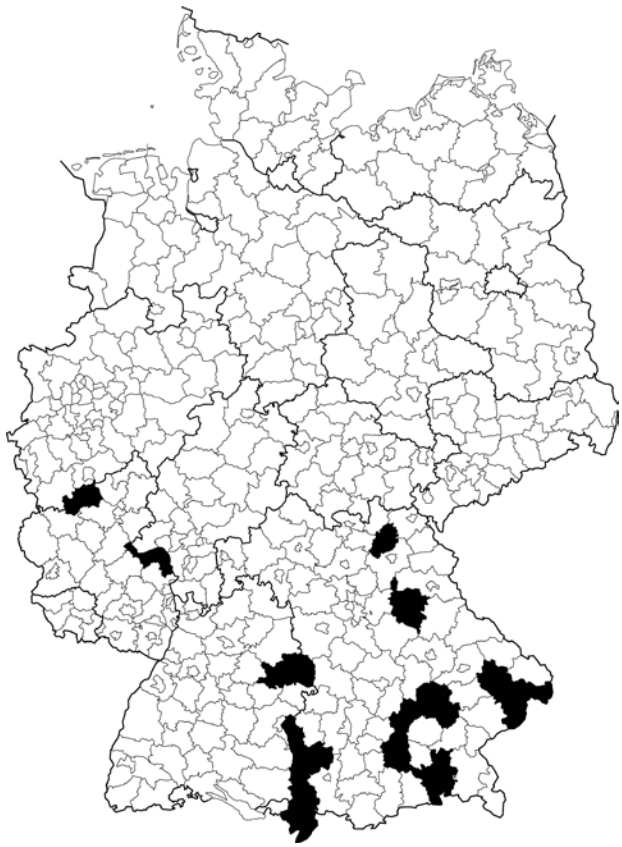

Fig. 1. Map of Germany showing the areas where rodent samples were collected from 2007 to 2010.

Table 1. Reaction conditions for the PCR amplification of DNA from *Leishmania infantum*. Total volume is 25µl, including 5µl template DNA from each sample (3 samples per reaction tube). Sequences of primers and probe follow Francino *et al*. (2006). Thermocycling conditions were 1 cycle at 95°C for 5 min. (activation of Hot Start Taq Plus), followed by 40 cycles at 95°C for 15 s (denaturation) and 60°C for 1 min. (annealing/elongation).

10x QIAGEN PCR buffer (at 15mM $MgCl_2$)	2.500µl
QIAGEN $MgCl_2$ (at 25mM)	1.785µl
QIAGEN dNTPs (10mM)	0.500µl
Forward primer (10µM)	2.250µl
Reverse primer (10µM)	2.250µl
Target specific fluorophore (10µM)	0.500µl
H_2O	0.090µl
QIAGEN Hot Start Taq Plus (5U/µl)	0.125µl

Results

A total of 1200 rodent samples from eight species within three families (Muridae, Arvicolidae and Sciuridae) from South Germany were analysed. All tested negative for *Leishmania infantum*. Details of species and numbers are given in Table 2. All positive controls produced a strong and typical positive amplification signal, whereas all negative samples produced a typical negative signal.

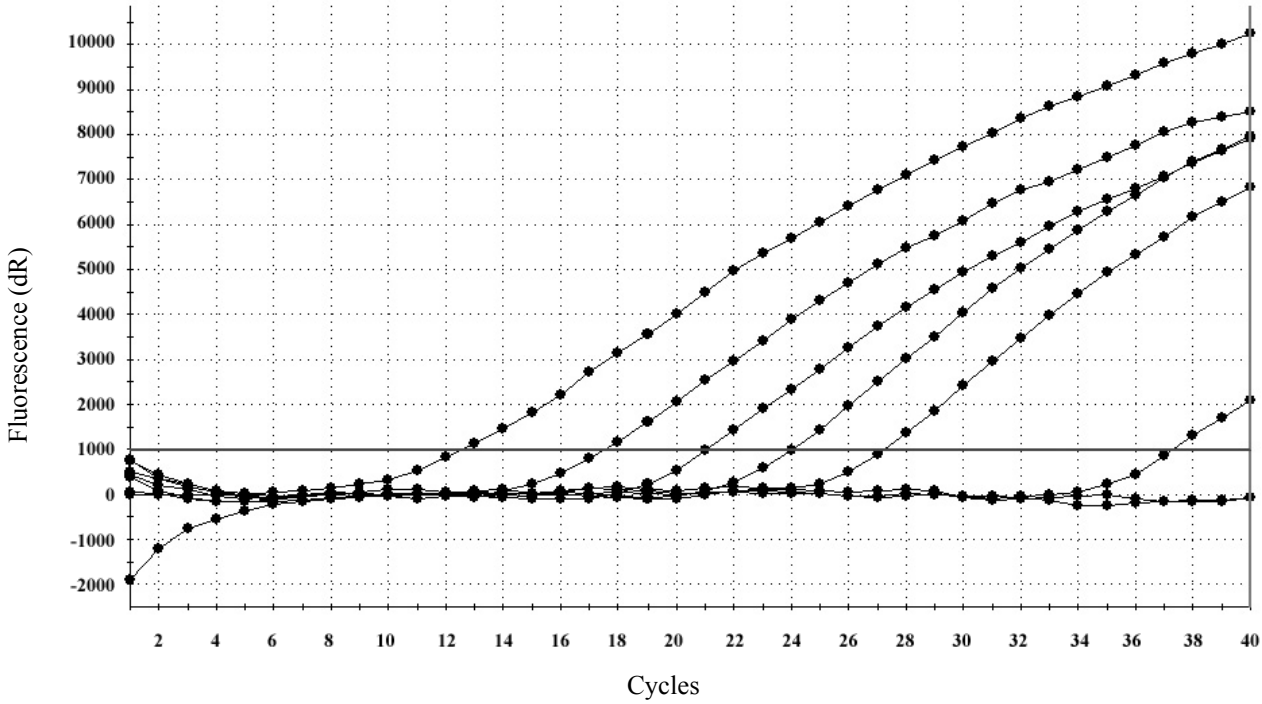

Fig. 2 Calibration curves obtained with 10-fold serial dilutions of the positive control. Ct values for undiluted positive control and dilutions 1:10 to $1:10^5$ are 12.49, 17.52, 21.00, 24.00, 27.20 and 37.30 respectively. There is no Ct value for dilution $1:10^6$ as detection threshold has been reached by then. The negative control produced a typical negative signal.

Table 2. Rodent species and number of individuals screened for *Leishmania infantum* by real-time PCR.

Species	Number tested
Apodemus flavicollis	337
Apodemus sylvaticus	39
Arvicola terrestris	40
Clethrionomys glareolus	107
Microtus arvalis	668
Mus domesticus	2
Rattus norvegicus	4
Sciurus vulgaris	3
Total	**1200**

Discussion

Absolutely all rodent samples yielded negative results despite real-time PCR being an extremely sensitive detection method, and despite ear tissue being the most suitable tissue sample for *Leishmania* diagnosis in rodents (Svobodová *et al.* 2003). The fact that each positive control yielded a strong positive signal indicates that DNA amplification was successful in each PCR run. Based on these results, there is currently no supportive evidence for wild cycles nor peri-domestic cycles of transmission of *L. infantum* involving rodents in Germany. This comes to no surprise as autochthonous leishmaniosis transmission cycles are unlikely to occur in Germany under the current environmental conditions. If true autochthonous foci of *Leishmania* infection occurred in Germany, one would expect a significant number of autochthonous cases of canine leishmaniosis to be observed in the endemic area; This is currently not the case. For an autochthonous focus of *Leishmania* transmission to occur in Germany, a concatenation of rare events must happen: A parasite must be introduced (e.g. through an imported dog) into one of the rare and geographically restricted autochthonous sand-fly populations. These sand-flies must happen to be a competent vector and transmit the parasite to a new competent reservoir host (e.g. a dog or a black rat). If such an improbable transmission cycle were to emerge in Germany, the biological characteristics of black rats would make them suitable candidates for the role of wild reservoir hosts. Indeed, they are widespread in warm areas (i.e. areas suitable for phlebotomine sand-flies), their numbers may be

locally high, and they are competent reservoirs for *Leishmania infantum*. Furthermore, asymptomatic rats effectively maintain a stock of infective amastigotes through the non-transmission season. Finally, rats usually live in close proximity to Humans and domestic animals. Therefore, the black rat, *Rattus rattus*, which is often reported as a reservoir for *L. infantum*, may efficiently be used as a sentinel species for monitoring the introduction and hypothetical establishment of leishmaniosis in Germany. Live-trapping of rats at strategically selected locations (e.g. reported *Phlebotomus* foci, or next to parking spaces for lorries transporting live animals on the motorway network) would make it possible to collect skin biopsies by means of ear punching, thus providing optimal DNA samples for diagnosis purposes without endangering non-target species. Furthermore, CMR (capture-marking-recapture) of rats at strategic locations would provide valuable information on the evolution through time of the epidemiological situation. This would also provide up to date data on rat population dynamics and density. Such monitoring would provide the local health authorities with an early warning system.

Individual contributions

Collection of samples in the field: PGM, AH, BW, JB and CD.
DNA extraction: PGM, CM and AH.
Molecular diagnosis (PCR): PGM.

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