Development of *Leishmania (Mundinia)* in guinea pigs

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Abstract

Background: Leishmaniasis is a human and animal disease caused by parasites of the genus *Leishmania*, which is now divided into 4 subgenera – *L. (Leishmania)*, *L. (Viannia)*, *L. (Sauroleishmania)* and *L. (Mundinia)*. Subgenus *Mundinia*, established in 2016, is geographically widely dispersed, its distribution covers all continents, except Antarctica. It consists of 5 species - *L. enriettii* and *L. macropodum* are parasites of wild mammals while *L. martiniquensis*, *L. orientalis* and unnamed *L. sp.* from Ghana are infectious to humans. There is very little information on natural reservoir hosts and vectors for any *Mundinia* species.

Methods: Experimental infections of guinea-pigs with all five *Mundinia* species were performed. Animals were injected intradermally with 10^7 culture-derived promastigotes into both ear pinnae. The courses of infections were monitored weekly; xenodiagnoses were performed at weeks 4 and 8 post infection using *Lutzomyia migonei*. The distribution of parasites in different tissues was determined *post mortem* by conventional PCR.

Results: No significant differences in weight were observed between infected animals and the control group. Animals infected with *L. enriettii* developed temporary lesions at the site of inoculation and were infectious to *Lu. migonei* in xenodiagnoses. Animals infected with *L. martiniquensis* and *L. orientalis* developed temporary erythema and dry lesions at the site of inoculation, respectively, but were not infectious to sand flies. Guinea pigs infected by *L. macropodum* and *L. sp.* from Ghana showed no signs of infection during experiments, were not infectious to sand flies and leishmanial DNA was not detected in their tissue samples at the end of experiments at week 12 post-inoculation.

Conclusions: According to our results, guinea pigs are not an appropriate model organism for studying *Mundinia* species other than *L. enriettii*. We suggest that for better understanding of *L. (Mundinia)* biology it is necessary to focus on other model organisms.

Background

Leishmaniasis are vector-borne diseases whose etiological agents are protozoan parasites of the genus *Leishmania* (Kinetoplastida: Trypanosomatidae). Known previously as the *L. enriettii* complex, the subgenus *Mundinia* was established recently and currently contains 5 species: *L. enriettii*, *L. macropodum*, *L. orientalis*, *L. martiniquensis* and the unnamed species from Ghana (1–3). According to phylogenetic analysis this subgenus is the first to branch from the other *Leishmania* subgenera, indicating that they are likely to represent the most ancient and divergent group of species within the *Leishmania* (2,4). The geographical distribution of these parasites covers all continents except Antarctica, which can be explained by the formation of individual species from their common ancestor after the breakup of Gondwana (2).

Many important details of the biology of these parasites are unknown. The identity of the insect vectors responsible for transmission of *L. (Mundinia)* has not been confirmed for any species yet. It has been

assumed that these parasites, similar to other *Leishmania*, would be transmitted by sand flies of the genus *Phlebotomus* and/or *Sergentomyia* in the Old World and *Lutzomyia* in the New World (Diptera: Phlebotominae), and this may be the case. Recently, however, *Forcipomyia* (*Lasiohelea*) biting midges (Diptera: Ceratopogonidae) were reported as likely vectors of *L. macropodum* in Australia (5), and laboratory experiments have revealed a high susceptibility of *Culicoides sonorensis* to *L. enriettii* (6). The observations raise the possibility of non-sand fly vectors for at least some of the *Mundinia*.

Similarly, there is little current information on the natural mammalian reservoir hosts for these parasites. *Leishmania enriettii*, is a parasite that has only ever been found in domestic guinea pigs in Brazil, first isolated in the 1940s (7). The natural host of *L. enriettii* is not known although often assumed to be a wild rodent of some kind. *L. macropodum* is a parasite first isolated from red kangaroos in Australia, but from a game park in a region where these animals are not found (5). There is evidence of *L. macropodum* infection in three other species of Australian macropods, which are more likely to be the true host(s) of this parasite (7). On the other hand, human cases have been described with *L. martiniquensis*, *L. orientalis* and *L. sp* from Ghana. *Leishmania martiniquensis* was firstly isolated from a HIV positive man on Martinique island in 1992 (8). According to recent findings, this *Leishmania* species has a worldwide distribution with single or multiple cases reported from various continents where these parasites were isolated from various hosts like horses, cows and humans (9–12). *Leishmania orientalis* was scientifically named in 2018 (13); in the past it was reported as “*L. siamensis*” (9,14) but this name is a *nomen nudum* and should not be used anymore. *Leishmania sp.* from Ghana is a species causing cutaneous leishmaniasis in the Volta region in Ghana (4). The last two species were not isolated from any mammalian species, except humans, and the identity of their reservoir hosts remains enigmatic.

Since very little is known about biology of these neglected parasites, the aim of our study was the establishment of model host organisms, which would enable testing their behaviour and properties in a mammalian host. Here we present results of experimental infections in guinea pigs with all five known *L. (Mundinia)* species.

Methods

Parasites and guinea pigs

Leishmania enriettii (MCAV/BR/45/LV90), *L. macropodum* (MMAC/AU/2004/AM-2004), *Leishmania sp.* from Ghana (MHOM/GH/2012/GH5), *L. orientalis* (MHOM/TH/2014/LSCM4) and two strains of *L. martiniquensis* (MHOM/MQ/1992/MAR1 and MHOM/TH/2011/CU1) were used. Parasites were maintained at 23°C in M199 medium supplemented with 10% fetal calf serum (Gibco), 1% BME vitamins (Sigma), 2% sterile urine and 250 µg/ml amikacin (Amikin, Bristol-Myers Squibb). In our laboratory both strains were maintained in the cryobank with 2-3 sub passages *in vitro* before experimental infections of guinea pigs and no passages in animals were performed. Before experimental infection, parasites were washed by centrifugation and resuspended in saline solution.

Female guinea pigs (Dunkin-Hartley) originating from AnLab s.r.o. were maintained in groups of 2 specimens in T4 boxes (58 x 37 x 20 cm, Velaz) equipped with bedding German Horse Span (Pferde), breeding material (Woodwool) and hay (Krmne smesi Kvidera), provided with a feed mixture V2233 Ms-H Guinea Pig maintenance (AnLab) and water ad libitum, with a 12 h light/12 h dark photoperiod, temperature 22-25°C and humidity 40-60%. In the beginning of experiments average weight of animals was 499 g and average age of animals was 7 weeks.

Infection and xenodiagnoses of guinea pigs

Eighteen guinea pigs (*Cavia porcellus*) anaesthetized with ketamin/xylazin (37.5 mg/kg and 1.5 mg/kg, respectively) were injected with 10^7 stationary - stage promastigotes in 5µl of sterile saline intradermally into the ear pinnae of both ears. The course of infection was recorded weekly. Three animals inoculated with the same volume of saline solution were used as a control for external signs of infection.

Xenodiagnoses were performed at weeks 4 and 8 post-infection (PI) using the permissive vector *Lutzomyia migonei* (15). Five to six-day-old *Lu. migonei* were placed into plastic vials covered by fine nylon mesh and allowed to feed on the ear pinnae of anaesthetized animals. Engorged individuals were maintained for two days at 25 °C and then stored in Tissue lysis buffer (Roche) at -20 °C in pools of 5 females for subsequent PCR. Altogether 192 sand fly pools were tested.

At the end of experiments, 12 weeks post inoculation (PI), the hosts were euthanized, dissected and tissues from ears, paws, ear-draining lymph nodes, spleens, livers and blood were stored at -20 °C for subsequent PCR.

Conventional PCR

Extractions of DNA from vectors and animal tissues were performed using a High Pure PCR Template Preparation Kit (Roche) according to the manufacturer's instructions. The total DNA was used as a template for PCR amplification with the primers for a 246 bp long ITS-1 sequence (forward primer 5' - AGATTATGGAGCTGTGCGACAA-3' and reverse primer 5' -TAGTTCGTCTTGGTGCGGTC-3'). Reactions were performed using EmeraldAmp® GT PCR Master Mix and the cycler was set to: step 1, 94 °C/3:30 min; step 2, 94 °C/30 sec; step 3, 60 °C/30 sec; step 4, 72 °C/20 sec; step 5, 72 °C/7 min; and cooled to 12 °C afterwards. Steps 2 – 4 were repeated 35 times. Samples were analysed using 1% agarose gels.

Statistical analysis

Statistical analyses were carried out using R software (<http://cran.r-project.org/>). Correlation of animal weights in different groups (infected and non-infected) and time was tested by co-variance analysis.

Results

No significant differences in weight were observed between infected animals and the control group (*L. macropodum*, $p = 0.70$; *L. enriettii*, $p = 0.12$; *L. martiniquensis* MAR1, $p = 0.77$; *L. martiniquensis* CU1, $p = 0.12$; *L. sp.* from Ghana, $p = 0.20$; *L. orientalis*, $p = 0.11$, see also Additional file 1).

Development of dry lesions was observed in animals infected with *L. enriettii*. The lesions appeared on ear pinnae (the site of inoculation) by week 2-3 PI, increased in size through to week 5-6 PI and then healed up, completely disappearing between weeks 8-12 PI (Fig. 1, Fig. 2A). Animals were efficiently infectious to sand flies on week 4 PI (9/16 positive pools) while infectiousness was reduced by week 8 PI (1/16 positive pools).

In animals infected with *L. martiniquensis* (MAR1), temporary erythema was observed at the site of inoculation between weeks 4 – 8 PI, but no lesions developed and animals were not infectious to sand flies (0/32 positive pools).

Erythema at the site of inoculation was observed also in guinea pigs infected with *L. orientalis*. The erythematic spot appeared by week 3 – 4 PI. The spot nodulated and the nodules subsequently transformed into dry lesions surrounded by a purple skin macula (Fig. 2B, Additional file 2). The lesions were healed up by week 7 – 8 PI. However, animals were not infectious to sand flies (0/32 positive pools) and no leishmanial DNA was detected in any of tested tissue samples.

Guinea pigs infected with *L. macropodum*, *L. sp.* from Ghana and *L. martiniquensis* (CU1) did not show any external signs of infection during the whole experiment, animals were not infectious to sand flies (0/96 positive pools consisting of 5 blood fed females each) and leishmanial DNA was not detected in any of tissue samples collected by the end of experiment on week 12 PI.

Discussion

Leishmaniasis of men and animals caused by *L. (Mundinia)* species are emerging all over the world. The human diseases are characterized by symptoms varying from self-healing skin lesions (2,12) to visceral forms. The latter prevail in HIV positive patients (8,16) but visceral disease was also observed in immunocompetent humans (12,17). Very little is known about the life cycle of these ancient and neglected species and appropriate animal model is necessary for closer understanding of their biology.

Guinea pigs were chosen for the experimental model in our study as they are the only known non-human mammalian hosts of *L. (Mundinia)* species, except for kangaroos, cows and horses (6,7,9,10,18), which are not practicable for most laboratory investigations. *Leishmania enriettii* was repeatedly isolated from domestic guinea pigs from various localities in Brazil (6,19). Interestingly, individual cases were separated by long time periods, which does not fit with the fact that guinea pigs are popular pets and, according to several studies, they are very susceptible to infection (5,20,21). We suggest that this rare incidence may be explained by two different manners. The prevalence of infection is actually much higher, but the owners of infected guinea pigs do not take them for veterinary checks and, therefore, parasites are not isolated. Alternatively, guinea pigs are only incidental hosts and the primary reservoir

hosts (and primary insect vectors) are not present in a close vicinity to households. In this case, secondary vectors and/or reservoirs may be temporarily involved in transmission to domestic localities and domestic guinea pigs.

Our experiments confirmed the susceptibility of guinea pigs to *L. enriettii*. All infected animals showed development of typical ear lesions and the animals were infectious to sand flies. The numbers of positive sand flies were significantly higher on week 4 PI than at the later time interval, week 8 PI. This decrease of infectivity was also observed previously by Seblova et al. (2015)(6). On week 12 PI, the animals did not show any more external signs of infection and *Leishmania* DNA was not detected in any of the examined tissue samples. Spontaneous healing of lesions was observed also by Paranaiba et al. (2015)(22). In their experiments initiated by intradermal inoculation of 10^5 promastigotes, a different virulence between the two strains used was observed. The Cobaia strain did not develop any lesions, while strain L88 developed lesions that were growing by weeks 4 – 6 PI, and then diminution of lesions was observed until the end of the experiment. The authors also described development of bigger lesions in groups where sand fly salivary glands were added to the inoculum.

However, the virulence of the *L. enriettii* parasite strain and the presence of sand fly salivary glands are not the sole factors influencing the degree of pathogenicity for guinea pigs. The outcome of infections is also dependent on the method of their initiation, i.e., on parasite numbers and stages (amastigotes vs. promastigotes) used as an inoculum. Thomaz-Soccol et al. (1996)(23) described development of serious symptoms of disease, like dissemination of parasites and subsequent death of all tested animals, when the inoculum consisted of amastigotes of a strain identical to L88 according to isoenzyme analyses. Wide dissemination of *L. enriettii* in animals was observed also by Paraense et al. (1953)(20), who infected guinea pigs with amastigotes from lesion homogenates, and by Seblova et al. (2015)(6) who infected animals with 10^7 culture derived promastigotes.

Development of *L. enriettii* has also been tested also in hamsters (*Mesocricetus auratus*), where infections were characterized by the development of temporary lesions at the site of inoculation and their subsequent healing (5,24). In experiments with wild guinea pigs (*Cavia aperea*), rhesus macaques and dogs (7) no animals showed any signs of infection, so domestic guinea pigs remain the best laboratory model for *L. enriettii* at present.

Here we compared the susceptibility of guinea pigs to four other *L. (Mundinia)* species. The infections were lost in animals infected with *L. macropodum*, *L. sp.* from Ghana and *L. martiniquensis* strain CU1. Animals infected with a second *L. martiniquensis* strain, MAR1, and with *L. orientalis* developed only temporary changes on the ears and the animals were not infectious to sand flies. However, PCR analysis showed no presence of leishmanial DNA by week 12 PI in any of tested samples. We suggest that *L. martiniquensis* and *L. orientalis* are capable of temporary survival at the site of inoculation, but they cannot disseminate to other tissues of guinea pigs.

We suggest that for the better understanding of *L. (Mundinia)* biology it is necessary to focus on other model host organisms. The first choice could be BALB/c mice or hamsters as the most common animal models for research used with many *L. (Leishmania)* and *L. (Viannia)* species. Infections of these standard laboratory animals with their controlled genetic background may bring valuable information. Alternatively, genetically polymorphic models like wild rodents mimicking natural hosts could be used. These less common models can allow a better understanding of the dynamics of infection and host-parasite relationships related more closely to the situation in the wild (25). On the other hand, when infected with *L. enriettii* and *L. orientalis*, guinea pigs could serve as a potential model for spontaneous healing, which could be informative for the design of vaccines.

Conclusions

Experimental infections showed that guinea pigs are not a good animal model for the subgenus *Mundinia*, with exception of *L. enriettii*. All other *Mundinia* species studied, *L. orientalis*, *L. martiniquensis*, *L. macropodum* and *L. (Mundinia)* sp. from Ghana, were not able to develop infections transmissible to sand flies.

Declarations

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Ethics approval and consent to participate

Animals were maintained and handled in the animal facility of Charles University in Prague in accordance with institutional guidelines and Czech legislation (Act No. 246/1992 and 359/2012 coll. on Protection of Animals against Cruelty in present statutes at large), which complies with all relevant European Union and international guidelines for experimental animals. All the experiments were approved by the Committee on the Ethics of Laboratory Experiments of Charles University in Prague and were performed under permission no. MSMT-1373/2016-5 of the Ministry of the Environment of the Czech Republic. Investigators are certificated for experimentation with animals by the Ministry of Agriculture of the Czech Republic.

Consent for publication

The manuscript does not contain any individual person's data in any form.

Availability of data and materials

All the data are included within the manuscript or are attached as supplementary data.

Competing interest

The authors declare that they have no competing interests.

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Authors' contributions

Experimental infections of animals and xenodiagnostic experiments TB, JS, molecular analysis TB, substantial contribution to conception and design JS, PV, drafting the article TB, JS, providing the parasite strains and revising the manuscript PS, PB. All authors read and approved the final manuscript.

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Abbreviations

PCR – Polymerase chain reaction

PI – post infection

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Figures

Figure 1

Development of lesions in guinea pigs infected with *L. enriettii*.

Figure 2

Dry lesions observed in animals infected with *L. enriettii* (A) and nodules transforming to dry lesions in animals infected by *L. orientalis* (B); both at week 5 PI.

Supplementary Files

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