Ticks as potential vectors of *Mycobacterium leprae*: Use of tick cell lines to culture the bacilli and generate transgenic strains

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Abstract

Leprosy is an infectious disease caused by *Mycobacterium leprae* and frequently resulting in irreversible deformities and disabilities. Ticks play an important role in infectious disease transmission due to their low host specificity, worldwide distribution, and the biological ability to support transovarial transmission of a wide spectrum of pathogens, including viruses, bacteria, and protozoa. To investigate a possible role for ticks as vectors of leprosy, we assessed transovarial transmission of *M. leprae* in artificially-fed adult female *Amblyomma sculptum* ticks, and infection and growth of *M. leprae* in tick cell lines. Our results revealed *M. leprae* RNA and antigens persisting in the midgut and present in the ovaries of adult female *A. sculptum* at least 2 days after oral infection, and present in their progeny (eggs and larvae), which demonstrates the occurrence of transovarial transmission of this pathogen. Infected tick larvae were able to inoculate viable bacilli during blood-feeding on a rabbit. Moreover, following inoculation with *M. leprae*, the *Ixodes scapularis* embryo-derived tick cell line IDE8 supported a detectable increase in the number of bacilli for at least 20 days, presenting a doubling time of approximately 12 days. As far as we know, this is the first *in vitro* cellular system able to promote growth of *M. leprae*. Finally, we successfully transformed a clinical *M. leprae* isolate by inserting the reporter plasmid pCHERRY3; transformed bacteria infected and grew in IDE8 cells over a 2-month period. Taken together, our data not only support the hypothesis that ticks may have the potential to act as a reservoir and/or vector of leprosy, but also suggest the feasibility of technological development of tick...
Author summary

Leprosy is a slow-progressing and extremely debilitating disease; the armadillo is the only animal model able to mimic the symptoms observed in humans. In addition, the causative agent, *Mycobacterium leprae*, is not culturable *in vitro*. Due to these constraints the chain of transmission is still not yet completely understood. We know, however, that at least two animals, armadillos in the Americas and red squirrels in the UK, are natural reservoirs of the bacillus, although their role in disease epidemiology is unclear. This information raised the following question: Can ticks carry leprosy from wild animals to humans? In the present study we demonstrated that artificially-infected female cayenne ticks are able to transmit the bacillus to their offspring, which were then able to transmit it to rabbits during bloodfeeding. We were able to grow *M. leprae* *in vitro* in a tick cell line for the first time. We also generated the first transgenic *M. leprae* strain, making the pathogen fluorescent in order to monitor its viability in real time. We believe that this new methodology will boost the screening of new drugs useful for control of leprosy, as well as improving understanding of how *M. leprae* causes disease.

Introduction

Leprosy, also known as Hansen’s disease, is a chronic infectious disease caused by the intracellular pathogen *Mycobacterium leprae*, manifested mainly as dermatoneurological signs and symptoms with high potential to progress causing physical disabilities and deformities.

Although it is currently agreed that leprosy is transmitted through the prolonged contact of genetically susceptible individuals with untreated multibacillary patients, the epidemiology of leprosy displays some features that are not well explained by this sole mode of transmission, particularly its geographic distribution. The disease, which during human history has spread throughout the globe [1], was eradicated in Europe almost a century before rifampicin discovery [2], and is now found mainly in tropical regions [3].

Several studies address the impact of environmental factors in the endemicity of leprosy, including factors such as poverty [4], human migration [5], population density [6], contact with armadillos [7] and environmental humidity [8]. One of the factors which is most strongly associated with the disease in Brazil is deforestation for agricultural activities [9]. In Brazil, new cases of leprosy are mainly concentrated in populations living on the agricultural borders, in the north and -west-central parts of the country, with 3.58 new cases / 10,000 inhabitants, contributing 68.18% of the new cases registered countrywide in 2015. These populations, who live in the countryside, are apparently more exposed to leprosy than the inhabitants of the great cities of the southeast region, where despite higher population density and public health vigilance, only 14.05% of new cases were notified, according to the Health Hazard Notification System (SINAN) of the Brazilian Ministry of Health. Although leprosy is not currently recognized as a zoonosis outside the southern USA [10] and the northern region of Brazil [11], several studies point to the potential of the armadillo *Dasypus novemcinctus* in other parts of the Americas [10], non-human primates around the world [12,13], and more recently red squirrels in the United Kingdom [14] as potential reservoirs of *M. leprae*. Some of these animals are
frequently infested by ticks. Since the beginning of the last century, studies have been performed in an attempt to implicate the action of hematophagous arthropods in the transmission of leprosy [15]. Although efforts have been made in describing the supposed role of mosquitoes, such as *Aedes aegypti* [16–18] and *Culex fatigans* [19], and flies [20] in leprosy transmission, these studies were based only on microscopic observations of acid-fast bacilli inside the insects’ digestive tracts, without concurrent *M. leprae* identification by molecular tools.

More recently, we have presented experimental evidence indicating that triatomine bugs, already associated with the transmission of another mycobacterial disease, Buruli ulcer [21], have potential as vectors of leprosy, being able to excrete infective bacilli in their feces at least 20 days after the blood meal [22]. The first report of the possibility of ticks acting as vectors of leprosy dates back to the 1940s, when acid-fast bacilli were observed in intestinal macerates of ticks of the genus *Amblyomma* after blood feeding on a skin lesion of a boy afflicted by the disease [23].

The three-host tick *Amblyomma sculptum* is a member of the *Amblyomma cajennense* sensu lato complex [24]; *A. cajennense* s.l. is distributed throughout tropical and sub-tropical areas of the American continent, from the southern US and Central America to northern Argentina, with the exception of Chile, Uruguay and the extreme south of Brazil, following the current distribution of leprosy in the Americas [25]. Humans are most commonly attacked by the immature stages (larvae and nymphs) of *A. sculptum*, while adults prefer to parasitize other mammals such as horses, cattle, tapirs, capybaras, domestic and wild canids, as well as birds, armadillos and rodents [26]. This tick species is implicated in the transmission of Rocky Mountain spotted fever to humans [26,27], and has been shown to have the ability to transfer pathogens such as *Rickettsia rickettsii* to its eggs [28]. Because of its wide geographic distribution and important role as a vector of human pathogens in South America, *A. sculptum* was chosen as a model for the present study.

Tick cell lines have often been used for the isolation and growth of fastidious or previously non-cultivable bacterial pathogens transmitted by ticks and other arthropods [29–33]. These lines exhibit some advantageous characteristics which could be critical for the *in vitro* cultivation of *M. leprae*, such as the ability to survive for long periods, measured in months or even years, in the same culture container requiring only regular changes of medium [34]. They also grow relatively slowly, at temperatures between 28˚C and 34˚C [30], the same range required by *M. leprae* for survival [35].

Here we provide evidence for the ability of *A. sculptum* to acquire and transmit, under controlled experimental conditions, the *M. leprae* bacillus during blood feeding. In addition, we describe a protocol for the *in vitro* production of *M. leprae* in the *Ixodes scapularis* tick cell line IDE8. Once we were able to grow this mycobacterium *in vitro*, we generated the first transgenic *M. leprae* strain to be described, through transfection with the reporter plasmid pCHERRY3. We believe that this methodology will change the paradigm of studies on leprosy, making possible continuous *in vitro* cultivation and generation of mutant strains of *M. leprae*, thereby enabling rapid progress in many areas including identification of virulence factors and drug discovery.

## Materials and methods

### *Amblyomma sculptum* source and maintenance

*A. sculptum* ticks from the Lab. de Doenças Parasitárias colony at the Federal Rural University of Rio de Janeiro (UFRRJ) were maintained on male rabbits (*Oryctolagus cuniculus*, aged between 60 and 90 days and weighing approximately 2 Kg, provided by Veterinary Institute Cuniculture, UFRRJ), by a modification of a previously-described method [36]. A small area
of the dorsal region was shaved, and bags of cotton cloth were glued to the rabbit’s skin. Within these bags were placed 20 pairs of adult male and female *A. sculptum*. To prevent removal of the bags by the rabbits, plastic cervical collars were placed on the necks of the animals as previously described [37].

**Mycobacterium leprae preparation**

*Mycobacterium leprae* Thai-53 strain [38], originally donated by Dr. James Krahenbuhl (National Hansen’s Disease Program, Laboratory Research Branch, Louisiana State University, Baton Rouge, LA, USA) was used to infect athymic nude mice bred in the Lauro de Souza Lima Institute (Bauru, São Paulo, Brazil) under germ-free conditions with $10^8$ bacilli in each posterior footpad. After six months the mice were sacrificed by a lethal dose of ketamine (300 mg/Kg) (Syntec, São Paulo, Brazil), skin and bones were removed from the footpads aseptically in RPMI medium, and the remaining tissues were cut into small pieces with sterilized scissors and tweezers. The tissues were then gently dissociated for 2 h at 33°C in a solution comprising 15 mg/mL collagenase type I, 7 mg/mL dispase, 50 μg/mL DNAse and 100 μg/mL ampicillin (Life Technologies, Carlsbad, CA, USA) dissolved in Milli-Q (Millipore, Darmstadt, Germany) ultrapure water. After digestion, the resultant suspension was transferred to 1.5 mL tubes, centrifuged at 12,000 x g for 5 min and the supernatant was discarded. The pellet was washed twice with 1.5 mL water at 12,000 x g for 5 min, resuspended in 0.1 N sodium hydroxide (Sigma, St. Louis, MI, USA) and immediately centrifuged at 12,000 x g for 5 min. The purified bacilli were washed twice with 1.5 mL RPMI medium (LGC, Cotia, SP, Brazil) and the pellet was mechanically dissociated by passing the suspension through a 26 gauge needle. *M. leprae* preparations supplemented with 10% fetal bovine serum (FBS) (Cripion, Andradina, SP, Brazil) were stored for up to 3 days at 4°C. For the transformation protocol, *M. leprae* isolated from a skin lesion of a multibacillary leprosy patient from Igarapé Açú, Pará, northern Brazil, was used. This strain, designated PA4, had been cultivated in nude mouse footpads for six months and genotyped as a 4N strain.

The viability of both *M. leprae* strains was determined by assessing membrane integrity using the LIVE/DEAD BacLight bacterial viability kit (Life Technologies, CA, USA), performed according to the manufacturer’s instructions [39]. The percentage of viable bacilli in the preparation were estimated by counting the numbers of living bacteria (Cyto9 green signal excited at 488nm) and dead bacteria (propidium iodide red signal excited at 470nm), using an Axio Observer Z1 Zeiss microscope, with a HXP light source. Twenty fields each presenting at least 50 bacteria were quantified per preparation. Only bacterial preparations with viability of at least 85% as determined by the LIVE/DEAD method were used in the present work. Sterility of *M. leprae* preparations was checked by inoculation onto BHI and 7H9 liquid media (BioCen, Campinas, SP, Brazil), and Lowenstein and blood agar plates (BioCen, Campinas, SP, Brazil), which were monitored daily for 72 h (BHI and blood agar) and 45 days (7H9 and Lowenstein) at 37°C in order to exclude the presence of other contaminating microorganisms, including mycobacteria.

**Artificial tick feeding and infection**

Nearly-engorged adult female *A. sculptum* were detached from rabbits and weighed before being artificially fed, using plastic pipette tips [40,41] as follows. The ticks were divided into two groups; one group received at least 400μL of citrated rabbit blood containing $10^7$/mL viable *M. leprae* Thai-53 strain, and the other group received the same volume of uninfected blood. The tick mouthparts were introduced into the tips at an angle of approximately 30° and the ticks were maintained at 37±1°C with relative humidity of 80% for 24 h, when they were
weighed again and ticks that had not gained at least 0.2 g were discarded. Engorged ticks were maintained at 80% humidity and 27˚C for between 2 h and 15 days prior to dissection or for oviposition. Artificially-fed ticks were dissected and tissue samples were collected individually and stored in TRIzol (Thermo Fisher Scientific, MA, USA) or 10% buffered formalin; midguts were collected after 2 h and 15 days, while ovaries were collected after 2 days. Pools of 0.2 g eggs laid by the artificially-fed ticks were collected on the second and third days after the blood meal and homogenized in TRIzol. Larvae were obtained from the same amounts of eggs after 30 days’ incubation in sterile syringes [42] at 27˚C with relative humidity approximately 80%. Two days after hatching, larvae were homogenized in TRIzol to investigate presence of M. leprae DNA and RNA.

Analysis of the potential of blood feeding larval ticks to transmit M. leprae
Two days after hatching, larvae originating from 0.2g of eggs laid by artificially-infected female A. sculptum were fed on a rabbit as described above for five days, the time required for this developmental stage to complete engorgement, and then removed. Subsequently, biopsies (5 mm punch) were performed in areas of the rabbit’s skin where there was evidence of previous insertion of the larval mouthparts. The biopsies were divided into two parts using a sterile scalpel; one part was extracted in TRIzol for quantitative PCR (qPCR) analysis of bacillary viability as described below, and the other part was fixed in 10% buffered formalin to perform immunolocalization of M. leprae as described below.

Analysis of M. leprae viability by qPCR
RNA and DNA were simultaneously extracted from test and control samples using TRIzol through a modification of the previously-described single-tube RNA extraction protocol [43]. Briefly, TRIzol was added to all samples to give a total volume of 1 ml. This solution was transferred to FastRNA tubes (FastRNA kit-Blue; MP Biomedicals, Santa Ana, CA). Samples were homogenized twice in the FastPrep FP 24 instrument (MP Biomedicals) at a speed setting of 6.5 for 45 s. Tubes were allowed to cool for 2 min between each round of homogenization. After homogenization, tubes were chilled on ice for 5 min, 200 μl chloroform-isoamyl alcohol (24:1) was added, and tubes were vortexed for 10 s and then centrifuged at 700 × g at 4˚C for 5 min. The supernatant was transferred to another tube and centrifuged again at 12,000 × g for 10 min at 4˚C. RNA was purified from 400 μl of the aqueous phase, any contaminating DNA was removed from the RNA preparations using the DNA-free kit (Ambion, Inc., Austin, TX) following the manufacturer’s instructions, the RNA was precipitated with isopropanol, washed with 70% ethanol, finally resuspended in 30 μl diethyl pyrocarbonate-treated H₂O, and stored at -80˚C until use [44]. DNA was purified from the remaining aqueous phase and interphase of the FastRNA tubes. Briefly, 100 μl of 10 mMTris-EDTA (pH 8.0) and 150 μl chloroform-isoamyl alcohol (24:1) were added to the remaining aqueous phase and interphase (500 μl) and homogenized in the FastPrep FP 24 instrument twice. After centrifugation at 12,000 × g for 10 min, the aqueous phase was transferred into another tube and precipitated with 0.1 volume of 3 M sodium acetate pH 5.2 and two volumes of cold ethanol. The DNA pellet was washed in 70% ethanol, dissolved in 30 μl of sterile distilled water, and stored at -80˚C until use. Qualitative RNA analysis was performed by agarose gel electrophoresis and quantification of both DNA and RNA was performed in a NanoDrop One apparatus (Thermo Fisher Scientific, MA, USA). The RNA was reverse-transcribed using random primers and Superscript III (Invitrogen, CA, USA) following the manufacturer’s instructions.

The levels of M. leprae 16S rRNA (as inferred from the resultant cDNA) and genomic DNA were determined in all tissues by qPCR, using as primer pair: sense 5’ GCA TGT CTT CIT TGT C...
GTG GAA AGC ‘3 and anti-sense 5’ CAC CCC ACC AAC AAG CTG AT ‘3, as described elsewhere [44]. The PCR protocol used was 50˚C for 2 min and 95˚C for 10 min, followed by 40 cycles of 95˚C for 15 seconds and 60˚C for 1 min, monitoring SYBR Green fluorescence in an ABI StepOne Plus System (Applied Biosystems, CA, USA). Viability of *M. leprae* was determined by calculating the qPCR 16S cDNA:DNA ratio, according to the formula $2^{-\Delta\Delta CT}$, where $\Delta CT = M. leprae$ 16S cDNA CT - *M. leprae* 16S DNA CT [44,45]. In order to convert $2^{-\Delta\Delta CT}$ qPCR signals into numbers of live *M. leprae* genomes, we generated standard curves by adding different concentrations of living *M. leprae*, ranging from $10^8$ to $10^3$, into uninfected rabbit skin biopsies, tick cell cultures and tissues: midguts dissected 2h and 15 days after the blood meal, pools of 0.2 g eggs harvested between 48h and 72h after the blood meal, and larvae originating from 0.2 g of eggs harvested 48h after hatching. We did not include ovarian tissue in this analysis due to difficulties in assuring absence of contamination from midgut contents during dissection. The angular coefficient of each of the five standard curves was used to determine the number of viable *M. leprae* in each condition. Control tissues and samples without the addition of DNA were negative for both cDNA and DNA targets.

**Immunolocalization of *M. leprae* by fluorescence microscopy**

As tick tissues are not well preserved by freezing protocols, *M. leprae* immunolocalization in tick tissues and rabbit skin biopsies was performed by fixation in 10% buffered formalin followed by standard paraffin embedding and sectioning as previously described [46]. The antigenic recovery was performed as follows: paraffin sections were dewaxed in xylol, hydrated in graded ethanol and distilled water, and heated in 10 mM sodium citrate buffer (pH 6.0) for 10 min in a 700 W microwave oven followed by 30 min to cool down to room temperature. *M. leprae* immunolocalization was performed using the monoclonal anti-lipoarabinomannan (LAM) antibody CS-35 reactive with *Mycobacterium* spp., kindly provided by the Biodefense and Emerging Infections Research Resources Repository: http://www.beiresources.org/TBVTRMResearchMaterials/tabid/1431/Default.aspx. Briefly, slides were permeabilized and blocked by 30 min incubation with 0.01% Triton X-100 and 10% FBS (Gibco/Life Technologies) in PBS pH 7.2 (permeabilization buffer). Then tissues were incubated for 2 h with the mouse IgG anti-LAM antibody CS-35 (diluted 1:50 vol/vol in permeabilization buffer) and nuclei were stained with 4’,6-diamidino-2-phenylindole (DAPI Sigma-Aldrich, St. Louis, USA). Secondary antibodies, IgG anti-mouse conjugated with Alexa 594 (Invitrogen, CA, USA) diluted 1:250 in permeabilization buffer, were incubated with the samples for an additional 2 h and the tissues were then mounted in Vectashield (Vector Laboratories, CA, USA) and observed using a Zeiss Axios Observer Z1 microscope with a Colibri illumination system using a 590 nm LED with a Zeiss fluorescence filter 50 (Carl Zeiss, Heidenheim, Germany).

**M. leprae** infection of tick and human cell lines

Tick cell lines AVL/CTVM17 derived from larval *Amblyomma variegatum* [47], HAE/CTVM8 derived from embryonic *Hyalomma anatolicum* [48] and IDE8 derived from embryonic *Ixodes scapularis* [49] were maintained at Lab. de Doenças Parasitárias, Institute of Veterinary Medicine (UFRRJ) as described previously [50]. All cell lines were seeded in 24-well culture plates at a density of 2x10⁵ cells / well in 1 mL of complete culture medium as follows. HAE/CTVM8 cells were cultivated in L-15/H-Lac medium comprising equal volumes of Leibovitz’ L-15 medium (Gibco/Life Technologies) supplemented with 20% FBS (Cripion, Andradina, SP, Brazil), 10% tryptose phosphate broth (TPB, Sigma-Aldrich, Missouri, USA) and 2mM L-glutamine (Sigma-Aldrich, Missouri, USA) (L-15), and Hanks’ balanced salt solution (HBSS, Sigma-Aldrich, Missouri, USA) supplemented with 20% FBS, 0.5% lactalbumin hydrolysate.
(Sigma-Aldrich, Missouri, USA) and 2 mM L-glutamine (L-Lac). IDE8 cells were grown in L-15B medium [51] supplemented with 10% FBS, 10% TPB, 0.1% bovine lipoprotein-cholesterol concentrate (MP Biomedicals, UK) and 2 mM L-glutamine (L-15B). AVL/CTVM17 cells were cultivated in medium comprising equal volumes of L-15, H-Lac and L-15B. Cells destined for microscopical analysis were seeded on coverslips, and all tick cells were maintained at 30˚C. Human monocyte THP-1 cells were obtained from the American Type Culture Collection and maintained in RPMI 1640 medium (LGC Bioscience, Cotia, SP) supplemented with 10% FBS (Cultilab, Campinas, SP), without antibiotics. THP-1 cultures were grown at 37˚C and transferred to 30˚C when infected, within a humidified 5% CO₂ atmosphere. Cells were subjected to infection with *M. leprae* Thai-53 strain at a multiplicity of infection of 50 bacteria per cell (MOI 50:1) at 30˚C. To examine association of *M. leprae* with tick cells, a total of 10⁷ freshly harvested *M. leprae* were stained with PKH67 dye (Sigma-Aldrich, Missouri, USA) following the manufacturer’s instructions with minor adaptations. Briefly 100 μL of diluent provided with the kit and 1 μL of PKH67 were added to the bacterial suspension. After 10 min incubation at room temperature with periodic mixing, 1 mL of FBS was added to the bacterial suspension for 1 min to stop the reaction. The suspension was centrifuged at 14,000 x g for 5 min and the bacteria were resuspended in 100 μL of complete L15-B medium and immediately added to cell cultures seeded as above. For microscopy, after 24 h incubation at 30˚C, cells in each well were fixed with 500 μL of 4% paraformaldehyde for 20 min, followed by three washes with HBSS and nuclear staining with DAPI. The coverslips were removed from the wells and placed on slides for analysis with a Zeiss Axio Observer Z1 fluorescence microscope as above. Samples were excited using 360 nm and 470 nm LEDs for respectively DAPI and PKH67, together with a Zeiss fluorescence filter 61. Images were acquired with a HMR Axiocam monochromatic camera controlled by Axiowision software version 3.2 (Carl Zeiss, Heidenheim, Germany), in which colors were arbitrarily attributed to the different channels. For flow cytometry, cells were gently washed with HBSS in order to remove any free bacilli, detached and fixed by the addition of 1% paraformaldehyde at 4˚C until analysis using a BD Accuri C6 cytometer (BD Biosciences, California, USA) with the FL1-A channel set for counting 10,000 events. In both techniques, microscopy and flow cytometry, the index of bacterial association was expressed as the percentage of fluorescent cells.

In order to determine *M. leprae* growth inside tick cells, 2x10⁵ cells of each of the three cell lines were seeded in wells of a 24-well cell culture plate and infected 24 h later with live *M. leprae* Thai-53 strain, at a MOI of 50:1. After 24 h, 10 days and 20 days of infection, cultures were washed twice with sterile PBS and the cells were resuspended. The cell suspensions were centrifuged at 12,000 x g for 5 min and resuspended in milk-formaldehyde, which was prepared by centrifuging 1.5 mL of skimmed milk (Nestlé, Vaud, Switzerland) for 15 min at 12,000 x g, and transferring 1 mL of the supernatant to another tube containing 150 μL of formaldehyde (Sigma-Aldrich, Missouri, USA) and 9 mL of ultra-pure water. The pellets were resuspended at a dilution of 1:10 v/v in milk-formaldehyde and 10 μL aliquots were each spread over a 10 mm diameter circle etched on a glass slide (Electron Microscopy Sciences, Hatfield, USA). The glass slides were stained using the Kinyoun method for microscopic analysis [52]. Twenty fields with at least 50 bacteria were quantified per cell culture. The number of bacilli was estimated based on the ratio of the area of the circle on the slide area to the area of the field of view of the microscope obtained with a Zeiss Objective Plan-Apochromat microscope 100x/1.40 Oil objective (Carl Zeiss, Heidenheim, Germany). To calculate this ratio, the radius (r) of each area was measured using a 1 mm microscope micrometer calibration slide ruler with 100 divisions (Cnscope, Beijing, China), and the areas were calculated using the formula π x r². Thus, the area of the circle on the slide and the area of the microscope field were determined as 78,539 mm² and 38 mm² respectively. This ratio (2.07x10⁴) was employed in
the following equation:

\[
\text{Number of bacilli per ml of cell culture} = A \times 2.07 \times 10^3 \times B \times 100
\]

when \(A\) = average number of bacilli in ten microscope fields, \(B\) = dilution correction and 100 = volume correction from 10 \(\mu\)L to 1 mL.

*M. leprae* viability inside cells was determined by qPCR using 16S rRNA as target as described above. Briefly, \(2 \times 10^5\) cells of each of the three tick cell lines and the human monocyte THP-1 cells were seeded in 24-well cell culture plates. The differentiation of monocytes to macrophages was achieved by adding 200 ng/ml PMA (Sigma-Aldrich, Missouri, USA) to the medium. After 24 h, undifferentiated monocytes and dead cells were washed out with PBS, medium was resupplied, and another 24 h incubation preceded the infection with live *M. leprae* Thai-53 strain at a MOI of 50:1. After 20 days of infection, cultures were washed twice with sterile PBS and intracellular *M. leprae* DNA and RNA were extracted by adding TRIzol reagent.

**Shepard's model test of viability of tick cell-derived *M. leprae***

An IDE8 cell culture was infected with *M. leprae* Thai-53 strain at a MOI of 50:1 at 30˚C. Culture medium was changed by removal and replacement of two-thirds of the total volume at weekly intervals. After 20 days of incubation, cells were washed and homogenized in 0.05% Tween 80 in sterile water. *M. leprae* was pelleted by centrifugation at 12,000 x g for 10 min, resuspended in 100 \(\mu\)L of PBS and counted as described above. Fifteen BALB/c mice were divided into three groups of 5 animals. Two groups were inoculated with 20 \(\mu\)L per foot pad of *M. leprae* suspension containing \(10^4\) bacilli harvested from the IDE8 culture in a conventional Shepard’s infection model [53]. A negative control was generated by treating animals in one of the two groups that received IDE8-derived *M. leprae* with 10mg/kg/week rifampicin by gavage. As a positive control, the third group were inoculated with the same number of *M. leprae* freshly purified from nude mouse foot pads. After six months, the mice were sacrificed by an intraperitoneal overdose of ketamine (300mg/kg) and xylazine (30mg/kg) and their foot pads were excised and macerated for bacillary counting after Kinyoun staining as above.

**M. leprae transformation***

An aliquot of \(10^8\) bacteria of the *M. leprae* strain PA4 was subjected to transformation by electroporation with 5\(\mu\)g of pCHERRY3 plasmid DNA, kindly provided by Dr Suzie Hingley-Wilson of the University of Surrey, UK. This non-integrative plasmid, with a size of 6.3 kb, harbors a hygromycin resistance gene, a mycobacterial replication cassette, and the far-red fluorescence gene *mCherry* with expression driven by the *Mycobacterium smegmatis* promoter *Psmyc* [54]. The BioRad Micropulser TM (Hercules, CA, USA) electroporator parameters were adjusted for tension of 2.5 kV and electric resistance of 1000 \(\Omega\). The bacilli were pelleted by centrifuging for 10 min at 12,000 x g. The supernatant was discarded, and the pellet was dissolved in 200 \(\mu\)L sterile MilliQ water with 0.05% Tween 80. After adding the plasmid, the mixture was homogenized and transferred to a sterile electroporation cuvette at room temperature. After applying the electric pulse, transformed *M. leprae* were recovered with a sterile Pasteur pipette and immediately transferred to a 25 cm\(^2\) culture flask containing \(10^7\) IDE8 cells, maintained with weekly medium changes as above. After one week, selection of transformed *M. leprae* was performed by adding 50 \(\mu\)g/mL of hygromycin. After 1 week, 2 weeks and 2 months, a small number of cells were gently detached from the culture flask by shaking, and \(10^5\) cells were placed in a glass bottomed 1.9 cm\(^2\) cell culture dish (CELLview, Greiner Bio-One). *mCherry* expression by *M. leprae* was monitored using the Zeiss Axio Observer Z1
microscope as above, using a 590 nm LED with a Zeiss fluorescence filter 61 (Carl Zeiss, Heidenheim, Germany). Time-lapse photographic analysis of *M. leprae*-mCherry was performed 2 weeks after transformation, using $10^5$ cells placed in a glass bottomed CELLview culture dish and the same microscope with the Zeiss Definitive Focus (Carl Zeiss, Heidenheim, Germany) accessory with stage temperature stabilized at 30°C. The medium was supplemented with 150 μg/mL ofloxacin (Sigma, St. Louis, MI, USA), and 5 h later the video was started, with acquisition of one frame every 5 min over a period of 3 h.

**Genotyping by multiple Locus Variable number analysis (MVLA)**

DNA extracts from *M. leprae*-infected tick cell cultures were submitted to genotyping by MLVA analysis of 16 variable-number tandem-repeat (VNTR) loci, as previously described [55]. For each sample, four multiplex PCR reactions were performed that generated 16 amplicons and allele copy number was determined by denaturation of amplicons and capillary gel electrophoresis on the sequencer ABI 3130 Genetic Analyzer, using the internal molecular weight sizing standards LIZ 500 (Thermo Fisher Scientific, MA, USA) and copy number definition with the Peak Scanner software (Applied Biosystems, MA, USA).

**Statistical analysis**

All numerical data were analyzed using nonparametric tests, with Dunn’s multiple comparisons test to compare relevant groups, or Mann Whitney test to compare continuous variables. All statistical analyses were performed with GraphPad Prism software.

**Ethics statement**

The use of rabbits in this study was supported by the authorization granted by the Committee on Ethics in the Use of Animals of UFRJ (CEUA-UFRJ) (Rio de Janeiro, Brazil) under No. 380/2013, process 23083.006255 / 2013–25. The use of mice was approved in license number 219/11 by the Animal Welfare Committee of Sagrado Coração University (CEUA-USC) (São Paulo, Brazil), responsible for animal care and inspections at the Instituto Lauro de Souza Lima where all mouse experiments were performed. All procedures were performed in accordance with the Brazilian guide for the production, maintenance and use of animals in teaching or scientific research activities from CONCEA (Conselho Nacional de Controle de Experimentação Animal). In the present work we used the *M. leprae* strain PA4 isolated from a skin lesion of an anonymous relapsed multibacillary patient from Igarapé Acu, Pará, Brazil, maintained in nude mice in the *M. leprae* Biobank at Lauro de Souza Lima since 2008. The Lauro de Souza Lima Research Ethics Committee (ILSL-CEP) was notified and permitted the use of this sample.

**Results and discussion**

The main requirement of a good arthropod vector is to be able to keep a pathogen viable in its tissues until the next blood meal, and to transmit it alive to the bloodstream or tissues of the host during feeding. Some tick-borne pathogens such as *Rickettsia parkeri* are capable of infecting the ovary of the vector arthropod [56], thereby spreading to the offspring which, in the case of the tick *A. sculptum*, comprise between 5000 and 8000 eggs per female [57].

Since *M. leprae* is only cultivable *in vivo*, rapid quantification of its viability in a non-sterile sample such as tick midgut is a challenge. In order to test the ability of *A. sculptum* to sustain *M. leprae* infection, we fed nearly-engorged adult female ticks with rabbit blood containing $10^7$ live *M. leprae/ml. This concentration was chosen to mimic heavily-infected armadillo blood, as reticuloendothelial tissue of these animals can contain more than $10^9$ *M. leprae*/g
[58], and was considered necessary to perform a reliable quantification of live *M. leprae* in the different samples. The amount of viable bacteria was determined in tick midguts at 2 h and 15 days by the method of Martinez and collaborators [44], in which the relationship between the number of copies of the 16S ribosomal RNA and the DNA coding for this bacterial gene is measured by qPCR (Fig 1). As PCR efficiency may vary between the various tissue samples due to their intrinsic differences, we performed titration curves of living bacilli for all analyzed materials, identifying the 16S rRNA:16S rDNA ratio corresponding to the number of viable *M. leprae* genomes in each tissue type. The 16S rRNA gene was used to test viability because it is the most sensitive and reliable target, due to its abundance and its being completely degraded 48h after *M. leprae* inactivation [59].

In our artificial feeding model, each tick would theoretically be able to receive ~2x10⁶ bacilli, since they usually ingested approximately 200 μL of blood per feed, although wide variation was observed in the amount of live *M. leprae* per tick midgut at 2 h post infection (Fig 1). This variation in *M. leprae* presence, reflecting differences in blood intake, was similarly seen in subsequent samples of eggs and larvae. Nevertheless, we showed that engorged female *A. sculptum* were able to maintain the viability of *M. leprae* in their midguts for at least 15 days after the blood meal, a time point equivalent to about half-way through the oviposition period [60].

At the second day after the blood meal, the artificially-fed females started to lay eggs. We demonstrated that many of the eggs were infected, by detection of *M. leprae* 16S rRNA in this material, as well as in larvae that hatched from eggs after 30 days of incubation at 28˚C in a humid chamber (Fig 1). More importantly, we allowed some of these larvae to feed on rabbits. After five days of exposure, the tick attachment sites were identified and biopsied. Analysis of this material also identified over 10⁷ live *M. leprae* in some of the bite sites (Fig 1), suggesting
that during blood feeding the larvae could have inoculated an amount of *M. leprae* sufficient to infect a susceptible armadillo [61].

Direct visualization of mycobacteria which we believed to be *M. leprae* in the various tissues was obtained through immunolocalization of LAM, a glycolipid exclusive to the genus *Mycobacterium*. At 15 days post feeding, when digestion of the blood meal would have been completed by the majority of the individual ticks [62], bacilli were visible in the basal region of the digestive cells (Fig 2), at a site where these cells are inserted between the midgut epithelial basophilic cells and make contact with the basal lamina, thereby gaining direct access to the hemocoel [63].

Demonstration of escape of *M. leprae* from midgut to hemocoel was provided by LAM immunolocalization revealing the presence of intact bacilli in both oocytes and the pedicels that attach the oocytes to the oviduct in infected females 2 days after feeding (Fig 3), as well as in the digestive tract of larvae that hatched from these eggs (Fig 4), suggesting vertical transmission of *M. leprae*, a phenomenon also reported for other tick-borne pathogens such as...
Rickettsia rickettsii [28]. This was further supported by the presence of mycobacteria at the larval bite sites, shown by immunolocalization of LAM in the rabbit skin biopsies (Fig 5). We did not observe any difference in the number of inflammatory cells infiltrating the host tissues, when comparing infected and control skin biopsies. The bacilli were observed mostly around the lesions generated by the tick mouthparts. The absence of any LAM signal in the tick and rabbit control samples (Figs 2–5) makes it very unlikely that the LAM-positive cells found in the tissues from infected samples might be attributed to another *Mycobacterium* species, since the ticks and rabbits in both groups (infected and control) came from the same colony and animal facility respectively.

The conclusion that *A. sculpturn* ticks are potential leprosy vectors led us to investigate whether *M. leprae* could infect embryonic or larval cells from different tick species in vitro. We used three different tick cell lines: AVL/CTVM17 (*A. variegatum*), HAE/CTVM8 (*H. anatolicum*) and IDE8 (*I. scapularis*), all of which are derived from human-biting tick species [64]. *A. variegatum* was chosen because there were no cell lines available from *A. sculpturn*, *H. anatolicum* is a tick species widely distributed in India where leprosy is endemic, and the *I. scapularis* cell line IDE8 is known to be highly permissive to infection with many different bacterial...
Tick cell cultures were inoculated with *M. leprae* labeled with the green fluorophore PKH67. We observed by flow cytometry (Fig 6A and 6B) and by microscopy (Fig 6C and 6D) that *M. leprae* was capable of associating with all three cell lines at rates between 45.3% (HAE/CTVM8) and 76.9% (AVL/CTVM17) (Fig 6B). The levels of *M. leprae*-cell association determined by quantification of fluorescence/cell through microscopy corroborated the flow cytometry analysis, confirming that AVL/CTVM17 and IDE8 cells were more susceptible to *M. leprae* attachment than HAE/CTVM8 cells (Fig 6D).

We then investigated whether *M. leprae* would be able to remain viable within these cells, grown at 30°C. For this, we inoculated the three tick cell lines with *M. leprae* and the total numbers of acid fast bacilli in the cultures were determined 2 h, 10 days and 20 days after infection (Fig 7). Due to their thick cell wall made up of mycolic acids, dead bacilli are not digested, and can remain within the culture seemingly intact for weeks. Thus, the observed levels of *M. leprae* at 20 days in the AVL/CTVM17 and HAE/CTVM8 cultures were possibly due to inactivated or dead bacteria, although these levels were higher than those seen in human species [30].

**Fig 5. Immunolocalization of *Mycobacterium* LAM cell wall antigen in skin biopsies from a rabbit infested with *Amblyomma sculptum* larvae.** Biopsies of bite sites collected from rabbits following 5 days’ infestation with larvae hatched from eggs laid by *A. sculptum* females after artificial feeding with uninfected (C, D) or *M. leprae*-infected (A, B) rabbit blood were fixed and sectioned. Panels A and C were generated through the merging of red channel image (anti-LAM + anti-mouse Alexa 594) with blue channel image (DAPI nuclear staining). Panels B and D were generated by merging with differential interference contrast images. In panels A and B, LAM-positive bacilli (arrows) can be seen in the area where the larval hypostome penetrated the rabbit skin. These images are representative of four biopsies obtained from two independent experiments. Scale bar represents 40 μm.

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THP-1 cells at the same time point (Fig 7D). In contrast, the IDE8 cell line was able to support a detectable increase in the number of bacilli over the 20 day period, showing an *in vitro* doubling time of approximately 12 days, similar to that observed *in vivo* in mouse foot-pads [35].

Fig 6. *Mycobacterium leprae* is able to associate with different tick cell lines. A) Histograms generated by flow cytometry showing association between *M. leprae* labelled with PKH67 and three tick cell lines: AVL/CTVM17, HAE/CTVM8 and IDE8. Gray lines represent uninfected tick cells and red lines represent cells associated with the fluorescent bacilli. B) Quantification of the histograms in (A) demonstrating a high level of association between the pathogen and cell lines AVL/CTVM17 and IDE8. C) Fluorescence microscopy 24h after infection of tick cell lines with *M. leprae* labelled with the green fluorophore PKH67. D) Quantification of the mean values of fluorescence intensity/cell in (C), for which sixty photos were analyzed from three independent experiments. Scale bar represents 20μm; ** = p <0.005 and * = P <0.05 by Dunn’s multiple comparisons test.

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**Table 1. Viability of tick cell-derived Mycobacterium leprae in the Shepard model.**

| Inoculum                                      | Mouse number |
|------------------------------------------------|--------------|
|                                                | 1  | 2    | 3    | 4    | 5    |
| Mice receiving *M. leprae* from IDE8 cells     |    |      |      |      |      |
|                                                | 2.5x10^6  | 8.9x10^5 | 6.1x10^4 | 8.8x10^4 | 5.5x10^5 |
| Mice receiving *M. leprae* from IDE8 cells     |    |      |      |      |      |
| followed by rifampicin treatment               | 0  | 0    | 0    | 0    | 0    |
| Mice receiving *M. leprae* from mouse footpads |    |      |      |      |      |
|                                                | 4.0x10^5  | 1.3x10^5 | 3.5x10^5 | 1.8x10^5 | 2.5x10^5 |

*Number of bacilli recovered from BalbC mouse footpads six months after inoculation with 10^4 bacteria harvested from IDE8 cells alone (test group) or with subsequent treatment with rifampicin (negative control group), or inoculation with 10^5 bacteria freshly harvested from mouse footpads (positive control group).*

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bacterial viability determined weekly by LIVE/DEAD staining ranged between 70 and 90% over 59 days in vitro. After two months IDE8 cells in the infected culture started to present apparent cytopathic effects, although intracellular *M. leprae*-mCherry maintained their ability to express mCherry (Fig 8).

At this point we developed a real-time viability analysis of *M. leprae*, by exposure of IDE8 cells infected with *M. leprae*-mCherry IDE8 cells to ofloxacin. After 5 h of incubation, time-lapse video microscopy showed, for the first time, real-time *M. leprae* inactivation by an

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**Fig 8.** Fluorescent *Mycobacterium leprae* generated by transfection with the plasmid pCHERRY3 within IDE8 tick cells. Fluorescence of the bacteria expressing mCherry was seen after 1 week (A, B), 2 weeks (C, D) and 2 months (E, F) after transformation; at all time points, the majority of fluorescent *M. leprae*-mCherry were in division (arrows). Panels A, C and E were generated by conventional fluorescence microscopy, using a 590 nm LED with a Zeiss fluorescence filter 61; panels B, D and F were generated by merging with differential interference contrast images. Scale bar represents 10 μm.

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antibiotic, determining precisely the timing of cell wall permeabilization and leak of the mCherry fluorescent probe, a process which started 6 h after exposure to the antibiotic and took approximately 1 h (Supporting Video 1). The mycobacteria cultured in IDE8 cells (Fig 7) and subsequently inoculated into BalbC mice (Table 1) or transformed by the pCHERRY3 plasmid (Fig 8), were positively identified as *M. leprae* by VNTR sequencing (Table 2). Cultivable contaminating microorganisms were not observed among any of these samples 72h after inoculation of blood agar plates, and uninfected IDE8 cells did not exhibit any fluorescence.

The distribution and prevalence of leprosy in different countries of the world challenges the hypothesis pointing to untreated multibacillary patients as being the only source of contagion, since countries that receive a large flow of immigrants from hyperendemic areas, such as the United Kingdom and USA, do not register high numbers of autochthonous cases of the disease [3]. Moreover, in hyperendemic countries such as Brazil, most of the new cases are not related to large agglomerations of poor citizens, such as are found in the periphery of large urban centers where the transmission of respiratory infections such as tuberculosis is facilitated. In Brazil leprosy is actually concentrated at the agricultural frontier [69]. In fact, a study carried out in the Brazilian Amazon successfully correlated detection rates of new cases of Hansen’s disease with deforestation activity [9]. Armadillos are recognized as a possible source of *M. leprae* infection in the southern USA and northern Brazil, where the disease is considered by some authors to be a zoonosis [11, 10]. Autochthonous US leprosy patients live in areas where infected armadillos can be found, and direct contact with these animals in Brazil and USA is also associated with a higher risk of being infected [7, 10, 11, 70]. We hypothesized that the numbers of new cases of leprosy recorded annually may be related to person-to-person transmission plus infection via ingestion of, and/or contact with, *M. leprae*-infected wild animals such as armadillos [11], squirrels [14], monkeys [13, 71] and hematophagous arthropods that feed on these animals such as kissing bugs [22] and ticks.

Ticks have a high potential to be vectors because they are long-lived and may take their blood meal from multiple animal species during their life cycle, plus the fact that many of the microorganisms that they harbor can be transmitted vertically from the mother tick to her progeny via the eggs. For these reasons, ticks are responsible for transmitting more species of pathogen to humans and domestic animals than any other arthropod. The IDE8 cell line, used successfully in this study to propagate *M. leprae* in vivo, was isolated from embryos of the blacklegged tick *I. scapularis*. Ticks (Acar: Ixodidae) have been reported in the literature to parasitize armadillos in several regions of the Americas [26,72–74]. Their role in the transmission of leprosy between armadillos, and from armadillos to humans, must be investigated. In the near future we intend to start the search for ticks naturally infected with *M. leprae*, in order to best understand the putative role of ticks in leprosy transmission among squirrels in the UK [14] and armadillos in the Americas [75–78].

Table 2. VNTR genotypes of *M. leprae* samples analyzed before (original samples) and after cultivation in the tick line IDE8 (Thai-53 strain) and in vitro transformation (PA84 strain).

| Samples                                    | 6–3 | AT17 | GGT5 | GTA9 | AC8B | AC8A | AT15 | 21–3 | TTC | 6–7 | 27–5 | 23–3 | 12–5 | 18–8 |
|--------------------------------------------|-----|------|------|------|------|------|------|------|-----|-----|-----|-----|-----|-----|
| THAI-53*                                   | 3   | 10   | 5    | 9    | 7    | 11   | 13   | 3    | 14  | 6   | 5   | 2   | 4   | 8   |
| THAI-53 after 20 days’ growth in IDE8      | 3   | 10   | 5    | 9    | ND   | 11   | ND   | 3    | ND  | 6   | 5   | 2   | ND  | 8   |
| PA84*                                      | 3   | 14   | 4    | 10   | 7    | ND   | 17   | 2    | 11  | 6   | 5   | 2   | 4   | 3   |
| *M. leprae*-mCherry PA84                   | 3   | 14   | 4    | 10   | 7    | ND   | 17   | 2    | 11  | 6   | 5   | 2   | 4   | 3   |

*Original samples.
ND: unable to characterize due to negative PCR.

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Throughout the last century, the search for a laboratory animal model able to support growth of *M. leprae* involved several species, but significant progress was only made in 1960, when Charles Shepard for the first time described local and limited growth of the pathogen in the mouse foot pad, mainly based on the low temperature of this environment [53]. This method evolved into the use of the athymic nude mouse CrI:NU(NCr)-Foxn1<sup>nu</sup> for the cultivation of *M. leprae* [79]. Another protocol to produce *M. leprae* involves intravenous inoculation of *Dasypus novemcinctus* armadillos. The low body temperature of this animal (30–35˚C) is also believed to be related to the success of this model in generating high bacillary numbers in internal organs [80]. Until now only animal models have been able to produce *M. leprae* with sufficiently high numbers and viability for experimental use [35,39].

On the other hand, many attempts have been made in the past to cultivate *M. leprae* axenically in different kinds of media. Recently, Amako and colleagues made some progress with a modified Kirchner Medium, replacing bovine serum with human plasma supplemented with egg-yolk extract, pyruvate and transferrin [81]. Although the authors successfully observed *M. leprae* genome duplication using droplet digital PCR, *M. leprae* growth using this axenic protocol appeared to be abnormally slow, with a doubling time close to 70 days [81]. In contrast, using IDE8 cells we observed an apparent doubling time of approximately 12 days, much closer to that observed in vivo (Fig 7C).

In a parallel to the revolution that occurred in research on bovine anaplasmosis in the 1990s, when the obligate intracellular bacterium *Anaplasma marginale* was first cultivated in tick cell lines [33], the *in vitro* production of *M. leprae* in the tick cell line IDE8 has potential for enormous impact in studies on the biology of the pathogen, large-scale production of its antigens and screening of new drugs. Production of *M. leprae* in cell bioreactors becomes a distinct possibility, generating relatively pure and LPS-free bacilli, which is difficult to achieve using nude mouse foot pads or armadillo liver and spleen. Our findings will facilitate site-directed mutagenesis studies in *M. leprae*, to determine the impact of genes of interest on the physiology of the bacillus. In addition, we now have the means and potential to understand the role of the abundant noncoding regions of the *M. leprae* genome, finally unravelling the secrets of one of the most enigmatic pathogens that still afflict humankind.

**Supporting information**

S1 Video. Visualization of antibiotic-mediated killing of intracellular *Mycobacterium leprae*. Time-lapse microscopy shows the exact moment of death of an *M. leprae*-mCherry bacterium in an IDE8 cell commencing 5 h after medium supplementation with 150 μg / mL ofloxacin. Scale bar represents 10 μm. (AVI)

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References

1. Monot M, Honoré N, Garnier T, Zidane N, Sherafi D, Paniz-Mondolfi A, et al. Comparative genomic and phylogeographic analysis of Mycobacterium leprae. Nat Genet. 2009 Dec; 41(12):1282–9. https://doi.org/10.1038/ng.477 PMID: 19881526

2. Meima A, Irgens LM, van Oortmarssen GJ, Richardus JH, Habbema JDF. Disappearance of leprosy from Norway: an exploration of critical factors using an epidemiological modelling approach. Int J Epidemiol. 2002 Oct; 31(5):991–1000. PMID: 12435774

3. Global leprosy update, 2014: need for early case detection. Relev Epidem Hebd. 2015 Sep; 90 (36):461–74.

4. Koba A, Ishii N, Mori S, Fine PEM. The decline of leprosy in Japan: patterns and trends 1964–2008. Lepr Rev. 2009 Dec; 80(4):432–40. PMID: 20306642

5. Nobre ML, Dupnik KM, Nobre PJJL, Freitas De Souza MC, Düppre NC, Sarno EN, et al. Human migration, railways and the geographic distribution of leprosy in Rio Grande do Norte State—Brazil. Lepr Rev. 2015 Dec; 86(4):335–44. PMID: 26964429
6. Queiroz JW, Dias GH, Nobre ML, De Sousa Dias MC, Araujo SF, Barbosa JD, et al. Geographic Information Systems and Applied Spatial Statistics Are Efficient Tools to Study Hansen’s Disease (Leprosy) and to Determine Areas of Greater Risk of Disease. Am J Trop Med Hyg. 2010 Feb; 82(2):306–14. https://doi.org/10.4269/ajtmh.2010.08-0675 PMID: 20134009

7. Sharma R, Singh P, Loughry WJ, Lockhart JM, Inman WB, Douthie MS, et al. Zoonotic Leprosy in the Southeastern United States. Emerg Infect Dis. 2015 Dec; 21(12):2127–34. https://doi.org/10.3201/eid2112.150501 PMID: 26583204

8. Argaw AT, Shannon EJ, Assefa A, Mikru FS, Mariam BK, Malone JB. A geospatial risk assessment model for leprosy in Ethiopia based on environmental thermal-hydrological regime analysis. Geospat Health. 2006 Nov; 1(1):105. https://doi.org/10.4081/gh.2006.285 PMID: 18686236

9. Silva DRX, Ignotti E, Souza-Santos R, Haco S de S. [Hansen’s disease, social conditions, and deforestation in the Brazilian Amazon]. Rev Panam Salud Publica. 2010 Apr; 27(4):268–75. PMID: 20512229

10. Truman RW, Singh P, Sharma R, Busso P, Rougemont J, Paniz-Mondolfi A, et al. Probable Zoonotic Leprosy in the Southern United States. N Engl J Med. 2011 Apr; 364(17):1626–33. https://doi.org/10.1056/NEJMoa1010536 PMID: 21524213

11. Da Silva MB, Portela JM, Li W, Jackson M, Gonzalez-Juarrero M, et al. Evidence of zoonotic leprosy in Pará, Brazilian Amazon, and risks associated with human contact or consumption of armadillos. 2018 [cited 2018 Sep 21]; Available from: https://doi.org/10.1371/journal.pntd.0006532 PMID: 29953440

12. Gormus BJ, Wolf RH, Baskin GB, Ohkawa S, Gerone PJ, Walsh GP, et al. A second sooty mangabey monkey with naturally acquired leprosy: first reported possible monkey-to-monkey transmission. Int J Lepr Other Mycobact Dis. 1988 Mar; 56(1):61–5. PMID: 3373087

13. Honap TP, Pfister L-A, Housman G, Mills S, Tarara RP, Suzuki K, et al. [Hansen’s disease, social conditions, and deforestation in the Brazilian Amazon]. Rev Panam Salud Publica. 2010 Apr; 27(4):268–75. PMID: 20512229

14. 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 Nov; 354(6313):744–7. https://doi.org/10.1126/science.aah3783 PMID: 27846605

15. Benchimol JL, Romero Sa M. Adolpho Lutz and controversies over the transmission of leprosy by mosquitoes. Hist Cienc Saude Manguinhos [Internet]. 2003 [cited 2018 Jun 25]; 10(Suppl 1):49–93. Available from: http://www.ncbi.nlm.nih.gov/pubmed/14650407 PMID: 14650407

16. Banerjee R, Banerjee BD, Chaudhury S, Hati AK. Transmission of viable Mycobacterium leprae by Aedes aegypti from lepromatous leprosy patients to the skin of mice through interrupted feeding. Lepr Rev. 1991 Mar; 62(1):21–6. PMID: 2034021

17. Narayanan E, Sreevatsa, Kirchheimer WF, Bedi BM. Transfer of leprosy bacilli from patients to mouse footpads by Aedes aegypti. Lepr India. 1977 Apr; 49(2):181–6. PMID: 333183

18. Saha K, Jain M, Mukherjee MK, Chawla NM, Chaudhary DS, Prakash N. Viability of Mycobacterium leprae genomes within the gut of Aedes aegypti after they feed on multibacillary lepromatous patients: a study by fluorescent and electron microscopes. Lepr Rev. 1985 Dec; 56(4):279–90. PMID: 3908862

19. Narayanan E, Sreevatsa, Raj AD, Kirchheimer WF, Bedi BM. Persistence and distribution of Mycobacterium leprae in Aedes aegypti and Culex fatigans experimentally fed on leprosy patients. Lepr India. 1978 Jan; 50(1):26–37. PMID: 349262

20. Geater JG. The fly as potential vector in the transmission of leprosy. Lepr Rev. 1975 Dec; 46(4):279–86. PMID: 1107727

21. Doannio JMC, Konan KL, Dosso FN, Koné AB, Konan YL, Sankaré Y, et al. [Microcota sp (Corixidae) and Diplonychus sp (Belostomatidae), two aquatic Hemiptera hosts and/or potential vectors of Mycobacteria ulcerans (pathogenic agent of Buruli ulcer) in Cote d'Ivoire]. Med Trop (Mars). 2011 Feb; 71(1):53–7. PMID: 20512229

22. Neumann A da S, Dias F de A, Ferreira J da S, Fontes ANB, Rosa PS, Macedo RE, et al. Experimental Infection of Rhodnius prolixus (Hemiptera, Triatominae) with Mycobacterium leprae Indicates Potential for Leprosy Transmission. Lanz-Mendoza H, editor. PLoS One. 2016 May; 11(5):e0156037. https://doi.org/10.1371/journal.pone.0156037 PMID: 27203082

23. Souza-Araujo HC de. Poderá o carrapato transmitir a lepra? Mem Inst Oswaldo Cruz. 1941; 36(4):577–84.

24. Nava S, Beatl L, Labruna MB, Cáceres AG, Mangold AJ, Guglielmone AA. Reassessment of the taxonomic status of Amblyomma cajennense (Fabricius, 1787) with the description of three new species, Amblyomma tonelliae n. sp., Amblyomma interandinum n. sp. and Amblyomma patinoi n. sp., and reinstatement of Amblyomma mixtum Koch, 1. Ticks Tick Borne Dis. 2014 Apr; 5(3):252–76. https://doi.org/10.1016/j.ttbdis.2013.11.004 PMID: 24356273
25. Estrada-Peña A, Guglielmone AA, Mangold AJ. The distribution and ecological “preferences” of the tick Amblyomma cajennense (Acari: Ixodidae), an ectoparasite of humans and other mammals in the Americas. Ann Trop Med Parasitol. 2004 Apr; 98(3):283–92. https://doi.org/10.1111/000348904225003316 PMID: 15119974

26. Bechara GH, Szabo MPJ, Almeida Filho W V, Bechara JN, Pereira RJG, Garcia JE, et al. Ticks associated with armadillo (Euphractus sexcinctus) and anteater (Myrmecophaga tridactyla) of Emas National Park, State of Goias, Brazil. Ann N Y Acad Sci. 2002 Oct; 969:290–3. PMID: 12381607

27. Smith MW. Some aspects of the ecology and lifecycle of Amblyomma cajennense (Fabricius 1787) in Trinidad and their influence on tick control measures. Ann Trop Med Parasitol. 1975 Mar; 69(1):121–9. PMID: 1124964

28. Soares JF, Soares HS, Barbieri AM, Labruna MB. Experimental infection of the tick Amblyomma cajennense, Cayenne tick, with Rickettsia rickettsii, the agent of Rocky Mountain spotted fever. Med Vet Entomol. 2012 Jun; 26(2):139–51. https://doi.org/10.1111/j.1365-2915.2011.00982.x PMID: 22007869

29. Bell-Sakyi L, Palomar AM, Bradford EL, Shkap V. Propagation of the Israeli vaccine strain of Anaplasma centrale in tick cell lines. Vet Microbiol. 2015 Sep; 179(3–4):270–6. https://doi.org/10.1016/j.vetmic.2015.07.008 PMID: 26210950

30. Bell-Sakyi L, Zweygarth E, Blouin EF, Gould EA, Jongejan F. Tick cell lines: tools for tick and tick-borne disease research. Trends Parasitol. 2007 Sep; 23(9):450–7. https://doi.org/10.1016/j.pt.2007.07.009 PMID: 17662657

31. Pornwiroon W, Pourciau SS, Foil LD, Macaluso KR. Rickettsia felis from cat fleas: isolation and culture in a tick-derived cell line. Appl Environ Microbiol. 2006 Aug; 72(8):5589–95. PMID: 16885313

32. Varela AS, Luttrell MP, Howertth EW, Moore VA, Davidson WR, Stallknecht DE, et al. First culture isolation of Borrelia lonestari, putative agent of southern tick-associated rash illness. J Clin Microbiol. 2004 Mar; 42(3):1163–9. https://doi.org/10.1128/JCM.42.3.1163-1169.2004 PMID: 15004069

33. Munderloh UG, Blouin EF, Kocan KM, Ge NL, Edwards WL, Kurtti TJ. Establishment of the tick (Acari: Ixodidae)-borne cattle pathogen Anaplasma marginale (Rickettsiales:Anaplasmataceae) in tick cell culture. J Med Entomol. 1996 Jul; 33(4):656–64. PMID: 8699463

34. Bell-Sakyi L, Darby A, Baylis M, Makepeace BL. The Tick Cell Biobank: A global resource for in vitro research on ticks, other arthropods and the pathogens they transmit. Ticks Tick Borne Dis. 2007 Sep; 23(9):450–7. https://doi.org/10.1016/j.pt.2007.07.009 PMID: 17662657

35. Truman RW, Krahenbuhl JL. Viable M. leprae as a research reagent. Int J Lepr Other Mycobact Dis. 2001 Mar; 69(1):1–12. PMID: 11480310

36. Neitz WO, Boughton F, Walters HS. Laboratory investigations on the life-cycle of Rhipicephalus theileri Bedford & Hewitt, 1925 (Ixodoidea: Ixodidae) parasites of the cattle tick Anaplasma marginale. Onderstepoort J Vet Res. 1972 Jun; 39(2):117–23. PMID: 4664319

37. Watts BP, Pound JM, Oliver JH. An adjustable plastic collar for feeding ticks on ears of rabbits. J Parasitol. 1972 Dec; 58(6):1105. PMID: 4641880

38. Matsuoka M. The history of Mycobacterium leprae Thai-53 strain. Lepr Rev. 2010 Jun; 81(2):137. PMID: 20825118

39. Lahiri R, Randhawa B, Krahenbuhl J. Application of a viability-staining method for Mycobacterium leprae derived from the athymic (nu/nu) mouse foot pad. J Med Microbiol. 2005 Mar; 54(3):235–42. PMID: 15004069

40. Ribeiro CCDU, de Azevedo Baêta B, de Almeida Valim JR, Teixeira RC, Cepeda PB, da Silva JB, et al. Use of plastic tips in artificial feeding of Dermacentor (Anocentor) nitens females Neumann, 1897 (Acari: Ixodidae). Ticks Tick Borne Dis. 2014 Oct; 5(6):689–92. https://doi.org/10.1016/j.ttbdis.2014.05.012 PMID: 25132536

41. Valim de A JR, Rangel CP, Baêta B de A, Ribeiro CCDU, Cordeiro MD, Teixeira RC, et al. Using plastic tips in artificial feeding of Rhipicephalus sanguineus sensu lato (Acari: Ixodidae) females. Rev Bras Parasitol Veterinaria. 2017 Mar; 26(1):110–4.

42. de Monteiro CM, Maturano R, Daemon E, Catunda-Junior FEA, Calmon F, de Souza Senra T, et al. Use of eugenol in artificial feeding of Dermacentor (Anocentor) nitens females Neumann, 1897 (Acari: Ixodidae).Ticks Tick Borne Dis. 2014 Oct; 5(6):689–92. https://doi.org/10.1016/j.ttbdis.2014.05.012 PMID: 25132536

43. Williams DL, Oby-Robinson S, Pittman TL, Scollard DM. Purification of Mycobacterium leprae RNA for gene expression analysis from leprosy biopsy specimens. Biotechniques. 2003 Sep; 35(3):534–6, 538, 540. https://doi.org/10.2144/03353st07 PMID: 14513559

44. Martinez AN, Lahiri R, Pittman TL, Scollard D, Truman R, Moraes MO, et al. Molecular Determination of Mycobacterium leprae Viability by Use of Real-Time PCR. J Clin Microbiol. 2009 Jul; 47(7):2124–30. https://doi.org/10.1128/JCM.00512-09 PMID: 19439537
63. Lara FA, Lins U, Paiva-Silva G, Almeida IC, Braga CM, Miguens FC, et al. A new intracellular pathway Caperucci D, Camargo Mathias MI, Bechara GH. Histopathology and ultrastructure features of the midgut of Amblyomma cajennense (Acari: Ixodidae) in various feeding stages and submitted to three infestations. Ultrastruct Pathol. 2009 Dec; 33(6):249–59. https://doi.org/10.1080/01913120903203638

62. Drummond RO, Whetstone TM. Oviposition of the Cayenne Tick, Amblyomma cajennense (F.), in the tick cell line Hyalomma anatolicum anatolicum. J Parasitol. 1991 Dec; 77(6):1006–8. PMID: 1779279

61. Munderloh UG, Liu Y, Wang M, Chen C, Kurtti TJ. Establishment, maintenance and description of cell lines from the tick Ixodes scapularis. J Parasitol. 1994 Aug; 80(4):533–43. PMID: 8064520

56. Cordeiro MD, de Azevedo Batista BA, Ferreira JS, Medeiros RC, Maya-Monteoime CRM, Lara FA, et al. Efficacy of sarolaner (Simparic) against induced infestations of Amblyomma cajennense on dogs. Parasit Vectors. 2017 Aug; 10:220. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5592192/

55. Munderloh UG, Kurtti TJ. Formulation of medium for tick cell culture. Exp Appl Acarol [Internet]. 1989 Jul; 10:1128–JCM.02014-08 PMID: 19386839

54. Carroll P, Schreuder LJ, Muwanguzi-Karugaba J, Wiles S, Robertson BD, Ripoll J, et al. Sensitive detection of Gene Expression in Mycobacterium under Replicating and Non-Replicating Conditions Using Optimized Far-Red Reporters. PLoS One [Internet]. 2010 [cited 2018 May 14]; 5(3). Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2843721/pdf/pone.0009823.pdf

53. Sheppard CC, Mcrae DH. Mycobacterium leprae in mice: minimal infectious dose, relationship between staining quality and infectivity, and effect of cortisone. J Bacteriol. 1965 Feb; 89(2):365–72.

52. Fandinho FC, Orsi-Souza AT, Salem JI. A comparison of the Ziehl-Neelsen and Kinyoun methods in staining smears from leprosy patients. Int J Lepr Other Mycobact Dis. 1990 Jun; 58(2):389–91.

51. Munderloh UG, Kurtti TJ. Formulation of medium for tick cell culture. Exp Appl Acarol [Internet]. 1989 Aug [cited 2018 Jun 25]; 7(3):219–29. Available from: http://www.ncbi.nlm.nih.gov/pubmed/2766897

50. Teixeira RC, Baêta BA, Ferreira JS, Medeiros RC, Maya-Monteiro CM, Lara FA, et al. Fluorescent membrane markers elucidate the association of Borrelia burgdorferi with tick cell lines. Brazilian J Med Biol Res. 2016; 49(7).

49. Munderloh UG, Kurtti TJ. Formulation of medium for tick cell culture. Exp Appl Acarol [Internet]. 1989 Jun 17; 58(2):1128–JCM.02014-08 PMID: 19386839

48. Bell-Sakyi L. Continuous cell lines from the tick Hyalomma anatolicum anatolicum. J Parasitol. 1991 Dec; 77(6):1006–8. PMID: 1779279

47. Bell-Sakyi L. Ehrlichia ruminantium grows in cell lines from Four Ixodid Tick Genera. J Comp Pathol [Internet]. 2004 May 1 [cited 2018 May 29]; 130(4):285–93. Available from: https://www.sciencedirect.com/science/article/pii/S0021997503001385?via%3Dihub https://doi.org/10.1016/j.jcpa.2003.12.002 PMID: 15053931

46. Rosenfeldt MT, Nixon C, Liu E, Mah LY, Ryan KM. Analysis of macroautophagy by immunohistochemistry. Autophagy. 2012 Jun; 8(6):963–9. https://doi.org/10.4161/auto.20186 PMID: 22562096

45. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001 Dec; 25(4):402–8. https://doi.org/10.1006/meth.2001.1262 PMID: 11846609

44. Estrada-Pena A, McGregor S, Jolly D, Lounibos LP. Ticks as potential leprosy vectors. Int J Trop Med Hyg [Internet]. 1985 Jan 1 [cited 2018 May 9]; 34(1):151–61. Available from: http://www.ajtmh.org/content/journals/10.4269/ajtmh.1985.34.151 PMID: 8882008

43. Capercucci D, Camargo Mathias MI, Bechara GH. Histopathology and ultrastructure features of the midgut of adult females of the tick Amblyomma cajennense Fabricius, 1787 (Acari: Ixodidae) in various feeding stages and submitted to three infestations. Ultrastruct Pathol. 2009 Dec; 33(6):249–59. https://doi.org/10.4109/01913120903296945 PMID: 19929171

42. Sanchez RM, Job CK, Hastings RC. Manifestations of Experimental Leprosy in the Armadillo. Am J Trop Med Hyg [Internet]. 1985 Jan 1 [cited 2018 May 9]; 34(1):151–61. Available from: http://www.ajtmh.org/content/journals/10.4269/ajtmh.1985.34.151 PMID: 296945

41. Caperucci D, Camargo Mathias MI, Bechara GH. Histopathology and ultrastructure features of the midgut of adult females of the tick Amblyomma cajennense Fabricius, 1787 (Acari: Ixodidae) in various feeding stages and submitted to three infestations. Ultrastruct Pathol. 2009 Dec; 33(6):249–59. https://doi.org/10.4109/01913120903296945 PMID: 19929171

40. Drummond RO, Whetstone TM. Oviposition of the Cayenne Tick, Amblyomma cajennense (F.), in the Laboratory.1. Ann Entomol Soc Am [Internet]. 1975 Mar 17 [cited 2018 May 9]; 47(7):2124 –30. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19439537 https://doi.org/10.1080/01913120903296945

39. Estrada-Pena A, Jongejans F. Ticks feeding on humans: a review of records on human-biting Ixodidae with special reference to pathogen transmission. Exp Appl Acarol. 1999 Sep; 23(9):865–715. PMID: 29601710
65. Lahiri R, Krahenbuhl JL. The role of free-living pathogenic amoeba in the transmission of leprosy: A proof of principle. Lepr Rev. 2008; 79:401–409.

66. Wheat WH, Casali AL, Thomas V, Spencer JS, Lahiri R, Williams DL, et al. Long-term survival and virulence of Mycobacterium leprae in amoebal cysts. PLoS Negl Trop Dis. 2014; 8:e3405.

67. Shepard CC. The experimental disease that follows the injection of human leprosy bacilli into foot-pads of mice. J Exp Med. 1960 Sep; 112(3):445–54. PMID: 19867175

68. Ehrt S, Guo X V, Hickey CM, Ryou M, Monteleone M, Riley LW, et al. Controlling gene expression in mycobacteria with anhydrotetracycline and Tet repressor. Nucleic Acids Res. 2005 Feb; 33(2):e21. https://doi.org/10.1093/nar/gni013 PMID: 15687379

69. Magalhães M da CC, Rojas Li. Diferenciação territorial da hanseníase no Brasil. Epidemiol e Serviços Saúde. 2007; 16(2):75–84.

70. Deps PD, Alves BL, Gripp CG, Aragao RL, Guedes B, Filho JB, et al. Contact with armadillos increases the risk of leprosy in Brazil: a case control study. Indian J Dermatol Venereol Leprol [Internet]. 2008 [cited 2018 May 9]; 74(4):338–42. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18797053 PMID: 18797053

71. Meyers WM, Gorman BJ, Walsh GP, Baskin GB, Hubbard GB. Naturally acquired and experimental leprosy in nonhuman primates. Am J Trop Med Hyg. 1991 Apr; 44(4 Pt 2):24–7.

72. Guglielmone AA, Estrada-Peña A, Luciani CA, Mangold AJ, Keirans JE. Hosts and distribution of Amblyomma auricularium (Conil 1878) and Amblyomma pseudoconcolor Aragão, 1908 (Acari: Ixodidae). Exp Appl Acarol. 2003; 29(1–2):131–9. PMID: 14580065

73. Medri IM, Martins JR, Doyle RL, Mourão G, Marinho-Filho J. Ticks (Acari: Ixodidae) from yellow armadillo, Euphractus sexcinctus (Cingulata: Dasypodidae), in Brazil’s Pantanal wetlands. Neotrop Entomol. 2010 Oct; 39(5):823–5. PMID: 21120394

74. Mertins JW, Vigil SL, Corn JL. Amblyomma auricularium (Ixodidae: Ixodidae) in Florida: New Hosts and Distribution Records. J Med Entomol. 2017 Jan; 54(1):132–41. https://doi.org/10.1093/jme/tjw159 PMID: 28082640

75. Amezcuia ME, Escobar-Gutiérrez A, Storrs EE, Dhople AM, Burchfield HP. Wild Mexican armadillo with leprosy-like infection. Int J Lepr Other Mycobact Dis. 1984 Jun; 4(2):252–5. PMID: 6539311

76. Cardona-Castro N, Beltrán JC, Ortiz-Bernal A, Vissa V. Detection of Mycobacterium leprae DNA in nine-banded armadillos (Dasypus novemcinctus) from the Andean region of Colombia. Lepr Rev. 2009 Dec; 80(4):424–31. PMID: 20306641

77. Frota CC, Lima LNC, Rocha A da S, Suffys PN, Rolim BN, Rodrigues LC, et al. Mycobacterium leprae in six-banded (Euphractus sexcinctus) and nine-banded armadillos (Dasypus novemcinctus) in Northeast Brazil. Mem Inst Oswaldo Cruz. 2012 Dec;209–13. PMID: 23283473

78. Balamayooran G, Pena M, Sharma R, Truman RW. The armadillo as an animal model and reservoir host for Mycobacterium leprae. Clin Dermatol. 2015 Jan; 33(1):108–15. https://doi.org/10.1016/j.clindermatol.2014.07.001 PMID: 25432816

79. Colston MJ, Hilson GR. Growth of Mycobacterium leprae and M. marinum in congenitally athymic (nude) mice. Nature. 1976 Jul; 262(5567):399–401. PMID: 795274

80. Kirchheimer WF, Storrs EE. Attempts to establish the armadillo (Dasypus novemcinctus Linn.) as a model for the study of leprosy. I. Report of lepromatoid leprosy in an experimentally infected armadillo. Int J Lepr Other Mycobact Dis. 39(3):693–702. PMID: 4948218

81. Amako K, Iida K-I, Saito M, Ogura Y, Hayashi T, Yoshida S-I. Non-exponential growth of Mycobacterium leprae Thai-53 strain cultured in vitro. Microbiol Immunol. 2016 Dec; 60(12):817–23. https://doi.org/10.1111/1348-0421.12454 PMID: 27925336