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Assessment of the Pathogenicity of *Rickettsia amblyommatis*, *Rickettsia bellii*, and *Rickettsia montanensis* in a Guinea Pig Model

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Abstract

Members of the genus *Rickettsia* range from nonpathogenic endosymbionts to virulent pathogens such as *Rickettsia rickettsii*, the causative agent of Rocky Mountain spotted fever. Many rickettsiae are considered nonpathogenic because they have been isolated from ticks but not vertebrate hosts. We assessed the ability of three presumed endosymbionts: *Rickettsia amblyommatis*, *Rickettsia bellii*, and *Rickettsia montanensis*, to infect a guinea pig animal model. These species were chosen because of their high prevalence in respective tick vectors or published reports suggestive of human or animal pathogenicity. Following intraperitoneal (IP) inoculation of cell culture suspensions of *R. rickettsii*, *R. amblyommatis*, *R. bellii*, or *R. montanensis* into guinea pigs, animals were monitored for signs of clinical illness for 13 days. Ear biopsies and blood samples were taken at 2- to 3-day intervals for detection of rickettsial DNA by PCR. Animals were necropsied and internal organ samples were also tested using PCR assays. Among the six guinea pigs inoculated with *R. amblyommatis*, fever, orchitis, and dermatitis were observed in one, one, and three animals respectively. In *R. bellii*-exposed animals, we noted fever in one of six animals, orchitis in one, and dermatitis in two. No PCR-positive tissues were present in either the *R. amblyommatis*- or *R. bellii*-exposed groups. In the *R. montanensis*-exposed group, two of six animals became febrile, two had orchitis, and three developed dermatitis in ears or footpads. *R. montanensis* DNA was detected in ear skin biopsies collected on multiple days from three animals. Also, a liver specimen from one animal and spleen specimens of two animals were PCR positive. The course and severity of disease in the three experimental groups were significantly milder than that of *R. rickettsii*. This study suggests that the three rickettsiae considered nonpathogenic can cause either subclinical or mild infections in guinea pigs when introduced via IP inoculation.

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Keywords

Rickettsia amblyommatis ; *Rickettsia bellii* ; *Rickettsia montanensis* ; endosymbiont; pathogenicity

Introduction

The genus *Rickettsia* consists of gram-negative, obligate intracellular coccobacilli that are primarily transmitted by fleas, lice, mites, and ticks (Azad and Beard 1998). This genus is divided into four groups; the largest of which is the spotted fever group (SFG). The SFG contains over 20 recognized species, ranging from putatively nonpathogenic to deadly agents of disease. In addition, there is the typhus group, which contains two species, *Rickettsia prowazekii* and *Rickettsia typhi* (Diop et al. 2018). Two more recent subdivisions are the ancestral group, which contains *Rickettsia bellii* and *Rickettsia canadensis*, and the transitional group, which includes *Rickettsia akari* and *Rickettsia felis* (Tang et al. 2015, Nováková and Šmajs 2018). Historically, the two rickettsial species primarily responsible for severe human rickettsioses in the United States are *Rickettsia rickettsii* and *R. prowazekii*, the causative agents for Rocky Mountain spotted fever (RMSF) and epidemic typhus, respectively.

While the pathogenicity of these two rickettsiae is well documented, the potential of many other rickettsial species to cause human illness remains uncharacterized. Numerous rickettsiae have been discovered in ticks and described as endosymbionts based on the absence of attributable clinical illness in animals or humans (Parola et al. 2013). Some of these endosymbionts, including *Rickettsia parkeri* and *Rickettsia slovaca*, were subsequently identified as human or animal pathogens based on diagnosable clinical infections, serological response, or PCR-positive tissues in animals (Raoult et al. 1997, Paddock et al. 2004). Many more poorly understood *Rickettsia* spp. are common in human-biting ticks, thus increasing the importance of determining the public health risk they may pose (Parola et al. 2005, Jado et al. 2007).

Rickettsia amblyommatis (formerly *Candidatus* Rickettsia amblyommii) is a transovarially and transstadially transmitted SFG rickettsiae found in a wide variety of ticks throughout the Americas, including several *Amblyomma*, *Rhipicephalus*, and *Dermacentor* spp. (Burgdorfer et al. 1981, Labruna et al. 2011). In the United States, the lone star tick (*Amblyomma americanum)* is its primary host, with rates of infection between 20% and 80% commonly reported in *A. americanum* populations (Mixson et al. 2006, Killmaster et al. 2014, De Jesus et al. 2019). Initially, *R. amblyommatis* was labeled as nonpathogenic because inoculation of the original type strain WB-8–2 T did not cause clinical signs of illness in guinea pigs (*Cavia porcellus*) (Burgdorfer et al. 1981). However, in a subsequent study with another strain (9-CC-3-1), the enlargement of testes was noted 2 days after *R. amblyommatis* exposure and tissues from these animals were PCR positive (Rivas et al. 2015, Karpathy et al. 2016).

To complement these reports, Levin et al. (2018) found that guinea pigs feeding naturally infected *A. americanum* nymphs presented with multiday scrotal edema in some animals

and *R. amblyommatis* DNA was present in detectable levels in ear skin biopsies. However, no gross abnormalities were noted upon necropsy in this study (Levin et al. 2018). In addition, Barrett and coauthors reported immunoglobulin G (IgG) antibody titers as high as 1/16,000 in dogs naturally exposed to infected ticks on multiple occasions, as well as PCR detection of *R. amblyommatis* DNA in whole blood samples taken during the study (Barrett et al. 2014). Evidence of exposure to *R. amblyommatis* based on seroconversion has been suggested in some human cases of rickettsiosis originally presumed to be RMSF. Retrospective serological assessment of patients showed a diagnostic titer (defined as a fourfold or higher titer increase) to *R. amblyommatis*, but not *R. rickettsii* (Apperson et al. 2008). Because *A. americanum* is a highly aggressive human-biting tick with a high prevalence of *R. amblyommatis* infection, it is important to understand the potential pathogenicity of this endosymbiont.

R. bellii was originally isolated from a pool of *Dermacentor variabilis* in 1966 and has since been isolated from both ixodid and argasid ticks across the United States as well as Central and South America (Horta et al. 2006). Many ticks harbor this bacterium, including multiple *Amblyomma* and *Dermacentor* spp., *Haemaphysalis leporispalustris*, *Ixodes loricatus*, and several soft tick species (Krawczak et al. 2018). The prevalence in tick populations varies widely from <1% to 80% (Parola et al. 2013).

Initial reports of animal inoculations with *R. bellii* (strain 369-C) labeled this species as nonpathogenic to Swiss white mice (*Mus musculus*), guinea pigs (*C. porcellus*), and voles (*Microtus pennsylvanicus*), although seroconversion was noted in some animals (Philip et al. 1983). In a later study, intradermal inoculation with *R. bellii* (strain 369L42-1) into guinea pigs led to eschars by day 3 postinoculation (La Scola et al. 2009). In addition, there is serological evidence for *R. bellii* exposure in capybaras, which suggests that at minimum it can be transmitted to vertebrate hosts during tick feeding (Pacheco et al. 2007). Notably, there has been no direct isolation of *R. bellii* from vertebrate hosts. As with *R. amblyommatis*, this *Rickettsia* spp. is widely disseminated and present in human-biting ticks and its potential pathogenicity warrants further assessment.

Rickettsia montanensis (formerly *Rickettsia montana*) is a member of the SFG and is found primarily in *D. variabilis* and *Dermacentor andersoni* in the United States and Canada. Its prevalence ranges from <1% to ~30% depending on the region (Dergousoff et al. 2009, St John et al. 2016, Hecht et al. 2019). This is the most prevalent *Rickettsia* spp. found in *D. variabilis* in the eastern United States. *D. variabilis* is also recognized as the primary vector of *R. rickettsii* in this region (Stromdahl et al. 2011). Results of animal inoculation studies have varied. In 1984, Norment and Burgdorfer found that dogs inoculated with *R. montanensis* (strain M/5–6 B) did not show any signs of illness following needle or tick inoculation (Norment and Burgdorfer 1984), whereas in another study, repeated exposure to tick bites led to seroconversion and detection of rickettsial DNA in the whole blood of exposed dogs (Barrett et al. 2014). After being suggested in a possible pediatric case of rickettsiosis, it seems possible *R. montanensis* could cause a mild SFG illness (McQuiston et al. 2012).

A. americanum and *D. variabilis* are two of the most common ticks collected during flagging and trapping in the southeastern United States, as well as in reports of human tick bites (Smith et al. 2010). Given the widespread nature of the symbiotic rickettsiae associated with these ticks, humans and animals bitten by *Amblyomma* and *Dermacentor* ticks likely often encounter their symbionts as well. With these considerations in mind, we exposed a guinea pig animal model to three widespread rickettsiae: *R. amblyommatis*, *R. montanensis*, and *R. bellii*, for assessment of their ability to infect a vertebrate host and cause clinical or subclinical infections.

Materials and Methods

This study was undertaken at a facility fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International. All procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals, eighth edition. All procedures of this study were preapproved by the Centers for Disease Control and Prevention (CDC) Institutional Animal Care and Use Committee.

Three groups of six tick-naïve, specific pathogen-free, Hartley strain, male guinea pigs (6–8 months old) were needle inoculated intraperitoneally (IP) in the lower right quadrant of the abdomen with *R. amblyommatis*, *R. bellii*, or *R. montanensis*. The fourth (positive control) group was used for clinical comparison and consisted of three guinea pigs needle inoculated with *R. rickettsii*. An additional group of three guinea pigs were needle inoculated with clean Vero cells to serve as a negative control. All isolates were propagated in Vero cells, frozen back for long-term storage, and thawed immediately before inoculation. The amount of rickettsiae in harvested cultures was assessed using a Pan-*Rickettsia* quantitative real-time PCR assay before inoculation (Kato et al. 2013).

Each of six guinea pigs in the first group was inoculated with 0.25 mL of *R. amblyommatis* (strain Lake Alexander, passage 4) culture containing 6.67×10^6 rickettsiae. In the second group, animals received 0.12 mL of *R. bellii* (strain Yolo, passage 2) culture containing 1.25×10^4 rickettsiae. In the third group, guinea pigs were inoculated with 0.25 mL of *R. montanensis* (strain OSU, passage 4) culture containing 5.10×10^6 rickettsiae. In the positive control group, each animal received 0.25 mL of *R. rickettsii* (strain AZ3, passage 8) culture containing 1.85×10^7 rickettsiae. In the negative control group, each animal was inoculated with 0.25 mL of clean Vero cells.

Guinea pigs were monitored daily for clinical signs of infection, including fever (defined as rectal temperature ≥39.8°C), scrotal edema, and dermatitis in ears or footpads for 13 days postinoculation (DPI) (Walker et al. 1977). In addition, bodyweight changes were measured as (weight loss/gain ÷ original bodyweight). Any of these signs during the study categorized an animal as clinically ill. Samples of whole blood collected from ear veins (100 μL) and ear skin biopsies (2 mm in diameter) were collected aseptically under anesthesia every 2–3 days to assess rickettsial infection status via real-time quantitative PCR (qPCR).

At 13 DPI, animals were euthanized and necropsied to assess internal pathology, including known rickettsial pathology such as necrotic lesions of the liver, splenomegaly, testicular

erythema, and lung petechiation. Samples of sera for indirect immunofluorescence assays (IFA) and internal organs for PCR assays were also collected during euthanasia and necropsy, respectively. Sampled internal organs included the liver, spleen, bladder, testes, lung, and heart tissues. Detection of rickettsial DNA in skin biopsies or internal tissues, or seroconversion in animals in the absence of observable clinical signs, was categorized as evidence of subclinical infection.

DNA extraction and PCR procedures were carried out in separate facilities to prevent contamination. DNA from ear skin biopsies and organ samples was extracted using the Qiagen DNeasy Blood and Tissue Kit (Valencia, CA) using the manufacturer's recommendations for purification of total DNA from Animal Tissues (Spin-Column Protocol) with one modification: the addition of a 10-min, 70°C pathogen inactivation step after the addition of Buffer AL but before the addition of 100% ethanol. DNA from 100 μL of whole blood was extracted using the Qiagen FlexiGene Blood and Tissue kit according to the manufacturer's recommendations. Final elution volumes for these extracts were 100 μL in both cases.

After DNA extraction, samples were first tested using a broad range Pan-*Rickettsia* spp. real-time PCR assay targeting a portion of the 23S rRNA gene (Kato et al. 2013). Pan-*Rickettsia*-positive samples were then assessed using real-time species-specific PCR assays for *R. amblyommatis*, *R. montanensis*, *R. rickettsii*, and *R. bellii* (Jiang et al. 2010, Smith et al. 2010, Kato et al. 2013, Hecht et al. 2016). See Table 1 for the complete list of PCR assays used in this study. For quantitation of rickettsiae in cultures used for inoculation, a plasmid produced by Blue Heron BioTech LLC (Washington) specific for the above Pan-*Rickettsia* assay was diluted from 10^8 down to 10^1 copies per reaction and used for quantitation.

All real-time PCRs were performed in duplicate on a BioRad CFX 96 thermal cycler with a final reaction volume of 20 μL (25 μL in the case of the *R. bellii*-specific assay). Five microliters of DNA was assessed in each reaction for Pan-*Rickettsia*, *R. montanensis*, and *R. rickettsii* assays, 4 μL of DNA in the case of the *R. bellii*-specific assay, and 2 μL of DNA in the *R. amblyommatis*-specific assay. Samples were considered positive if both duplicates had a cycle threshold ≤36. One negative control and one positive control were included in each PCR run, where 2–5 μL water was used as the negative control and 2–5 μL of species-specific genomic DNA was used as a positive control depending on the assay.

All samples positive by the 23S Pan-*Rickettsia* assay were further screened using a seminested conventional PCR assay targeting a portion of the *omp*A gene specific for SFG rickettsiae (Eremeeva et al. 2006). An alternate gltA assay was used in the case of *R. bellii* samples as it is not part of the SFG (Labruna et al. 2004). DNA amplicons were visualized on 1.5% agarose gels containing 0.1 μg/mL of ethidium bromide. Amplicons were then extracted and purified using the Promega Wizard SV Gel and PCR Clean-up System (Promega, Madison, WI). Products were bidirectionally sequenced using the BigDye Terminator v3.1 kit on an ABI 3130xl genetic analyzer (Applied Biosystems, Carlsbad, CA) and assembled using SeqMan Pro 14 software. A BLASTn analysis comparing the

assembled sequences with sequences available in GenBank identified the *Rickettsia* spp. present in the PCR-positive samples.

Serum samples were tested by an indirect IFA technique for IgG antibodies reactive with whole-cell antigens of the specific species to which the experimental animal was exposed: *R. rickettsii* (Sheila Smith), *R. bellii* (Yolo), *R. amblyommatis* (Lake Alexander), or *R. montanensis* (OSU). Vero cell-inoculated animals were assessed on *R. rickettsii* (Sheila Smith) antigen-coated slides. Samples were screened at initial dilutions of 1/16 and 1/256 and subsequently diluted serially to the endpoint titer if seropositive upon the initial screen. A fluorescein isothiocyanate-labeled goat anti-guinea pig conjugate reactive with the heavy and light chains of IgG (Kirkegaard and Perry Laboratories) was used at a 1/150 dilution.

Positive control serum for slides was derived from animals previously exposed to each specific agent (but not necessarily the same strain): *R. rickettsii* (Sheila Smith), *R. amblyommatis* (Darkwater), *R. bellii* (Yolo), and *R. montanensis* (OSU). Titers were expressed as the reciprocal of the last dilution exhibiting specific fluorescence to the specific rickettsial antigen. Specimens with IgG titers ≥128 were considered seropositive for the study as defined by the 2020 CDC IFA guidelines for humans. Specimens with IgG titers = 64 were considered supportive evidence of clinical infection based on the updated 2020 CDC IFA guidelines for humans.

Results

Vero cells

All three guinea pigs in the Vero cell-inoculated group remained healthy and absent of all clinical signs throughout the study. All animals gained weight during the study (9.3–11.4%) and all ear skin biopsies and blood samples remained PCR negative throughout the study. Upon necropsy, no signs of infection were noted during gross examination, and all organ samples were PCR negative. All animals remained seronegative as of 13 DPI.

R. rickettsii (strain AZ3)

All three guinea pigs in the *R. rickettsii*-exposed group presented with signs clinically compatible with rickettsiosis. Table 2 presents the clinical and molecular findings for animals inoculated with *R. rickettsii*. Two animals lost weight (between 0.25% and 2.20% of the original bodyweight), whereas one animal gained weight (5.7%). All animals exhibited decreased activity levels, including a lack of grooming. All three animals had fevers on multiple days with an onset at 3 DPI for all animals and ending on 8–11 DPI. All animals had dermatitis of the ear pinna with onset at 3–7 DPI and ending on 9 DPI for one animal while lasting to the termination of the study on 13 DPI for the other two. All animals presented with footpad dermatitis starting at 5–6 DPI, two animals had persistent footpad dermatitis through the end of the study, and one had dermatitis on 6 and 9 DPI only.

All three animals had notable internal pathology at the time of necropsy, including widespread necrotic liver lesions, lung petechiation, and splenomegaly. Testicular erythema was not observed in any animal. All three animals in this group had PCR-positive ear skin samples on multiple days, with two animals having detectable amounts of rickettsial DNA

starting at 3–7 DPI through termination at 13 DPI. In addition, all animals had PCR-positive liver and spleen samples. One animal also had a PCR-positive testis and lung specimen, and another had a PCR-positive bladder sample. All amplicons had *Rickettsia* identity confirmed using an *R. rickettsii*-specific assay and all but two samples were also identified by DNA sequencing as *R. rickettsii* using the ompA seminested assay (GenBank accession no. MT035856). Seroconversion to *R. rickettsii* IFA antigen occurred in all three animals with antibody titers ranging from 1/1024 to 1/2048.

R. amblyommatis (strain Lake Alexander)

Table 3 presents the clinical and molecular findings for animals inoculated with *R. amblyommatis*. Four of six animals in this group presented with clinical illness based on described criteria. All guinea pigs inoculated with *R. amblyommatis* gained weight throughout the study (between 2.9% and 24.3% gain) despite two guinea pigs (Ra-4 and 5) becoming notably ill, displaying decreased activity levels, poor grooming, scrotal edema, and dermatitis. One of these two animals also had mild fever on 6 DPI. These two animals also had the lowest weight gains among the *R. amblyommatis*-inoculated group. In two other animals, the only recognizable signs were mild edema and dermatitis in the ears or footpads on a single day.

Upon euthanasia and necropsy at 13 DPI, three of six animals (all with observable clinical signs of infection) presented with necrotic lesions on the liver. Two of these animals presented with other pathological signs: testicular erythema in one case and lung petechiation in the other. No animals had detectable rickettsial DNA in any tissues throughout the study. One animal seroconverted at a titer of 1/64 against *R. amblyommatis* (strain Lake Alexander) antigen, which was considered supportive evidence of infection when paired with clinical signs present in this animal.

R. montanensis (strain OSU)

Table 4 presents the clinical and molecular findings for animals inoculated with *R. montanensis*. Four of six animals in this group presented with clinical illness and one had evidence of subclinical infection. Five of the guinea pigs inoculated with *R. montanensis* gained between 4.3% and 26% bodyweight during the 13-day observation period, whereas one animal lost 29.9% of its bodyweight. Two of six animals had fevers lasting 1 day; one of these had a notable spike to 40.6°C on 3 DPI. These two animals also had multiple days of ear and footpad dermatitis and one of the two additionally presented with scrotal edema over multiple days. Another animal had multiple days of footpad dermatitis and scrotal edema along with 1 day of ear dermatitis, and the last of the four clinically ill animals had a single day of footpad dermatitis on 7 DPI. Only one animal had notable necropsy findings, with bladder and testicular erythema present.

Rickettsia spp. DNA was detected in multiple tissue types. The three animals with ear dermatitis had PCR-positive ear skin samples on at least 1 day during the study and in one case repeatedly through the end of the study. One guinea pig with PCR-positive ear skin biopsies on multiple days had a PCR-positive spleen specimen. Interestingly, the animal with a PCR-positive liver and spleen specimen was the animal that lost almost a third of its

bodyweight but showed no other clinical signs of infection. The liver and spleen samples from Rm-2, as well as the ear biopsies from all animals, were PCR positive using the specific *R. montanensis* assay, and ompA seminested product sequencing was successful on all samples (GenBank accession nos. MT035857, MT035858). Seroconversion occurred in four animals, with titers against *R. montanensis* (strain OSU) antigen ranging from 1/64 to 1/1024.

R. bellii (strain Yolo)

Table 5 presents the clinical and molecular findings for animals inoculated with *R. bellii*. Animals inoculated with *R. bellii* received a smaller dose of rickettsiae than the other two groups due to the availability of inoculum. Four of six animals presented with at least one clinical sign at some point throughout the study. All animals gained weight during the study period (3.0–16.3% weight gain) and no behavioral changes were noted. One of six animals had a single-day fever of 40.3°C on 4 DPI but showed no other clinical signs during the study. In another guinea pig, the only recognizable sign was mild scrotal edema. Ear dermatitis was present for a single day in one animal (at 3 DPI) and for over a week (from 4 to 12 DPI) in another.

At necropsy, internal pathological changes were noted in three animals: one guinea pig had necrotic liver lesions, another had liver lesions as well as testicular erythema and lung petechiae, and the third had just lung petechiation. The animal with multiple days of fever showed no internal pathological changes, whereas the two animals with a single day of clinically evident illness both showed internal changes. No animals had PCR-positive tissues at any point throughout the study. Two animals seroconverted against *R. bellii* (strain Yolo) antigen with titers of 1/64 for both: one was considered supportive evidence of infection (in Rb-5), whereas one cannot be considered evidence of infection due to the lack of any corresponding clinical signs or PCR confirmation (in Rb-1).

Discussion

R. amblyommatis, *R. bellii*, and *R. montanensis* are the three rickettsial species that are highly prevalent in human biting ticks in the Americas. In addition, *R. amblyommatis* and *R. montanensis* have been reported as possibly causing mild SFG rickettsioses (Apperson et al. 2008, McQuiston et al. 2012, Delisle et al. 2016). Given this information, we inoculated guinea pigs with infected cell cultures of three prevalent rickettsiae to assess whether they are capable of causing clinical or subclinical infections in laboratory animals challenged with relatively large doses of these agents.

Signs of rickettsial infection in guinea pigs are notoriously variable between individual animals (Levin et al. 2020). However, in this study, all *R. rickettsii*-infected guinea pigs presented with clear signs of rickettsiosis and provided a reference point for assessing clinical signs in the groups of guinea pigs inoculated with the endosymbiotic rickettsiae. Overall, each of the three experimental agents assessed in this study was able to establish a generalized infection in the model vertebrate host; all the clinical and pathological observations in individual guinea pigs within each group varied widely from prominent to inapparent illness. The lack of clinical signs in Vero cell-inoculated animals indicates that

these clinical signs are likely not caused by inoculation of the cells, but the *Rickettsia* within them. Although no scrotal edema was noted in the control group, which would suggest that the inoculated *Rickettsia* was responsible for inflammation, it is still possible that the scrotal edema reported in other exposed groups was due to IP inoculation rather than rickettsial infection.

R. amblyommatis (strain Lake Alexander) infections, at the most severe, caused a single-day mild fever, multiday scrotal edema, and ear dermatitis. Rickettsial DNA could not be detected in any animals and serological conversion occurred in one animal, although at a low level. The animal with a supportive positive IFA titer was one of the animals with the most conspicuous clinical signs within this group. Internal pathological changes were noticed in the organs of some animals, with necrotic liver lesions being the most common finding. This indicates that to some extent, *R. amblyommatis* (Lake Alexander) can be pathogenic to guinea pigs, as seen previously in Rivas et al. (2015), although causing a much milder infection than *R. rickettsii* (Rivas et al. 2015). A similar course of infection was seen when guinea pigs were exposed to naturally infected *A. americanum* nymphs (Levin et al. 2018). However, infections did not seem to adversely affect the animals' activity levels or overall health for long and are much more akin to a subclinical or mild infection that could be easily missed than a case of RMSF.

R. montanensis-infected animals exhibited the most notable pathological changes and outward signs of illness among the experimental groups. While guinea pigs' fevers were short lived, scrotal edema as well as ear and footpad dermatitis persisted for multiple days in three animals. In addition, *R. montanensis* DNA was detected in ear skin and organ tissue samples from four of six animals. This was the only experimental cohort where *Rickettsia* spp. PCR-positive samples were identified at the species level by qPCR and/or sequencing. However, despite observation of tangible signs of illness during clinical observation, very few changes were noted internally upon necropsy. This is interesting considering we detected rickettsial DNA in one liver sample and two spleen samples.

One notable occurrence in this cohort was the 30% bodyweight loss in one animal. This animal was diagnosed with a gastrointestinal disorder upon necropsy but showed no clinical signs of rickettsiosis during the study or upon necropsy and had an IFA titer below cutoff. However, the animal had an *R. montanensis* PCR-positive liver and spleen sample. It is unclear whether the gastrointestinal condition was present before the study and was exacerbated by the exposure to an infectious agent. It is possible it had some effect on the course of infection in this animal, which otherwise showed no recognizable clinical signs of rickettsial infection.

Animals infected with *R. bellii* (strain Yolo) received a significantly lower dose of inoculum than other cohorts and thus could not be directly compared with the other experimental groups. The most common finding in this cohort was ear dermatitis, and at the most severe, animals had a 1-day fever or multiday ear dermatitis (from 4 to 12 DPI). Necropsy results were inconsistent between animals, with one animal having liver necrosis, testicular erythema, and lung petechiation despite showing no clinical signs of illness outside of mild ear dermatitis on 6 DPI. Despite a lower dose of inoculum, just as many animals

seroconverted (and at similar titers) as in the *R. amblyommatis*-infected group. These data indicate that *R. bellii* can cause pathological changes to IP-inoculated animals internally, but that recognition of external clinical signs of infection may be difficult as illness seems to be largely subclinical and transient.

Overall, our clinical, pathological, molecular, and serological data lead to a conclusion that the three assessed species of *Rickettsia*—despite being considered endosymbionts—can infect guinea pigs and cause either clinical or subclinical infections. This suggests the possibility of these three *Rickettsia* spp. being capable of infecting other mammalian hosts as well. *R. montanensis* appears capable of proliferating in guinea pigs after IP inoculation based on consistent detection in ear skin samples and in some organ samples at the termination of the study. The evidence for *R. amblyommatis* and *R. bellii* proliferation appears less strong; while clinical signs were observed in some animals, pathogen DNA detection in samples was not reported. It is also possible, however, that these three rickettsiae have different time courses of infection and the 13 DPI mark was favorable for identifying internal pathological changes in model animals inoculated with some agents, but not others.

Another possibility that could affect the proliferation of *Rickettsia* spp. in guinea pigs is that previously frozen cultures were used for inoculation. A previous study found that *Ehrlichia chaffeensis* isolates cryopreserved and thawed immediately before inoculation caused only mild clinical signs in dairy calves and less effective detection of the pathogen in blood samples via PCR, while the use of active cultures of the same strain led to more severe clinical signs in animals (Delos Santos et al. 2007). While this is a different bacterium, the general concept of cryopreservation affecting the progress of infection could be pertinent.

It should be noted that the IP inoculation mode of transmission cannot be directly equated to tick-borne pathogen transmission that occurs naturally during feeding. Tick saliva is known to contain anti-inflammatory and immunomodulating molecules that can change the profile of pathogen transmission (Ramachandra and Wikel 1992, Esteves et al. 2019). There is also some evidence that tick saliva potentiates the transmission of tick-borne agents and enhances their virulence in vertebrate hosts (Simo et al. 2017). This study assessed the innate ability of the three endosymbiotic *Rickettsia* spp. to infect an animal model without the assistance of salivary transmission. In addition, other modes of inoculation are utilized in studying the effects of various rickettsiae in model guinea pigs. These include subcutaneous, intradermal, and intravenous inoculation routes, among others. As the goal of this study was to determine if purported nonpathogenic *Rickettsia* spp. could cause clinical signs in model animals, the ensured exposure was of more concern than the mode of delivery.

To build on this pilot study, tick-borne transmission studies should be performed using naturally infected ticks to confirm this mode of transmission. Another direction of interest may be to compare the pathogenicity of various individual strains of these rickettsiae using model animals such as guinea pigs or mouse models. While some strains of *R. amblyommatis* have been reported to only cause seroconversion, some others have caused active clinical infections in model animals (Karpathy et al. 2016). There appears to be some variation between the strains of the same species in disease-causing capacity. This has also

been seen in *R. rickettsii* where much more work has been done on characterizing various strains (Paddock et al. 2014, Clark et al. 2015). Here we can show that *R. amblyommatis* (Lake Alexander) and *R. bellii* (Yolo) can infect guinea pigs and cause clinical or subclinical signs of rickettsiosis but may not proliferate very effectively, while *R. montanensis* (OSU) appears to cause clinical signs of infection and proliferates in model animals during infection.

Conclusions

In this study, we investigated the pathogenic potential of several rickettsiae commonly found in aggressive, human-biting ticks of the Americas. After IP inoculation and a 13-day period of clinical monitoring, we found that all three rickettsiae are capable of causing clinical or subclinical illnesses in guinea pigs. *R. amblyommatis* inoculation led to a variety of outcomes in guinea pigs ranging from no clinical signs to a 1-day mild fever, multiday scrotal edema, and ear dermatitis. *R. montanensis* inoculation also led to no active infections in some guinea pigs and mild fever along with multiday scrotal edema, and ear and footpad dermatitis in others. *R. bellii*-infected guinea pigs had either no or very mild external clinical signs but had notable internal pathology in some guinea pigs.

These data suggest that these three rickettsiae can be pathogenic, to varying extents, but present as a very mild illness compared with RMSF. This supports previously reported data where either animals or humans presented with mild clinical illnesses or positive serology after being exposed to *R. amblyommatis*, *R. montanensis*, or *R. bellii*. Future studies should aim to assess the clinical outcomes in model animals exposed to these agents via the more natural route of tick inoculation as tick saliva can change the profile of pathogen transmission.

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Table 1.

Conventional and Real-Time PCR Assays (Table view)

Assay	Primer/probe name	Primer/probe sequence (5'→3')	Gene target	Reference
Pan-Rickettsia real-time PCR	PanR8_F	AGC TTG CTT TTG GAT CAT TTG G	23S rRNA	Kato et al. (2013)
	PanR8_R	TTC CTT GCC TTT TCA TAC ATC TAG T		
	PanR8_P	FAM[a]-CCT GCT TCT ATT TGT CTT GCA GTA ACA CGC CA-BHQ1		
Rickettsia amblyommatis real-time PCR	Ra477F	GGT GCT GCG GCT TCT ACA TTA G	ompB	Jiang et al. (2010)
	Ra618R	CTG AAA CTT GAA TAA ATC CAT TAG TAA CAT		
	Ra532Probe	FAM-CGC GAT CTC CTC TTA CAC TTG GAC AGA ATG CTT ATC GCG-BHQ1		
Rickettsia bellii real-time PCR	RBELLII-F	ATC CTG ATT TGC TGA ATT TTT T	gltA	Hecht et al. (2016)
	RBELLII-R	TGC AAT ACC AGT ACT GAC G		
	RBELLII-P	CALRED610[b]-ATG ATG TTT GCC ACA CCT TGT GAA AA-BHQ2		
Rickettsia montanensis real-time PCR	RMF2832	GCG GTG GTG TTC CTA ATA C	ompB	Smith et al. (2010)
	RMR2937	CCT AAG TTG TTA TAG TCT GTA GTG		
	RMB2875	FAM-CGG GGC AAA GAT GCT AGC GCT TCA CAG TTA CCC CG-BHQ1		
Rickettsia rickettsii real-time PCR	RRi6_F	AAA TCA ACG GAA GAG CAA AAC	Hypothetical protein A1G_04230	Kato et al. (2013)
	RRi6_R	CCC TCC ACT ACC TGC ATC AT		
	RRi6_P	FAM[a]-TCC TCT CCA ATC AGC GAT TC-BHQ1		
SFG *Rickettsia* conventional seminested PCR	RR190.70F	ATG GCG AAT ATT TCT CCA AAA A	ompA	Eremeeva et al. (2006)
	RR190.602R	AGT GCA GCA TTC GCT CCC CCT		
	RR190.701R	GTT CCG TTA ATG GCA GCA TCT		
Rickettsia spp. conventional PCR	CS-78	GCA AGT ATC GGT GAG GAT GTA AT	gltA	Labruna et al. (2004)
	CS-323	GCT TCC TTA AAA TTC AAT AAA TCA GGA T		

[a]Modified from original fluorescein (Fl) to FAM.

[b]Modified from original FAM to CALRED610.

FAM, 6-carboxyfluorescein; SFG, spotted fever group.

Table 2.

Clinical, PCR, and Immunofluorescence Assay Results from Guinea Pigs Inoculated with *Rickettsia rickettsii* (Days Postinoculation) (Table view)

Animal No.	Rr-1	Rr-2	Rr-3
Fever	+ (3–11)	+ (3–8)	+ (3–9)
Weight change	−0.25%	−2.2%	+5.7%
Scrotal edema	+ (3–12)	+ (5, 7–11)	+ (8–9)
Ear dermatitis	+ (3, 5–13)	+ (6–13)	+ (7–9)
Foot dermatitis	+ (5–13)	+ (5–13)	+ (6, 9)
Liver necrosis	+	+	+
Testicular erythema	–	–	–
Lung petechiation	+	+	+
Splenomegaly	+	+	+
Ear skin PCR	+ (3–13)	+ (3–13)	+ (7–10)
Blood PCR	–	–	–
Organ PCR	+ (liver, spleen, testes, lung)	+ (liver, spleen, bladder)	+ (liver, spleen)
IFA titer	2048	1024	1024

IFA, immunofluorescence assay.

Table 3.

Clinical, PCR, and Immunofluorescence Assay Results from Guinea Pigs Inoculated with *Rickettsia amblyommatis* (Days Postinoculation) (Table view)

Animal No.	Ra-1	Ra-2	Ra-3	Ra-4	Ra-5	Ra-6
Fever	–	–	–	+ (6)	–	–
Weight change	+22.8%	+13.8	+24.3%	+8.5%	+6.3%	+2.9%
Scrotal edema	–	–	–	+(7, 8, 10–13)	–	–
Ear dermatitis	–	–	+ (7)	+ (5–7)	+ (4–8, 11–13)	–
Foot dermatitis	–	–	–	–	+ (5, 7)	+ (13)
Liver necrosis	–	–	–	+	+	+
Testicular erythema	–	–	–	–	+	–
Lung petechiation	–	–	–	–	–	+
Splenomegaly	–	–	–	–	–	–
Ear skin PCR	–	–	–	–	–	–
Blood PCR	–	–	–	–	–	–
Organ PCR	–	–	–	–	–	–
IFA titer	–	–	–	–	64	–

Table 4.

Clinical, PCR, and Immunofluorescence Assay Results from Guinea Pigs Inoculated with *Rickettsia montanensis* (Days Postinoculation) (Table view)

Animal No.	Rm-1	Rm-2	Rm-3	Rm-4	Rm-5	Rm-6
Fever	–	–	–	+ (3)	–	+ (4)
Weight change	+ 26%	−29.9%	+ 10.4%	+ 4.3%	+ 8.5%	+ 8.0%
Scrotal edema	–	–	–	+ (3–8, 10)	+ (3–6)	–
Ear dermatitis	–	–	+ (7)	+ (5–7)	+ (4–8, 11–13)	–
Foot dermatitis	–	–	–	+ (4–6, 10–12)	+ (6)	+ (6, 8–10, 12–13)
Liver necrosis	–	–	–	–	–	–
Testicular erythema	–	–	–	–	+	–
Lung petechiation	–	–	–	–	–	–
Splenomegaly	–	–	–	–	–	–
Ear skin PCR	–	–	–	+ (3–10)	+ (3–10, 13)	–
Blood PCR	–	–	–	–	–	–
Organ PCR	–	+ (liver, spleen)	–	–	+ (spleen)	–
IFA titer	–	–	64	512	1024	512

Table 5.

Clinical, PCR, and Immunofluorescence Assay Results from Guinea Pigs Inoculated with *Rickettsia bellii* (Days Postinoculation) (Table view)

Animal No.	Rb-1	Rb-2	Rb-3	Rb-4	Rb-5	Rb-6
Fever	–	+ (4)	–	–	–	–
Weight change	+16.5%	+7.2%	+16.1%	+3.0%	+8.6%	+4.1%
Scrotal edema	–	–	–	+ (8)	–	–
Ear dermatitis	–	–	–	–	+ (6)	+ (4–12)
Foot dermatitis	–	–	–	–	–	–
Liver necrosis	–	–	–	+	+	–
Testicular erythema	–	–	–	–	+	–
Lung petechiation	–	–	–	–	–	–
Splenomegaly	–	–	–	–	–	–
Ear skin PCR	–	–	–	–	–	–
Blood PCR	–	–	–	–	–	–
Organ PCR	–	–	–	–	–	–
IFA titer	64	–	–	–	64	–