



Isolate-Dependent Differences in Clinical, Pathological, and Transcriptional Profiles following *In Vitro* and *In Vivo* Infections with *Rickettsia rickettsii*

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ABSTRACT *Rickettsia rickettsii*, the etiological agent of Rocky Mountain spotted fever (RMSF), a life-threatening tick-borne disease that affects humans and various animal species, has been recognized in medicine and science for more than 100 years. Isolate-dependent differences in virulence of *R. rickettsii* have been documented for many decades; nonetheless, the specific genetic and phenotypic factors responsible for these differences have not been characterized. Using *in vivo* and *in vitro* methods, we identified multiple phenotypic differences among six geographically distinct isolates of *R. rickettsii*, representing isolates from the United States, Costa Rica, and Brazil. Aggregate phenotypic data, derived from growth in Vero E6 cells and from clinical and pathological characteristics following infection of male guinea pigs (*Cavia porcellus*), allowed separation of these isolates into three categories: nonvirulent (Iowa), mildly virulent (Sawtooth and Gila), and highly virulent (Sheila Smith[†], Costa Rica, and Tiaçu). Transcriptional profiles of 11 recognized or putative virulence factors confirmed the isolate-dependent differences between mildly and highly virulent isolates. These data corroborate previous qualitative assessments of strain virulence and suggest further that a critical and previously underappreciated balance between bacterial growth and host immune response could leverage strain pathogenicity. Also, this work provides insight into isolate-specific microbiological factors that contribute to the outcome of RMSF and confirms the hypothesis that distinct rickettsial isolates also differ phenotypically, which could influence the severity of disease in vertebrate hosts.

KEYWORDS RMSF, *Rickettsia rickettsii*, isolate-dependent, phenotypes, virulence

Rocky Mountain spotted fever (RMSF) is a life-threatening tick-borne disease that affects humans and various animal species (1). Infected patients demonstrate multiple signs and symptoms that generally include high fever, severe headache, generalized rash, and myalgias. This disease, caused by *Rickettsia rickettsii*, can evolve rapidly to multiorgan system failure and death from systemic vasculitis (2). In the United States, the estimated case fatality rate of RMSF has ranged from 5% to 10% since the discovery in the 1940s of tetracycline-class antibiotics (3, 4). For reasons not yet understood, case fatality rates of RMSF are considerably higher in many Latin American countries, and contemporary estimates in Mexico and Brazil are approximately 30 to 40% (5–7). Interestingly, previous studies identified genetic differences between various isolates of *R. rickettsii* that correlate directly with their geographical origin (8–13).

Isolate differences in *R. rickettsii* virulence have been recognized for more than

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TABLE 1 Clinical signs of infection at 6 days postinoculation^a

Clinical sign	% of animals infected					
	Iowa	Sawtooth	Gila	Sheila Smith ^T	Costa Rica	Taiacu
Fever ^b	50	100	100	100	100	100
Footpad dermatitis	0	20	10	90	20	30
Scrotal edema	0	25	80	100	30	90
Wt loss	0	80	90	100	100	100
Splenomegaly ^c	100	100	100	100	100	100

^a*n* = 10 for Iowa, Sawtooth, Gila, Sheila Smith^T, and Taiacu; *n* = 9 for Costa Rica.

^bTemperature above 39.5°C during the 6 days of the experiment.

^cAbove 0.13 index of total body weight.

60 years (14). Less virulent isolates of *R. rickettsii*, such as Sawtooth, highly virulent isolates, such as the type strain Sheila Smith, and the avirulent Iowa isolate were described previously, although the phenotypic and molecular basis for their differences have not been fully characterized (8–11, 14–17). Here, we describe an *in vitro* and *in vivo* analysis of several phenotypic and transcriptional differences among six geographically distinct isolates of *R. rickettsii* from the United States, Costa Rica, and Brazil, including three isolated from patients with severe or fatal disease, and three highly pathogenic, minimally pathogenic, or nonpathogenic isolates from ticks (15, 16, 18). These findings provide further insight into isolate-specific microbiological factors and corresponding pathology that contribute to RMSF.

RESULTS

Clinical and pathological characteristics of guinea pigs infected with different *R. rickettsii* isolates. To characterize the clinical and pathological differences among isolates of *R. rickettsii*, 6- to 9-week-old, pathogen-free, male guinea pigs (*Cavia porcellus*) were infected with standardized amounts of each bacterial isolate (Iowa, Sawtooth, Gila, Sheila Smith^T, Costa Rica, and Taiacu) and the course of infection was documented daily by clinical scoring for 6 days. Sheila Smith^T-, Costa Rica-, and Taiacu-infected animals exhibited consistent and similar signs and lesions of severe disease, as indicated by the clinical parameters, gross abnormalities, and histopathologic findings. In contrast, Sawtooth- and Gila-infected animals showed clinical and pathological findings compatible with mild disease, and animals infected with Iowa remained minimally symptomatic or asymptomatic.

Desquamating dermatitis of the footpads was observed in 20% of Sawtooth-infected, 10% of Gila-infected, 20% of Costa Rica-infected, and 30% of Taiacu-infected animals, and it was present in the majority (90%) of Sheila Smith^T-infected animals (Table 1). In addition, 80 to 100% of Sheila Smith^T-, Taiacu-, and Gila-infected animals exhibited significant scrotal edema, including the presence of necrotic areas in Sheila Smith^T- and Taiacu-infected animals. Only 40% of Sawtooth- and 30% of Costa Rica-infected animals presented these scrotal findings.

All Sawtooth-, Gila-, Costa Rica-, Taiacu-, and Sheila Smith^T-infected animals presented with fever $\geq 39.5^\circ\text{C}$ (Fig. 1). Only 50% of the Iowa-infected animals had low-grade fever (40.0°C to 40.5°C) on days 1 and 2 and returned to normal temperature by day 3. Negative-control animals did not develop fever. Each of the Sheila Smith^T-, Costa Rica-, and Taiacu-infected animals presented with fever beginning on day 2 post-infection and continued with high fever between 40.5°C and 41°C until the end of the study. Sawtooth- and Gila-infected animals defervesced after day 4 of infection.

Animal weights were measured at the time of inoculation and at day 6. Both absolute and percent changes in body weight were analyzed. As the results agreed qualitatively, percent change in body weight and confidence intervals were used to provide general interpretation of the comparisons (Fig. 2). By day 6 postinfection, weights were statistically lower among Gila-, Sheila Smith^T-, Costa Rica-, and Taiacu-infected animals than in Iowa-infected animals and the negative-control group, which either maintained weight (*n* = 1 in each group) or gained weight (*n* = 9 of each group) over the course of

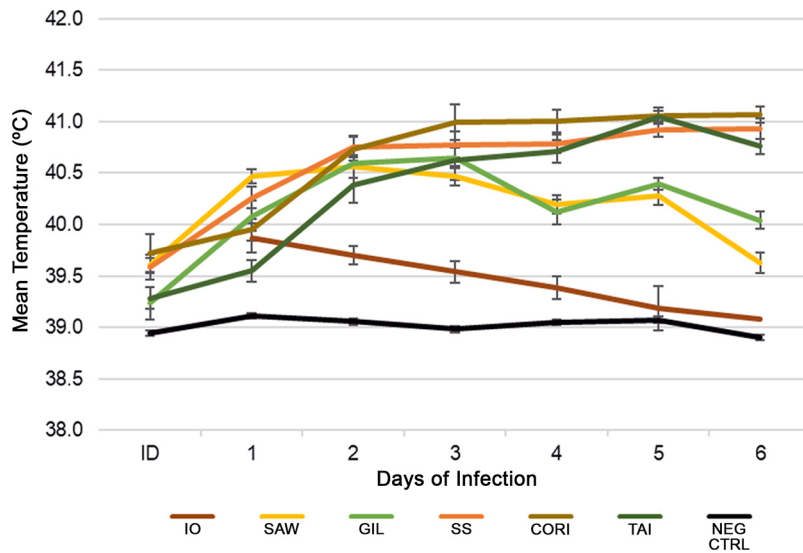


FIG 1 Body temperature of *Rickettsia rickettsii*-infected guinea pigs. Temperatures were monitored daily. Each point represents the mean temperature of 10 animals, except for Costa Rica ($n=9$), and standard errors of the means (SEM) are shown. Controls received the same amount of noninfected Vero cells. ID, inoculation day; IO, Iowa; SAW, Sawtooth; GIL, Gila; SS, Sheila Smith^T; CORI, Costa Rica; TAI, Tiaçu; NEG CTRL, negative control. The ID data point for Iowa was not collected.

the study (Fig. 2A). Weight loss in the Sawtooth- and Gila-infected animals was also statistically significant, although Sheila Smith^T-, Costa Rica-, and Tiaçu-infected-animal losses were about 5% greater than the combined average for Sawtooth- and Gila-infected animals (Fig. 2A and B). We also identified a statistically significant greater day 6 spleen-to-weight index among Sawtooth-, Gila-, Sheila Smith^T-, Costa Rica-, and Tiaçu-infected animals than in the Iowa-infected and negative-control groups (Fig. 2C and D).

During necropsy, gross abnormalities were observed in the spleens and lungs of the infected animals as well as in livers and testes, as shown in Fig. 3. Pale foci in the liver were identified in 40% to 50% of all Sheila Smith^T-, Costa Rica-, and Tiaçu-infected animals, while congested lungs were observed in 30% of these animals (Table 1 and Fig. 3). Prominent congestion and erythema of the testes were identified in almost all individuals infected with the Sheila Smith^T ($n=10$ of 10) or Tiaçu ($n=9$ of 10) isolates (Table 1 and Fig. 3). These lesions were observed in a few Sawtooth-infected ($n=4$ of 10) and Costa Rica-infected ($n=3$ of 9) animals. No gross abnormalities were observed in Iowa-infected or negative-control animals. All infected animals had enlarged spleens compared to negative-control animals at day 6 of infection.

Histopathological and immunohistochemical evaluation of spleens, livers, lungs, and testes obtained at necropsy from guinea pigs infected with *R. rickettsii* revealed a spectrum of pathology that was tissue and isolate dependent (Fig. 4). None of the animals infected with the Iowa isolate showed abnormal histopathologic features or definitive immunohistochemical evidence of infection in any of the tissues examined. For the remaining isolates, inflammatory lesions were identified in the testes and epididymides of all Sheila Smith^T-, Costa Rica-, and Tiaçu-infected animals, comprising lymphohistiocytic vasculitis of the tunics and interstitium (Fig. 4A and B). Focal to extensive immunohistochemical staining was identified in inflamed foci of all animals infected with these three isolates but was most abundant with Costa Rica (Fig. 4C). Testes and epididymides were inflamed in only half of Sawtooth- and Gila-infected animals. Infiltrates in these animals were typically less abundant, and rickettsial antigens were identified in only those animals with inflammatory foci. Pulmonary lesions, comprising predominantly mild interstitial mononuclear inflammatory cell infiltrates, were identified in all Sheila Smith^T-infected and almost all Costa Rica- and Tiaçu-infected

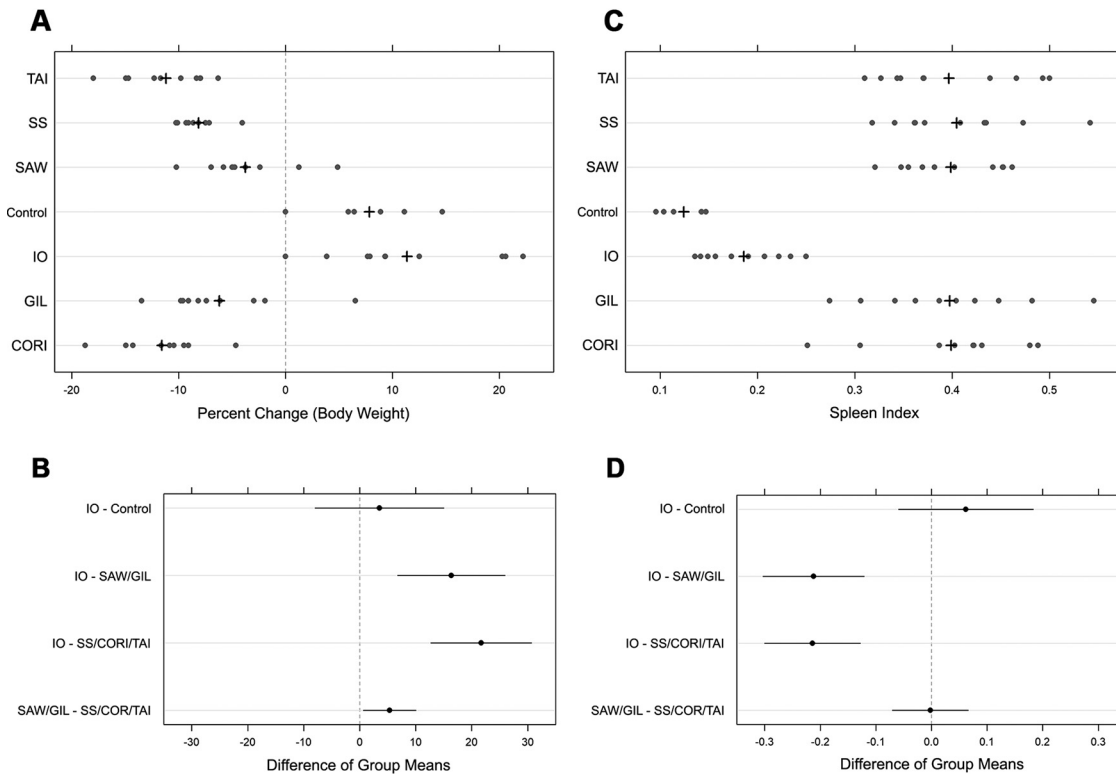


FIG 2 Body weight change and spleen size evaluation of all infected and noninfected samples. (A) Percentage of *Rickettsia rickettsii*-infected guinea pig body weight change. The weight was measured on days 1 and 6 of infection. (C) Spleen index at 6 days postinoculation. The spleen index was calculated based on the final body weight. +, mean value. (B and D) Comparative pairwise differences (95% CI) in mean percentage change in body weight and spleen index, respectively, with the null reference line at 0. Differences in CIs that do not contain 0 are considered statistically significant at 5% significance. IO, low; SAW, Sawtooth; GIL, Gila; SS, Sheila Smith^T; CORI, Costa Rica; TAI, Tiaçu.

animals, whereas only half of the animals infected with Sawtooth or Gila demonstrated similar lesions. Interestingly, the lungs of most Gila-infected animals also demonstrated multifocal capillaritis, often accompanied by fibrin thrombi (Fig. 4D).

Antigens of *R. rickettsii* were detected in inflamed foci of all Sheila Smith^T-infected, almost all Costa Rica- and Tiaçu-infected, and only half of Sawtooth- or Gila-infected animals. The predominant hepatic lesion in animals infected with all isolates except lowa was vacuolar degeneration of hepatocytes, scattered single-cell hepatocyte necrosis, and small lobular foci of mixed inflammatory cell infiltrates (Fig. 4E). Immunohistochemistry revealed generally rare, focal staining of antigens of *R. rickettsii* in Kupffer cells of all animals infected with Sheila Smith^T, Costa Rica, and Tiaçu and half of those infected with Gila but only one animal infected with Sawtooth. Diffuse histiocytic infiltrates were identified in the splenic red pulp of all animals infected with Sheila Smith^T and Costa Rica and 40% infected with Tiaçu. In all Sawtooth-infected and 30% of Gila-infected animals, these infiltrates coalesced to form loose granulomas (Fig. 4F). Rare rickettsial antigens were detected by immunohistochemistry (IHC) staining in the spleens of all Costa Rica- and Tiaçu-infected and almost all Sawtooth-, Gila-, and Sheila Smith^T-infected animals.

Infection load. To identify tissue differences in *R. rickettsii* load among isolates, we evaluated spleens, livers, lungs, and testes from animals infected with all isolates on day 6 postinfection using a multivariate analysis of variance (MANOVA). The *Rickettsia*-specific real-time quantitative PCR multivariate data are shown in pairwise plots in Fig. 5. Spleen, liver, and lung samples from all animals infected with all isolates except lowa contained rickettsial DNA, with a positive correlation between bacterial loads in livers and lungs. While testes of all animals infected with Sheila Smith^T, Costa Rica, and

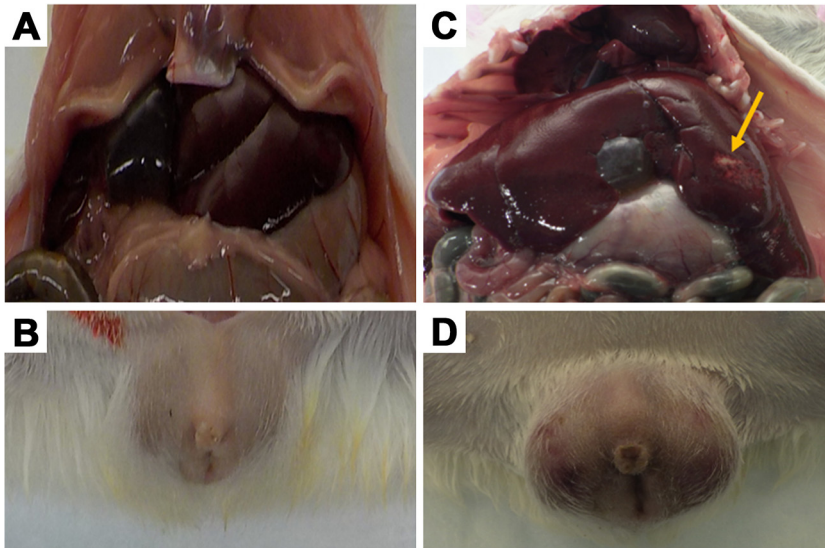


FIG 3 Gross pathological findings in one isolate of *Rickettsia rickettsii* 6 days postinfection. (Top) Internal organs from the abdominal area; (bottom) scrotal area. (A and B) Negative controls; (C and D) Taiacu infection. The arrow indicates the pale foci in the liver (C). Edema, erythema, and necrotic lesions on infected testes can be seen (D).

Taiacu contained DNA of *R. rickettsii*, testes from only 70% of Sawtooth- and 60% of Gila-infected animals demonstrated molecular evidence of infection. To evaluate the overall difference in rickettsial load among the isolates, combined tissue data for each isolate were analyzed (Fig. 6). Statistically significant differences were also identified when Sawtooth- and Gila-infected tissues were individually compared to Costa Rica- and Taiacu-infected tissues (Fig. 6). Interestingly, when tissues were analyzed individually, significant differences in bacterial loads in the testes were seen between Sawtooth- and Gila-infected animals and Sheila Smith^T- and Costa Rica-infected animals (see Fig. S1 in the supplemental material). Variation in rickettsial load was less pronounced in the spleen than in other tissues and ranged from 3.63×10^2 to 9.29×10^2 rickettsiae/ μg of extracted DNA (Fig. 5 and Fig. S2). Sawtooth- and Gila-infected livers, lungs, and testes had bacterial loads 10- to 100-fold lower than those in Sheila Smith^T-, Costa Rica-, and Taiacu-infected groups (Fig. 5).

To determine bacterial viability within individual tissues, we inoculated Vero E6 cells with all testicular tissues collected at day 6 of infection and monitored cell cultures for 4 weeks or until the cytopathic effect exceeded 70%. We identified statistically significant differences in isolation rates comparing Sawtooth- to Sheila Smith^T-infected ($P < 0.01$), Sawtooth- to Costa Rica-infected ($P < 0.05$), and Sawtooth- to Taiacu-infected ($P < 0.05$) tissue replicates. Viable *R. rickettsii* was recovered from 80% of Sheila Smith^T-, 77.77% of Costa Rica-, and 80% of Taiacu-infected samples, while fewer Sawtooth-infected (10%) and Gila-infected (20%) samples contained live *Rickettsia*. No bacteria were recovered from the Iowa-infected samples or negative-control animals (data not shown).

Based on aggregate phenotypic data, we grouped the 6 studied isolates into three categories: a nonvirulent isolate (Iowa), two mildly virulent isolates (Sawtooth and Gila), and three highly virulent isolates (Sheila Smith^T, Costa Rica, and Taiacu).

Virulence factor gene expression. To identify gene expression differences that could account for differential virulence *in vitro* and *in vivo* experiments, we compared the transcriptional profiles of one representative of the mildly virulent (Sawtooth) and one representative of the highly virulent (Sheila Smith^T) groups. Initially, both isolates were analyzed *in vitro*, tracking the infected Vero E6 cell cultures for 6 days. Both infections were established successfully, although differences in the number of bacteria per

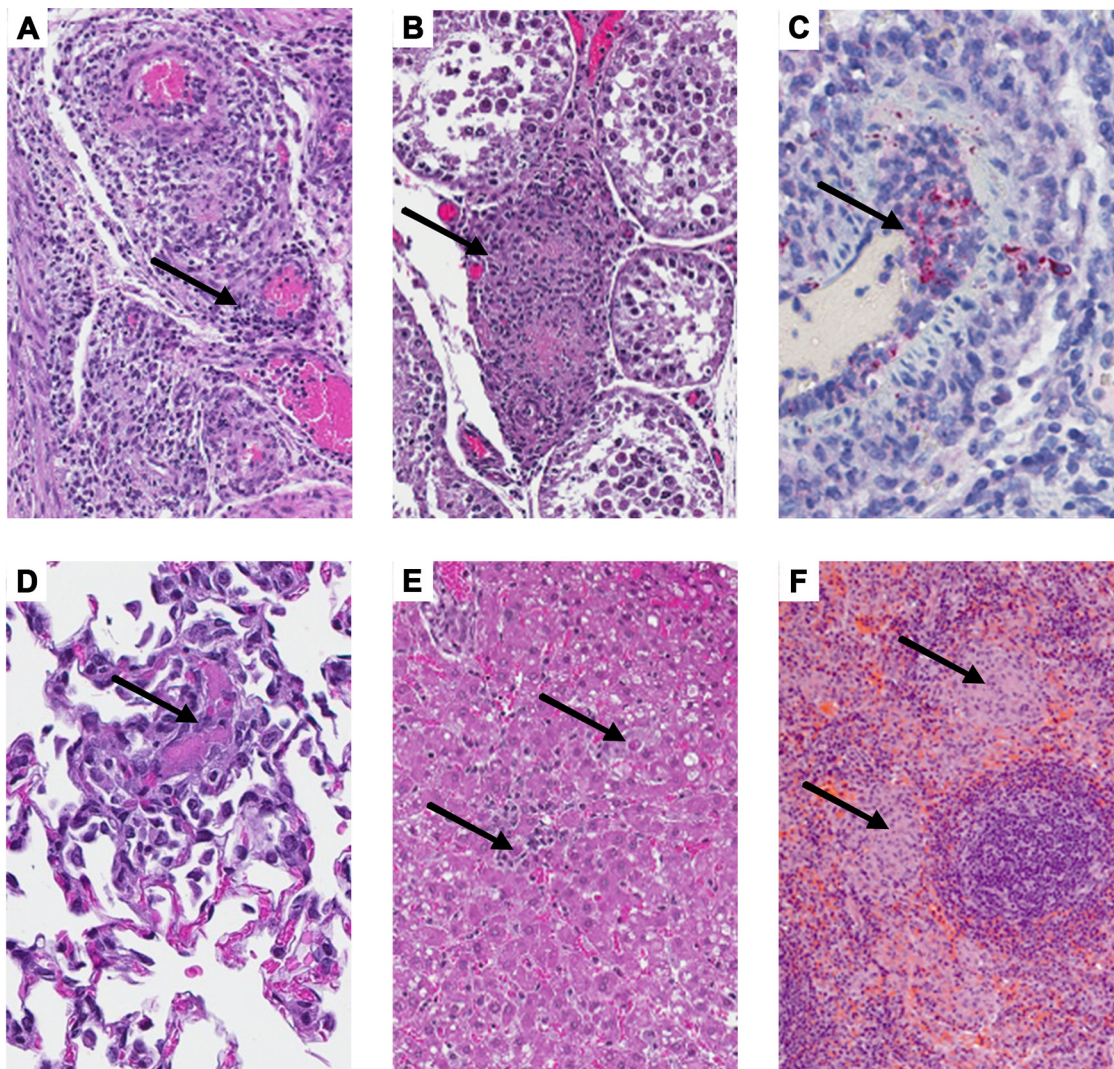


FIG 4 Histopathological and immunohistochemical findings in tissues from animals infected intraperitoneally with *Rickettsia rickettsii*. Testes stained with hematoxylin and eosin showing lymphohistiocytic vasculitis of the tunics and interstitium (A and B). Immunohistochemical staining showing focal to extensive rickettsial antigens (red) in Costa Rica-infected testes (C). Capillaritis with fibrin thrombus in the lung of a Gila-infected animal (D). Mild vacuolar degeneration, rare single-cell necrosis, and mild lobular inflammation, comprising the predominant hepatic lesions produced following infection with all isolates except Iowa (E). Spleen histiocytes forming loose granulomas in a Sawtooth-infected animal (F). Magnifications, $\times 50$ (A, B, and E), $\times 100$ (C and D), and $\times 25$ (F).

Vero E6 cell were identified over the course of the experiment when the ratio of each isolate per Vero cell (actin) was calculated. Sheila Smith^T- and Sawtooth-infected-cell ratios (*Rickettsia* to actin) were compared. Over time, a statistically significant increase in the number of Sheila Smith^T organisms per infected cell relative to the number of Sawtooth organisms per infected cell was identified (Fig. 7A). The ratio of the number of Sheila Smith^T organisms per infected cell to the number of Sawtooth organisms per infected cell, a ratio of ratios (Sheila Smith^T [*Rickettsia* to actin] to Sawtooth [*Rickettsia* to actin]), increased from 0.64 (95% confidence interval [CI], 0.41 to 1.00) at day 1 to 5.70 (95% CI, 3.64 to 8.95) at day 6 of infection, revealing that Sheila Smith^T was five times more abundant on a per-cell basis than Sawtooth by day 6. As the rickettsial load per cell was significantly different during the infection, we compared the difference in transcriptional profiles of both isolates at days 1 and 6 of the experiment (Fig. 7B). No transcriptional differences between Sawtooth and Sheila Smith^T were identified among most of the 11 potential virulence factors at day 1, including several previously

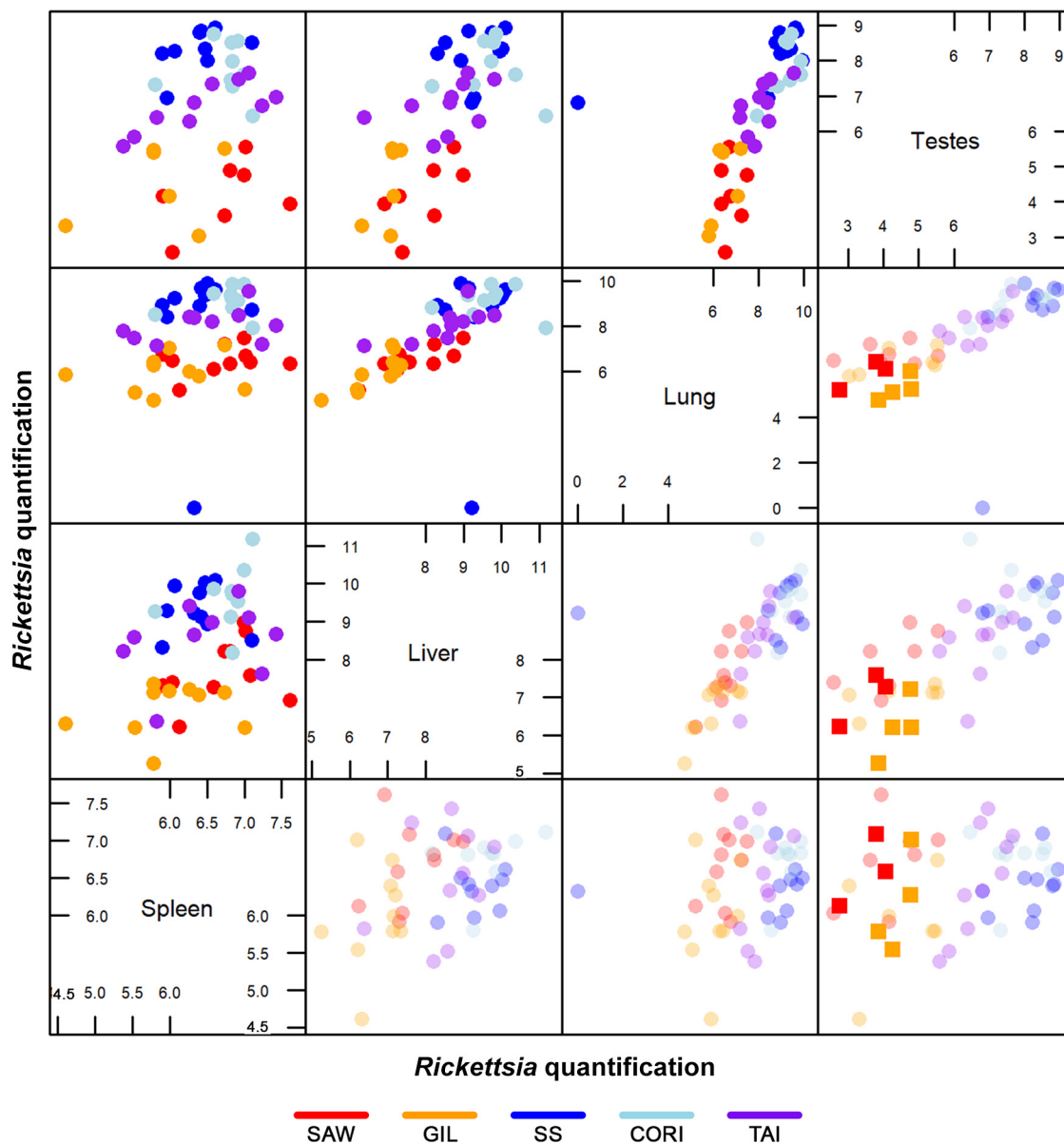


FIG 5 Pairwise plots of a multivariate analysis of *Rickettsia rickettsii* load in all biological replicates from the five virulent isolates infecting animals 6 days postinfection. Plots represent the original quantification data (top left) and original with mean of multiply imputed quantification data (bottom right) for spleens, livers, lungs, and testes. In the lower half, the means of the multiply imputed values are presented in full color intensity, overlaid on the transparent original data. The imputation of the missing data for Sawtooth- and Gila-infected testes confirmed the patterns already identified in the original data set. Axes represent the log quantity of bacteria obtained by qPCR data. Each dot represents one biological replicate. SAW, Sawtooth; GIL, Gila; SS, Sheila Smith^T; CORI, Costa Rica; TAI, Tiaçu.

associated with rickettsial virulence following infections *in vitro* and *in vivo* (19, 40–44, 50–51). However, a strong induced profile was identified at day 6 in Sawtooth- compared to Sheila Smith^T-infected samples, associated mainly with components of secretion systems such as type 4 secretion systems (*virB8*, *virB10*, and *virB11*) as well as the Sec apparatus (*secE*).

We then evaluated the transcriptional profiles of these targets in the livers, lungs, and testes of Sawtooth- and Sheila Smith^T-infected animals. These tissues were selected because they presented significant pathology and the bacterial load was higher than in the spleen. Similar to the *in vitro* evaluation, there was a significant difference in the number of bacteria per tissue cell within all tested tissues. The calculated

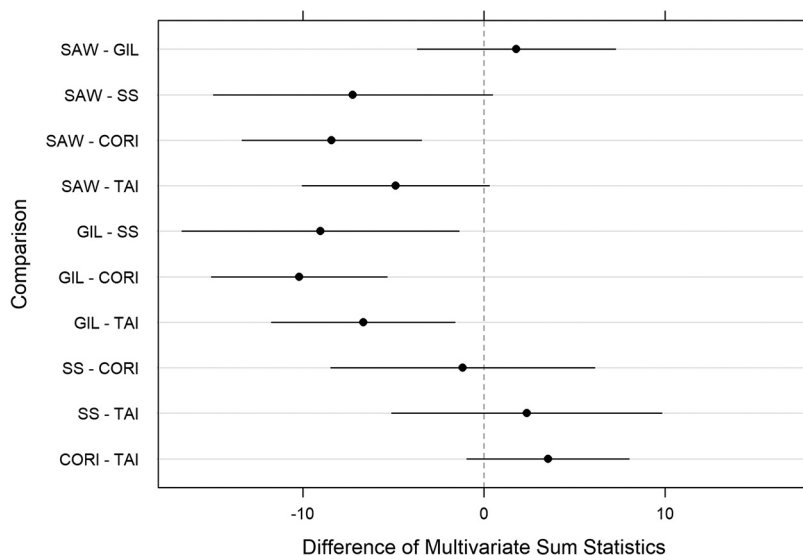


FIG 6 *Rickettsia rickettsii* load differences among isolates, considering all tissues. Pairwise differences (with 95% CI) of multivariate sum mean statistics. The sum of the isolate pairwise differences of the means over the 432 multivariate measurements [log(qPCR)] were used for the four tissues. CIs were adjusted for multiple comparisons and multiple imputation. Estimates were sorted in increasing order of the difference. Differences in CIs that do not contain 0 are considered statistically significant at 5% significance. SAW, Sawtooth; GIL, Gila; SS, Sheila Smith^T; CORI Costa Rica; TAI, Tiaçu.

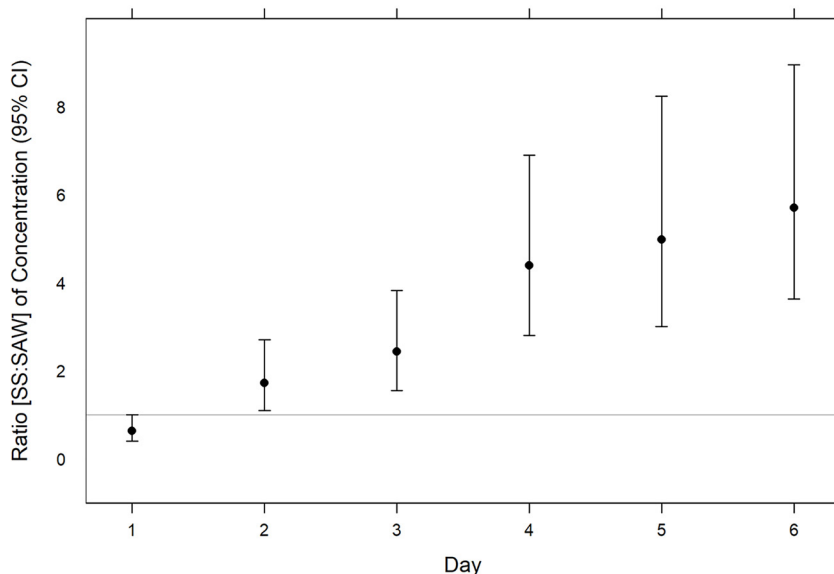
ratio of *Rickettsia* to actin for Sheila Smith^T was significantly higher than that for Sawtooth in all three tissues (Fig. 8A), ranging from 4.79 to 50 times more Sheila Smith^T in livers and testes than Sawtooth. However, the absolute number of organisms was lower in the Sawtooth samples. As transcription analyses was carried out in those *in vivo* samples, transcripts of most of Sawtooth target genes were not detected at day 6 postinoculation, which may be due to the lower number of bacteria in each sample. In contrast, transcripts of 54.55% of the examined genes in liver, 100% in lungs, and 90.91% in testes of animals infected with the Sheila Smith^T isolate were detected at day 6 (Fig. 8B).

DISCUSSION

In this study, we demonstrated distinct and measurable differences in clinical and pathological characteristics of disease in guinea pigs following infection with standardized inocula from six distinct isolates of *R. rickettsii*. Intraperitoneal (i.p.) inoculation of guinea pigs with *R. rickettsii* has been used as a convenient and reproducible animal model system for RMSF for almost 100 years (27–30). Although i.p. inoculation does not reflect a natural route of infection, guinea pigs exposed to *R. rickettsii* in this manner nonetheless develop clinical signs largely identical to those observed in animals exposed to the pathogen via tick bite, including fever, scrotal edema and erythema, footpad dermatitis, and splenomegaly (31, 32). Interestingly, despite being a different animal model, a recent intravenously infected RMSF C3H/HeN mouse model (17) confirmed the rickettsial tissue distribution and load identified herein.

We identified specific differences in bacterial growth and transcriptional profiles of the different isolates following *in vitro* and *in vivo* infections. Previous investigators identified distinct genetic differences among various isolates of *R. rickettsii* (9, 33) that correlate with geographical origin (8–13). However, a recent comparison of four complete genomes of *R. rickettsii* obtained from eastern and western regions of the United States did not identify any specific genetic factors that could satisfactorily explain geographical differences in virulence (9) among regions reporting fatality rates ranging from 5% to 80% (3, 5–7, 13). To assess the phenotypic differences among isolates that could be associated with differences in disease outcome, we evaluated infections with

A



B

	1 day		6 days	
	log ₂ -Fold Change	95% Confidence Interval	log ₂ -Fold Change	95% Confidence Interval
<i>in vitro</i> sca0	0.63	-0.52, 1.77	2.93	0.48, 5.37
<i>in vitro</i> sca5	0.00	-1.21, 1.34	4.20	1.16, 7.24
<i>in vitro</i> RARP-1	-1.10	-2.08, -0.31	1.10	-0.82, 3.01
<i>in vitro</i> secA	-0.30	-1.09, 0.36	2.30	-0.26, 4.88
<i>in vitro</i> secE	1.00	0.19, 1.96	4.40	1.97, 6.86
<i>in vitro</i> sca2	0.40	-0.35, 1.27	1.70	-0.78, 4.33
<i>in vitro</i> rickA	0.30	-0.68, 1.29	3.40	1.91, 4.98
<i>in vitro</i> pat b1	1.50	0.24, 2.82	3.60	0.94, 6.28
<i>in vitro</i> virB8	2.90	1.96, 3.86	8.90	8.01, 9.87
<i>in vitro</i> virB10	2.00	0.65, 3.45	5.30	2.35, 8.25
<i>in vitro</i> virB11	1.70	0.43, 3.11	3.60	0.95, 6.25

Magnitude of change	0	0.87	1.74	2.61	3.48	4.35	5.22	6.09	6.96	7.83	>8.7
Greater than zero											
Less than zero											

FIG 7 *In vitro* infection of *Rickettsia rickettsii*. (A) Daily comparative quantitative ratios of Sheila Smith^T (SS) to Sawtooth (SAW) per Vero E6 cell for 6 days. (B) *In vitro* relative gene expression (quantified as log₂ fold change) of Sawtooth/Sheila Smith^T at days 1 and 6 of infection. Expression levels were determined by reverse transcriptase quantitative PCR (RT-qPCR). 95% CIs for the log₂ fold change are shown for each time point and target gene.

six isolates originating from widely separated regions of United States, Costa Rica, and Brazil that included isolates obtained from human and tick hosts. The Iowa strain was isolated from a *Dermacentor variabilis* tick in 1941; it initially exhibited a mildly virulent phenotype in guinea pigs and became avirulent after multiple passages in embryonated chicken eggs (16). The Sawtooth strain was isolated from adult *Dermacentor andersoni* ticks in western Montana in 1961 (11), is considered virulent (14), and causes a mild cell injury response *in vitro* (10). Sheila Smith^T, isolated in 1952 from a patient with severe RMSF from Montana (15), is perhaps the best-characterized isolate and exhibits a highly virulent phenotype *in vitro* and *in vivo* (8–11, 15, 17). The Costa Rica strain also represents a highly virulent phenotype and was isolated in 1980 from the blood of a patient with a fatal case of RMSF from the Limón region of Costa Rica (34). Taiaçu was isolated in 2002 from an *Amblyomma aureolatum* tick from Taiaçupeba,

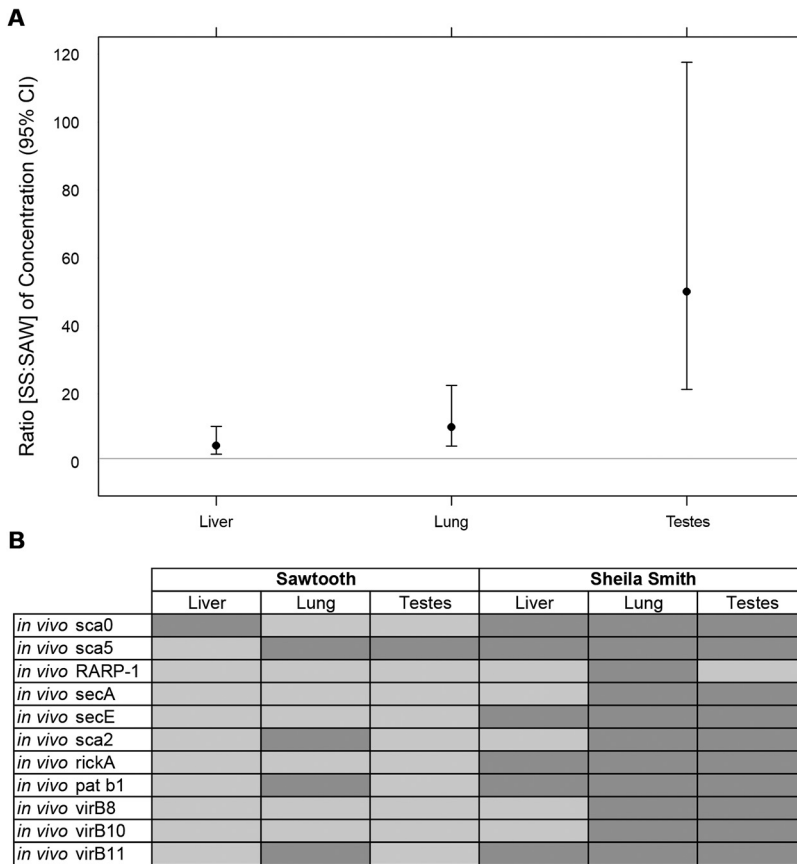


FIG 8 *Rickettsia rickettsii* *in vivo* infection of livers, lungs, and testes. (A) Comparative quantitative ratios of Sheila Smith^T (SS) to Sawtooth (SAW) per liver, lung, and testis cell at day 6 of infection. (B) Detected transcripts (dark gray) and nondetected transcripts (light gray).

Brazil (35), where RMSF is endemic (<http://www.saude.gov.br/saude-de-a-z/febre-maculosa>). The Gila strain was isolated in 2016 from a patient with fatal RMSF in Arizona, USA (this study).

Differences among RMSF phenotypes were initially evaluated *in vivo* using clinical signs and histopathological findings. Three distinct phenotypes were observed: (i) a nonvirulent phenotype in which no animal was successfully infected (Iowa), (ii) a mildly virulent phenotype that produced mild clinical signs (Sawtooth and Gila), and (iii) a highly virulent phenotype characterized by animals presenting signs of severe disease and extensive tissue inflammation (Sheila Smith^T, Costa Rica, and Taiacu). Iowa-infected animals did not exhibit fever, significant splenomegaly, or weight change by the end of the study. In addition, no bacteria were detected in any organ from any biological replicate, which was confirmed by the unsuccessful reisolation of the organism *in vitro*. Indeed, the absence of RMSF phenotype in the same animal model was reported previously (8), although antibody titers were identified then but were not investigated in our study. Thus, as the Iowa strain is unable to successfully establish itself in the guinea pig host and cause disease but may promote seroconversion, it could be considered a possible candidate for a *R. rickettsii* vaccine. However, further studies are needed in order to identify the molecular factors associated with its apparent nonvirulence (36). All animals infected with the other five isolates developed fever and demonstrated significant weight loss and splenomegaly. Sawtooth- and Gila-infected animals exhibited weight loss, only transient fever, and significantly fewer rickettsiae in each tissue than animals infected with each of the highly virulent isolates. Consequently, these isolates were classified as mildly virulent in our animal model. Although bacterial passage

TABLE 2 *Rickettsia rickettsii* isolate information

Isolate	CRIRC identification	Host origin (yr of isolation)	Geographical origin	Reference(s) ^a	Passage history ^b
Iowa	RRI010	<i>Dermacentor variabilis</i> (1941)	USA	8, 12	271 EP, 1 GP, 9 VR, 1 BC, 16 VR
Sawtooth	RRI025	<i>Dermacentor andersoni</i> (1961)	USA	7, 13, 14	8 VR, 1 MS, 3 VR
Gila	RRI017	Human (2016)	USA	Our unpublished data	3 VR
Sheila Smith ^T	RRI001T	Human (1952)	USA	9, 12–15	2 VR
Costa Rica	RRI020	Human (1980)	Costa Rica	16, 17	4 VR
Taiacu	RRI011	<i>Amblyomma aureolatum</i> (2002)	Brazil	18	5 VR, 12 BME26, 2 VR

^aReference where disease severity associated with the host can be found.

^bPassage history after acquisition of the strain by CDC. EP, egg passage; GP, guinea pig; VR, Vero cells; BC, BALB/c mouse; MS, mouse; BME26, *Rhipicephalus microplus* cells.

history and isolate origin have been associated previously with changes in virulence (37), the passage histories of these isolates did not appear to affect disease outcome in our guinea pig model. Most of the isolates of *R. rickettsii* used in this evaluation had extensive passage histories (Table 2), while each behaved as described previously in our animal model with respect to having greater virulence (8, 15, 17) or mild or no virulence (10, 16). In comparison, the minimally passaged Gila isolate exhibited relatively mild virulence despite originating from a patient with fatal disease.

Overall, the variation in rickettsial loads in all tissues among the different virulence groups corroborated the histological differences identified in this study. Fewer animals infected with mildly virulent isolates exhibited inflammatory responses than animals infected with highly virulent isolates. Surprisingly, spleens exhibited low bacterial loads relative to other organs regardless of virulence category, although there was significant splenomegaly associated with histiocytic infiltrates, which could promote rickettsial clearance (38, 39). Indeed, the granulomas detected in all Sawtooth-infected spleen samples could reflect slower growth by this isolate, as identified *in vitro*. Similarly, testes had lower bacterial loads than lungs and livers, despite prominent vasculitis of the tunics and interstitium in animals infected with highly virulent isolates. The intraperitoneal needle inoculation route used in the study likely affected subsequent bacterial distribution as well as distinct tissue-specific immune responses; nonetheless, this route allowed complete control of the volume of inoculum administered in each biological replicate, which was key in this experimental setting. In contrast to spleens and testes, high and correlative bacterial loads were identified in livers and lungs, suggesting critical roles for these organs during the pathogenesis of this systemic disease.

Differences in rickettsial load among mildly and highly virulent isolates were identified *in vivo* and *in vitro* and could reflect differences in the growth rates among individual isolates. When Sawtooth and Sheila Smith^T were compared, a lower growth rate and a lower bacterial load per cell in Sawtooth cultures were identified. When the transcription of potential virulence factors previously associated with rickettsial motility (RickA and Sca2) (40, 41), secretion system components or effectors (T4SS, Sec, and RARP-1) (42, 43), and host cell adhesion, attachment, and/or invasion (sca0 and sca5) (19, 44) were investigated, an increase in overall Sawtooth gene expression at the end of the *in vitro* study was identified, whereas a similar pattern was observed significantly earlier during infection with Sheila Smith^T. These molecular patterns of potential virulence factors strengthen the hypothesis of a slower and constant growth of Sawtooth, compared to the rapid and more destructive growth of Sheila Smith^T. Indeed, smaller amounts of Sawtooth and the same pattern of bacterium/cell ratios were detected *in vivo*. This could explain why so few Sawtooth transcripts were identified *in vivo*. A low growth rate was previously hypothesized to play a role in the propagation of *R. prowazekii* (20). As a confirmation of Sawtooth's slow growth *in vivo*, granulomas were identified exclusively in Sawtooth-infected spleen samples and in no other tissue from animals infected with any other isolate, suggesting that relatively slow growth of this isolate allowed a robust host response. The important equilibrium between host response and bacterial growth is supported additionally by the recent confirmation of fatal RMSF in a human host caused by an Hlp strain of *R. rickettsii* (13). Guinea pigs infected with the first described Hlp strains exhibited milder disease than those

infected with a classically virulent strain of *R. rickettsii*, including longer incubation periods, shorter febrile intervals, diminished scrotal pathology, and, most notably, the absence of fatal outcomes in the H1p-infected animals (21). In this context, it is plausible that even subtle changes in host immunity can affect the outcome of infection with mildly virulent strains of *R. rickettsii*.

The data from this investigation, obtained under highly controlled settings with standardized inocula and collected during the log phase of bacterial replication, illuminate quantitative phenotypic and transcriptional differences among geographically distinct *R. rickettsii* isolates within the classically recognized animal model of infection. Collectively, this information supports previously recognized qualitative assessments of strain virulence and suggests a critical and previously underappreciated balance between bacterial growth and host immune response that leverages strain pathogenicity. Finally, these data indicate that an overall assessment of strain virulence based on geographical origin should be undertaken in the context of regional host factors that influence the immune response to infections with *R. rickettsii*.

MATERIALS AND METHODS

Ethics statement. All animals were maintained in a facility fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International. Care and husbandry of the animals were performed in accordance with the recommendations in the *Guide for the Care and Use of Laboratory Animals* (22). The procedures of the study were approved by the Centers for Disease Control and Prevention Institutional Animal Care and Use Committee under protocol 2834LEVGUIC and monitored by a veterinarian stationed on site, following the U.S. Animal Welfare Act and all associated regulations.

Inocula and animal host. All isolates used in the study—Iowa, Sawtooth, Gila, Sheila Smith^T, Costa Rica, and Taiacu—are included in the CDC Rickettsial Isolate Reference Collection (CRIRC) as described in Table 2. These isolates were selected by geographical origin, previous genotype characterization (13), and previous use *in vitro* or *in vivo* evaluations of virulence, when available (8–11). All isolates were cultured in African green monkey (*Cercopithecus aethiops*) kidney cells (Vero E6) at 34°C in a 5% CO₂. To prepare the inocula, the bacteria were initially grown in 150-cm² flasks of confluent Vero E6 cells until they reached the log phase of replication. Cells were then harvested and processed as described previously (23, 24) with a few modifications. Briefly, cells were detached using sterile 3-mm glass beads, transferred to 50-ml centrifuge tubes and centrifuged at 4°C and 12,000 × *g* for 20 min to resuspend pellets in cold Snyder-1 medium (25, 26). The suspension was transferred to a 20-ml syringe, passed through a 20-gauge needle 10 times, and filtered through a 2-μm syringe filter. The number of viable bacteria was determined by counting the bacteria stained with a LIVE/DEAD BacLight bacterial viability kit (Molecular Probes) in a Petroff-Hausser bacterial counting chamber (Hausser Scientific) and examined using a Zeiss microscope (45) in order to standardize all inocula at 10⁷ viable bacteria. This inoculum was selected based on data acquired from an initial pilot study using Sheila Smith^T which showed systemic tissue distribution and significant morbidity in guinea pigs at 7 days postinfection (data not shown). To standardize the volume of inoculum, the *Rickettsia* suspension was then diluted in Snyder-1 (1:1). Groups of 10 (9 in the case of Costa Rica) 6- to 9-week-old pathogen-free male Hartley guinea pigs (*Cavia porcellus*) weighing 400 to 500 g (Charles River Laboratories) were intraperitoneally (i.p.) inoculated with an isolate. Negative-control animals received noninfected Vero cells in a volume equivalent to that of the inoculum given to the infected groups.

***Rickettsia rickettsii* infection and sample collection.** Animals were followed for 6 days postinoculation. Clinical signs, including body temperature, scrotal edema (presence/absence), and footpad dermatitis (presence/absence), were recorded daily (46), while body weight was measured on days 1 and 6 of the experiment. Six days after inoculation, animals were euthanized and necropsied. Three samples of each spleen, liver, lung, and testis were collected (i) in RNAlater solution (Thermo Fisher Scientific) for nucleic acid extraction, (ii) in 10% formalin for histopathology processing, and (iii) in sterile sucrose-phosphate-glutamic acid (SPG) buffer for bacterial isolation. The spleen weight and final body weight were used for calculations as described previously (47).

Histopathology and immunohistochemistry. Representative portions of spleens, livers, lungs, and testes were fixed in 10% neutral buffered formalin for 48 h and then embedded in paraffin. Three-micrometer sections were cut from paraffin blocks, stained with hematoxylin-eosin, and also stained by an immunohistochemical phosphatase technique with a polyclonal rabbit anti-*R. rickettsii* antibody diluted at 1/500 and counterstained with hematoxylin (48).

DNA and RNA extraction. Simultaneous extraction of genomic DNA (gDNA) and total RNA was performed individually on all samples using the Qiagen AllPrep DNA/RNA minikit according to the manufacturer's instructions. A 30-mg sample of each tissue was disrupted and homogenized using sterile 5-mm stainless steel beads (Qiagen) on a TissueLyser II system (Qiagen) for two cycles of 1 min at 30 Hz. Extracted gDNA was stored at –20°C until needed for quantitative real-time PCR (qPCR), while the total RNA was stored at –80°C until required for transcription assays.

***In vitro* infection.** For *in vitro* growth analysis, *R. rickettsii* Sawtooth and Sheila Smith^T were each cultured in a T25 flask of confluent Vero E6 cells at 34°C in the presence of 5% CO₂ for 6 days, at a

TABLE 3 Primers used in gene expression analysis

Gene ID	Annotation	Sequence (5'–3')		Annealing temp (°C)	Reference ^a
		Forward	Reverse		
A1G_06990	190-kDa cell surface antigen	ACCAGCCATTAACCTGACCTGT	GACGTGTTATTGCAACGACACT	60	This work
RRR_00670	Surface cell antigen Sca2	GCATCAGCCCCGATGGTTA	GAGGGGTATGGATTAGCGGC	61	This work
A1G_06030	Outer membrane protein B (cell surface antigen sca5)	AGTAGTACCGCCGCTAAAA	TGGTGCAGGATTACAAGGAA	60	51
A1G_04855	Preprotein translocase subunit SecA	TACCCCGTCTGCCATATTA	AAACGCCAAATTTTCATGAGC	61	51
A1G_01005	Preprotein translocase subunit SecE	TGTTGCTTCAACGTTAGTAGTGG	GCCGATATTAAGCAAAAGCTG	61	51
A1G_02210	VirB8 protein	GGCATATCAGGTGATGTATTGG	ACTTTTGAATCACTGGCAAAAA	60	51
A1G_02230	VirB10 protein	CGCCGGTCTTACCTCCTACT	TGCATCGCTCTCAACTAACG	61	51
A1G_02235	Type IV secretion system ATPase VirB11	GAGGCCTGTTTGCCTTAAAG	TGAACCGGGATGACCTGTAT	60	51
Rrlowa_1080	Arp2/3 complex activation protein	TGCAACCGGTTTTCTGTAT	GGAAAATCAACCAGCTCCTTC	61	51
A1G_05085	Patatin b1 precursor	CGATTTTACTGGAGGGACCA	TTGCGCTGCACTGAATAAAG	61	51
A1G_01760	Hypothetical protein/ankyrin repeats (RARP-1)	GCCGCTTATACCGTTGTTG	ATTTGGAGAGCTTGCCGGAG	60	This work

^aWhere the design description can be found.

multiplicity of infection (MOI) of 3. Samples were collected daily to evaluate infection progression via acridine orange (Becton Dickinson) staining and qPCR as described below. Simultaneous extraction of DNA and RNA was performed, as described above, in all biological triplicates at all time points.

Quantification assays. For the *Rickettsia*-specific qPCR, the rickettsial 50S ribosomal protein L16 was targeted as described previously (49). For the guinea pig-specific qPCR, the *C. porcellus* beta-actin gene (*actB*) was targeted using 0.8 μ M concentrations each of the forward primer 5'-CCTGTATGCCTCTGGCCGCA-3' and reverse primer 5'-GGACTTCGAGCAGGAGATGG-3' in addition to a 40 nM concentration of the fluorescence-labeled probe 5'-(Quasar 670)ACTGTGCCCATCTACGAG-3' as described previously (50) for mouse actin with a few modifications on the forward and reverse primer sequences to match the *C. porcellus* sequence, generating a 260-bp amplicon. The reaction conditions were a 15-min incubation at 95°C, followed by 55 cycles of 95°C for 15 s, 53°C for 15 s, and 72°C for 30 s. For the Vero cell-specific qPCR, the *C. aethiops* (AB004047) beta-actin was targeted using 0.8 μ M concentrations of the forward primer 5'-TGAAGTGTGACGTGGACATCCATA-3' and the reverse primer 5'-AGGATGCAGAAGGAGATTACTGCC-3' in addition to a 40 nM concentration of the fluorescence-labeled probe 5'-(Quasar 670)TGGCACCACCATGTACCCTGGCATTGCT-3', generating a 98-bp amplicon, also described previously (50), with a single modification on the forward primer sequence to match the *C. aethiops* sequence available in NCBI. In order to perform an absolute quantification, control plasmids were created for all targets described above by cloning each amplicon into a pCR 4-TOPO TA vector system (Thermo Fisher Scientific), followed by sequencing to confirm the plasmid construction. All reactions were performed with QuantiTect Probe PCR master mix (Qiagen) using a CFX96 real-time system (Bio-Rad), while the analysis was conducted with CFX Manager software. Three technical replicates were analyzed for each sample.

***Rickettsia rickettsii* isolation.** Samples of testes collected during necropsies from all isolate-infected animals were kept at –80°C in sterile SPG buffer until needed. After thawing, 50 mg of each tissue was individually disrupted and homogenized using a Covidien Precision disposable tissue grinder system in 0.5 ml supplemented minimal essential medium (MEM) (5% fetal bovine serum, 0.1 mM MEM nonessential amino acids, 2 mM L-glutamine, 10 mM sodium pyruvate, 10 mM HEPES buffer, 10 U/ml penicillin G, and 10 μ g/ml streptomycin sulfate). The inoculum was placed on a confluent monolayer of Vero E6 cells in a T25 tissue culture flask and incubated at 34°C in a 5% CO₂-in-air atmosphere. Cultures were monitored weekly for rickettsial infection by acridine orange (Becton Dickinson) staining and real-time PCR, described below.

RT-qPCR. The transcriptional profile of 11 selected *R. rickettsii* genes was determined by quantitative reverse transcription-PCR (RT-qPCR). Some have been previously associated with rickettsial virulence *in vitro* and in vertebrate or invertebrate hosts (19, 40–44, 51, 52). They are the genes encoding the 190-kDa cell surface antigen, surface cell antigen Sca2, outer membrane protein B (cell surface antigen sca5), preprotein translocase subunit SecA, preprotein translocase subunit SecE, VirB8 protein, VirB10 protein, type IV secretion system ATPase VirB11, Arp2/3 complex activation protein, patatin b1 precursor, and hypothetical protein/ankyrin repeats (RARP-1). Primers used for the experiment (Table 3) were designed at conserved regions among 10 *R. rickettsii* genomes (Sheila Smith¹, Iowa, Arizona, Colombia, Hlp#2, Hino, Hauke, Morgan, R, and Brazil) available in the National Center for Biotechnology Information (NCBI) database using Primer3 (53) or as described previously (51). Two hundred nanograms of DNA-free RNA was used as the template for reverse transcription (RT) in cDNA using SuperScript III reverse transcriptase (Life Technologies) according to the manufacturer's protocol. To determine the efficiency of each primer (0.8 μ M each), standard curves were generated using six serial dilutions of a unique gDNA sample composed of a pool of gDNA from the biological samples. The curves were tested at annealing temperatures of 60°C, 61°C, and 62°C. Primers presenting efficiencies between 92 and 100% at a specific tested temperature were included in the analysis. PCRs were run as follows: 95°C for 15 min, followed by 40 cycles of 94°C for 15 s, 60 to 62°C for 30 s, and 72°C for 30 s, followed by a melting curve. QuantiTect SYBR

green PCR master mix (Qiagen) was used. Reactions were performed using a CFX96 real-time system (Bio-Rad), while the analysis was conducted with CFX Manager software. Three technical replicates were analyzed for each sample. *R. rickettsii* gDNA was used for calculation of the standard curve. qPCR-derived fold change values are expressed using the equation described previously (54).

Statistical analysis. To compare percent change in body weight and spleen indexes among isolates, a standard one-way fixed-effects model was fitted with the factor isolate, using generalized least squares (equivalent to maximum likelihood). Different variance parameters for each isolate were included to account for potential differences in variability by isolates, and the model was fitted with generalized least squares. These two models were compared considering both the Akaike information criterion (AIC), for which smaller values indicate a better fit, and the likelihood ratio test. Ninety-five percent confidence intervals (CI) were computed for model parameters, and all pairwise differences of estimated means (95% CIs) were computed using Tukey's method to adjust for multiple comparisons and incorporated heterogeneity if indicated. Standard model diagnostics were used, including graphical evaluation of homoscedasticity and normality of the residuals.

For bacterial load analysis, the multivariate data were recorded as log(qPCR value) or as log(qPCR value + 1) for qPCR values of 0, presented as pairwise plots (55). Multiple imputation (56) was applied as a standard approach for modeling in the presence of missing data, assuming the Gaussian distributions for the log(qPCR) data together with linear models. We used four completed data sets based on standard diagnostics. To evaluate differences in bacterial loads among isolates, multivariate analysis of variance (MANOVA) was used to compare the multivariate means (i.e., mean vectors). As heterogeneity among isolates was anticipated, methods of standard MANOVA were used that have been adapted to this case (57). These methods were implemented in the R package MANOVA.RM. For comparison of the mean vectors for multivariate responses, the sum of the isolate pairwise differences of the means over the multivariate measurements [log(qPCR)] were used for the four tissues. Simultaneous 95% CIs for these isolates' pairwise differences were computed using the parametric bootstrap by computing appropriate quantile (q^*) of the estimated difference statistics, as described in reference 57. To employ this approach while accounting for the uncertainty associated with multiple imputation, we averaged \bar{q}^* , which represents the computed quantiles from the analysis of each multiple imputation completed data set. Further, we calculated a revised value for the quantile from the method of Friedrich and Pauly adapted to include Rubin's rules by multiplying \bar{q}^* by the ratio of the t value with degrees of freedom computed using Rubin's rules to the "plain" t value with degrees of freedom computed assuming no adjustments for simultaneous inference (so using, generically, $n + m - 2$). This ratio of t values captures the adjustment to the standard pairwise t comparison made by the multiple imputation adjustment using Rubin's Rules, and multiplying it by \bar{q}^* by the method described in reference 57 preserves the simultaneous and distribution-free nature of their approach.

We compared the proportion of reisolated samples among the isolates using Fisher's exact followed by pairwise comparisons adjusted for multiple comparisons using the Holm procedure (58), as implemented in the RVAideMemoire package in R.

To compare the number of bacteria per host cell, we fitted a linear model to the within-sample differences between the logarithms of the loads [log(*Rickettsia*) – log(actin)], including day and isolate as covariates for the *in vitro* studies comparing Sheila Smith^T (SS) to Sawtooth (SAW) and also for the *in vivo* study comparing Sheila Smith^T to Sawtooth for livers, lungs, and testes. Standard diagnostics were performed to evaluate model assumptions. Linear functions of the model coefficients were used to estimate the effect of isolate by day (*in vitro*) and tissue (*in vivo*), and the corresponding simultaneous 95% CIs were computed using methods detailed previously (58). Resulting estimates and CIs were exponentiated to provide inference for the ratio of the ratios of concentrations, i.e., the SS *Rickettsia*-to-actin ratio to the SAW *Rickettsia*-to-actin ratio, as a means of summarizing the differential growth for the isolates.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

SUPPLEMENTAL FILE 2, PDF file, 0.1 MB.

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M.F.B.M.G., S.K., and C.P., conceived the hypothesis. M.F.B.M.G. designed and developed the project. M.F.B.M.G., J.H., and S.K. carried out the *in vivo* experiments. J.R. and C.P. carried out the histological analysis. B.B. performed the statistical analyses. M.F.B.M.G., S.K., and C.P. wrote the manuscript. All authors reviewed and revised the final version of the manuscript.

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We declare no conflict of interest.

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