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Genetic Identification of Rickettsial Isolates from Fatal Cases of Brazilian Spotted Fever and Comparison with *Rickettsia rickettsii* Isolates from the American Continents

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Fifteen bacterial isolates from spotted fever group rickettsiosis in Brazil were genetically identified as *Rickettsia rickettsii*. In a phylogenetic analysis with other *R. rickettsii* isolates from GenBank, the Central/South American isolates showed low polymorphism and formed a clade distinct from two North American clades, with the North American clades having greater in-branch polymorphism.

The tick-borne bacterium *Rickettsia rickettsii* is the etiologic agent of Rocky Mountain spotted fever (RMSF), the most severe rickettsiosis affecting humans in the Western Hemisphere (1). In Brazil, the disease has been referred to as Brazilian spotted fever (BSF). While RMSF fatality rates are usually 5% to 10% in the United States, general rates of 20% to 40% have been reported in Brazil (1).

In the state of São Paulo in southeastern Brazil, where BSF is a disease for which notification is compulsory, various spotted fever group (SFG) rickettsial isolates have been obtained from human clinical cases during the past few years (2, 3). Although all these isolates were confirmed to be SFG rickettsiae through indirect immunofluorescence assays using anti-*R. rickettsii* human sera (4), they were not genetically identified through molecular analysis. Herein, we performed genetic identification and molecular characterization of these rickettsial isolates and compared their genetic profiles with those of isolates from ticks in Brazil and with those of *R. rickettsii* isolates from other American countries.

Blood clot or skin lesion biopsy specimens from BSF-suspected cases were processed by the shell vial technique, as described previously (5). Once rickettsiae were visualized within Vero cells through an immunofluorescence assay using anti-*R. rickettsii* human polyclonal serum (4, 6), 1st-passage-infected cells were harvested, and one aliquot was used for DNA extraction through the PureLink genomic DNA kit (Invitrogen, Carlsbad, CA). Extracted DNA was assayed according to PCR protocols using primers targeting the rickettsial genes *gltA*, *ompA*, and *ompB* and the RR0155-*rpmB*, RR1240-*tlc5*^{b}, and *cspA-ksgA* intergenic regions, as shown in Table 1. In addition, DNA extracted from 3rd-passage-infected cells of three *R. rickettsii* isolates, previously isolated from *Amblyomma sculptum* (reported as *Amblyomma cajennense*), *Amblyomma aureolatum*, and *Rhipicephalus sanguineus* ticks in Brazil (12–14), was tested according to intergenic region PCR protocols. PCR products were sequenced in an ABI automated sequencer (model ABI 3500 Genetic Analyzer; Applied Biosystems/Thermo Fisher Scientific, Foster City, CA) with the same primers used for PCR. The generated sequences were compared with each other and submitted to BLAST analyses (www.ncbi.nlm.nih.gov/blast) to infer the closest similarities available in GenBank.

Phylogenetic analyses were performed using PAUP version 4.0b10 (15) to maximum parsimony (MP); the confidence values for individual branches of the resulting tree were determined by bootstrap analysis with 1,000 replicates. Bayesian analysis (BA) was performed with MrBayes version 3.1.2 (16) software with 1,000,000 generations using the GTR+I+G substitution model. Partial DNA sequences obtained from the amplified PCR products (*gltA*, RR0155-*rpmB*, RR1240-*tlc5*^{b}, *cspA-ksgA*, and *ompB*) were concatenated and aligned with corresponding sequences of different strains of *R. rickettsii* available in GenBank using CLUSTAL X (17) and adjusted manually using GeneDoc (18). Partial sequences of the *ompA* gene were not included because the region of the gene that was amplified in the present study showed no polymorphism among the *R. rickettsii* isolates. Corresponding sequences of *R. rickettsii* strain Hlp#2 (CP003311) and *Rickettsia philipii* strain 364D (CP003308) were used as outgroups.

Fifteen rickettsial isolates (designated IAL 1 to 15) from BSF patients (13 fatal cases) in the state of São Paulo, southeastern Brazil, were identified as *R. rickettsii*, since their *gltA* (737 nt), *ompA* (491 nt), and *ompB* (787 nt) DNA sequences were 100% identical to each other and to corresponding sequences on the genome of the *R. rickettsii* strain Brazil (GenBank accession no. CP003305). While the *ompA* partial sequences were also 100% identical to corresponding sequences of *R. rickettsii* strains from North America (e.g., CP000848 and CP000766), the *ompB* partial sequences were 100% identical to that of the North American Sheila Smith strain (CP000848) and, at the same time, differed from those of other North American strains (e.g., CP000766 and CP003307) by one single nucleotide polymorphism. The *gltA* sequences of the 15 Brazilian human isolates differed by an extra codon (CGG) compared to those of several North American strains, such as Sheila Smith (CP000848) and Bitterroot (U59729). This extra

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TABLE 1 Primer pairs used for amplification of rickettsial genes (*gltA*, *ompA*, and *ompB*) or intergenic regions (RR0155-*rpmB*, RR1240-*tlc5*[b], and *cspA-ksgA*)

Primer pair	Target	Primer	Primer sequence (5′ to 3′)	Amplicon size (bp)	Reference
1	*gltA*	CS-239	GCTCTTCTCATCCTATGGCTATTAT	834	7
		CS-1069	CAGGGTCTTCGTGCATTTCTT		7
2	*ompA*	Rr190.70p	ATGGCGAATATTTCTCCAAAA	530	8
		Rr190.602n	AGTGCAGCATTCGCTCCCCCT		8
3	*ompB*	120-M59	CCGCAGGGTTGGTAACTGC	862	9
		120–807	CCTTTTAGATTACCGCCTAA		9
4	RR0155-*rpmB*	Forward	TTTCTAGCAGCGGTTGTTTTATCC	290	10
		Reverse	TTAGCCCATGTTGACAGGTTTACT		10
5	RR1240-*tlc5*[b]	Forward	CGGGATAACGCCGAGTAATA	357	11
		Reverse	ATGCCGCTCTGAATTTGTTT		11
6	*cspA-ksgA*	Forward	CATCACTGCTTCGCTTATTTT	405	10
		Reverse	ATTTCTTTTCTTCCTCTTCATCAA		10

codon was also present in the *R. rickettsii* tick isolates (Taiaçu, Itu, and Rs1) from Brazil (12–14). The sequences of three intergenic regions (249 nucleotides [nt] for RR0155-*rpmB*, 315 nt for RR1240-*tlc5*[b], and 362 nt for *cspA-ksgA*) were determined for the 15 human isolates and for the three tick isolates (Taiaçu, Itu, and Rs1) from Brazil. For each intergenic region, the sequences were 100% identical to each other (no polymorphisms were detected), and when subjected to BLAST analysis, they were 100% identical to the corresponding sequences of the *R. rickettsii* strain Brazil (CP003305).

For the concatenated phylogenetic analysis, which included a total of 2,392 nt, the sequences of the 15 human isolates (IAL 1 to 15) and 3 tick isolates (Taiaçu, Itu, and Rs1) from Brazil were aligned with the corresponding sequences from 10 other *R. rickettsii* isolates available in GenBank (6 from the United States, 1 from Costa Rica, 2 from Colombia, and 1 from Brazil) (Table 2). The 15 human and 3 tick isolates of *R. rickettsii* from Brazil formed a clade under high bootstrap support (99% to 100%), with all three South American isolates available in GenBank (1 from Brazil and 2 from Colombia) and with the Central American isolate from Costa Rica (Fig. 1). This Central/South American clade, ecologically associated with 4 different tick species (namely, *A. aureolatum*, *Amblyomma patinoi*, *A. sculptum*, and *R. sanguineus*) (Table 2), had a sister group formed by the North American Sheila Smith and Bitterroot strains that were ecologically associated with the tick vector *Dermacentor andersoni*. A more divergent clade was

TABLE 2 Isolates of *Rickettsia rickettsii* used in the phylogenetic analysis of the present study

Isolate	Isolation source	Clinical outcome	Source of DNA sequences for rickettsial genes (GenBank accession no. or reference no.) for:					Haplotype[a]	Tick[b]
			gltA	RR0155-*rpmB*	RR1240-*tlc5*[b]	*cspA-ksgA*	*ompB*		
Iowa	Tick		CP000766	CP000766	CP000766	CP000766	CP000766	A	*Dermacentor variabilis*
Hino	Human	Fatal	CP003309	CP003309	CP003309	CP003309	CP003309	A	*D. variabilis*
Hauke	Human	Fatal	CP003318	CP003318	CP003318	CP003318	CP003318	A	*D. variabilis*
Arizona	Human	Fatal	CP003307	CP003307	CP003307	CP003307	CP003307	B	*Rhipicephalus sanguineus sensu lato*
Sheila Smith	Human	Fatal	CP000848	CP000848	CP000848	CP000848	CP000848	C	*Dermacentor andersoni*
Bitterroot	Tick		RRU59729	EF216032	EF215983	EF215860	X16353	C	*D. andersoni*
Costa Rica	Human	Fatal	19, 27	EF216038	EF215987	EF215872	27	D	?
I12B (Villeta)	Tick		KJ735644	KJ735647	KJ735648	KJ735649	KJ735646	D	*Amblyomma patinoi*
Rs1	Tick		13	This study	This study	This study	13	D	*Amblyomma sculptum*
Itu	Tick		KF742602	This study	This study	This study	KF742604	D	*A. sculptum*
Taiaçu	Tick		DQ115890	This study	This study	This study	12	D	*Amblyomma aureolatum*
IAL 1–2[c]	Human	Fatal	This study	This study	This study	This study	This study	D	*A. aureolatum*
IAL 4, 9[d]	Human	Cure	This study	This study	This study	This study	This study	D	*A. sculptum*
IAL 3, 5–8, 10–15[e]	Human	Fatal	This study	This study	This study	This study	This study	D	*A. sculptum*
Brazil	Human	Unknown	CP003305	CP003305	CP003305	CP003305	CP003305	D	*A. sculptum*
Colombia	Human	Fatal	CP003306	CP003306	CP003306	CP003306	CP003306	E	*A. patinoi*

[a] Refers to the concatenated haplotypes shown in the phylogenetic tree (Fig. 1).

[b] For tick isolates, refers to the tick species from which the isolate was obtained; for human isolates, refers to the incriminated vector of *R. rickettsii* to humans in the area of origin of the isolate, according to Ogrzewalska et al. (32) for *Amblyomma aureolatum*; A. A. Faccini-Martínez, F. B. Costa, T. E. Hayama-Ueno, A. Ramírez-Hernández, J. Cortés-Vecino, M. B. Labruna, and M. Hidalgo (submitted for publication), Nava et al. (33), and Pacheco et al. (13) for *Amblyomma patinoi* and *Amblyomma sculptum*; and Karpathy et al. (10) for *Dermacentor variabilis*, *Dermacentor andersoni*, and *Rhipicephalus sanguineus sensu lato*. The question mark represents an unknown vector, according to Hun et al. (27).

[c] Geographic origins (municipalities in the state of São Paulo, Brazil) of these isolates: São Paulo, IAL 1; São Bernardo do Campo, IAL 2.

[d] Geographic origins (municipalities in the state of São Paulo) of these isolates: Piracicaba, IAL 4; Valinhos, IAL 9.

[e] Geographic origins (municipalities in the state of São Paulo) of these isolates: Valinhos, IAL 3, 5, 8, 11, 12, and 15; Campinas, IAL 6 and 13; Jaguariúna, IAL 7; Piracicaba, IAL 10; Limeira, IAL 14.

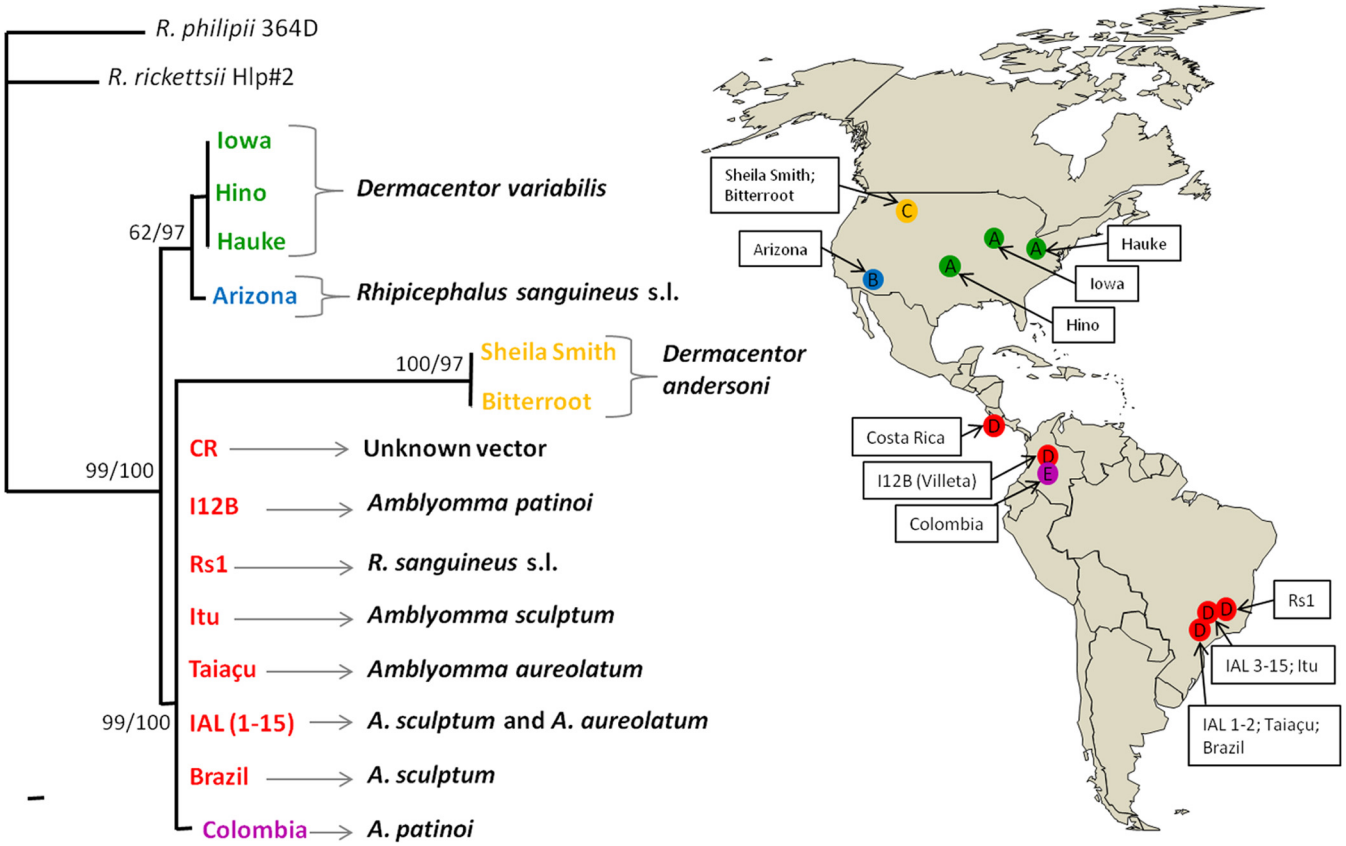

FIG 1 Molecular phylogenetic analysis of *Rickettsia rickettsii* isolates from the United States, Costa Rica, Colombia, and Brazil. A total of 2,392 unambiguously aligned nucleotide sites of two rickettsial genes (*gltA* and *ompB*) and three intergenic regions (RR0155-*rpmB*, RR1240-*tlc5*^{b}, and *cspA-ksgA*) were concatenated and subjected to analysis by maximum-parsimony and Bayesian methods. Corresponding sequences of *Rickettsia philipii* strain 364D and *R. rickettsii* strain Hlp#2 were used as an outgroup. Numbers at nodes are support values derived from bootstrap and posterior probability for MP and BA analyses (MP/BA). Sequence codes A to E, each with a different color, represent the five haplotypes generated from the 28 isolates of *R. rickettsii* described in Table 2. Gray braces or arrows at the clades indicate the tick species that has been ecologically associated with the *R. rickettsii* isolates. The geographical region of origin of the 28 isolates and their corresponding haplotypes (A to E) are indicated on the map of the American continents. (The map is reprinted from http://www.usgs.gov/.)

composed of North American isolates that have been ecologically associated with the ticks *Dermacentor variabilis* and *R. sanguineus*.

As reported in previous studies (10, 19–21), the North American isolates of *R. rickettsii* presented relatively high polymorphism compared to that of the Central/South American isolates. Our concatenated analysis showed the formation of 3 North American haplotypes (A, B, and C), each associated with a different tick species (Fig. 1, Table 2). Conversely, excluding the Colombia strain (haplotype E), there was a single haplotype (D) in Central/South America, although it was associated with 4 different tick species. Geographic distances cannot be inferred from this discrepancy because the distance between Costa Rica and southeastern Brazil is much higher than the distances between distinct North American isolates (Fig. 1). Interestingly, while low-, mild-, and high-virulent strains of *R. rickettsii* have been reported in the eastern and western parts of the United States (20, 22), only high-virulent strains, responsible for high fatality rates, have been reported in Central/South America, regardless of the tick vector (2, 23–29). While our results of no polymorphisms among Central/South American isolates was biased because most of these isolates were derived from fatal cases, a recent study reported the same clade distribution as shown in our Fig. 1 when analyzing the intergenic regions of *R. rickettsii* derived from fatal cases from the United States, Mexico, and Central/South America (21).

The relatively high level of polymorphism among North American isolates of *R. rickettsii* and the contrasting low level of polymorphism in Central/South America suggest that *R. rickettsii* radiated in North America and was introduced into South America during more recent periods. This scenario may also explain why there is a mixture of highly and less virulent *R. rickettsii* strains in North America (due to longer coevolving periods with vertebrates), while only highly virulent strains have been found in South America.

It has been suggested that the higher fatality rates of BSF, compared to those of RMSF in the United States, are related to delayed treatment, the use of less effective antibiotics, and more virulent *R. rickettsii* strains occurring in Brazil (2, 30, 31). The present study corroborates previous studies (10, 19–21) that provided genetic evidence for a very low level of polymorphism occurring among *R. rickettsii* isolates from South America. This fact should be a significant reason for the much higher fatality rates of BSF, although the others discussed above may also be contributing factors.

Nucleotide sequence accession numbers. The GenBank nucleotide sequence accession numbers of the partial sequences of *R.*

rickettsii generated in this study are KJ994337, KJ994338, and KJ994339 for the *gltA*, *ompA*, and *ompB* genes, respectively, and KJ994340, KJ994341, and KJ994342 for the RR0155-*rpmB*, RR1240-*tlc5*^{b}, and *cspA-ksgA* intergenic regions, respectively.

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REFERENCES

1. **Parola P, Paddock CD, Socolovschi C, Labruna MB, Mediannikov O, Kernif T, Abdad MY, Stenos J, Bitam I, Fournier PE, Raoult D.** 2013. Update on tick-borne rickettsioses around the world: a geographic approach. Clin. Microbiol. Rev. **26:**657–702. http://dx.doi.org/10.1128/CMR.00032-13.
2. **Angerami RN, Nunes EM, Mendes Nascimento EM, Ribas Freitas A, Kemp B, Feltrin AF, Pacola MR, Perecin GE, Sinkoc V, Ribeiro Resende M, Katz G, Jacintho da Silva L.** 2009. Clusters of Brazilian spotted fever in São Paulo State, southeastern Brazil: a review of official reports and the scientific literature. Clin. Microbiol. Infect. **15**(Suppl 2):S202–S204. http://dx.doi.org/10.1111/j.1469-0691.2008.02637.x.
3. **Angerami RN, Câmara M, Pacola MR, Rezende RC, Duarte RM, Nascimento EM, Colombo S, Santos FC, Leite RM, Katz G, Silva LJ.** 2012. Features of Brazilian spotted fever in two different endemic areas in Brazil. Ticks Tick Borne Dis. **3:**346–348. http://dx.doi.org/10.1016/j.ttbdis.2012.10.010.
4. **Nascimento E.** 2006. Cultivo celular de riquétsias no Brasil, p 131–132. 2nd Simposio Lationamericano de Ricketsioses. Colégio Brasileiro de Parasitologia Veterinária, Ribeirão Preto, Brazil.
5. **Marrero M, Raoult D.** 1989. Centrifugation-shell vial technique for rapid detection of Mediterranean spotted fever rickettsia in blood culture. Am. J. Trop. Med. Hyg. **40:**197–199.
6. **Melles HH, Colombo S, de Lemos ER.** 1999. Isolamento de *Rickettsia* em cultura de células vero. Rev. Soc. Bras. Med. Trop. **32:**469–473. http://dx.doi.org/10.1590/S0037-86821999000500001.
7. **Labruna MB, Whitworth T, Horta MC, Bouyer DH, McBride JW, Pinter A, Popov V, Gennari SM, Walker DH.** 2004. *Rickettsia* species infecting *Amblyomma cooperi* ticks from an area in the state of São Paulo, Brazil, where Brazilian spotted fever is endemic. J. Clin. Microbiol. **42:**90–98. http://dx.doi.org/10.1128/JCM.42.1.90-98.2004.
8. **Regnery RL, Spruill CL, Plikaytis BD.** 1991. Genotypic identification of rickettsiae and estimation of intraspecies sequence divergence for portions of two rickettsial genes. J. Bacteriol. **173:**1576–1589.
9. **Roux V, Raoult D.** 2000. Phylogenetic analysis of members of the genus *Rickettsia* using the gene encoding the outer membrane protein rOmpB (*ompB*). Int. J. Syst. Evol. Microbiol. **50:**1449–1455. http://dx.doi.org/10.1099/00207713-50-4-1449.
10. **Karpathy SE, Dasch GA, Eremeeva ME.** 2007. Molecular typing of isolates of *Rickettsia rickettsii* by use of DNA sequencing of variable intergenic regions. J. Clin. Microbiol. **45:**2545–2553. http://dx.doi.org/10.1128/JCM.00367-07.
11. **Fournier PE, Zhu Y, Ogata H, Raoult D.** 2004. Use of highly variable intergenic spacer sequences for multispacer typing of *Rickettsia conorii* strains. J. Clin. Microbiol. **42:**5757–5766. http://dx.doi.org/10.1128/JCM.42.12.5757-5766.2004.
12. **Pinter A, Labruna MB.** 2006. Isolation of *Rickettsia rickettsii* and *Rickettsia bellii* in cell culture from the tick *Amblyomma aureolatum* in Brazil. Ann. N. Y. Acad. Sci. **1078:**523–529. http://dx.doi.org/10.1196/annals.1374.103.
13. **Pacheco RC, Moraes-Filho J, Guedes E, Silveira I, Richtzenhain LJ, Leite RC, Labruna MB.** 2011. Rickettsial infections of dogs, horses and ticks in Juiz de Fora, southeastern Brazil, and isolation of *Rickettsia rickettsii* from *Rhipicephalus sanguineus* ticks. Med. Vet. Entomol. **25:**148–155. http://dx.doi.org/10.1111/j.1365-2915.2010.00915.x.
14. **Krawczak FS, Nieri-Bastos FA, Nunes FP, Soares JF, Moraes-Filho J, Labruna MB.** 2014. Rickettsial infection in *Amblyomma cajennense* ticks and capybaras (*Hydrochoerus hydrochaeris*) in a Brazilian spotted fever-endemic area. Parasit. Vectors **7:**7. http://dx.doi.org/10.1186/1756-3305-7-7.
15. **Swofford DL.** 2002. PAUP: phylogenetic analysis using parsimony. Beta Version 4.0b10. Sinauer and Associates, Sunderland, MA.
16. **Huelsenbeck JP, Ronquist F.** 2001. MrBayes: Bayesian inference of phylogenetic trees. Bioinformatics **17:**754–755. http://dx.doi.org/10.1093/bioinformatics/17.8.754.
17. **Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG.** 1997. The CLUSTAL_X Windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. **25:**4876–4882. http://dx.doi.org/10.1093/nar/25.24.4876.
18. **Nicholas KB, Nicholas HB, Jr.** 1997. GeneDoc: analysis and visualization of genetic variation. EMBNEW News **4:**14.
19. **Eremeeva ME, Klemt RM, Santucci-Domotor LA, Silverman DJ, Dasch GA.** 2003. Genetic analysis of isolates of *Rickettsia rickettsii* that differ in virulence. Ann. N. Y. Acad. Sci. **990:**717–722. http://dx.doi.org/10.1111/j.1749-6632.2003.tb07449.x.
20. **Eremeeva ME, Dasch GA.** 2009. Closing the gaps between genotype and phenotype in *Rickettsia rickettsii*. Ann. N. Y. Acad. Sci. **1166:**12–26. http://dx.doi.org/10.1111/j.1749-6632.2009.04526.x.
21. **Paddock CD, Dension AM, Lash RR, Liu L, Batten BC, Dahlgren FS, Kanamura CT, Angerami RN, Pereira Dos Santos FC, Brasil Martines R, Karpathy SE.** 23 June 2014. Phylogeography of *Rickettsia rickettsii* genotypes associated with fatal Rocky Mountain spotted fever. Am. J. Trop. Med. Hyg. http://dx.doi.org/10.4269/ajtmh.14-0146.
22. **Topping NH.** 1947. The epidemiology of Rocky Mountain spotted fever. N. Y. State J. Med. **47:**1585–1587.
23. **Patino-Camargo L.** 1941. Nuevas observaciones sobre un tercer foco de fiebre petequial (maculosa) en el hemisferio americano. Bol. Oficina. Sanit. Panam. **20:**1112–1124.
24. **Dias E, Martins AV.** 1939. Spotted fever in Brazil: a summary. Am. J. Trop. Med. **19:**103–108.
25. **de Rodaniche EC, Rodaniche A.** 1950. Spotted fever in Panama; isolation of the etiologic agent from a fatal case. Am. J. Trop. Med. Hyg. **30:**511–517.
26. **Hidalgo M, Orejuela L, Fuya P, Carrillo P, Hernandez J, Parra E, Keng C, Small M, Olano JP, Bouyer D, Castaneda E, Walker D, Valbuena G.** 2007. Rocky Mountain spotted fever, Colombia. Emerg. Infect. Dis. **13:**1058–1060. http://dx.doi.org/10.3201/eid1307.060537.
27. **Hun L, Cortés X, Taylor L.** 2008. Molecular characterization of *Rickettsia rickettsii* isolated from human clinical samples and from the rabbit tick *Haemaphysalis leporispalustris* collected at different geographic zones in Costa Rica. Am. J. Trop. Med. Hyg. **79:**899–902.
28. **Hun-Opfer L.** 2008. Las fiebres manchadas y su importancia en Costa Rica. Acta Méd. Costarric. **50:**77–86.
29. **Tribaldos M, Zaldivar Y, Bermudez S, Samudio F, Mendoza Y, Martinez AA, Villalobos R, Eremeeva ME, Paddock CD, Page K, Smith RE, Pascale JM.** 2011. Rocky Mountain spotted fever in Panama: a cluster description. J. Infect. Dev. Ctries. **5:**737–741. http://dx.doi.org/10.3855/jidc.2189.
30. **Angerami RN, Resende MR, Feltrin AF, Katz G, Nascimento EM, Stucchi RS, Silva LJ.** 2006. Brazilian spotted fever: a case series from an endemic area in southeastern Brazil: clinical aspects. Ann. N. Y. Acad. Sci. **1078:**252–254. http://dx.doi.org/10.1196/annals.1374.044.
31. **Walker D.** 2013. The challenge of rickettsial diagnosis, research, and awareness in Latin America. Acta Méd. Costarric. **55**(Suppl 1):S4–S6.
32. **Ogrzewalska M, Saraiva DG, Moraes-Filho J, Martins TF, Costa FB, Pinter A, Labruna MB.** 2012. Epidemiology of Brazilian spotted fever in the Atlantic Forest, state of São Paulo, Brazil. Parasitology **139:**1283–1300. http://dx.doi.org/10.1017/S0031182012000546.
33. **Nava S, Beati L, Labruna MB, Cáceres AG, Mangold AJ, Guglielmone AA.** 2014. 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*, and *Amblyomma sculptum* (Ixodida: Ixodidae). Ticks Tick Borne Dis. **5:**252–276. http://dx.doi.org/10.1016/j.ttbdis.2013.11.004.