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Immunoproteomic profiling of *Rickettsia parkeri* and *Rickettsia amblyommii*☆

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ABSTRACT

Rickettsia parkeri is an *Amblyomma*-associated, spotted fever group *Rickettsia* species that causes an eschar-associated, febrile illness in multiple countries throughout the Western Hemisphere. Many other rickettsial species of known or uncertain pathogenicity have been detected in *Amblyomma* spp. ticks in the Americas, including *Rickettsia amblyommii*, "*Candidatus* Rickettsia andeanae" and *Rickettsia rickettsii*. In this study, we utilized an immunoproteomic approach to compare antigenic profiles of low-passage isolates of *R. parkeri* and *R. amblyommii* with serum specimens from patients with PCR- and culture-confirmed infections with *R. parkeri*. Five immunoreactive proteins of *R. amblyommii* and nine immunoreactive proteins of *R. parkeri* were identified by matrix-assisted laser desorption ionization tandem time-of-flight mass spectrometry. Four of these, including the outer membrane protein (Omp) A, OmpB, translation initiation factor IF-2, and cell division protein FtsZ, were antigens common to both rickettsiae. Serum specimens from patients with *R. parkeri* rickettsiosis reacted specifically with cysteinyl–tRNA synthetase, DNA-directed RNA polymerase subunit alpha, putative sigma (54) modulation protein, chaperonin GroEL, and elongation factor Tu of *R. parkeri* which have been reported as virulence factors in other bacterial species. Unique antigens identified in this study may be useful for further development of the better serological assays for diagnosing infection caused by *R. parkeri*.

1. Introduction

Tick-borne spotted fever group (SFG) *Rickettsia* species are obligate intracellular α-Proteobacteria maintained within ticks by vertical transmission (female to progeny), and between tick populations by horizontal transmission via vertebrate hosts. Infections in humans by different species of SFG *Rickettsia* reveal a wide range of severity from life-threatening disease to self-limited or subclinical infections (Macaluso and Paddock, 2014). The factors that enhance or limit transmission to humans are poorly defined. In addition to complex vector traits, rickettsial-derived attributes are likely associated with successful vertebrate infection.

Recent studies have identified three SFG *Rickettsia* species that are associated with human biting *Amblyomma* species in the United States. *Rickettsia parkeri* transmitted predominantly by Gulf Coast ticks, *Amblyomma maculatum*, causes an eschar-associated rash illness in the southern United States that is milder than classical Rocky Mountain Spotted Fever (RMSF) (Paddock et al., 2008, 2004; Whitman et al., 2007). Molecular detection of *R. parkeri* from *A. maculatum* ranges from approximately 1–43% (Cohen et al., 2009; Ferrari et al., 2012; Fornadel et al., 2011; Jiang et al., 2012; Paddock et al., 2010; Sumner et al., 2007; Trout et al., 2010; Varela-Stokes et al., 2011; Wright et al., 2011), with a recent comprehensive multi-year study identifying prevalence between approximately 42–56% in Virginia (Nadolny et al., 2014). Currently, more than 35 cases of *R. parkeri* rickettsiosis have been identified in the United States (Paddock and Goddard, 2015). *Rickettsia amblyommii* is a SFG *Rickettsia* of undetermined pathogenicity and a ubiquitous resident of many populations of Lone Star ticks, *Amblyomma americanum*. This species has been identified in as many as 80% of *A. americanum* surveyed throughout the southern and eastern United States

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(Mixson et al., 2006); however, recent screening of *A. maculatum* collected in Arkansas for rickettsiae demonstrated that 26% had *R. amblyommii*, and only 1.4% had *R. parkeri* (Trout et al., 2010). Apperson et al. (2008) have proposed that much of the seroreactivity to SFG *Rickettsia* antigens in the United States is caused by mild infections with *R. amblyommii*. In addition to *R. parkeri* and *R. amblyommii*, a newly recognized and incompletely characterized *Rickettsia* species, *Candidatus* Rickettsia andeanae was detected in *A. maculatum* (Ferrari et al., 2012; Fornadel et al., 2011; Jiang et al., 2012; Paddock et al., 2010; Sumner et al., 2007). Currently, the only Amblyomma-associated SFG *Rickettsia* that is a confirmed pathogen of humans is *R. parkeri*.

Comparative genetics of rickettsial species has identified a potential genetic basis of rickettsial pathogenicity, notably in the outer membrane surface proteins, which are involved in host cell interactions and probably necessary for infection of vertebrate hosts (Ellison et al., 2008; Felsheim et al., 2009). Through genetic manipulation, functional characterization studies of suspected rickettsial virulence determinants have provided intriguing results, whereby an attenuated rickettsial phenotype can be created for some, but not all targets, suggesting multiple or redundant virulence factors (Driskell et al., 2009; Kleba et al., 2010; Noriea et al., 2015). An alternative approach of dissecting rickettsial virulence is to examine the presence of differentially produced rickettsial proteins during infection. While proteomic and immunoreactive profiles have been described for several rickettsial species (Chao et al., 2004, 2007; Hajem et al., 2009; Ogawa et al., 2007; Pornwiroon et al., 2009; Renesto et al., 2005; Sears et al., 2012; Tucker et al., 2011) and closely related tick-borne bacterial pathogens such as *Anaplasma* (Lopez et al., 2005) and *Ehrlichia* (Seo et al., 2008), little has been done with comparative antigenic analysis of different rickettsial species. In this study, a comparative immunoproteomic approach using sera from *R. parkeri*-infected patients was employed for the identification and distinction of several immunoreactive proteins from *R. parkeri* and *R. amblyommii*.

2. Materials and methods

2.1. Cell culture and rickettsiae

African green monkey kidney (Vero E6) cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 5% fetal bovine serum (Hyclone, Logan, UT) in a humidified 5% CO_2 incubator at 34 °C. *R. parkeri* (strain Portsmouth) originally isolated from a skin biopsy specimen (Paddock et al., 2004) and *R. amblyommii* (strain Darkwater) isolated from a female *A. americanum* tick collected in Wakulla County, FL in 2006 (CD Paddock, unpublished data) were propagated separately in Vero E6 cells. Rickettsiae were passaged <10 times for use in this study. Rickettsial infection was assessed by Diff-Quik (Dade Behring, Deerfield, IL) staining according to the manufacturer's protocol.

2.2. Purification of rickettsiae

When more than 90% of Vero E6 cells were infected, cells from four 175-cm^2 flasks were scraped and collected by centrifugation at 500 × *g* for 15 min at 4 °C. Rickettsiae were purified by using Renografin density gradient ultracentrifugation as previously described (Pornwiroon et al., 2009). The homogeneity of *Rickettsia* was examined by Diff-Quik staining, and the *Rickettsia* pellet was stored in protease inhibitor cocktail (Roche, Indianapolis, IN) at −80 °C until use.

2.3. Two-dimensional polyacrylamide gel electrophoresis (2D PAGE)

Rickettsial protein extracts were prepared from purified bacteria as previously described (Pornwiroon et al., 2009), and 30 μg of protein were subjected to isoelectric focusing (IEF) in an immobilized pH gradient strip (7 cm, linear pH 3 to 10; Bio-Rad, Hercules, CA) using a PROTEAN IEF cell apparatus (Bio-Rad) at 20 °C as described previously (Pornwiroon et al., 2009). The IEF strip was sequentially equilibrated for 15 min each in 1× NuPAGE LDS sample buffer (Invitrogen) containing 1% (w/v) dithiothreitol and 1× NuPAGE LDS sample buffer containing 2.5% (w/v) iodoacetamide. After the equilibration, the strip was placed on top of NuPAGE Novex 4–12% Bis–Tris ZOOM gel (Invitrogen) and sealed with 0.5% agarose. Electrophoresis was performed in the XCell SureLock Mini-Cell System (Invitrogen) at 100 V until the tracking dye reached the gel bottom. Gels were fixed in fixative solution (10% methanol/7% acetic acid) for 1 h and overnight stained with SYPRO Ruby protein gel stain (Bio-Rad). Gels were digitized using Molecular Imager Gel Doc XR System (Bio-Rad) and stored at 4 °C for spot excision and protein identification.

2.4. Immunoblotting

The 2D PAGE resolved proteins were transferred onto Immun-Blot polyvinylidene difluoride (PVDF) membranes (Bio-Rad) by electroblotting on the XCell II Blot Module (Invitrogen) according to the manufacturer's protocol. The membranes were blocked for 2 h with 3% skim milk in Tris-buffered saline–0.1% Tween 20 (TBST; 20 mM Tris–HCl [pH 7.5], 500 mM NaCl, 0.1% Tween 20) and incubated with primary antibodies at a dilution of 1:100 or 1:200 for 2 h at room temperature. Serum specimens collected from three patients with PCR- or culture-confirmed *R. parkeri* rickettsiosis were used as primary antibodies. Each sample contained IgG antibodies that reacted at high titers (≥256) with antigens of *R. parkeri* and with those of *Rickettsia akari*, *Rickettsia rickettsii*, and *R. amblyommii* when evaluated by using indirect immunofluorescence antibody (IFA) test (Table 1) (Paddock et al., 2008, 2004). Each serum sample was analyzed on a separate membrane. After being rinsed and washed twice for 10 min each time with TBST, the membranes were incubated with horseradish peroxidase-conjugated rabbit anti-human IgG (Sigma, St. Louis, MO) at a 1:80,000 dilution for 1 h at room temperature. This secondary antibody was tested to have no reactivity with *R. parkeri* and *R. amblyommii* proteins (data not shown). Following a rinse and two washes with TBST, immunoreactive protein spots were detected using SuperSignal West Pico Chemiluminescent Substrate kit (Pierce, Rockford, IL). The same exposure time was used for every blot. After Western blot analysis, the membranes were stained with MemCode Reversible Protein Stain kit (Pierce), according to the manufacturer's instruction, to match location of protein spots on the membrane with the Western blot signal. The immunoreactive antigens were then mapped by alignment protein spots on the membrane with a SYPRO Ruby-stained gel using ImageMaster 2D Platinum software (version 5.0) (Amersham Biosciences, Piscataway, NJ), then excised, and analyzed by matrix-assisted laser desorption ionization tandem time of flight mass spectrometry (MALDI-TOF/TOF MS).

2.5. In-gel digestion and MALDI-TOF/TOF MS

In-gel digestion and MS were performed by the Nevada Proteomics Center, University of Nevada as follows: excised protein spots were reduced, alkylated, and in-gel digested with trypsin as previously described (Pornwiroon et al., 2009). Digested protein samples were spotted onto a MALDI target with ZipTipu-C18 (Millipore, Billerica, MA). Samples were aspirated, dispensed three times,

Table 1
IFA titers of serum samples collected from three patients with confirmed *Rickettsia parkeri* rickettsiosis.

Patient	IFA titer (IgG antibody)				Reference
	R. akari	*R. amblyommii*	*R. parkeri*	*R. rickettsii*	
1	1024	512	2048	1024	Paddock et al. (2004)
2	256	256	512	256	Paddock et al. (2008)
3	1024	512	1024	1024	Paddock et al. (2008)

eluted with 70% acetonitrile and 0.2% formic acid, and overlaid with 0.5 μl of 5 mg ml^{-1} MALDI matrix (α-Cyano-4-hydroxycinnamic acid) and 10 mM ammonium phosphate. All mass spectrometric data were collected using an ABI 4700 MALDI-TOF/TOF (Applied Biosystems, Foster City, CA). The data were acquired in reflector mode from a mass range of 700–4000 Da and 1250 laser shots were averaged for each mass spectrum. Each sample was internally calibrated on trypsin's autolysis peaks. The eight most intense ions from the MS analysis, which were not on the exclusion list, were subjected to MS/MS. For MS/MS analysis the mass range was 70 to precursor ion with a precursor window of −1 to 3 Da with an average 5000 laser shots for each spectrum. The data were stored in an Oracle database. The data were extracted from the Oracle database and a peak list was created by GPS Explorer software (Applied Biosystems) from the raw data generated from the ABI 4700. This peak list was based on signal to noise filtering and an exclusion list and included de-isotoping. The resulting file was then searched by Mascot (Matrix Science, Boston, MA). Database search parameters include a mass tolerance of 20 ppm, one missed cleavage, oxidation of methionines, and carbamidomethylation of cysteines. Only the matched proteins with the significant scores ($P < 0.05$) were reported.

3. Results

To analyze and compare antigenic profiles of *R. parkeri* and *R. amblyommii*, protein extracts were prepared, resolved by 2D PAGE, and transferred to PVDF membrane and probed with serum specimens from three patients with confirmed *R. parkeri* rickettsiosis. Serum samples reacted to six protein spots of *R. amblyommii* and 12 of *R. parkeri* with apparent molecular masses ranging from 240 to 22 kDa (Fig. 1). The 240-, 150-, and 100-kDa antigens of both rickettsial species (spots RP1, RP2, RP4, and RA1–RA3) were detected in at least two sera tested. Antigenic differences between two rickettsial species were observed. Protein spots on a SYPRO-Ruby stained gel corresponding to signals obtained from Western blot analysis were excised and identified by MALDI-TOF/TOF MS. Their identities, matching scores, number of peptides matched, predicted molecular masses/isoelectric points (pIs), and percentages of sequence coverage are shown in Table 2. The matched peptide sequences of each protein spot are provided in Supplementary Table S1.

Supplementary table related to this article can be found, in the online version, at doi:10.1016/j.ttbdis.2015.07.012

Six *R. amblyommii* immunoreactive spots, corresponding to five proteins, were successfully identified: OmpA (spot RA1), OmpB (spots RA2 and RA5), translation initiation factor IF-2 (spot RA3), cell division protein FtsZ (spot RA4), and seryl-tRNA synthetase (spot RA6) (Table 2). Serum specimens reacted to five additional antigens of *R. parkeri*: cysteinyl–tRNA synthetase (spot RP6), DNA-directed RNA polymerase subunit alpha (spots RP8 and RP9), putative sigma (54) modulation protein (spot RP10), chaperonin GroEL (spot RP11), and elongation factor Tu (EF-Tu; spot RP12). To confirm that the lack of signal for *R. amblyommii* proteins was not due to the absence of expressed proteins, spots assigned as RA7 and RA8 (Fig. 1E), which were located at the same positions relative to that of *R. parkeri* 2D gel, were subjected to MALDI-TOF/TOF analysis and identified as chaperonin GroEL and EF-Tu, respectively. The results indicate these proteins were expressed in *R. amblyommii*.

Except for the major outer membrane proteins (OmpA and OmpB), no *R. parkeri* antigens were consistently recognized by immune serum specimens of each patient with confirmed *R. parkeri* rickettsiosis; however, one or more patient serum samples reacted specifically with five immunoreactive proteins (cysteinyl–tRNA synthetase, DNA-directed RNA polymerase subunit alpha, putative sigma (54) modulation protein, chaperonin GroEL, and EF-Tu) expressed by *R. parkeri*, but not with any of the protein homologs expressed by *R. amblyommii* (Table 3).

4. Discussion

In this study, a comparative immunoproteomic analysis was carried out to characterize antigens of two low-passage isolates of *Amblyomma*-associated rickettsiae, *R. parkeri* and *R. amblyommii*. We identified nine immunoreactive proteins of *R. parkeri* and five of *R. amblyommii* that were recognized by serum specimens from three patients with confirmed *R. parkeri* rickettsiosis. The theoretical and experimentally observed molecular mass and pI values of the identified proteins were in general agreement, except for spots RP1, RP7, and RA5. Spot RP1, identified as OmpA, had a much higher molecular mass than predicted because the matching peptides from the sequence database contain only partial protein sequences. While the molecular masses of spots RP7 and RA5 corresponding to OmpB were much lower than predicted as also observed for *Rickettsia felis* (Ogawa et al., 2007). This may be due to post-translational proteolytic cleavage of OmpB (Hackstadt et al., 1992). OmpA and OmpB were the major antigenic proteins of both *Amblyomma*-associated rickettsiae recognized by all sera tested. These immunodominant surface-exposed proteins have been implicated in the rickettsial adhesion to and invasion of host cells (Li and Walker, 1998; Martinez et al., 2005; Uchiyama, 2003; Uchiyama et al., 2006). Protective immunity has been demonstrated with OmpA and OmpB (Bourgeois and Dasch, 1981; Crocquet-Valdes et al., 2001; Sumner et al., 1995; Vishwanath et al., 1990); they are the principal candidates for vaccines against rickettsial infections. The antigenicity of translation initiation factor IF-2 and cell division protein FtsZ homologs, as common antigens of both rickettsial species identified in this study, has been reported in previous studies of several bacteria (Boonjakuakul et al., 2007; Nowalk et al., 2006; Zhang et al., 2008). Moreover, the FtsZ-like protein has been suggested to be involved in pathogenesis of *Bacillus anthracis* (Tinsley and Khan, 2006).

Among five immunoreactive proteins uniquely identified for *R. parkeri* including cysteinyl–tRNA synthetase, DNA-directed RNA polymerase subunit alpha, putative sigma (54) modulation protein, chaperonin GroEL, and EF-Tu, only chaperonin GroEL and EF-Tu were described previously as rickettsial antigens (Hajem et al., 2009; Qi et al., 2013; Renesto et al., 2005). DNA-directed RNA polymerase subunit alpha has been identified as immunoreactive protein of other gram-negative bacteria (Altindis et al., 2009; Lin et al., 2006). Proteins with homology to antigens of *R. parkeri* have been shown to play roles in bacterial pathogenesis. The involvement of EF-Tu in *Francisella tularensis* adhesion and entry into the

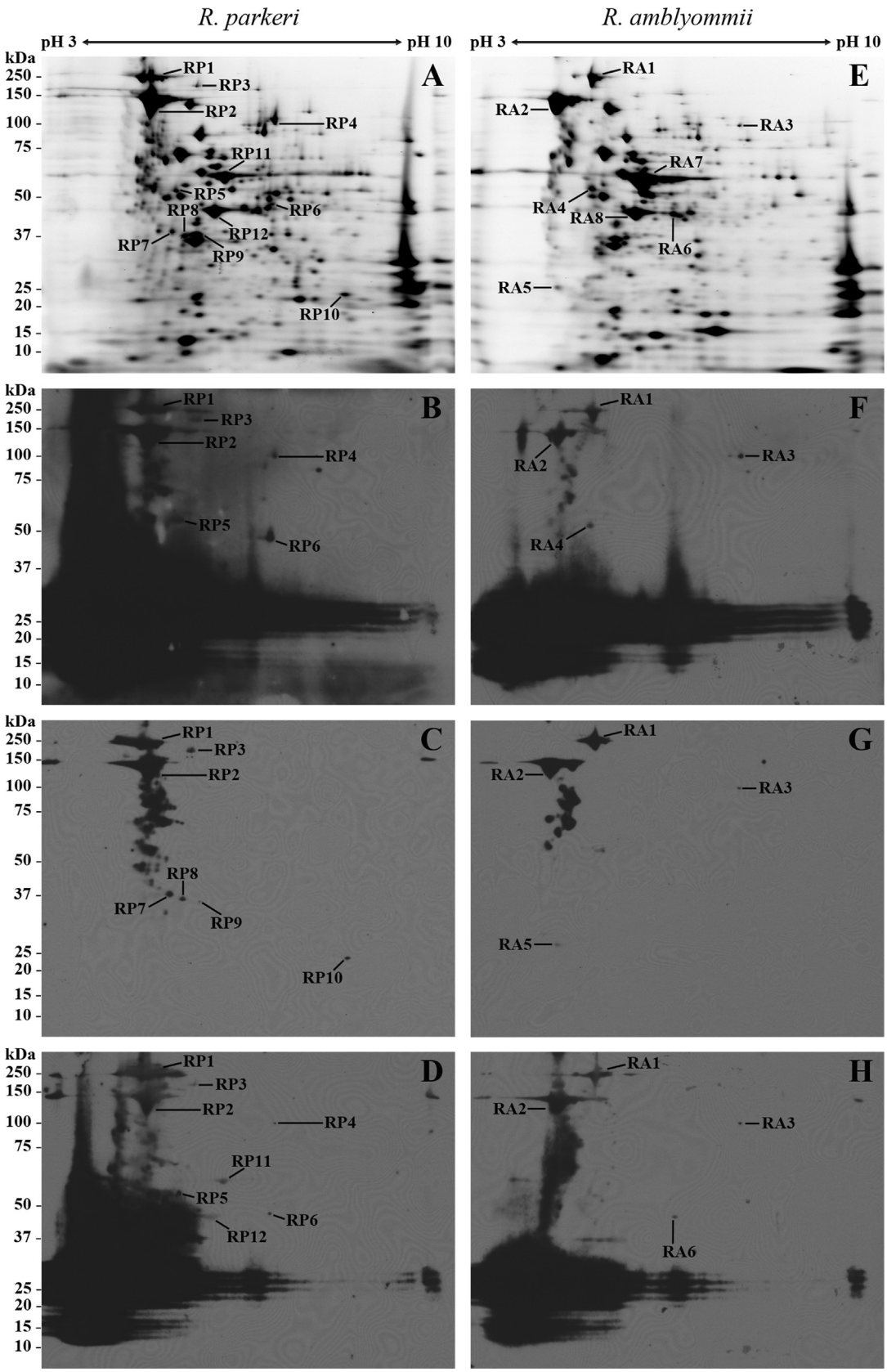

Fig. 1. 2D gels and immunoblots of *Rickettsia parkeri* (strain Portsmouth) and *Rickettsia amblyommii* (strain Darkwater) protein extracts. Isoelectric focusing was performed with protein extracts of rickettsiae using 7-cm pH 3 to 10 immobilized pH gradient strips, followed by sodium dodecyl sulfate PAGE on 4–12% Bis–Tris gels. The proteins resolved by 2D PAGE were either stained with SYPRO Ruby protein stain (A and E) or transferred to PVDF membranes and probed with serum specimens from three patients with confirmed *R. parkeri* rickettsiosis; patient 1 (B and F), patient 2 (C and G), and patient 3 (D and H). Representative images of two independent experiments are shown. The numbers refer to the protein identities shown in Table 2. The molecular masses of the Precision Plus Protein Kaleidoscope standards (Bio-Rad) are indicated on the left.

Table 2
Rickettsial proteins identified by matrix-assisted laser desorption ionization tandem time of flight mass spectrometry.

Spot no.	GenInfo identifier no.	Protein description[a]	Score	No. of peptides matched	Predicted molecular mass (kDa)	Predicted pI	Sequence coverage (%)
R. parkeri							
RP1	1778893	rOmpA	96	2	110.11	5.30	3
RP2	383484405	Outer membrane protein OmpB	439	3	168.13	5.49	4
RP3	383484405	Outer membrane protein OmpB	249	8	168.13	5.49	6
RP4	383484151	Translation initiation factor IF-2	397	9	91.10	6.27	17
RP5	383484344	Cell division protein FtsZ	443	10	48.48	4.94	37
RP6	383483461	Cysteinyl-tRNA synthetase	98	3	53.24	6.23	6
RP7	383484405	Outer membrane protein OmpB	333	5	168.13	5.49	4
RP8	157828836	DNA-directed RNA polymerase subunit alpha	92	4	38.47	5.24	12
RP9	157828836	DNA-directed RNA polymerase subunit alpha	686	16	38.47	5.24	47
RP10	383483976	Putative sigma (54) modulation protein	209	8	22.04	7.71	66
RP11	157828821	Chaperonin GroEL	992	16	58.59	5.62	41
RP12	157828862	Elongation factor Tu	828	15	42.97	5.50	47
R. amblyommii							
RA1	154125523	OmpA	133	2	191.22	5.46	8
RA2	383313022	Outer membrane protein OmpB	634	7	167.85	4.98	6
RA3	383312712	Translation initiation factor IF-2	595	12	90.98	6.89	19
RA4	383312969	Cell division protein FtsZ	377	7	48.33	4.81	27
RA5	383313022	Outer membrane protein OmpB	406	5	167.85	4.98	4
RA6	383313138	Seryl-tRNA synthetase	675	13	48.62	5.95	39
RA7	383312927	Chaperonin GroEL[b]	1070	16	58.68	5.54	45
RA8	383312964	Elongation factor Tu[b]	922	14	42.92	5.49	57

[a,b] Proteins that were expressed by *R. amblyommii*, but not recognized by serum samples from patients with *R. parkeri* rickettsiosis.

Table 3
Reactivity of serum samples from three patients with *R. parkeri* rickettsiosis to each rickettsial antigen.

Protein	*Rickettsia* sp.	Serum sample		
		Patient 1	Patient 2	Patient 3
OmpA	*R. parkeri*	+	+	+
	R. amblyommii	+	+	+
OmpB	*R. parkeri*	+	+	+
	R. amblyommii	+	+	+
Translation initiation factor	*R. parkeri*	+	–	+
IF-2	*R. amblyommii*	+	+	+
Cell division protein FtsZ	*R. parkeri*	+	–	+
	R. amblyommii	+	–	–
Seryl-tRNA synthetase	*R. parkeri*	–	–	–
	R. amblyommii	–	–	+
Cysteinyl-tRNA synthetase	*R. parkeri*	+	–	+
	R. amblyommii	–	–	–
DNA-directed RNA polymerase	*R. parkeri*	–	+	–
subunit alpha	*R. amblyommii*	–	–	–
Putative sigma (54)	*R. parkeri*	–	+	–
modulation protein	*R. amblyommii*	–	–	–
Chaperonin GroEL	*R. parkeri*	–	–	+
	R. amblyommii	–	–	–
Elongation factor Tu	*R. parkeri*	–	–	+
	R. amblyommii	–	–	–

human monocyte-like THP-1 cells has been demonstrated (Barel et al., 2008). The YhbH protein, a member of the sigma (54) modulation protein family, has been reported as the virulence factor of *Bacillus cereus* (Oosthuizen et al., 2002) and *F. tularensis* (Carlson et al., 2007).

For more than 30 years, IFA techniques using whole-cell rickettsial antigens have been considered the reference standard for serologic diagnosis of spotted fever rickettsioses (Parola et al., 2005; Philip et al., 1977). These assays, despite a high level of sensitivity, are specific to SFG *Rickettsia*, but cannot be used to ascribe a species-specific etiology. This limitation assumes increasing importance as the spectrum of distinct SFG *Rickettsia* infections in the United States continues to expand, presently including at least five pathogenic SFG *Rickettsia* with overlapping distributions and cross-reacting antigens: *R. rickettsii*, *R. akari*, *R. parkeri*, *Rickettsia* sp. 364D, and *Rickettsia massiliae* (Eremeeva et al., 2006; Paddock et al., 2008, 2004; Shapiro et al., 2010; Whitman et al., 2007). Other SFG *Rickettsia*, such as *R. amblyommii*, *Ca.* R. andeanae and *Rickettsia rhipicephali*, occur in several species of human biting ticks and represent candidate pathogens, although conclusive evidence linking these rickettsiae with human infections is lacking (Apperson et al., 2008; Jiang et al., 2012; Paddock, 2009; Paddock et al., 2010).

For many years, serologic assays that measured reactivity to antigens of *R. rickettsii* were often misinterpreted as evidence of recent or past infection with the agent of RMSF, one of the most severe of all tick-borne infectious diseases (Taylor et al., 1985). A more nuanced view, recognizing a broader group of *Rickettsia* sp. that collectively elicit group-specific antibodies, has emerged as the number of recognized SFG *Rickettsia* pathogens increases and clinical distinctions among the various diseases become more apparent. Indeed, several contemporary surveys of U.S. populations have identified serologic reactivity to SFG *Rickettsia*, as assessed by IFA assays using *R. rickettsii* as antigen, in 4–12% of study subjects (Graf et al., 2008; Hilton et al., 1999; Marshall et al., 2003; Taylor

et al., 1985); these almost certainly do not represent cases of RMSF, given the rarity of *R. rickettsii* infection in ticks in nature, and the absence of severe, moderate, or in some cases, any disease among most of the tested individuals (Paddock, 2009). Further studies with larger panels of serum specimens from patients with PCR- or culture-confirmed spotted fever rickettsioses, including RMSF, rickettsialpox, and *R. parkeri* rickettsiosis, and pedigreed samples from persons infected with *R. amblyommii*, as well as serum samples from persons with no known exposure to *R. parkeri*, will be needed to better define the specificity and diagnostic utility of these immunoreactive proteins for differentiation of rickettsial infections.

In conclusion, we identified *R. parkeri* antigens distinct from *R. amblyommii* antigens by comparative immunoproteomic analysis using *R. parkeri* rickettsiosis patient sera. The results may be useful for further development into a better diagnostic test in order to distinguish *R. parkeri* infection from other possible *Amblyomma*-associated *Rickettsia* infection in humans. Studying distinct rickettsial antigens, which may be useful for vaccine development, will facilitate our understanding of rickettsial pathogenesis in humans.

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