

Seroepidemiologic Study of Human Infections with Spotted Fever Group *Rickettsiae* in North Carolina

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Increasing entomologic and epidemiologic evidence suggests that spotted fever group rickettsiae (SFGR) other than *Rickettsia rickettsii* are responsible for spotted fever rickettsioses in the United States. A retrospective seroepidemiologic study was conducted on stored acute- and convalescent-phase sera that had been submitted for Rocky Mountain spotted fever testing to the North Carolina State Laboratory of Public Health. We evaluated the serologic reactivity of the paired sera to *R. rickettsii*, *Rickettsia parkeri*, and *Rickettsia amblyommii* antigens. Of the 106 eligible pairs tested, 21 patients seroconverted to one or more antigens. Cross-reactivity to multiple antigens was observed in 10 patients, and seroconversions to single antigens occurred in 11 patients, including 1 against *R. rickettsii*, 4 against *R. parkeri*, and 6 against *R. amblyommii*. Cross-absorption of cross-reactive sera and/or Western blots identified two presumptive cases of infection with *R. parkeri*, two presumptive cases of infection with *R. rickettsii*, and one presumptive case of infection with *R. amblyommii*. These findings suggest that species of SFGR other than *R. rickettsii* are associated with illness among North Carolina residents and that serologic testing using *R. rickettsii* antigen may miss cases of spotted fever rickettsioses caused by other species of SFGR.

Rocky Mountain spotted fever (RMSF), caused by the bacterium *Rickettsia rickettsii*, is the most commonly reported fatal tick-borne disease in the United States. The incidence of spotted fever rickettsioses in the United States (including RMSF) has been rising rapidly in recent years, from 2.5 cases per million in 2001 to 9.5 cases per million in 2011 (1). Mounting evidence suggests that infections with other species of spotted fever group rickettsiae (SFGR) may be at least partly responsible for the apparent increase in cases, which may reflect an increase in reporting or incidence or both. Seroepidemiologic surveys of adults and children in the United States show that seroprevalence to SFGR is between 6% and 12% (2, 3). Because subclinical infection or mild infections with *R. rickettsii* are considered to be rare or even nonexistent and there is cross-reactivity among SFGR in serologic tests, exposure to other species of SFGR could account for this relatively high seroprevalence (4). For many years, *Rickettsia parkeri* was considered to be a nonpathogenic SFGR, until it was isolated from a patient in Virginia in 2002 (5). *R. parkeri* is now recognized as a human pathogen, causing an illness characterized by formation of an eschar at the site of inoculation and generally milder symptoms than classic RMSF (6, 7). In California, *Rickettsia* 364D has been implicated in causing an eschar-associated illness decades after it was first identified in *Dermacentor occidentalis* ticks (8). Recent studies have suggested that *Rickettsia amblyommii*, which is present in a large percentage of Lone Star ticks (*Amblyomma americanum*), may cause a mild rickettsiosis in humans (9–15). In this study, we evaluated the reactivity of paired sera from North Carolina patients who had been tested for RMSF to a panel of SFGR, including *R. rickettsii*, *R. amblyommii*, and *R. parkeri*.

MATERIALS AND METHODS

Selection of case patients. Case patients were identified from a database of patients who were tested for RMSF at the North Carolina State Labo-

ratory of Public Health (NCSLPH) between 2008 and 2010. Samples were submitted from across the entire state, with the majority from patients in the Piedmont region (central North Carolina). Eligibility criteria included having paired sera available (from the acute and convalescent phases) and at least one sample with a titer of $\geq 1:64$ against *R. rickettsii* in the original test. Sera from patients tested for SFGR that do not demonstrate reactivity are not routinely retained for long-term storage at the NCSLPH and were therefore unavailable for testing. Patients were excluded from the study if there was only a single serum specimen available, if there were not sufficient sera remaining for additional testing, or if the sera could not be located.

Antigens. Indirect immunofluorescence assay (IFA) testing completed at the North Carolina State Laboratory of Public Health (NCSLPH) utilized rickettsiae grown in chicken egg yolk (*R. rickettsii* Sheila Smith) or Vero cells (*R. parkeri* Portsmouth and *R. amblyommii* Darkwater) provided by William Nicholson, Rickettsial Zoonoses Branch, CDC (16). Antigen preparations of infected Vero cells (*R. parkeri* and *R. amblyommii*) or cell-free bacteria in egg yolk suspension (*R. rickettsii*) were spotted onto the wells of glass templated slides using capillary tubes. Antigen spots were allowed to air dry and then fixed in acetone for 10 to 15 min at room temperature. Slides were then stored in sealed boxes at -70°C until they were ready to be used. IFA testing conducted at the Tennessee Vector-

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Borne Disease Laboratory (TNVBDL) utilized slides prespotted with rickettsiae grown in Vero cells (*R. rickettsii* Sheila Smith, *R. parkeri* Portsmouth, and *R. amblyommii* WB-82-like North Texas) and corresponding Renografin-purified rickettsial lysates for cross-absorption and Western blotting, prepared by Nicole Mendell, Department of Pathology, University of Texas Medical Branch at Galveston (17). Rickettsiae were cultivated in confluent Vero cells and monitored until the cells were determined to be approximately 70 to 80% infected. The infected monolayer was scraped, harvested, washed with 0.1 M phosphate-buffered saline (PBS) by centrifugation at $500 \times g$ for 5 min, and resuspended in PBS with 1% bovine calf serum (BCS). The cell suspension was pipetted onto poly-L-lysine-coated 12-well Teflon masked IFA slides at 10 μ l per well, allowed to dry, and fixed for 10 min with acetone. The rickettsial antigen lysate was generated by scraping heavily infected (80 to 100%) Vero cell monolayers and centrifugation at $17,000 \times g$ for 15 min to pellet the infected Vero cells. The infected Vero cells were resuspended in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 2 mM sucrose and lysed by sonication on ice for four 15-s pulses at 60% amplitude to release the rickettsiae. Host cell debris was removed by centrifugation at $1,000 \times g$ for 5 min. The supernatant was passed through a 0.22- μ m-pore syringe-driven filter, and cell-free rickettsiae were pelleted $17,000 \times g$ for 15 min and resuspended in PBS with 0.05% sodium azide. Rickettsiae were sonicated on ice for eight 15-s pulses at 60% amplitude to lyse the bacteria.

IFA. Assays were conducted using previously published methods for rickettsial diagnosis using the IFA (18). Briefly, frozen slides were placed at room temperature and allowed to warm while serial dilutions were prepared. The primary dilution of 1:64 was prepared in 3% egg yolk for the *R. rickettsii* antigen and 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for *R. parkeri* and *R. amblyommii*. The remaining serial 2-fold dilutions were made in PBS with 1% BSA for all antigens. Sera were diluted to a final dilution of 1:2,048, except for positive-control sera, which were diluted to 1:16,384. Positive-control sera from a patient with a known titer against *R. rickettsii* antigen were included in each run (including dilutions from 1:64 to 1:16,384). Negative controls, including PBS only, PBS with BSA, and a negative-control serum that is nonreactive to *R. rickettsii* (primary dilution only) were also included in each run. Dilutions were applied to the slides and incubated in a humidity chamber at 37°C for 30 min. Slides were rinsed in PBS, washed in PBS for 10 min, and then washed in distilled water for 10 min and air dried. Fluorescein isothiocyanate (FITC)-labeled goat anti-human IgG conjugate (Scimedx, Danville, NJ) with Eriochrome black counterstain was applied to each well, and the wells were incubated in a humid chamber at 37°C for 30 min. Slides were washed as described above, and once dried, a drop of buffered glycerol was added to each well and overlaid with a coverslip. Slides were stored in the dark until they were read (within 24 h).

Reading of slides and interpretation of results. Paired sera were always tested and read together by the same technician. Slides were read on a UV epifluorescence microscope, and wells were initially examined at low power (100 \times) and then high power (400 \times). After the negative-control wells were read, the positive-control and test samples were read from the highest dilution to the lowest dilution. Staining for negative controls was confirmed by the absence of specific fluorescence. Runs for which the positive control was within one dilution of the known titer were considered acceptable. Fluorescence was scored according to brightness and consistency of staining throughout the well on a scale of 4+, 3+, 2+, 1+, +/-, and -, with 4+ being the most intense fluorescence. Endpoint titers were recorded as the reciprocal of the dilution with 1+ fluorescence, unless the fluorescence at the 1:2,048 dilution was 1+ or greater, in which case the endpoint titer was recorded as $\geq 1:2,048$. If staining intensity did not increase as expected from higher dilutions to lower dilutions or if nonspecific staining patterns were observed, then this was noted, and no endpoint titer could be determined. If a sample had a questionable result or if problems with the staining were noted, the senior techni-

cian reevaluated the slide and either made the final call on the endpoint titer or suggested that the test be repeated. If a test needed to be repeated, both samples (acute and convalescent phases) were repeated together. For samples that did not yield a titer against *R. rickettsii* that was within one dilution of the original titer, the assay was repeated, and the titer that was found in the majority of the assays was accepted as the correct titer.

A seroconversion was defined as a 4-fold or greater change in IgG titer against an antigen between acute- and convalescent-phase samples. For each antigen, the patient was classified as either a seroconversion, stationary titer (lack of 4-fold or greater change in titer), or unknown (if one or more of the samples had nonspecific fluorescence or were unreadable).

Cross-absorption and IFA. A subset of samples for which patient clinical data and sufficient sera were available were selected for additional analyses at the TNVBDL, in an attempt to identify the presumptive causative agent. Additional testing included Western blots and cross-absorption followed by IFA. To cross-absorb sera, equal volumes of diluted sera (1:32) and antigen lysate (2 mg/ml) were incubated for 20 h at room temperature on a rotator. Sera were then centrifuged at $10,000 \times g$ for 11 min, and the supernatant was collected for use in IFAs (19). IFAs were completed as described above with the following modifications (according to the protocol used at the TNVBDL): serum dilutions were prepared in PBS with 1% BSA, 0.01% sodium azide, and 0.1% Tween 20, the wash buffer was PBS with 0.1% Tween 20, FITC-labeled goat anti-human IgG conjugate was purchased from KPL, and slides were counterstained with 1% Evans blue. A presumptive agent was identified when cross-absorption resulted in the removal (or at least 4-fold decrease) of both homologous and heterologous antibodies, while cross-absorption with the cross-reactive species removed homologous antibodies only.

Western blotting. Purified antigen (30 μ g) was denatured with Run-Blue dithiothreitol (DTT) reducer (Expedeon, San, Diego, CA) at 70°C for 10 min and separated by SDS-PAGE with a 4-to-12% gradient gel (Expedeon) using a Dual Cool electrophoresis system (CBS Scientific, San Diego, CA) at 180 V for 1 h. Resolved antigens were transferred to nitrocellulose membranes (Expedeon) using a semidry blotter (Amersham Biosciences) at 22 V for 1 h. Membranes were blocked in Tris-buffered saline (TBS; 10 mM Tris-HCl [pH 7.5], 150 mM NaCl) with 5% nonfat dry milk for 1 h at room temperature. Sera, diluted 1:125 in blocking buffer, were incubated with membranes overnight at room temperature. Membranes were washed once in TBS with 0.05% Tween 20 for 10 min and once in TBS for 10 min, followed by incubation with phosphatase-labeled goat anti-human IgG purified antibody (KPL, Gaithersburg, MD) diluted 1:20,000 in blocking buffer for 1 h at room temperature. Membranes were washed twice in TBS for 10 min, and bands were visualized using the BCIP/NBT (5-bromo-4-chloro-3-indolylphosphate-nitroblue tetrazolium) phosphatase substrate (KPL) and washed in distilled water to stop the reaction. Membranes were examined for the presence of the typical rickettsial lipopolysaccharide (LPS) antigen washboard pattern at 20 to 60 kDa and specific protein antigens (SPAs) in the 110- to 140-kDa region (20, 21).

Surveillance reports. Surveillance reports from the North Carolina Division of Public Health (NCDPH) were requested for all patients included in this study. Sample numbers from the NCSLPH were cross-referenced with event numbers in the North Carolina Electronic Disease Surveillance System (NCEDSS), and all available reports were provided in paper format (deidentified) by the North Carolina Public Health Data Group. A form was created in Qualtrics, and relevant fields were entered into the form for each patient.

Data analysis. Standard descriptive statistics were calculated for demographic and clinical variables. Positive percentage of agreement was calculated for each antigen using seroconversion against *R. rickettsii* as the referent comparison. All analyses were performed using SAS (version 9.2, SAS Institute, Inc., Cary, NC).

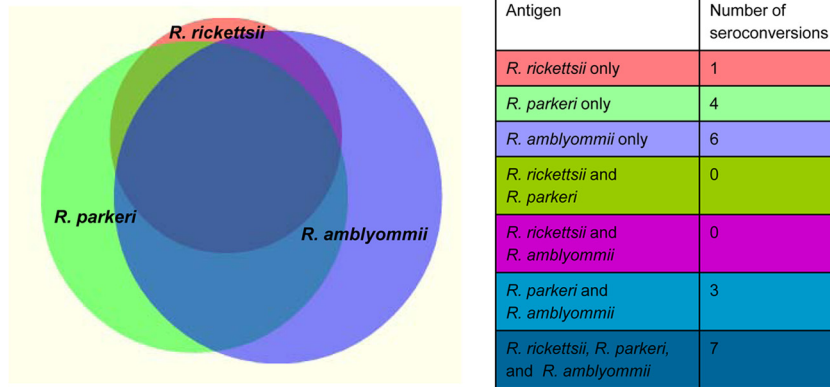


FIG 1 Proportional Venn diagram showing seroconversions to *R. rickettsii*, *R. parkeri*, and *R. amblyommii* antigens and degree of cross-reactivity by indirect immunofluorescent assay. The diagram was generated by a program available at <http://www.cmbi.ru.nl/~timhulse/venn/>.

Human subjects. This research was approved by the Institutional Review Board at the University of North Carolina at Chapel Hill. The use of the data was approved by the North Carolina Division of Public Health through a Data Use Agreement. Since all data were deidentified, informed consent was not required as the risk to subjects was minimal.

RESULTS

Case patients. IFA test results from patients in the NCSLPH database with paired sera that had been tested for RMSF between 2008 and 2010 were reviewed ($n = 311$). Of these patients, 126 (40.5%) had at least one sample with a titer of $\geq 1:64$ against *R. rickettsii*. Results for testing against *Rickettsia typhi* were also reviewed for these patients, all of which were negative. Samples from 8 patients were no longer available. Twelve patients were excluded from the analysis due to nonspecific fluorescence or an unreadable result, and assays for 22 subjects were repeated for which endpoint titers were all resolved. Endpoints were not reached for 11 samples, and these titers were recorded as $\geq 1:2,048$. Of the 106 patients included in the study, surveillance reports were available for 53. The majority of these patients were white males, with a median age of 50 (range, 1 to 80) (see Table S1 in the supplemental material). The demographic profile was similar for patients who seroconverted to at least one of the SFGR antigens and patients with stationary titers.

Seroconversions. Of the 106 eligible pairs tested, 10 patients seroconverted to *R. rickettsii* antigen in the original testing. In our subsequent testing, only 8 of the 10 seroconverted to *R. rickettsii* antigen. The two patients that seroconverted to *R. rickettsii* antigen in the original testing but did not meet the criteria for seroconversion to *R. rickettsii* in subsequent testing both produced titers that were within the one-dilution allowance for intra-assay variability of the IFA. In our subsequent testing, one of these patients seroconverted to *R. amblyommii* antigen and the other did not seroconvert to any of the SFGR antigens.

In this study, 21 patients seroconverted to one or more of the SFGR antigens. The frequency of seroconversions to each antigen and cross-reactivity between antigens is depicted in Fig. 1. Of the eight patients that seroconverted to *R. rickettsii*, seven also seroconverted to both *R. parkeri* and *R. amblyommii*. Eleven patients had seroconversions against a single antigen: 1 against *R. rickettsii*, 4 against *R. parkeri*, and 6 against *R. amblyommii*. Three patients had seroconversions against both *R. parkeri* and *R. amblyommii* antigens.

The ability to detect seroconversions to *R. parkeri* and *R. amblyommii* using *R. rickettsii* antigen was poor, as measured by positive percentages of agreement of 50% (95% confidence interval [CI], 24.0 to 76.0%) and 43.8% (95% CI, 20.8 to 69.4%), respectively. The majority of positive agreement between seroconversion classifications was due to broad cross-reactivity to all three antigens.

Cross-absorption and Western blot assays. Convalescent-phase sera from seven patients were cross-absorbed against all three antigens followed by IFA. Patterns in IFA titers consistent with *R. parkeri* infection were identified in patients 3 and 42, *R. rickettsii* infection in patients 56 and 58, and *R. amblyommii* infection in patient 88 (Table 1). IFA patterns were indeterminate in cross-absorbed sera from patients 68 and 70. Western blots of all seven patient sera showed reactivity to LPS antigen from all three species, but in many cases, specific protein antigens were not visible or were extremely faint (e.g., patients 68 and 88) (Fig. 2). In contrast, the convalescent-phase serum from patient 42 was clearly reactive with an ~ 150 -kDa protein of *R. parkeri* (Fig. 2), supporting the results of the cross-absorption and IFA testing.

Clinical characteristics of seroconverters. Surveillance reports were available for half of the patients ($n = 53$). Of these, 12 reports were available from patients who seroconverted to at least one of the SFGR antigens. Fever, thrombocytopenia, leukopenia, elevated liver enzymes, and acute renal failure occurred more frequently among seroconverters, while skin rash was more common among patients who did not seroconvert (see Table S1 in the supplemental material). None of the patients who seroconverted exclusively to *R. parkeri* and/or *R. amblyommii* antigens reported a rash, compared to three of five patients with seroconversions to all three antigens. Hospitalization occurred in two seroconverters: in a patient who seroconverted to *R. parkeri* (patient 70) and in another patient who seroconverted to *R. amblyommii* (patient 31, who also had a confirmed case of ehrlichiosis). Both *Ehrlichia chaffeensis* and *R. amblyommii* share a tick vector (*A. americanum*), thus this case was a potential coexposure.

DISCUSSION

The purpose of this study was to evaluate reactivity of paired sera from suspected RMSF patients against *R. rickettsii*, *R. parkeri*, and *R. amblyommii* antigens concurrently. These antigens are likely to represent the species of SFGR that occur in tick vectors most fre-

TABLE 1 IFA titers and clinical characteristics for patients who seroconverted to *R. rickettsii*, *R. parkeri*, and/or *R. amblyomii* antigens^a

Patient no. (n = 21)	IFA titer (acute, convalescent phase)			Presumptive agent by:		Symptom(s)									
	<i>R. rickettsii</i>	<i>R. parkeri</i>	<i>R. amblyomii</i>	Cross-absorption with IFA	Western blot	Fever	Rash	Headache	Myalgia	Other	Hospitalized	Discharge diagnosis			
Patients who seroconverted to all 3 antigens															
3	<1:64, 1:256	<1:64, 1:1,024	<1:64, 1:512	<i>R. parkeri</i>	IND	+	+	-	-	-	-	Presumed RMSF			
42	1:128, 1:1,024	1:512, >1:2,048	1:128, >1:2,048	<i>R. parkeri</i>	<i>R. parkeri</i>	+	+	+	+	-	-	NA			
56	<1:64, 1:512	<1:64, ≥1:2,048	<1:64, ≥1:2,048	<i>R. rickettsii</i>	IND	+	+	+	+	-	-	Lethargy, thrombocytopenia			
58	<1:64, 1:1,024	<1:64, 1:512	1:64, 1:512	<i>R. rickettsii</i>	IND	+	-	+	+	-	-	Nausea, vomiting			
65	<1:64, 1:512	<1:64, 1:256	<1:64, 1:256	IND	IND	NA	NA	NA	NA	NA	NA	NA			
68	<1:64, 1:2,048	<1:64, ≥1:2,048	<1:64, 1:2,048	IND	IND	-	+	-	-	-	-	NA			
85	<1:64, 1:2,048	1:64, 1:1,024	1:64, 1:1,024	IND	IND	NA	NA	NA	NA	NA	NA	NA			
Patients who seroconverted to 2 antigens															
55	1:64, 1:64	1:256, 1:1,024	1:256, 1:2,048			NA	NA	NA	NA	NA	NA	NA			
88	1:512, 1:256	1:1,024, 1:256	1:1,024, 1:256	<i>R. amblyomii</i>	IND	+	-	+	+	+	-	NA			
109	1:256, 1:128	1:256, 1:1,024	1:256, 1:1,024	<i>R. amblyomii</i>	IND	+	-	NA	NA	NA	-	Multiple ^d			
Patients who seroconverted to 1 antigen															
5	1:128, 1:64	1:64, 1:64	1:64, 1:64			NA	NA	NA	NA	NA	NA	NA			
7	1:256, 1:256	1:256, <1:64	1:256, <1:64			NA	NA	NA	NA	NA	NA	NA			
19	1:512, 1:512	≥1:2,048, 1:1,024	1:2,048, 1:512			NA	NA	NA	NA	NA	NA	NA			
25	1:128, 1:1,024	1:256, 1:256	1:256, 1:256			NA	NA	NA	NA	NA	NA	NA			
29	1:64, 1:128	1:128, 1:512	1:128, 1:512			NA	NA	NA	NA	NA	NA	NA			
31 ^c	1:128, 1:256	1:256, 1:256	1:256, 1:256			+	-	-	-	-	+	Multiple ^d			
44	1:128, 1:256	1:512, 1:1,024	1:64, 1:512			NA	NA	NA	NA	NA	-	Multiple ^d			
70	1:256, 1:256	1:256, 1:1,024	1:256, 1:1,024			+	-	+	+	+	+	Multiple ^d			
76	1:512, 1:256	1:1,024, 1:2,048	1:512, 1:2,048			-	-	-	+	+	-	Fatigue			
93	1:512, 1:512	1:2,048, 1:512	1:1,024, 1:1,024			-	-	-	-	-	-	NA			
116	1:128, 1:128	1:128, 1:64	1:512, 1:128			NA	NA	NA	NA	NA	NA	NA			

^a Seroreversions are highlighted in bold. For subjects with sufficient remaining sera, the presumptive agent was determined from the results of cross-absorption of convalescent-phase sera followed by IFA and/or Western blotting.

IND, indeterminate; NA, not available; +, positive; -, negative.

^b Nausea, diarrhea, weakness, cough, and sweats.

^c In the original testing, this patient seroconverted to *E. chaffeensis* with acute- and convalescent-phase titers of 1:128 and 1:8,192.

^d Elevated liver enzymes, acute renal failure, thrombocytopenia, leukopenia, and anemia.

^e Elevated liver enzymes, thrombocytopenia, leukopenia, and anemia.

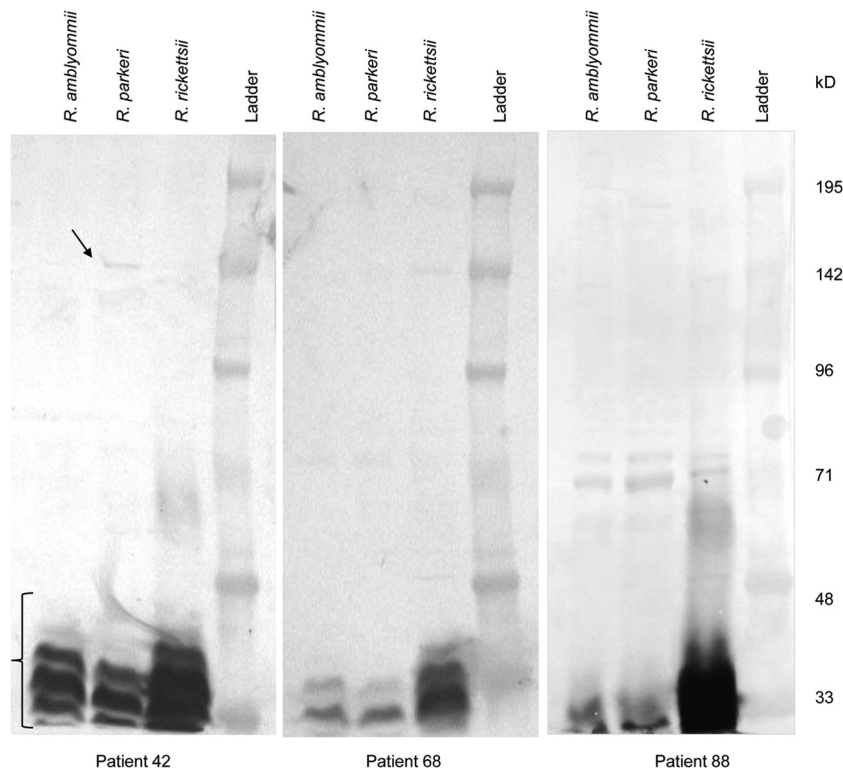


FIG 2 Western blots of rickettsial antigens reacted with convalescent-phase patient sera (1:125 dilution). The bracket in the lower left indicates reactivity to nonspecific LPS antigen, and the arrow indicates reactivity with a specific protein antigen.

quently in North Carolina, based on current knowledge. Although the seroconversions observed cannot be used to infer etiology, the greater number of unique seroconversions to *R. parkeri* and *R. amblyommii* than to *R. rickettsii* indicates that species of SFGR other than *R. rickettsii* may be causing infections among North Carolina residents. This theory is supported by identification of *R. parkeri* as the presumptive agent in two patients and *R. amblyommii* in one patient using cross-absorption and Western blot assays. The low positive percentage of agreement for *R. parkeri* and *R. amblyommii* seroconversion classification also suggests that serologic testing using *R. rickettsii* antigen may result in missed cases of spotted fever rickettsioses caused by other species of SFGR. Thus, even the current “gold standard” for serologic diagnosis of SFGR has serious limitations.

The large relative frequency of seroconversions to *R. amblyommii* was not unexpected in the context of previous work by Apperson et al. (12). Active surveillance for tick-borne diseases in a central North Carolina county resulted in the identification of several patients with mild illness in which initial testing failed to confirm RMSF. Upon further testing with both *R. rickettsii* and *R. amblyommii* antigens, three patients seroconverted against *R. amblyommii* in that study. At this time, *R. amblyommii* has not been recognized as a human pathogen, but the high infection prevalence in *A. americanum*, the most ubiquitous and aggressive human-biting tick in this region, creates ample opportunity for potential infection of human and animal hosts (10, 13, 15). In Oklahoma, canine infection with *R. amblyommii* was demonstrated among dogs naturally exposed to ticks (22). In a longitudinal study of outdoor workers in North Carolina, more than 90% of ticks removed from subjects were *A. americanum* (23), while

less than 5% of ticks were *D. variabilis* and less than 1% of ticks were *Amblyomma maculatum* (Charles Apperson, unpublished data). The predominant rickettsial species found in ticks removed from subjects in that study was *R. amblyommii* (51.6%), while *R. rickettsii* was found in only one tick, an *A. americanum* nymph (Apperson, unpublished).

We also anticipated that we would detect seroconversions to *R. parkeri*, a known human pathogen that was found in 29% of gulf coast ticks (*Amblyomma maculatum*) in a recent entomologic survey in North Carolina (24). A recent study identified *R. parkeri* in Lone Star ticks from Tennessee and Georgia, suggesting that *A. americanum* may serve as a vector for *R. parkeri* (25). Seroconversions to *R. parkeri* and *R. amblyommii* among ill patients in this study provide further evidence that infections caused by these species of SFGR are likely to account for some of the increase in spotted fever rickettsioses reported in this region.

It is well known that there is serologic cross-reactivity between species of SFGR, and as a result, serologic tests cannot be used to infer etiology (26). Serologic cross-reactivity between *R. rickettsii* and *R. parkeri* antigens has been described previously. Raoult and Paddock retested sera from 15 patients diagnosed with RMSF using class-specific IFA with *R. rickettsii* and *R. parkeri* antigens (27). Equal titers were observed for six patients, while 4 patients had higher titers to *R. rickettsii* and 5 patients had higher titers to *R. parkeri*. Western blot analysis of 4 patients with higher titers against *R. parkeri* provided additional evidence of infection with *R. parkeri*. Paddock et al. conducted comparative class-specific IFA with samples from 6 confirmed and 6 probable cases of *R. parkeri* rickettsiosis using *R. parkeri*, *R. rickettsii*, *R. amblyommii*, and *Rickettsia akari* antigens (28). IgG geometric mean titers were

higher against *R. rickettsii* than for any other antigen. These studies illustrate that comparison of single titers is unreliable for distinguishing cases of RMSF and *R. parkeri* rickettsiosis. By evaluating reactivity of acute- and convalescent-phase paired sera, we found that some patients showed rising or falling titers to multiple rickettsiae, which is consistent with the cross-reactivity observed in previous studies. It is also possible that patients could have been coinfecting with multiple species of rickettsiae or with species not included in the testing for this study. Notably, we found that some patients seroconverted to only a single antigen, indicating that extensive cross-reactivity between SFGR antigens may not be present in all cases.

Limitations of this study include the lack of clinical data for all patients and the variability in the level of completeness of those for which data are available. The small number of patients with surveillance reports available prevents us from making conclusions on the association between seroconversion to specific antigens and clinical signs and symptoms. Due to the eligibility requirement of having paired sera, which represents a small minority of patients, the patients in this study are likely to have suffered from more severe illnesses, which led the medical provider to order repeated testing for tick-borne pathogens. By limiting our study to these patients, it is possible that we excluded many cases of mild disease caused by SFGR. Curiously, there were some patients with paired sera who had no reported signs or symptoms. In several surveillance reports, it was noted that testing was done as a precaution after multiple tick bites, although none of those patients seroconverted in this study. The scarcity of appropriately timed paired samples with accompanying clinical information further compounds the diagnostic confusion for spotted fever rickettsioses, indicating a need for acceptability criteria for serologic testing of patient samples (29).

It is clear that while *R. rickettsii* is still circulating and causing disease among people living in the southern and southeastern United States, residents are also being infected with other SFGR carried by ticks in these regions. Some of these SFGR, such as *R. parkeri*, have been shown to cause human disease, while the pathogenic potential of others, such as *R. amblyommii*, *R. montanensis*, and *R. rhipicephali*, has yet to be determined. Until more specific serologic diagnostic methods are developed that can distinguish between species of SFGR or the use of molecular detection techniques becomes routine, the relative contributions of different species of SFGR to human morbidity will remain unclear. Active surveillance for cases of suspected tick-borne illness, which include paired serology and molecular detection, are needed to determine the etiologies of SFGR infections in this region. If current serological methods continue to be the standard for diagnosis and surveillance of spotted fever group rickettsioses, inclusion of antigens for all species known to cause human disease in the relevant geographic region should be considered to prevent missed cases.

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