

Antibodies Reactive to *Rickettsia rickettsii* Among Children Living in the Southeast and South Central Regions of the United States

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Background: The reported annual incidence of Rocky Mountain spotted fever in the United States is 2.2 per million, but studies have suggested that human infection with *Rickettsia rickettsii* may be more common. This study estimated the prevalence of antibodies reactive to *R rickettsii* among children living in the southeastern and south central United States.

Study Design: Approximately 300 specimens were obtained from children at each of 7 pediatric referral centers (N=1999). Serum was tested for *R rickettsii* antibodies by means of indirect immunofluorescence antibody assay. Three different cutoff titers (≥ 64 , ≥ 128 , and ≥ 256) represented increasing levels of stringency to define positive specimens.

Results: Overall, 12.0% of children had *R rickettsii* antibody titers of at least 64; 7.3%, at least 128; and 4.3%, at least 256. Strong relationships were seen between increasing age and seroprevalence at each cutoff titer. Re-

markably, 6.4% of children aged 13 to 17 years had titers of at least 256. Age-adjusted seroprevalence rates at titers of at least 64 varied from 21.9% in Little Rock, Ark, to 3.5% in Louisville, Ky. At titers of at least 256, seroprevalence ranged from 7.7% in Nashville, Tenn, to 1.8% in Winston-Salem, NC. Only site and age group were strong predictors of seropositivity; a weak association was seen with nonurban residence.

Conclusions: To our knowledge, this is the largest serosurvey of rickettsial infection in children in the United States. Within the limitations of the immunofluorescence antibody assay, these data suggest that infections with *R rickettsii* or antigenically related spotted-fever group rickettsiae may be common and subclinical. The results also have implications for the interpretation of single immunofluorescence antibody assay titers in children with suspected Rocky Mountain spotted fever.

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EMERGING TICK-BORNE diseases represent a particular problem for pediatricians because the habitats of children and ticks increasingly converge.¹ In fact, Rocky Mountain spotted fever (RMSF), a prototypical tick-borne rickettsiosis caused by *Rickettsia rickettsii*, occurs most commonly in children aged 5 to 9 years.² Unfortunately, early recognition of the disease can be impeded by the overlap of symptoms such as fever, headache, myalgia, and rash with common benign viral syndromes.³ This can lead to delayed empirical antibiotic therapy and increased mortality.⁴⁻⁷

Although RMSF is the most common fatal tick-borne disease in the United States, it remains relatively rare. From 1993 to 1996, only 1253 cases were reported to the Centers for Disease Control and Prevention, for an average annual incidence of 2.2 per million.² Human contact with ticks, however, is undoubtedly common, and in

regions where the prevalence of *R rickettsii*-infected ticks may be high,⁸ such contact is likely to lead to exposure and infection. The discrepancy between the number of reported cases and the number of probable exposures has led to the suggestion that many cases of RMSF are self-limited and subclinical or misdiagnosed.⁹⁻¹⁴

The present study is, to our knowledge, the largest and most geographically extensive serosurvey of rickettsial infection in children in the United States. Even if one uses the most stringent criteria to define seropositive individuals, the results suggest widespread infection with *R rickettsii* or related spotted-fever group rickettsia.

METHODS

STUDY POPULATION

Details of the study population have been published.¹⁵ Briefly, residual serum or plasma from patients who had their blood drawn for any rea-

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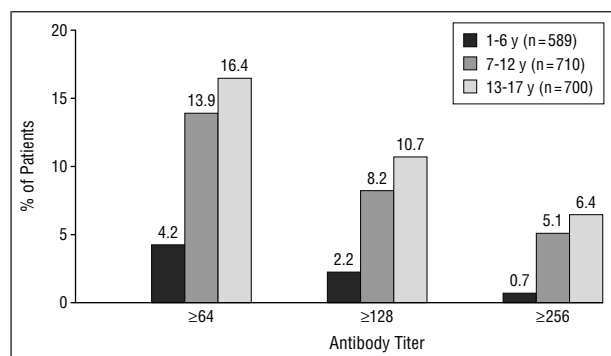


Figure 1. *Rickettsia rickettsii* reactivity by age group. Differences between groups were significant for each cutoff titer ($P < .001$).

son was collected at each of 7 pediatric centers. These specimens were considered waste, and since they were anonymously linked to demographic data, the need for informed consent was waived at each institution. A total of 1999 subjects (1015 boys) aged 1 to 17 years of age were studied from the following sites: Winston-Salem, NC (n=312); Louisville, Ky (n=300); Nashville, Tenn (n=299); Memphis, Tenn (n=302); Little Rock, Ark (n=296); Kansas City, Mo (n=194); and Oklahoma City, Okla (n=296). The age distribution was skewed toward older patients, because they have more residual serum after clinical tests are performed. Sites differed with respect to subject age, racial distribution, the proportion living in an urban setting, and the source of specimens (hospital admission, emergency department visit, or other outpatient visit).¹⁵

SEROLOGY

Specimens were tested for antibodies to *R. rickettsii* using an indirect fluorescence antibody assay (IFA) (PanBio InDx, Inc, Baltimore, Md). Each slide had 12 wells containing fixed whole Vero cells infected with *R. rickettsii* (Sheila Smith strain). For screening, serum samples stored at -20°C were thawed, centrifuged at 1730g for 10 minutes at 4°C , and diluted 1:64 in phosphate-buffered saline (PBS; pH, 7.4); 20 μL was then pipetted onto each well. Slides were incubated in a moist chamber at room temperature for 30 minutes, after which they were rinsed with PBS, washed in PBS for 10 minutes, and rinsed again with distilled water. Slides were then air-dried, and the wells were reacted with 20 μL of fluorescein isothiocyanate-conjugated polyclonal goat antiserum reactive with human IgG heavy and light chains diluted 1:100 in PBS with eriochrome black counterstain. After 30 minutes in a moist chamber at room temperature, slides were rinsed and washed with PBS, overlaid with 80% glycerol in PBS (pH, 8.0) and a cover slip, and observed using epifluorescence microscopy at a magnification of $\times 400$. Results were determined to be positive if the specimens showed readily identifiable apple green fluorescing coccobacillary organisms. Specimens that were positive at the screening dilution were retested at dilutions of 1:64, 1:128, and 1:256 to determine the antibody titer. Positive and negative control serum samples were run with each assay.

All slides were read independently by 2 individuals (G.S.M. and G.G.S.) who were masked to information about the specimens (except site of origin). Disagreements were resolved by consensus after reexamination of the slides. To monitor for problems in specimen shipping, handling, and storage and to assess the accuracy of signal detection, 4 vials of control serum (shipping controls) were randomly included in the sequence of patient specimens from each site; 2 were from a patient with RMSF who was seropositive for antibodies to *R. rickettsii*, and

2 were from a seronegative individual. All serum samples were also tested for *Ehrlichia chaffeensis* and *Rickettsia typhi* antibody as previously described.^{15,16}

ANALYSIS

Most studies of *R. rickettsii* seroprevalence have used IFA cutoff titers of at least 64 to define positive specimens,⁹⁻¹⁴ and the Centers for Disease Control and Prevention case definition¹⁷ considers a single titer of this magnitude to be supportive of infection in the proper clinical context. Because standards for interpreting IFA results in seroepidemiological studies do not exist, the present data were analyzed at 3 different cutoff titers (≥ 64 , ≥ 128 , and ≥ 256) that represent increasing levels of stringency and specificity for *R. rickettsii* infection.

Data were stored and analyzed on a Macintosh G4 computer (Apple Computer, Inc, Cupertino, Calif) running OS-9.1 and StatView 5.0 (SAS Institute Inc, Cary, NC). Age was collapsed into the following 3 categories: 1 to 6 years; 7 to 12 years; and 13 to 17 years. The ZIP code of residence was demographically categorized as urban or nonurban on the basis of 1990 census data as previously described.¹⁵ Preliminary analyses sought bivariate associations between categorical variables using the χ^2 test (the χ^2 test for trend was used when age group was a variable); in all cases, expected cell frequencies were greater than 5. In the case of 2×2 tables, Fisher exact test was used. Because age was found to be a significant predictor of seropositivity, and because age distribution differed from site to site, seroprevalence rates were adjusted for age using the entire study population as the reference. Multiple logistic regression was performed by adding the following variables in sequence: site, age group, source, residence, and sex. For each variable, the element with the lowest seroprevalence rate was used as the reference level. Significance for all analyses was defined at a P level of .05.

RESULTS

In the initial screening, there were 1620 agreements as negative, 321 agreements as positive, and 58 discrepancies, for a κ of 0.9, indicating excellent interrater reliability. All shipping controls were correctly identified.

Overall, 12.0% of children had *R. rickettsii* antibody titers of at least 64; 7.3%, at least 128; and 4.3%, at least 256. **Figure 1** shows the strong relationship between age and seroprevalence. Reactive antibodies were detected at a titer of at least 64 in 4.2% of children aged 1 to 6 years, 13.9% of those aged 7 to 12 years, and 16.4% of those aged 13 to 17 years ($P < .001$). Similarly robust associations were seen at titers of at least 128 and at least 256 ($P < .001$ for each cutoff value). Remarkably, 6.4% of children aged 13 to 17 years had antibody titers of at least 256. Age-adjusted seroprevalence rates for each site are given in **Figure 2**. At titers of at least 64, 21.9% of children in Little Rock were positive compared with 3.5% in Louisville. At titers of at least 256, seroprevalence ranged from a high of 7.7% in Nashville to a low of 1.8% in Winston-Salem. Differences between groups were significant for each cutoff titer ($P < .001$).

As seen in **Table 1**, other bivariate associations with seropositivity were seen at various cutoff titers, including the source of the specimen and nonurban residence. However, after multiple logistic regression, the only strong independent predictors of seropositivity at each cutoff titer were site and age group (**Table 2**). Much weaker

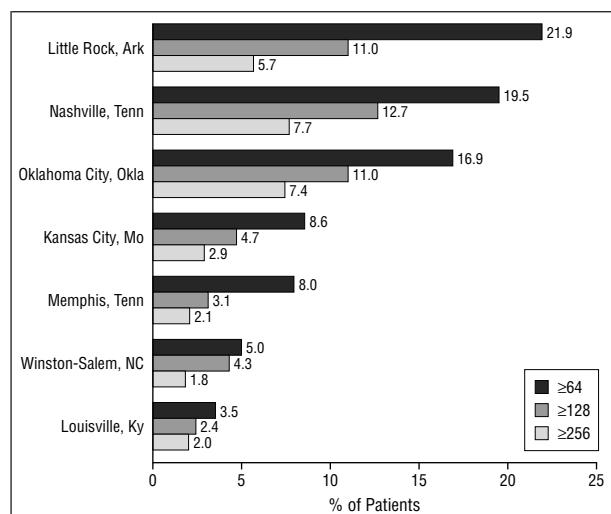


Figure 2. Age-adjusted seroprevalence by geographic site and antibody titers. Differences between groups were significant for each cutoff titer ($P < .001$).

associations were seen between seropositivity and specimen origin in the emergency department, male sex, and nonurban residence.

No subjects with *R. rickettsii* antibodies had *R. typhi* antibodies (tested at a dilution of 1:64). However, as seen in **Table 3**, 31 (13.0%) of the 239 subjects with *R. rickettsii* antibodies had *E. chaffeensis* titers of 80, and 11 (4.6%) had *E. chaffeensis* titers of at least 160 (7 subjects had high titers to both organisms). Of the 42 children with antibodies to both organisms, 17 were from Little Rock; 8, Nashville; 6, Winston-Salem; 5, Oklahoma City; 3, Kansas City; 2, Memphis; and 1, Louisville ($P < .001$). Three of these subjects were aged 1 to 6 years; 13, 7 to 12 years; and 26, 13 to 17 years ($P < .001$).

COMMENT

The present study suggests that exposure to *R. rickettsii* or related rickettsiae is much more common than indicated by disease reports. At a cutoff titer of at least 64, approximately one fifth of children living in Little Rock and Nashville had antirickettsial antibodies. Even when the stringency of the analysis was increased by raising the cutoff titer to at least 256, more than 7% of children living in some areas were seropositive. At each cutoff titer, age was significantly related to seroprevalence, which is consistent with the expected accumulation of exposures over time at the population level and the persistence of antibody after infection.

Microimmunofluorescence slides for the diagnosis of RMSF have been in worldwide commercial distribution since the early 1980s. They constitute the standard for serodiagnosis of RMSF and have been used in numerous seroprevalence studies of RMSF⁹⁻¹⁴ and other spotted-fever group rickettsiae.¹⁸⁻²⁰ The slides used in this study have been cleared by the US Food and Drug Administration for diagnostic use and have been used in previous seroprevalence studies.¹⁴ Nevertheless, immunofluorescence is not an ideal method for seroepidemiological studies because nonspecific staining can occur, as can

Table 1. Bivariate Associations*

Factor	<i>Rickettsia rickettsii</i> Antibody Titer		
	≥64 (n = 239)	≥128 (n = 146)	≥256 (n = 85)
Source			
Hospital admission	7.8†	5.3‡	3.3
Outpatient visit	13.7	7.7	4.4
Emergency department	14.1	9.6	5.9
Sex			
Female	10.6	6.3	3.7
Male	13.3	8.3	4.8
Residence			
Urban	10.4§	6.4	3.5
Nonurban	14.5	8.8	5.6

*Data are expressed as percentage of patients with seropositive test results.

† $P < .001$.

‡ $P = .04$.

§ $P = .009$.

|| $P = .03$.

cross-reactivity between spotted-fever group rickettsiae.²¹ In this regard, antibodies to some human rickettsial pathogens, including *Rickettsia conorii* (Mediterranean spotted fever), *Rickettsia akari* (rickettsialpox), *Rickettsia sibirica* (Siberian tick typhus), and *Rickettsia australis* (North Queensland tick typhus), would not be expected because these infections are geographically circumspect. Cross-reactions with typhus group rickettsiae are less likely, and indeed were not demonstrated here with *R. typhi*. Some of the antibodies detected in this study may have been directed against other spotted-fever group rickettsiae not presently thought to be pathogenic, such as *Rickettsia montanensis*, *Rickettsia belli*, *Rickettsia parkeri*, *Rickettsia peacockii*, *Rickettsia amblyommii*, and *Rickettsia rhipicephali*.²²

A significant number of children (42 [17.6%]) with *R. rickettsii* antibodies also had antibodies to *E. chaffeensis*. Since cross-reactions between these organisms are not appreciable, these children might have had an infection with each organism at some point in the past. Dual infections have been described.²³⁻²⁵ Children in the present study with dual seropositive findings were older and more likely to live in regions with the highest prevalence of both diseases.

Several biases may have been operative in the acquisition of this convenience sample.¹⁵ However, after controlling for all other variables, strong independent relationships persisted between seroprevalence and site and age. The association of seroprevalence with site generally fit the national distribution of reported cases of RMSF,² except for Winston-Salem, where the age-adjusted seroprevalence was unexpectedly low. North Carolina has the highest statewide disease-reporting rate in the country (23.0 per million) and records the largest number of deaths due to RMSF east of the Mississippi.⁷ Potential explanations for this discrepancy include the following:

1. Sampling at this site might have selected against tick-exposed children. However, the high proportion of

Table 2. Multiple Logistic Regression Analysis*

Factor	<i>Rickettsia rickettsii</i> Antibody Titer		
	≥64	≥128	≥256
Site			
Louisville, Ky	1.0	1.0	1.1 (0.4-3.4)
Winston-Salem, NC	1.5 (0.7-3.4)	1.9 (0.8-4.9)	1.0
Memphis, Tenn	2.2 (1.0-4.8)	1.2 (0.4-3.3)	0.9 (0.3-3.1)
Kansas City, Mo	3.1 (1.3-7.3)	2.8 (1.0-7.8)	2.2 (0.7-7.4)
Oklahoma City, Okla	6.4 (3.1-13.1)	5.8 (2.5-13.5)	5.0 (2.1-12.3)
Nashville, Tenn	6.9 (3.4-14.0)	6.3 (2.7-14.6)	4.6 (1.9-11.2)
Little Rock, Ark	8.1 (4.0-16.5)	5.3 (2.2-12.3)	3.0 (1.2-7.5)
Age, y			
1-6	1.0	1.0	1.0
7-12	3.7 (2.4-6.0)	4.0 (2.1-7.4)	8.5 (3.0-24.2)
13-17	5.1 (3.2-8.2)	5.9 (3.2-11.0)	11.4 (4.0-32.3)
Source			
Hospital admission	1.0	1.0	1.0
Outpatient visit	1.5 (1.1-2.2)	1.2 (0.8-1.9)	1.2 (0.7-2.0)
Emergency department	1.8 (1.1-2.9)	2.0 (1.1-3.5)	2.1 (1.0-4.3)
Sex			
Female	1.0	1.0	1.0
Male	1.5 (1.1-2.0)	1.6 (1.1-2.3)	1.5 (1.0-2.4)
Residence			
Urban	1.0	1.0	1.0
Nonurban	1.5 (1.1-2.1)	1.5 (1.0-2.2)	1.9 (1.2-3.1)

*Data are expressed as odds ratios (95% confidence intervals).

Table 3. Specimens With Antibody to *Rickettsia rickettsii* and *Ehrlichia chaffeensis**

<i>R rickettsii</i> Titer	<i>E chaffeensis</i> Titer			Total
	80	≥160	Negative	
64	15	3	75	93
128	8	1	52	61
≥256	8	7	70	85
Total	31	11	197	239

*Data are expressed as number of subjects.

nonurban dwellers (64%) and children aged 13 to 17 (46%) should have, if anything, biased the seroprevalence rate upward.

- There may be fewer infections in North Carolina but proportionately more disease reporting. This seems unlikely, especially since dogs, which are effective sentinels for human disease, have seroprevalence rates as high as 60% to 70% in some parts of the state.^{26,27} Similarly unlikely explanations include increased virulence of local strains leading to more identifiable disease, failure to detect antibodies to local strains of *R rickettsii*, and more infections with nonpathogenic rickettsiae in North Carolina compared with other areas in the southeast.
- Increased awareness might prompt early empirical treatment in suspected cases, resulting in blunted antibody responses.^{23,25,28} However, it is not clear why similar factors would not be operative at other sites with high endemicity, and evidence from challenge experiments suggests that early therapy does not affect antibody production.²⁹

- North Carolina has a high incidence of ehrlichiosis (4.7 per million),³⁰ and children in the Winston-Salem area have a correspondingly high prevalence of *Ehrlichia* antibodies.¹⁵ Since symptoms of RMSF are indistinguishable from those of ehrlichiosis, some of what gets reported as RMSF may actually be ehrlichiosis. However, it would be difficult to explain why the same phenomenon would not occur in areas such as Little Rock, which also has endemic ehrlichiosis³⁰ and a high seroprevalence rate.¹⁵
- The geographic distribution of RMSF is spotty, and hyperendemic foci have been reported.^{2,11,31,32} Therefore, the sample from Winston-Salem may have come predominantly from areas in North Carolina with lower endemicity than other parts of the state. To address this hypothesis, reported cases by county for 1990 through 1999 were obtained from the surveillance unit of the North Carolina Department of Health and Human Services (Raleigh), and annual incidence rates were calculated using the average US Census population counts from years 1990 and 2000. Each of the 8 counties contributing 10 or more specimens to the study had incidence rates below the calculated statewide rate of 1.79 per 100 000. These counties constituted 16.3% of the state's population. The 2 most represented counties, Forsyth (n=104) and Guilford (n=31), had incidence rates of 0.66 and 0.94 per 100 000, respectively. Inasmuch as reported cases reflect infection rates, these data support the notion that counties with lower endemicity were oversampled.

It is counterintuitive that a strong association was not seen between nonurban residence and seropreva-

What This Study Adds

Fewer than 1000 confirmed cases of Rocky Mountain spotted fever (RMSF) are reported to the Centers for Disease Control and Prevention, Atlanta, Ga, each year, yet previous serological studies suggest that rickettsial infection may be much more common. In this large, multicenter serosurvey, 12.0% of children were found to have antibodies reactive to *Rickettsia rickettsii* at titers of at least 64 and 4.3% at titers of at least 256. Age-adjusted seroprevalence rates at titers of at least 256 approached 8% in some areas. Widespread serological reactivity could be the result of unrecognized infection with *R. rickettsii* or related rickettsiae and has implications for the interpretation of one-time diagnostic titers in children with suspected RMSF.

lence, although this was true for ehrlichiosis as well.¹⁵ Although the ZIP code demographic classification might have been inaccurate, and travel to rural areas by urban dwellers could have occurred, it seems more likely that foliage density and the proximity of animal hosts is a more relevant factor than is urbanization per se.

Two earlier studies looked at *R. rickettsii* seroprevalence in children; both were limited to sixth graders in one geographic region, and both used IFA assays with a cutoff titer of at least 64. The first study⁹ found a 1% seroprevalence rate among 508 children in Forsyth County, North Carolina, and the second¹¹ showed a 9% seroprevalence rate among 368 children living in Johnson and Tarrant counties in Texas. Other studies of various populations in North Carolina (this study included some children),¹⁰ Arkansas,¹³ and New York¹⁴ yielded seroprevalence estimates in the range of 4% to 10%. The present study supports these earlier observations and suggests that infection with *R. rickettsii* or related rickettsiae is much more common than would be expected from passive case reporting. Most rickettsial infections may thus be subclinical or mild and self-limited.

The possibility that the IFA reactivity reported herein was not the result of *R. rickettsii* infection makes the interpretation of diagnostic IFA assays in children with suspected RMSF problematic. For example, approximately one fifth of children living in Little Rock may be seropositive for *R. rickettsii*. Therefore, patients with a viral syndrome characterized by fever, myalgia, headache, and rash could easily meet the Centers for Disease Control and Prevention case definition for probable RMSF,¹⁷ which requires a single *R. rickettsii* IFA titer of at least 64 in the presence of a compatible clinical syndrome. This finding highlights the importance of performing acute and convalescent serology, polymerase chain reaction analysis, tissue immunofluorescence, or organism isolation for definitive diagnosis. Prospective studies of seroconversion, surveillance for incident disease, and the development of more specific serological reagents could answer many of the questions raised by this study.

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