ISOLATION OF RICKETTSIA AKARI FROM ESCHARS OF PATIENTS WITH RICKETTSIALPOX

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Abstract. Rickettsialpox is a cosmopolitan, mite-borne, spotted fever rickettsiosis caused by Rickettsia akari. The disease is characterized by a primary eschar, fever, and a papulovesicular rash. Rickettsialpox was first identified in New York City in 1946 and the preponderance of recognized cases in the United States continues to originate from this large metropolitan center. The most recently isolated U.S. strain of R. akari was obtained more than a half century ago. We describe the culture and initial characterization of five contemporaneous isolates of R. akari obtained from eschar biopsy specimens from New York City patients with rickettsialpox. This work emphasizes the importance and utility of culture- and molecular-based methods for the diagnosis of rickettsialpox and other eschar-associated illnesses.

INTRODUCTION

Rickettsialpox, a mite-borne zoonosis caused by Rickettsia akari, is one of only a few spotted fever group (SFG) rickettsioses with a cosmopolitan distribution. Culture-confirmed cases of rickettsialpox have been reported from the United States, the Ukraine, and Croatia. Human infections with R. akari are also suggested by serologic surveys and case reports of patients from other locations, including Bosnia, Costa Rica, France, Italy, South Africa, and Turkey. The disease in humans is characterized by an ulcerated, approximately 0.5–1.5 cm primary lesion (the eschar) at the site of inoculation, followed approximately one week later by a systemic illness that includes high fever, severe headache, and a generalized papulovesicular rash.

Benjamin Shankman, a New York City physician, is credited with the initial recognition of rickettsialpox during an epidemic in the borough of Queens during the spring and summer of 1946. Remarkably, the causative agent was isolated, characterized, and described in the scientific literature within five months of the first reports of the illness to the New York City Department of Health. The MK (Kaplan) strain of R. akari was isolated in mice from blood obtained from a 20-year-old patient of Shankman’s named Marjory Kaplan. Additional isolates were subsequently obtained from patients in New York City and from patients in the Ukraine. Isolates of R. akari were also recovered from the arthropod vector, the house mouse mite (Liponyssoides [formerly Allodermomyssus] sanguineus), and the principal rodent host, the house mouse (Mus musculus), in New York City, Boston, and Hartford, Connecticut, from the brown rat (Rattus norvegicus), M. musculus, and L. sanguineus in the Ukraine, and from the reed vole (Microtus fortis pelliceus) in Korea.

In the decades after the sentinel New York City epidemic, the disease fell into relative obscurity in the United States. However, from 2001 through 2003, an increase in the number of clinical samples submitted to the Centers for Disease Control and Prevention (CDC) for laboratory confirmation of rickettsialpox followed the malicious release of Bacillus anthracis during 2001, and enhanced public and clinician awareness of eschar-associated febrile illness. When possible, cutaneous biopsy specimens obtained during and after this occurrence were evaluated by culture- and molecular-based diagnostic assays at the CDC. We report the first isolations of R. akari from patients in the United States since the late 1940s and discuss the importance of culture as a diagnostic adjunct to the diagnosis of rickettsialpox and other spotted fever rickettsioses.

METHODS

Patient samples. From November 2001 through March 2003, fresh cutaneous biopsy specimens were obtained from seven patients in New York City with eschar-associated febrile illnesses, who prospectively or retrospectively were suspected of having rickettsialpox on the basis of initial clinical findings or laboratory tests. These diagnostic biopsy specimens were obtained by using a 2.5–6.0-mm skin punch, placed in gauze moistened with sterile non-bacteriostatic saline, sent by overnight delivery to the CDC, and evaluated by using various confirmatory laboratory assays for R. akari. These samples were frozen at -80°C or refrigerated at 4°C until diagnostic assays were performed. Most specimens were bi-}

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were stained with hematoxylin and eosin and by using an immunoalkaline phosphatase technique with a hyperimmune rabbit antibody to R. rickettsii at a dilution of 1:500. The antibody to R. rickettsii reacts strongly with several different species of spotted fever group rickettsiae indigenous to the United States, and has been used previously to identify various pathogenic rickettsiae from this group, including R. akari, R. rickettsii, and R. parkeri in formalin-fixed tissue specimens.

**Cell culture.** Isolation of R. akari was attempted only from eschar biopsy samples because immunohistochemical (IHC) stains had previously showed that rickettsiae were more abundant in these specimens than in biopsies of papular rash lesions. Samples were triturated in 250 μL of cell culture medium (RPMI 1640 supplemented with 5% fetal bovine serum and 2 mM L-glutamine). Residual tissue fragments were allowed to sediment and the remaining mixture was transferred to a confluent monolayer of Vero E6 cells in a 25-cm² cell culture flask and centrifuged at 140 × g for 30 minutes at room temperature. After centrifugation, 5 mL of cell culture medium containing 10 U/mL of penicillin G sodium and 10 μg/mL of streptomycin sulfate was added to the monolayer. Cells were incubated at 34.5°C in a 5% CO₂-enriched atmosphere. The medium was removed after 48 hours and replaced with 5 mL of fresh medium containing no antibiotics. Thereafter, the medium was replaced 1–2 times each week. Cultures were monitored for evidence of infection by examining cytocentrifuged preparations of Vero E6 cells fixed in absolute methanol and stained with a 0.01% solution of acridine orange at pH 3.5. Cultures were evaluated for 6–8 weeks before being considered negative for growth of rickettsiae.

**Molecular analyses.** DNA was extracted in a final volume of 200 μL from residual eschar biopsy triturates and from cell culture supernatants by using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA). Five microliters of DNA extract was tested using a nested polymerase chain reaction (PCR) assay designed to amplify a segment of the rickettsial 17-kD antigen gene. Primers R17-122 and R17-500 were used in the primary reaction; 1 μL of the primary reaction product was then evaluated by using primers TZ15 and TZ16 in the nested reaction. The reactions were performed using Ready-To-Go Beads with puReTaq (Amersham Biosciences UK Limited, Little Chalfont, United Kingdom) and primers were used at a final concentration of 1 μM. Thermal cycling conditions consisted of an initial denaturation step for 5 minutes at 95°C, followed by a three-step cycling profile of 40 cycles at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 1 minute. The cycling profile was followed by a final extension at 72°C for 5 minutes. Products of the expected size were excised from a 1.6% low-melting-point agarose gel containing ethidium bromide after electrophoresis. Amplicon DNA was purified using Wizard PCR Prep (Promega, Madison, WI). Purified products were sequenced using a BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA) and detected using an Applied Biosystems 310 automated sequencer. Nucleotide homologies were established using the BLAST program at the National Center for Biotechnology Information (Bethesda, MD).

**Purification of rickettsiae and protein electrophoresis.** For analysis of whole-cell protein profiles by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), all spotted fever group rickettsiae except R. bellii were grown at 34.5°C in a 5% CO₂-enriched atmosphere in Vero E6 cells with RPMI 1640 medium supplemented with 2% fetal bovine serum and 1% L-glutamine. Rickettsia bellii was grown at 28°C in Xenopus XTC-2 cells with L-15 medium supplemented with 2% fetal bovine serum and 10 mM HEPES. Infected cells were harvested with 5-mm glass beads and centrifuged at 10,000 rpm for 20 minutes. Pellets were suspended at 1 mL/150-cm² cell culture flask in a mixture containing sucrose phosphate with 5 mM glutamate, 5 mM MgCl₂, and 1% Renografin 76, pH 7.1 (SRM). Suspensions (30 mL) were homogenized 5 times in a 15-mL glass homogenizer with a motorized Teflon pestle at 1,000 rpm and diluted to 50 mL with SRM before the crude cell debris and nuclei were removed by centrifugation at 1,000 rpm. The pellet was washed by suspension in 50 mL of SRM and centrifuged at 1,000 rpm for 10 minutes. The combined supernatants were filtered through an AP-25 glass fiber filter (Millipore Corporation, Bedford, MA) to remove remaining nuclei. The rickettsiae were centrifuged at 12,000 rpm for 10 minutes and resuspended in 3 mL of SRM. This suspension was layered on a continuous 22.5%–47.5% (w/v) 33-mL Renografin density gradient and centrifuged at 25,000 rpm for 1 hour. The rickettsial bands were collected with 14-gauge canula, diluted in SRM, and centrifuged at 12,000 rpm for 15 minutes. The pellets were resuspended in 20 mL of SRM and their concentration was estimated by their turbidity at 420 nm. Purified rickettsiae were centrifuged at 12,000 rpm and resuspended at a concentration of 10 mg/mL in water in 50-μL aliquots and stored at −80°C. Rickettsia prowazekii, R. typhi, and R. canadensis were grown in embryonated chicken eggs and purified with a Renografin density gradient previously described. Rickettsia akamibyomii (strain AaT16) was isolated in cell culture from eggs of lone star ticks (Amblyomma americanum) collected from Panola Mountain State Park, Georgia, (Dasch GA and others, unpublished data). Electrophoresis was performed as described previously. Proteins were solubilized by boiling for 5 minutes in 2 × Laemmli buffer containing 5% 2-mercaptoethanol at a concentration of 1 μg of protein/μL. Twenty microliters of solubilized protein was placed into each well of a 5% stacking gel. Proteins were separated in an 8–15% gel by electrophoresis at 80 V at 4°C and visualized by staining with Coomassie brilliant blue.

**RESULTS**

Eschar biopsy specimens were submitted for six patients with clinical diagnoses that included rickettsialpox and for one (patient 1) for whom cutaneous anthrax was suspected clinically; patient 1 was subsequently diagnosed with rickettsialpox by use of an IHC test. The patients (three men and four women) ranged in age from 24 to 73 years and lived in five neighborhoods in the New York City boroughs of Manhattan and the Bronx (Table 1). Three (patients 1, 3, and 4) had recently seen mice in their home or workplace. All patients had febrile illnesses associated with eschars; all except patient 6 were aware of a primary lesion before seeking medical attention. Duration of fever at the time of biopsy ranged from 4–8 days and estimated age of the eschar at the time of biopsy was approximately 1–2 weeks. Eschars were localized...
to the ankle, lower leg, thigh, buttock, chest, or arm (Figure 1A). Histopathologic examination of these lesions showed various amounts of ulceration, dermal necrosis, and hemorrhage, and predominantly lymphohistiocytic, perivascular, inflammatory cell infiltrates. All but patient 1 had an erythematous papulovesicular rash. Two (patients 3 and 5) had been given a cephalosporin antibiotic prior to biopsy. Six patients responded rapidly to therapy with doxycycline; patient 4 recovered without specific antibiotic treatment after an illness of approximately two-weeks duration.

Acute-phase serum samples were collected a median of six days after onset of fever (range = 4–8 days) and a median of 10 days after the initial appearance of the eschar (range = 7–14 days). Diagnostic titers of IgG antibodies reactive with R. akari were detected in the initial serum samples of patients 2, 4, 5, and 6 (Table 2). Patient 2, the only patient for whom acute- and convalescent-phase serum samples were collected, showed a 16-fold increase in antibody titer reactive with R. akari in a serum sample collected 53 days after the onset of fever. Spotted fever group rickettsiae were identified by IHC staining in eschar biopsy specimens of all patients (Figure 1B). Intact, predominantly cocoid rickettsiae were identified in the cytoplasm and occasionally in the nuclei of macrophages (Figure 1C) in the perivascular infiltrates of the superficial and deep dermis. The IHC staining of rickettsial antigens was occasionally identified in a few dermal fibrohistiocytic cells (Figure 1D) and rarely in capillary endothelial cells. Rickettsiae were also localized by IHC staining in perivascular mononuclear cells in the papulovesicular rash biopsy specimens of patients 2, 5, 6, and 7; however, rickettsial antigens were consistently more abundant in the eschars than in the rash lesions. A 208-basepair segment of the rickettsial 17-kD antigen gene was amplified from residual eschar biopsy triturates from patients 1, 2, 3, 5, and 6 (Table 2).

Five isolates of R. akari, designated Columbia 1, 2, and 3, Manhattan, and Bronx, were obtained in cell culture from eschar biopsy specimens of patients 1, 2, 3, 5, and 6, respectively (Table 2). Viable rickettsiae were isolated in Vero E6 cells from 4 skin specimens stored at 4°C for 1–4 days and from 1 stored at −80°C for approximately 9 months after the biopsy procedures. Abundant, predominantly diplobacillary bacteria were identified by using acridine orange stain 13–15 days after the primary inoculation of Vero E6 cells; no evidence of infection was noted for any of the primary isolates evaluated by acridine orange staining during the first week of incubation. Attempts to isolate rickettsiae from 2 eschar biopsy specimens that were refrigerated at 4°C for 4 and 17 days (patients 4 and 7, respectively) after the biopsy procedure were not successful. A 208-basepair segment of the rickettsial 17-kD antigen gene was amplified from each isolate and all sequences were identical to the corresponding sequence of R. akari (Hartford strain, GenBank accession no. AF445383). Each isolate remained stable and grew abundantly in culture after three passages in Vero E6 cells. No apparent differences in growth rate or cytopathic effect in Vero E6 cells were observed among the five isolates during the first three passages.

The SDS-PAGE analysis (Figure 2) of the five new isolates of R. akari showed protein patterns identical to those of the existing U.S. reference strains of R. akari, and distinct from the profiles of each of the other U.S. spotted fever and typhus group rickettsiae evaluated, e.g., R. rickettsii, R. montanensis, R. rhipicephali, R. parkeri, R. amylovorii, R. prowazekii, R. typhi, R. canadensis, and R. bellii.

### DISCUSSION

The five isolates described in this report represent the only record of R. akari cultured from the cutaneous lesions of patients with rickettsialpox, and the first isolates of this pathogen from the Western Hemisphere reported in > 50 years. Only three extant, autochthonous U.S. isolates existed prior to these endeavors: MK, isolated from the blood of a patient from New York City in 1946; Fuller #7, isolated from a house mouse collected from Boston in 1950; and Hartford, isolated from house mouse mites collected from West Hartford, Connecticut, in 1952. The protein profiles of these isolates, which appear identical to the new isolates described in this report by one-dimensional protein electrophoresis, suggest that R. akari has maintained a stable protein phenotype since its discovery in the mid 1940s; however, more sensitive techniques (e.g., two-dimensional gel electrophoresis) may ultimately demonstrate subtle differences in the protein compositions of the new isolates of R. akari. In this context, the existence of five contemporaneous, low-passage isolates provides novel opportunities to compare specific biologic and genetic characteristics of these strains with older, high-passage isolates.

Isolation of R. akari was frequently attempted by investigators during the decade after the discovery of the pathogen and was typically accomplished by inoculating whole blood from febrile patients into the peritoneal cavity of mice and...
subsequent passages in guinea pigs and chicken yolk sacs to obtain a non-contaminated, robust isolate.\textsuperscript{1,2,13} More recently, \textit{R. akari} has been obtained in culture by using Vero cells, a broadly permissive cell line commonly used to isolate viruses.\textsuperscript{3,21} In this context, biosafety level 2 laboratories that routinely perform viral cultures should be able to isolate \textit{R. akari} or other rickettsiae from clinical specimens in cell culture; however, biosafety level 3 practices would be required for subsequent propagation of any established isolate.

Penicillin and streptomycin were added to the medium with the primary inoculum to diminish or eliminate growth of bacteria that often colonize skin and could potentially contaminate the culture of rickettsiae. \textit{Rickettsia akari} is relatively resistant to these antibiotics \textit{in vitro}\textsuperscript{2,32} and early investiga-
Results of confirmatory laboratory assays for patients with rickettsialpox from New York City, evaluated by culture isolation for *Rickettsia akari* at the Centers for Disease Control and Prevention, 2001–2003*

<table>
<thead>
<tr>
<th>Patient</th>
<th>Isolate designation</th>
<th>IHC stain</th>
<th>IFA titer(s) to <em>R. akari</em></th>
<th>PCR</th>
<th>T7KD antigen gene</th>
<th>PCR</th>
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<tr>
<td>1</td>
<td>Manhattan</td>
<td>Eschar</td>
<td>+</td>
<td>&lt; 32</td>
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<td>+</td>
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<tr>
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<td>Columbia 1</td>
<td>Eschar</td>
<td>+</td>
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<td>+</td>
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<tr>
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<td>Eschar</td>
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<td>Eschar</td>
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<td>&lt; 32</td>
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*IHC = immunohistochemical, IFA = indirect immunofluorescence antibody; PCR = polymerase chain reaction; NA = no specimen available; NS = attempt to isolate rickettsiae in cell culture was not successful.† All initial serum samples were obtained at the same time as the biopsy specimens.

Clinical suspicion and appropriate geographic context are generally sufficient for an astute clinician to develop a presumptive diagnosis of rickettsialpox and to initiate empirical therapy; unfortunately, confirmatory laboratory methods including serologic analysis, IHC staining, PCR, and cell culture seldom provide timely data for patient care and management during the acute phase of the illness. Nonetheless, the differential diagnosis for the ill patient with an eschar is broad and includes spider bites, and cutaneous infections caused by *Aspergillus, Fusarium,* and *Mucor* spp., herpes simplex viruses, varicella zoster virus, parapoxviruses, *Streptobacillus moniliformis,* and various agents associated with bioterrorism (e.g., *Francisella tularensis, Yersinia pestis,* *Burkholderia pseudomallei,* and *B. anthracis,*22,44,45 and concerted efforts to establish a specific etiologic diagnosis are not simply academic exercises. Several patients initially presumed to have cutaneous anthrax after intense national concern regarding a bioterrorism attack during 2001 were eventually proven to have rickettsialpox.21,22,44 Eschars have also been described in North American travelers who become ill with scrub typhus or African tick bite fever after returning to the United States from countries where these diseases are endemic.46,47 In this context, a multidisciplinary laboratory approach to testing eschar biopsy specimens from patients with imported rickettsioses can be used to establish pathogen-specific diagnoses in a manner similar to that described for rickettsialpox.

From an ecologic and epidemiologic perspective, populations of the vector mite and rodent host are documented throughout the United States, and various studies suggest that substantial rates of infection occur among inhabitants of other urban areas;23 however, every U.S. case series of rickettsialpox published during the last 50 years describes a patient cohort that resided within the limited geographic boundaries of New York City.21,22,42,43,48 This observation suggests that most clinicians beyond the borders of this large metropolitan center are relatively unfamiliar with rickettsialpox despite historical, ecologic, and clinical evidence to suggest that *R. akari* is able to infect and disseminate in the United States from countries where these diseases are endemic.
occurs in several other U.S. cities, including Boston, Philadelphia, Cleveland, and Baltimore. The magnitude and geographic distribution of rickettsialpox in the United States and elsewhere may be expanded by intensified efforts to isolate rickettsiae from cutaneous lesions to identify cases of rickettsialpox that might otherwise be attributed to various infectious and non-infectious causes.

Culture-focused diagnostics also provides the opportunity to characterize newly recognized causes of spotted fever rickettsioses. Since 1984, at least 10 other eschar-producing SFG rickettsiae have been identified as pathogens of humans using molecular and culture-based diagnostic methods. Multiple and distinct pathogenic spotted fever rickettsiae coexist in various geographic regions. As examples, at least three agents, *R. conorii*, *R. sibirica mongolotimonae*, and *R. slovaca*, cause eschar-associated rickettsioses in France, and *R. conorii* and *R. akari* cause spotted fever in Croatia. This paradigm also exists in the United States where *R. parkeri* was identified recently as a cause of human illness when an isolate of the agent was obtained from an eschar of a patient presumed to have had rickettsialpox. Group-specific serologic and IHC assays have identified additional U.S. cases of rickettsialpox, *R. parkeri* rickettsiosis, or possibly other novel rickettsioses in patients with eschar-associated illnesses from various U.S. states, including California, Florida, Mississippi, and North Carolina.

In 1991, rickettsiologist Joseph McDade wrote, “For decades, rickettsial isolation was the sine qua non for disease diagnosis, but as we approach the end of the 20th century, isolation is being supplanted by newer methodologies. This is indeed a very unfortunate trend, because it is based on the supposition that all of the pathogenic rickettsiae have been isolated and that other techniques are sufficient for detecting evidence of rickettsial infection.” Traditional microbiologic methods (e.g., serologic analysis and cell culture isolation), combined with contemporary assays (e.g., IHC and PCR), offer the greatest opportunity to accurately diagnose rickettsialpox and other rickettsioses and enhance clinician and public awareness of these poorly recognized but widespread infections.

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