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# Routine argyrophil techniques detect *Rickettsia rickettsii* in tissues of patients with fatal Rocky Mountain spotted fever

**Christopher D. Paddock**[1], **Jeanine H. Sanders**[2], **Amy M. Denison**[2], **Atis Muehlenbachs**[2], **Sherif R. Zaki**[2]

[1]Rickettsial Zoonoses Branch, Centers for Disease Control and Prevention, Atlanta, GA, USA

[2]Infectious Diseases Pathology Branch, National Center for Emerging and Zoonotic Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, GA, USA

## Abstract

*Rickettsia rickettsii*, a bacterial tickborne pathogen that causes Rocky Mountain spotted fever (RMSF), stains poorly or not at all with conventional tissue Gram techniques, and contemporary visualization of the pathogen in formalin-fixed, paraffin-embedded tissues has relied almost entirely on immunohistochemical staining methods that are generally limited to specialized research laboratories or national reference centers. To our knowledge, previously described argyrophil-based histochemical techniques have not successfully detected rickettsiae in formalin-fixed, paraffin-embedded tissues. To investigate the ability of standard silver impregnation techniques to demonstrate the occurrence and distribution of *R. rickettsii* in tissues of patients with RMSF confirmed by molecular and immunohistochemical methods, three widely recognized and commercially available silver impregnation methods (Warthin–Starry, Steiner, and Dieterle's) were applied to various tissues obtained at autopsy from 10 patients with fatal RMSF. *R. rickettsii* bacteria were demonstrated in one or more tissues of all patients, using each of the argyrophil-based methods, and appeared as small, dark brown-to-black lanceolate rods, often in pairs and occasionally surrounded by a faint halo. Rickettsiae were identified most consistently in small arteries and arterioles of liver, kidney, and leptomeninges, and were localized predominantly to the cytoplasm of endothelial cells and less often within the internal elastic lamella and smooth muscle of the media. This validation of argyrophilic techniques to detect *R. rickettsii* demonstrates the utility of inexpensive core histochemical methods in the diagnosis of infectious agents in pathology specimens and may have utility in certain resource-limited settings where RMSF is endemic.

### Keywords

Argyrophil; Dieterle's; *Rickettsia rickettsii*; Steiner; Rocky Mountain spotted fever; Warthin–Starry

Correspondence to: Christopher D. Paddock, Rickettsial Zoonoses Branch, Centers for Disease Control and Prevention, Mailstop G-13, 1600 Clifton Road, Atlanta, GA 30333, USA. cdp9@cdc.gov.

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## Introduction

Rocky Mountain spotted fever (RMSF), a nationally notifiable disease caused by the tickborne bacterium *Rickettsia rickettsii*, is one of the most lethal infectious diseases in the western hemisphere. During 1983–2012, 300 fatal cases of RMSF were reported to the Centers for Disease Control and Prevention, which likely represents an underestimate of the actual number of deaths in the United States attributable to this disease.[1–3] In several countries of Latin America where RMSF is endemic, including Argentina, Brazil, Colombia, Costa Rica, Mexico, and Panama, the case fatality rates for RMSF are considerably higher than in the United States.[4–12] Efforts to assess the magnitude of fatal RMSF in the Americas are curtailed by limited access to costly diagnostic techniques in many of these regions.

Most bacterial species have the ability to adsorb silver ions from a solution. When the adsorbed silver is chemically reduced, the metallic form becomes visible and the morphology of the bacterium is revealed. Several histochemical techniques, including those developed during the early twentieth century by Warthin and Starry,[13] Dieterle,[14] and Steiner,15 use this principle to detect bacteria that otherwise stain weakly or not at all by conventional tissue Gram stains. During the last 100 years, argyrophil-based methods have been used widely by pathologists to demonstrate various pathogenic bacteria, including *Treponema pallidum*, *Legionella pneumophila*, *Bartonella henselae*, *Borrelia burgdorferi*, *Helicobacter pylori*, and *Klebsiella* (*Calymmobacterium*) *granulomatis* in formalin-fixed, paraffin-embedded patient tissues.[16–20]

Shortly following the discovery of RMSF during the early 1900s, the pathologist S.B. Wolbach used a modified Giemsa stain to identify *R. rickettsii* in endothelial and smooth muscle cells in small blood vessels of patients with fatal RMSF (Fig. 1(A)).[21,22] Other pathologists effectively used this technique during the first half of the twentieth century to further characterize the pathology of fatal RMSF.[23–25] Nonetheless, Giemsa stain has not been consistently or successfully used in contemporary histopathological studies of RMSF, and some investigators have noted limitations in its interpretation, particularly the non-specific staining of cellular debris.[26,27] *R. rickettsii* and other species of pathogenic spotted fever group *Rickettsia* (SFGR) bacteria stain poorly with tissue Gram methods and, during the last 40 years, pathologists relied almost exclusively on antibody-based staining techniques, including immunofluorescence, immunoperoxidase, and immunoalkaline phosphatase methods, to demonstrate *R. rickettsii* in tissues of patients with RMSF.[28–30] More recently, pathologists have developed molecular methods to detect SFGR in formalin-fixed tissues.[31] Although antibody-based and molecular assays are highly sensitive and specific for SFGR, these methods characteristically require specialized reagents, instrumentation, and expertise that are often limited to research institutions and national or international reference centers.

A silver impregnation technique, described as the method of Morozov, was used by early Russian investigators to examine *Rickettsia* species in smear preparations made from infected arthropods and animal tissues,[32] but there are no descriptions of SFGR in formalin-fixed, paraffin-embedded human tissues using standard argyrophil-based techniques. Herein

we report the validation of these methods to demonstrate the distribution of *R. rickettsii* in tissues of patients with fatal RMSF.

## Methods and Materials

### Patients

Tissues were obtained at autopsy from patients from 10 US states who died from RMSF during 2005–2013. The 10 patients ranged in age from 1–59 years (median, 5 years), comprised four females and six males, and died 6–12 days (median, 9 days) following illness onset. The tissues evaluated included lung, liver, kidney, spleen, skin, adrenal gland, testis, and central nervous system. Infection with *R. rickettsii* was confirmed in all of the decedents using immunohistochemical and molecular techniques.

### Molecular methods

To confirm the presence of DNA of *R. rickettsii* in patient tissues, four 16-μm sections were cut from each of one or more paraffin blocks from each patient and placed in microcentrifuge tubes. The sections were de-paraffinized with xylene and washed twice with absolute ethanol. Tissues were then incubated overnight at 56 °C in 180 μL buffer ATL and 20 μL proteinase K (QIAGEN, Valencia, CA). Extraction of the supernatant was completed using a Qiagen QIAamp deoxyribonucleic acid (DNA) Micro Kit with a final elution volume of 50 μL. A real-time polymerase chain reaction (PCR) assay, adapted to detect rickettsial nucleic acids obtained from formalin-fixed tissues,[31] was used to amplify a segment of a hypothetical protein (A1G_04230) gene of *R. rickettsii*.[33] Reactions included 12.5 μl of Qiagen QuantiTect Multiplex PCR Master Mix, 0.2 μM of each of primers RRi6_F and RRi6_F and probe RRi6_P, and 2.5 μl of DNA extract in a 25 μl volume.[31] Cycling conditions consisted of one cycle of 95°C for 15 min, and 45 cycles of 94°C for 1 min, and 60°C for 90 s using an Agilent Mx3005P real-time thermal cycler. All PCR reactions included appropriate positive and negative controls, and samples were considered positive if the $C_t$ value was ≤40.

### Histochemical methods

Serial sections were cut at 4 -μm thickness from formalin-fixed, paraffin-embedded tissue blocks and evaluated by Warthin–Starry, Dieterle's, and Steiner techniques using commercially available reagents and according to the manufacturer's recommendations. The Warthin–Starry Stain Kit was optimized for use on the Artisan™ Staining System (Dako, Carpenteria, CA) and is an automated technique comprising three reagents: gelatin in deionized water containing 0.5% ProClin™ 300, a 1% silver nitrate solution, and a 0.13% hydroquinone reducing solution. The Dieterle's Stain Kit (American MasterTech, Lodi, CA) supports a manual procedure comprising six reagents: Wiley's Solution, Solution A, Solution B, hydroquinone capsules, 10% zinc formalin, and alcoholic gum mastic. The Steiner Stain Kit (American Mastertech, Lodi, CA) supports a manual procedure comprising six reagents: an oxidizer solution, 0.1% silver nitrate, hydroquinone capsules, 10% zinc formalin, 2.5% gum mastic, and 1% silver nitrate. Vero E6 cells infected with *R. rickettsii* were pelleted by centrifugation, fixed in 10% neutral buffered formalin, and embedded in paraffin for use as a positive control sample.

### Immunohistochemistry

Three-μm sections cut from formalin-fixed, paraffin-embedded tissue specimens were stained using an immunoalkaline phosphatase technique with naphthol fast-red and Mayer's hematoxylin counterstain. The primary antibody for this assay is a rabbit polyclonal anti-*R. rickettsii* antiserum diluted at 1/500.[30] This technique is specific for SFGR species and does not react with other bacterial pathogens of vascular endothelium, including typhus group *Rickettsia* species or *Orientia tsutsugamushi* (the agent of scrub typhus).

## Results

DNA of *R. rickettsii* was detected using a real-time PCR assay and rickettsiae were identified by immunohistochemical (IHC) stain in tissue specimens of each of the ten patients evaluated in this study. Rickettsiae were also demonstrated in one or more tissues of all patients, as well as the *R. rickettsii*-infected Vero E6 cell control, using argyrophil-based methods. With these techniques, *R. rickettsii* appeared as small, dark brown-to-black lanceolate rods, often in pairs and occasionally surrounded by a faint halo. The appearance and distribution of rickettsiae using argyophil techniques resembled closely those seen by the IHC method (Fig. 2), except for additional IHC staining of fragmented antigens that co-localized frequently with intact bacteria.

By use of Warthin–Starry, Dieterle's and Steiner methods, *R. rickettsii* bacteria were found most consistently in small arteries and arterioles of infected tissues. In these vessels, rickettsiae localized to the cytoplasm of endothelial cells and less often within the internal elastic lamella and smooth muscle of the media (Figs. 1(B), 2(A and C), 3(A and B). Rickettsiae were also clearly identified in endothelium lining alveolar capillaries (Fig. 4(A)) and hepatic sinusoids (Fig. 4(B)). Abundant intracellular rickettsiae were frequently identified in sloughed, spindle-shaped cells within the lumens of small arteries and arterioles and occasionally in sinusoids of the liver (Fig. 5). Of the multiple tissues evaluated, rickettsiae were most easily detected in arterioles and small arteries in the portal areas of the liver, interlobular arteries of the kidneys, and arterioles and venules of the leptomeninges. No distinct rickettsiae were identified in the single segments of skin evaluated in this study. Of the methods assessed, the Warthin–Starry technique generally resulted in the least background staining, particularly in the spleen and liver, making it easier to interpret the slides.

## Discussion

Silver impregnation methods provide detailed resolution of *R. rickettsii* in intact vascular endothelium and smooth muscle, as well as in detached intravascular endothelial and mononuclear cells in multiple tissues of patients with fatal RMSF. The morphology and distribution of *R. rickettsii* in the microvasculature of the patients described in this study are identical to the features documented by Wolbach nearly a century ago.[21,22] Wolbach's meticulously detailed descriptions provided the first definitive identification of the pathogen in human tissues and established the microbiological and histological foundation for the pathogenesis of this infection. Wolbach accomplished these observations using a slightly alkalinized preparation of Giemsa's stain applied to tissues fixed in Zenker's fluid. Wolbach

emphasized that it was almost impossible to demonstrate the bacteria using this stain if the tissues were processed in Schaudin's fixative, as originally recommended by Giemsa.[22] In this context, it is likely that subtleties of an effective and relatively simple histochemical technique were lost over time, prompting rickettsiologist and pathologist D.H. Walker to comment that the '… diagnosis of rickettsial disease by staining of the organisms in tissues … is virtually a lost art'.[34] Indeed, more recently, argyrophil techniques have been applied, albeit unsuccessfully, to cases of fatal RMSF.[35,36] To our knowledge, this is the first application of these methods to consistently identify SFGR bacteria in formalin-fixed human tissues.

Wolbach did not address specifically the process by which *R. rickettsii* are disseminated to various tissues and organs of the body, and even today, the sequence of events leading to systemic infection remains incompletely characterized.[37,38] Infection of endothelium by SFGR causes varied disruptions in the form and function of these cells, including discontinuity of the intercellular adherens junctions >36 h following infection.[39] This study and others identified *R. rickettsii* within detached, intraluminal and intrasinusoidal endothelial cells. Detached endothelial cells are frequently packed with rickettsiae, creating infectious 'rafts' that might be distributed to other tissues and organs to lodge in capillary beds, or adhere to uninvolved endothelium of distant small-caliber vessels. Indeed, investigators have detected circulating endothelial cells infected with *Rickettsia conorii* in the peripheral blood of patients with Mediterranean spotted fever.[40]

One or more of these techniques is available to most diagnostic pathology laboratories; these methods may be useful in establishing a presumptive diagnosis of RMSF, particularly when autopsy tissues are available for evaluation in cases of fatal disease, and in resource-challenged settings where rapid access to immunohistochemical staining or molecular techniques to detect spotted fever group *Rickettsia* species is limited or non-existent.[4,5,9–12,41,42] The automated Warthin–Starry technique was the most simple to perform and provided the least background; nonetheless, many laboratories may not have access to this type of instrumentation. In the absence of automated techniques, the Steiner method was the simplest of these methods to perform.

Many other infectious conditions, including rat bite fever, *Staphylococcus aureus* sepsis, meningococcemia, leptospirosis, infective endocarditis, malaria, and dengue, as well as some non-infectious diseases, such as thrombotic thrombocytopenic purpura, cause life-threatening illnesses that may be confused clinically with RMSF.[43–47] Silver impregnation techniques will accentuate most pathogenic bacteria in tissues, including *Streptobacillus moniliformis, Staphylococcus aureus, Neisseria meningitidis*, *Leptospira* species, and other bacteria that cause sepsis syndromes; nonetheless, spotted fever and typhus group *Rickettsia* species, and *O. tsutsugamushi* are unique among bacterial pathogens of humans in that these organisms localize predominantly to vascular endothelium.[38] In this context, identification of small, argyrophilic, rod-shaped bacilli in the endothelium of small vessels in a patient with a fatal, rash-associated illness provides strong supportive evidence of RMSF. Further studies are needed to determine if *O. tsutsugamushi* or other pathogenic *Rickettsia* species are similarly demonstrated in tissues by silver impregnation methods.

Argyrophil techniques accentuate other cellular structures and foreign material, including carbon particles, lipofuscin, and neurosecretory granules which occasionally hinders interpretation of these methods. This study was limited by a focused evaluation of tissues obtained from patients with fatal disease; the application of silver impregnation methods to skin biopsy specimens from patients with less severe disease may be far less sensitive. Nonetheless, this evaluation demonstrates the continued utility of core histochemical methods in the diagnosis of infectious agents in pathology specimens, and may be particularly applicable in many regions of the western hemisphere where RMSF is endemic but where access to immunohistochemical or molecular methods may be limited.

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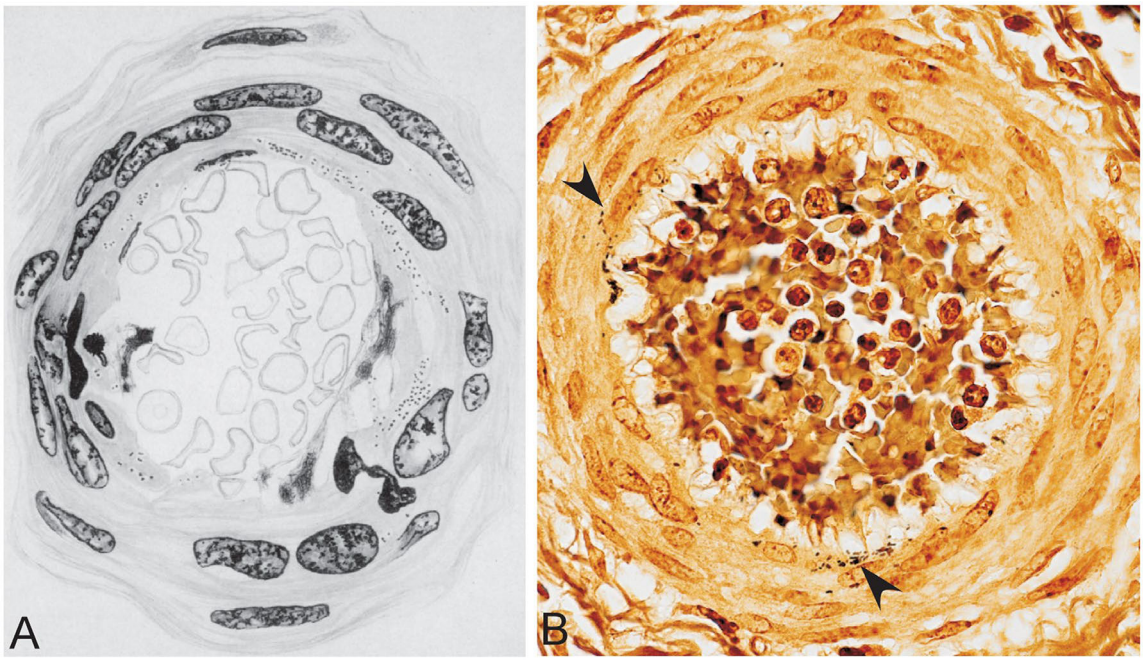


**Figure 1.**

(A) Pen and ink drawing by E.R. Piotti of an arteriole from the skin of a man who died from Rocky Mountain spotted fever (RMSF) during the early twentieth Century. Reproduced from Wolbach, SB. Studies on Rocky Mountain spotted fever. J Med Res. 1919;41:1–197 © Public Domain. (B) Minute, rod-shaped Rickettsia rickettsii bacteria (arrowheads), demonstrated by the Warthin–Starry method, in the cytoplasm of endothelium and smooth muscle cells of the media of an interlobular artery in the kidney of an infant who died from RMSF (original magnification ×100). Image produced by author and adapted from Plate 19, Figure 66 in reference [22]

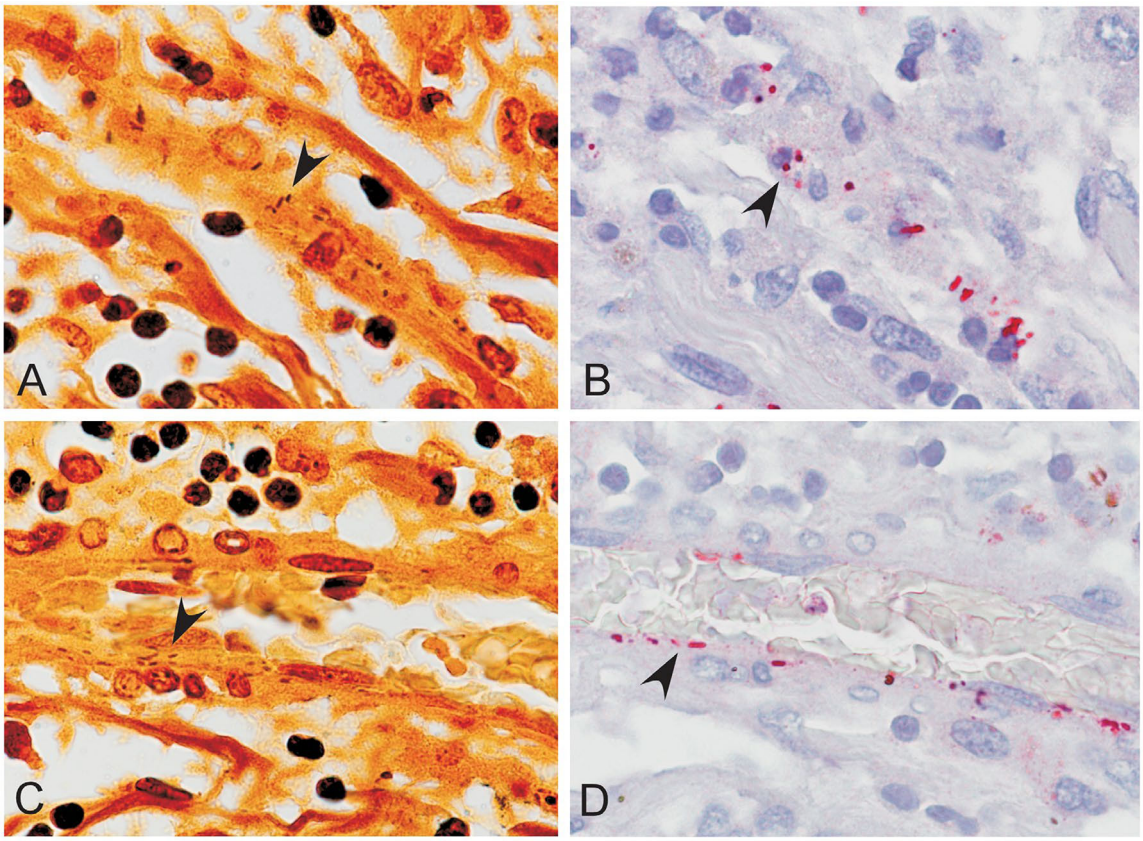


**Figure 2.**

Demonstration of *Rickettsia rickettsii* bacteria (arrowheads) in the liver of a man who died from Rocky Mountain spotted fever using the Warthin–Starry silver impregnation method (A and C) and an immunohistochemical stain specific for spotted fever group rickettsiae (B and D). The distribution and appearance of the minute, rod-shaped rickettsiae, localized to the cytoplasm of endothelial cells in a portal area capillary (A and B) and portal arteriole (C and D), are identical in sequentially stained sections (original magnifications ×158)

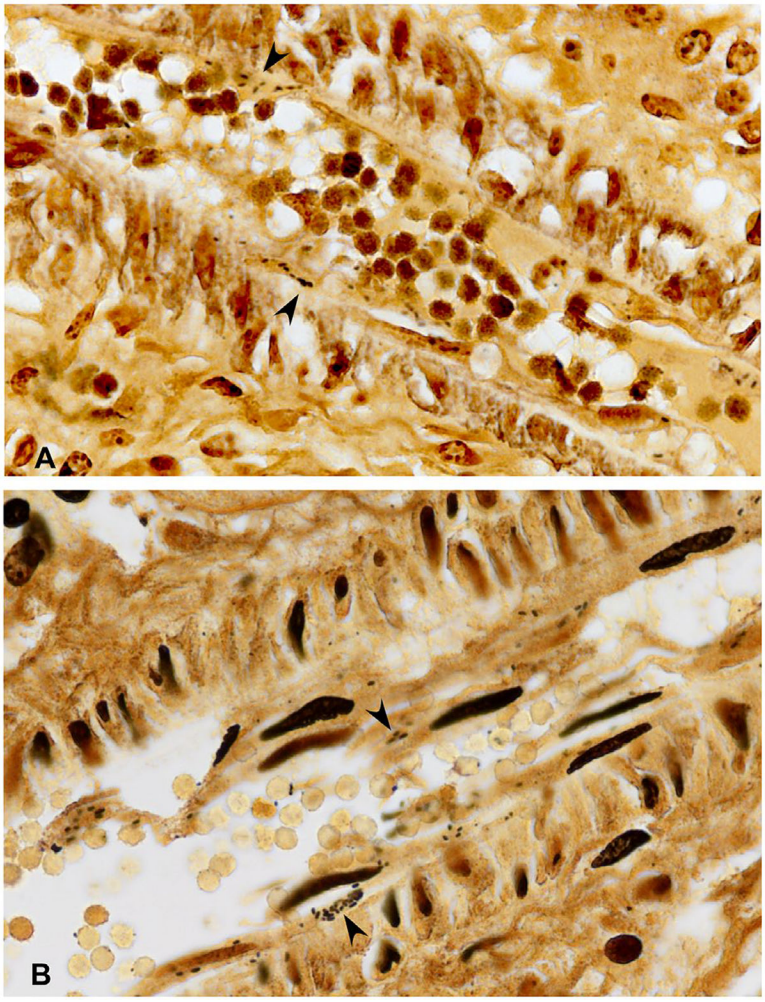


**Figure 3.**
Abundant intracellular *Rickettsia rickettsii* bacteria (arrowheads) in endothelial cells of an interlobular artery from the kidney of a child who died from Rocky Mountain spotted fever, demonstrated using Warthin–Starry (A) and Dieterle's (B) silver impregnation methods (original magnifications ×158)

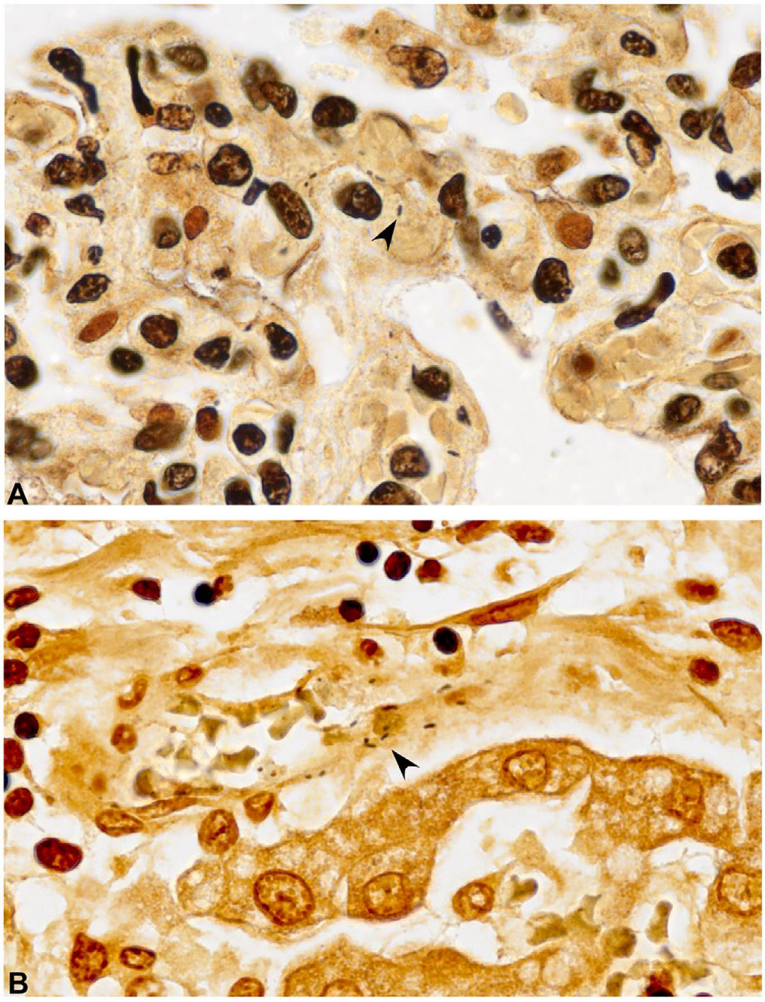


**Figure 4.**

A. *Rickettsia rickettsii* bacteria (arrowhead) in alveolar capillary endothelial cells from the lung of a child who died from RMSF, as demonstrated using the Dieterle's method. B. *R. rickettsii* bacteria (arrowhead) in sinusoidal endothelial cells in the liver of a man with fatal RMSF, Warthin–Starry method (original magnifications ×158)

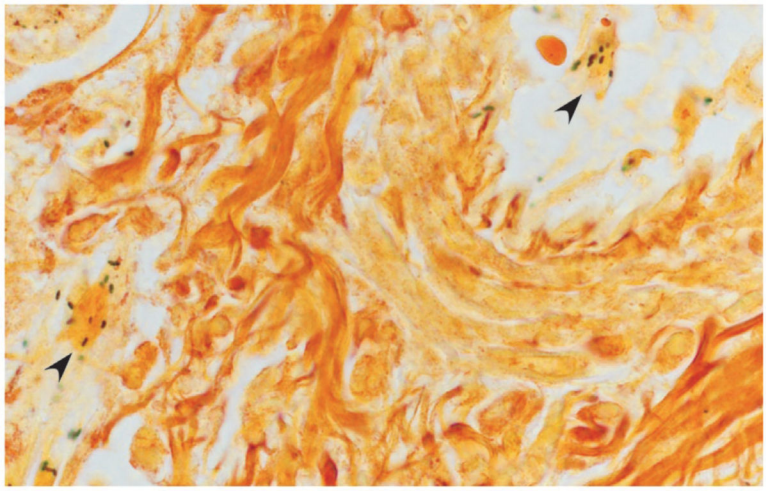

**Figure 5.**

Minute rod-shaped *Rickettsia rickettsii* bacteria (arrowheads) in sloughed endothelial cells in a portal arteriole (upper right) and in an adjacent hepatic sinusoid of a man with fatal RMSF (Steiner method, original magnification ×158)