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Diagnosis of Mediterranean Spotted Fever by Cultivation of *Rickettsia conorii* from Blood and Skin Samples Using the Centrifugation-Shell Vial Technique and by Detection of *R. conorii* in Circulating Endothelial Cells: a 6-Year Follow-Up

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***Rickettsia conorii*, an obligate intracellular bacterium that infects vascular endothelial cells, is the etiologic agent of Mediterranean spotted fever. We correlated the results of 205 *R. conorii* blood and skin cultures for 157 patients and the results of 48 detections of *R. conorii* in circulating endothelial cells (CEC) for 41 patients with relevant serological, clinical, and therapeutic data. *R. conorii* was cultured from 40% of patients and 29.8% of samples. *R. conorii* was detected in CEC in 50% of samples, representing 46.2% of patients. When these calculations were limited to the samples from untreated patients prior to their seroconversion to *R. conorii*, the sensitivity of culture was 59%, whereas it remained at 50% for detection in CEC. We also performed PCRs for the detection of *R. conorii* on eight shell vial supernatants from positive cultures and on 43 blood samples. Only nonfrozen supernatants from fresh cultures were positive. The methods described in this report are suitable for use in all laboratories. Our findings suggest that for samples to be suitable for culture they must be collected prior to the initiation of an antibiotic regimen, as early as possible in the course of the disease, and be inoculated onto shell vials with minimal delay, if *R. conorii* is to be successfully isolated. For patients who have been treated or who have a delayed diagnosis, detection of *R. conorii* in CEC remains helpful.**

Mediterranean spotted fever (MSF) is an acute, febrile, tick-transmitted rickettsiosis caused by *Rickettsia conorii*, an obligate intracellular bacterium. The disease is widely distributed in Mediterranean countries, including the south of France, where it is endemic in the summer months. Diagnosis is based on clinical findings early in the course of the disease. MSF should be suspected in patients presenting with a fever of >39°C, a so-called "tache noire", and a maculopapular rash of the palms and soles. MSF may be easily misdiagnosed in patients outside the area of endemicity (10) or during winter (22). A serological confirmation of infection is possible only late in the illness (21). A severe form of MSF, with a mortality rate of 33%, has been described in patients with underlying conditions such as diabetes mellitus, alcoholism, chronic liver disease, and glucose-6 phosphate dehydrogenase deficiency (17). Higher mortality rates are also correlated with delays in consulting a physician (>5 days) and delays in the administration of appropriate antibiotic therapy (>10 days) (17). Similar prognosis factors have been reported for Rocky Mountain spotted fever (24). In order to enhance laboratory diagnosis, several techniques have been developed, such as demonstration of rickettsiemia by centrifugation-shell vials technique (11), genomic amplification of rickettsial DNA by PCR (6, 23), direct demonstration of *R. conorii* in skin biopsy specimens by using direct immunofluorescence (13), and, more recently, indirect immunofluorescence of *R. conorii* in circulating endothelial cells (CEC) isolated with monoclonal antibody-coated immunomagnetic beads (3). This last technique allows the confirmation of MSF, but only blood culture on human embryonic lung (HEL) cells by the shell vial technique can be used to easily establish isolates of *R. conorii*. Between January 1989 and November 1995, we attempted to isolate *R. conorii* from 157 patients with suspected MSF. We correlated the presence of positive culture with serological status, prior antibiotic therapy, and delay between collection of the sample and inoculation of shell vials. The diagnostic usefulness of the detection of *R. conorii* in CEC was compared with that of the shell vial assay. A preliminary assessment of this method has been previously described (3); however, our study, unlike the earlier one, evaluated the technique while in routine use. We also performed PCR amplification, retrospectively and prospectively (year 1995), on sera and shell vial supernatants from several patients with positive cultures, in order to evaluate its usefulness for the diagnosis of MSF.

MATERIALS AND METHODS

Patients and clinical samples. Our center, located in Marseille (southern France), is the National Reference Center for Rickettsiosis. Sera are received from all areas of France but primarily from the southeastern region, where the disease is endemic. The usefulness of the shell vial technique is now more well recognized in our region than it was in the past, and clinicians often send us a 5-ml heparinized blood sample or a tissue skin biopsy specimen suitable for use in this assay, along with serum samples, when MSF is suspected. If serum samples alone are received, we will contact the sender and arrange for a blood sample to be sent. At this point we are also able to obtain relevant clinical and epidemiological data on the patient.

The sample of heparinized blood is sedimented for 1 h, and 1 ml of the supernatant is collected for inoculation into shell vials. Skin biopsy specimens are triturated in cell culture medium prior to inoculation. In about 25% of cases, clinicians also send a blood sample in a siliconized tube containing EDTA (1 mg/ml) suitable for the detection of *R. conorii* in CEC. PCRs are performed on frozen (−20°C) serum samples and on shell vial supernatants, and in 1995, we also performed PCRs on all serum samples we received with culture requests.

Case definition. A final diagnosis of MSF was confirmed in patients with a score of >25 (Table 1) by the MSF diagnostic score previously described (16).

Culture procedure. Isolation of *R. conorii* by cell culture is performed routinely in our laboratory, since the usefulness of this procedure was demonstrated.

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TABLE 1. Diagnostic score for MSF[a]

Diagnostic criterion	Points
Epidemiologic criteria	
Life or recent travel in endemic area	2
Onset between May and September	2
Contact with dog ticks	2
Clinical criteria	
Fever higher than 39°C	5
"Tache noire"	5
Maculopapular or purpuric eruption	5
Two of the three clinical criteria	3
All three clinical criteria	5
Unspecific biological criteria	
Platelet count of $<150 \times 10^9$/liter	1
Liver enzymes of >50 IU/liter	1
Bacteriological criteria	
Detection of *R. conorii* in skin biopsy by using immunofluorescence assay	25
Isolation of *R. conorii* from blood	25
Serological criteria (immunofluorescence)	
Single serum sample with total Ig of ≥1:128	5
Single serum sample with IgG of ≥1:128 and IgM of ≥1:64	10
Two serum samples with fourfold titer elevation within 2 weeks	20

[a] A total of >25 is consistent with a presumptive diagnosis of Mediterranean spotted fever.

Culture is performed by the centrifugation-shell vial technique with HEL fibroblasts as described previously (5, 11). In the present study, three confluent shell vials were inoculated with each blood sample. Detection of *R. conorii* on the coverslip was carried out, while it remained inside the shell vial, by immunofluorescence after 3 and 6 days of incubation. If immunofluorescence was negative, the culture was reported as negative. If immunofluorescence was positive after 3 and 6 days of incubation, the culture was reported as positive. The supernatants of positive shell vials and of the third shell vial were inoculated on confluent layers of HEL cells in 150-cm^2 culture flasks in order to obtain isolates of *R. conorii.*

Serological procedures. Sera were analyzed by using a microimmunofluorescence assay as previously described (21). All sera were diluted 1/32, 1/64, and 1/128. After screening for total immunoglobulins (Ig), all sera found to be positive were serially diluted (twofold dilutions initially ranging from 1/32 to 1/2,048 and more if needed), and the titers of IgG and IgM were determined. In order to remove IgG, rheumatoid factor absorbent (RF-absorbent; Behringwerke AG, Marburg, Germany) was used prior to IgM determination, according to the manufacturer's instructions.

Detection of *R. conorii* in CEC. CEC were isolated from whole blood by using immunomagnetic beads, and *R. conorii* was detected by using indirect immunofluorescence, as previously described (3).

PCR amplification of *R. conorii* 190-kDa antigen and RFLP analysis. DNA for PCR analysis was extracted from 200 μl of serum in the case of blood samples or 200 μl of cell culture medium in the case of shell vial supernatants by using the QIA ampBlood kit (Qiagen, Hilden, Germany) under the conditions recommended by the manufacturer. PCR amplification was performed by using previously described (18) oligonucleotide primer pairs Rr 190.70p and Rr 190.602n generated from the 190-kDa antigen of *Rickettsia rickettsii* (Eurogentec, Seraing, Belgium). This primer pair was shown to allow amplification of a 190-kDa antigen from all the strains of *R. conorii* previously tested (4). Each of the 35 cycles of amplification consisted of denaturation at 95°C for 20 s, annealing at 48°C for 30 s, and sequence extension at 60°C for 2 min, according to the protocol described by Regnery et al. (18). A total of 100 μl of the reaction mixture, which contained 10 μl of prepared sample, 59.5 μl of distilled H_2O, 10 μl of 10× *Taq* buffer (Boehringer Mannheim, Meylan, France), 10 μl of deoxynucleotide triphosphates (2% ATP, 2% TTP, 2% CTP, 2% GTP [Boehringer Mannheim]) in distilled water, 5 μl of each component of the primer pair, and 0.5 μl of *Taq* polymerase (5,000 U/ml; Boehringer Mannheim), was prepared and processed by using a thermocycler PEC 9600 (Perkin-Elmer Cetus, Norwalk, Conn.). To verify the result of the PCR amplification, 10 μl of the amplified material was electrophoresed at 100 V in a 1% agarose gel (Sigma, St. Louis, Mo.) in 1× Tris-borate-EDTA buffer for 1 h, stained with ethidium bromide, and transilluminated (365 nm). The size of the amplified product (650 bp) was verified by using the DNA molecular mass marker VI (Boehringer Mannheim). Aliquots of 23.3 μl of the amplified product were digested with 1 μl (10 to 20 U) of *Rsa*I and *Pst*I restriction endonucleases (New England Biolabs, Beverly, Mass.) for 2 h at 37°C, and the restriction products were separated in a 8% polyacrylamide gel that was run at 100 V for 4 h, stained with ethidium bromide, and transilluminated. DNA molecular mass marker V (Boehringer Mannheim) was run simultaneously with the samples to determine the molecular masses of the observed DNA fragments. The computer program QGel-1D (Quantigel Corporation, Madison, Wis.) was used to calculate the fragment sizes as a function of their relative electrophoretic mobilities. For negative and positive controls, we used noninfected and *R. conorii* (Moroccan strain)-infected cells, respectively.

Quality indicators. Each protocol was assessed by four criteria: sensitivity [true positive/(true positive + false negative)], specificity [true negative/(false positive + true negative)], predictive value of a positive result (PPV) [true positive/(true positive + false positive)], and predictive value of a negative result (NPV) [true negative/(false negative + true negative)] (1).

RESULTS

Patients and samples. From January 1989 to November 1995, we received 205 samples (28 skin biopsy samples and 177 blood samples) from 157 patients. Ninety-one samples were collected from 80 patients for whom the final diagnosis was not MSF. None of these patients had concurrent serum antibody titers of ≥1:32. We performed 114 cell cultures for 77 patients for whom the final diagnosis was MSF, 14 from skin biopsy samples and 100 from blood samples.

For 41 patients we also received 48 blood specimens for the detection of *R. conorii* in CEC, of which only 32 specimens were from patients for whom the final diagnosis was MSF. Sixteen samples were from 16 patients for whom the final diagnosis was not MSF. PCR amplification was performed for 8 shell vial supernatants from 8 positive cultures, 5 of which were previously frozen. PCR amplification was also performed on 18 frozen serum samples from 18 patients for whom the final diagnosis was MSF. In 1995, 24 sera were tested for 20 patients; 15 of these sera were from patients for whom the final diagnosis was MSF.

Detection of *R. conorii* in CEC. Detection of *R. conorii* was difficult, primarily because cellular boundaries were difficult to observe despite counterstaining. Some cells appeared as ghost cells, and in such instances, the intracellular location of fluorescence was difficult to assess. *R. conorii* was, however, easily identifiable in endothelial cells, giving clear fluorescence (Fig. 1). For the 16 samples from the patients not having MSF, CEC with fluorescence suggestive of rickettsial infection was observed in only one case (Fig. 2). *R. conorii* was also detected in 16 of the 32 blood specimens from patients having MSF. *R. conorii* was detected in two successive blood samples for four patients. In the group of blood samples collected from patients without prior antibiotic therapy, *R. conorii* was detected in 14. Seven of these were collected from patients in whom concurrent serum antibody titers of ≥1:32 were found (four of whom were also culture positive), and the other seven were collected from patients with serum antibody titers of <1:32, although five were culture positive. Interestingly, *R. conorii* was detected in CEC of two patients receiving specific antibiotic therapy and having concurrent serum antibody titers of ≥1:32. Neither of these patients yielded positive cultures.

For the 48 blood samples collected from patients suspected of having MSF, the sensitivity of CEC detection was 50%, the specificity was 93.8%, the PPV was 94.1%, and the NPV was 48.4%. When these quality indicators were calculated for patients rather than specimens, the sensitivity was 46.2%, the specificity was 93.8%, the PPV was 92.3%, and the NPV was 51.2%. Interestingly, the sensitivity was similar for samples from the group of untreated patients with concurrent antibody titers of <1:32 and for samples from the group of seropositive or treated patients (Table 2).

Cultures. Positive cultures were easily recognized by direct immunofluorescence staining (Fig. 3). No positive cultures were yielded from the 91 samples from the patients who did not have MSF (Fig. 4). Cultures were also negative for the 35

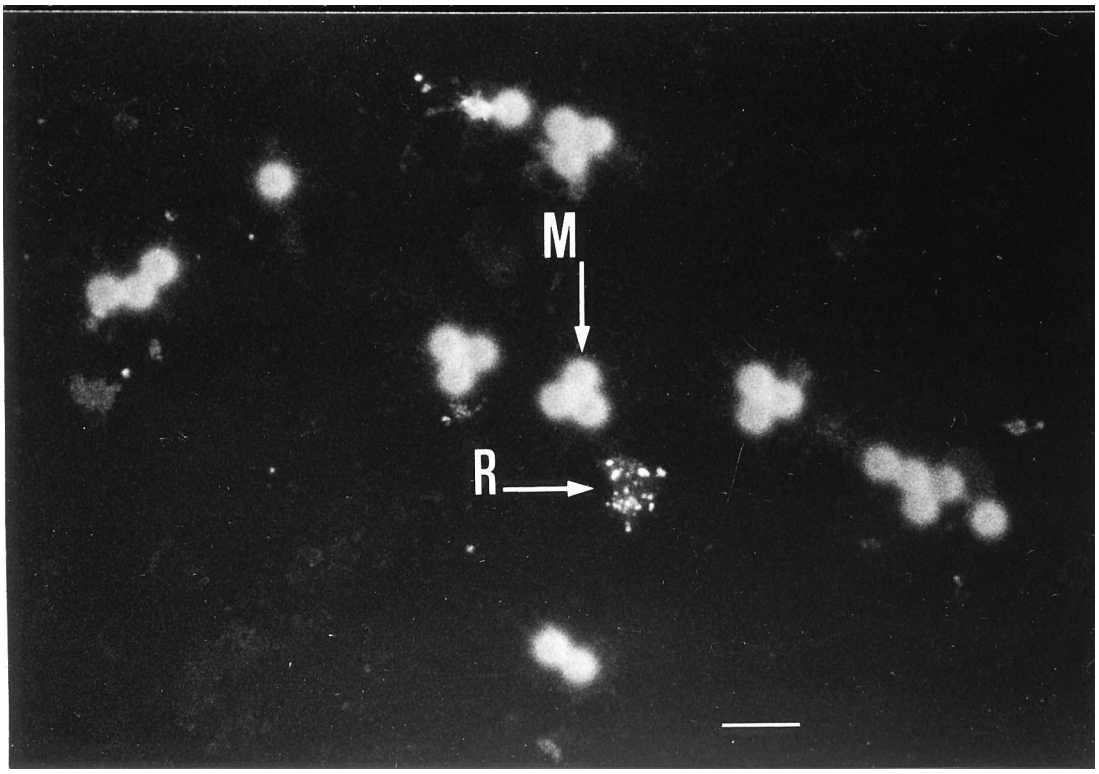

FIG. 1. Indirect immunofluorescence staining of *R. conorii* within circulating endothelial cells (R) isolated by using monoclonal antibody S-endo 1-coated magnetic beads (M). Bar, 4 μm. Magnification, ×400.

samples collected from the patients with MSF and prior antibiotic therapy. In contrast, for the 79 samples collected from the untreated patients, 34 yielded positive cultures. The origins and serologic statuses of the samples were studied for this last group. From the 49 samples related to the patients with a concurrent antibody titer of <1:32, culture was positive for three skin biopsy samples (out of a total of six skin biopsy samples) and for 26 blood samples (out of a total of 43 blood

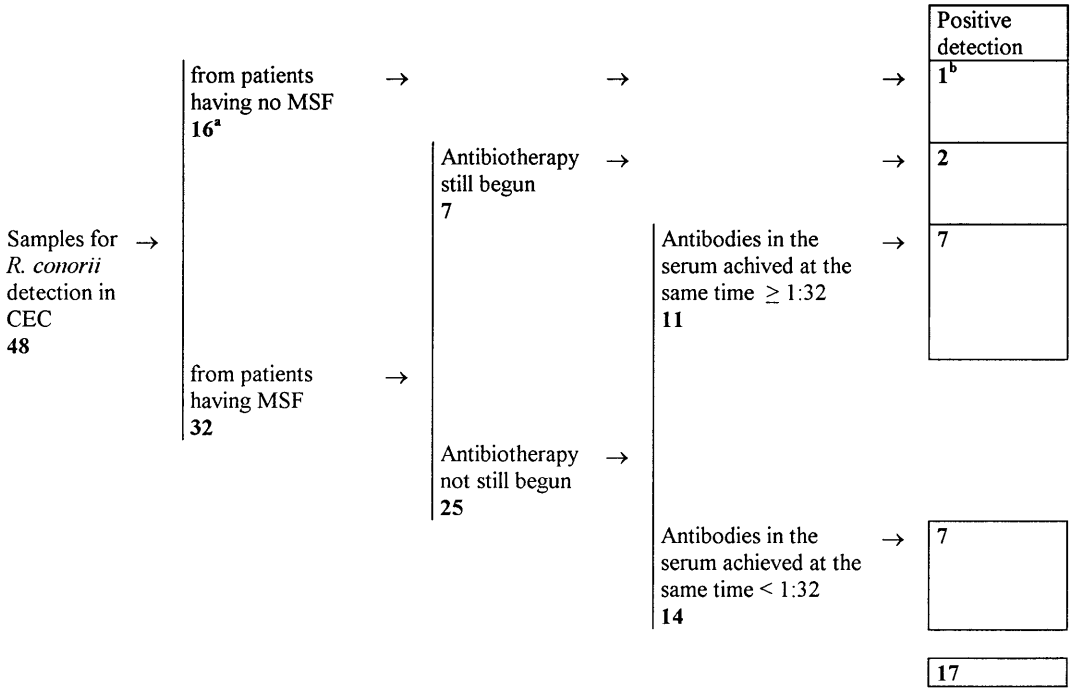

FIG. 2. Results of *R. conorii* detection in CEC according to final diagnosis, prior specific antibiotic therapy, and concurrent serum antibody titers. a, prior specific antibiotic therapy for five patients; b, one false positive.

TABLE 2. Quality indicators for cell cultures and detection of *R. conorii* in CEC[a]

Quality indicator	Cell culture			Detection of *R. conorii* in CEC		
	Raw data (%)	Early samples (%)	Late samples (%)	Raw data (%)	Early samples (%)	Late samples (%)
Sensitivity	29.8	59	7.1	50	50	50
Specificity	100	100	100	93.8	91.7	100
PPV	100	100	100	94.1	87.5	100
NPV	53.2	75	32.3	48.4	61.1	35.7

[a] Raw data include all samples, early samples refer to samples collected from untreated patients with concurrent serum antibody titers of <1:32, and late samples refer to samples collected either from patients treated at the time the sample was taken or from patients with concurrent serum antibody titers of ≥1:32.

samples). Of the 30 samples obtained from patients with a concurrent antibody titer of ≥32, 5 blood samples yielded positive cultures (out of a total of 24 blood samples); no positive cultures were obtained from the 6 biopsy samples included in this group. Considering all 205 samples from our 157 patients who were suspected of having MSF, the sensitivity of culture was 29.8% and the NPV was 53.2%. When considered in terms of patients, the sensitivity was 40% and the NPV was 63.5%. If the calculation of quality indicators is limited to patients with no prior antibiotic therapy and a concurrent antibody titer of <1:32, the sensitivity and NPV reach 59% (69% for patients) and 75% (79.7% for patients), respectively. Contrary to these results, for samples from the group of treated patients or from patients with a concurrent antibody titer of ≥1:32, the sensitivity decreases to 7.1% (Table 2). For the 34 positive cultures, detection of *R. conorii* was achieved on the first stained shell vial (day 3) in 28 cases. Of the samples from untreated patients with a concurrent antibody titer of <1:32, 15 were not inoculated onto shell vials on the day of collection. All failed to yield positive cultures. Most positive cultures resulted from vial inoculation within a few hours of specimen collection. *R. conorii* was established on successive cell cultures on 15 of the 34 positive cultures, of which one was derived from a skin biopsy specimen and 14 were from blood samples. In the remaining 19 cases, the bacterial strain died when shell vial supernatants were inoculated onto confluent monolayers of HEL cells in 150-cm^2 culture flasks. None of the five positive cultures from patients with an antibody titer of ≥1:32 was successfully established.

PCR amplification of *R. conorii* 190-kDa antigen and RFLP analysis. The 650-bp Rr 190.70p-Rr 190.602n DNA fragment was amplified from the positive control and from the three unfrozen shell vial supernatants. These four amplified fragments had the same *Rsa*I and *Pst*I restriction profiles as previously reported (4, 18). All other samples tested were negative.

DISCUSSION

The rapid diagnosis of MSF is very important for prognosis, since mortality is directly related to a delay in the initiation of appropriate antibiotic therapy (17). Although the microimmunofluorescence assay is sensitive and specific for MSF, seroconversion often does not occur until 30 days after onset of symptoms (21). In order to achieve an acute-phase diagnosis of MSF, several techniques have been proposed. Direct immunofluorescence of skin biopsy specimens was first proposed for direct diagnosis of Rocky Mountain spotted fever (25, 26, 28) and was later proposed for the diagnosis of MSF (13). This test is specific and sensitive if done before the initiation of antimi-

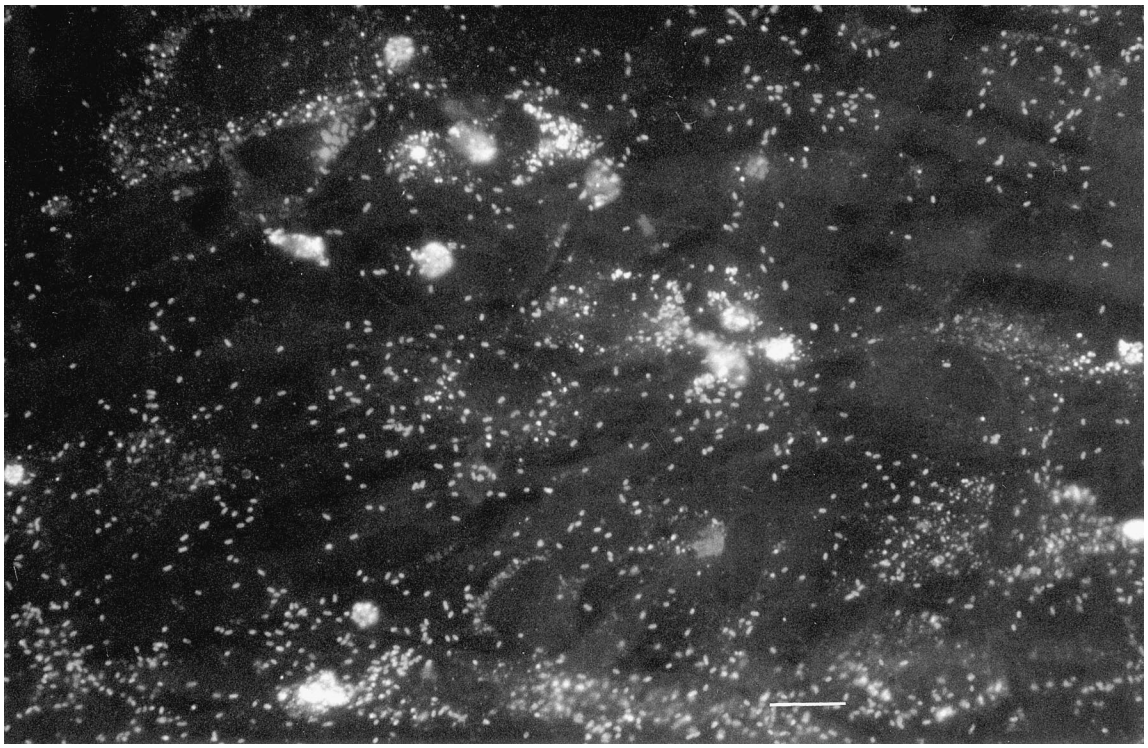

FIG. 3. Indirect immunofluorescence staining of *R. conorii* in HEL cells by using the shell vial assay. Bar, 4 μm. Magnification, ×400.

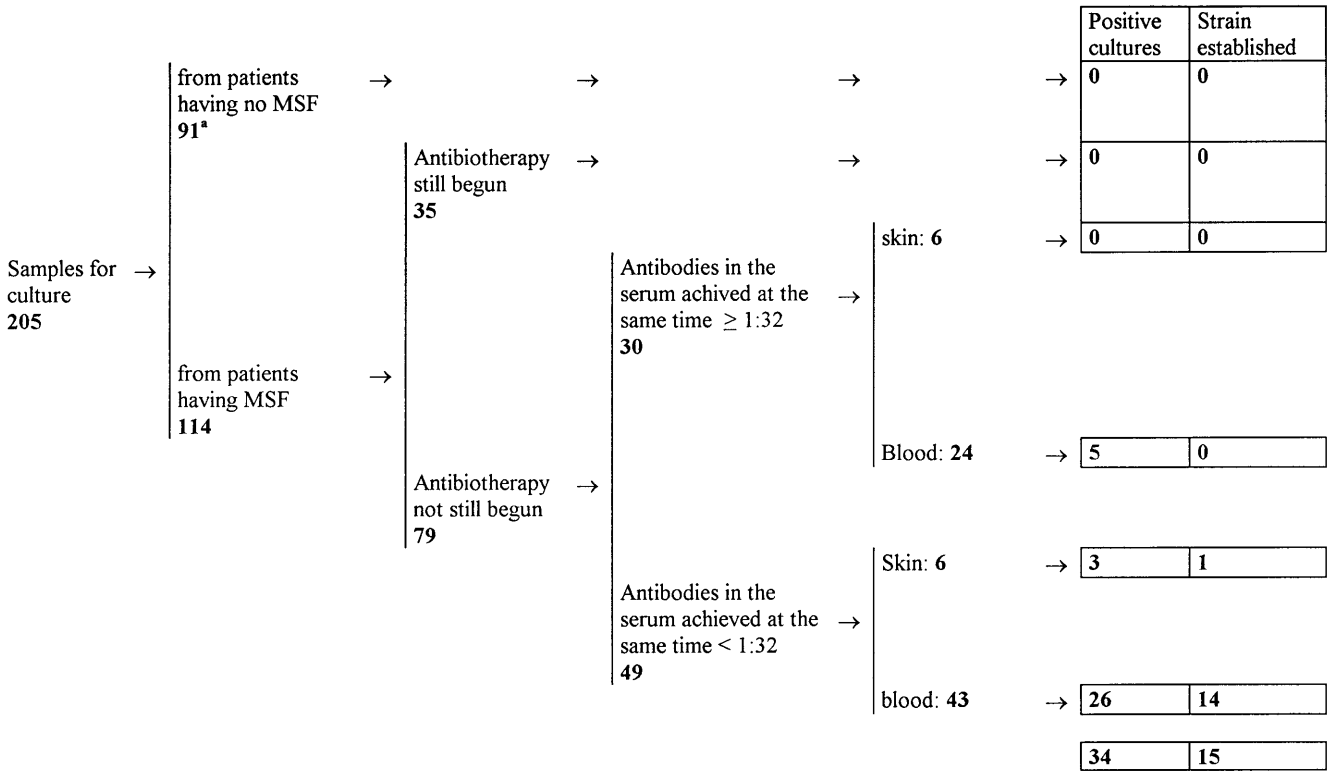

FIG. 4. Results of culture and strain establishment according to final diagnosis, prior specific antibiotic therapy, concurrent serum antibody titers, and type of sample. a, prior specific antibiotic therapy for 31 patients.

crobial therapy and before the 10th day of the disease. It is, however, also time-consuming, requires an experienced pathologist, and is difficult to apply under routine conditions. The centrifugation-shell vial assay and the detection of *R. conorii* in CEC have been subsequently proposed. The centrifugation-shell vial assay can detect rickettsiae in blood samples or skin biopsy specimens within 2 to 3 days of receipt of samples (11), whereas the result of the detection of *R. conorii* in CEC is available within 3 h (3). After preliminary demonstration of their usefulness, these two new techniques have now been introduced into routine use in our laboratory. The purpose of this study was to evaluate the usefulness of these procedures under these conditions between January 1989 and November 1995.

Detection of *R. conorii* in CEC appears to be a sensitive method, giving 50% positive results. The sensitivity remained 50% even in the group of samples collected from patients either under specific antibiotic therapy or with concurrent serum antibodies. A positive result appears to be indicative of MSF (PPV 94.1%), although a negative result does not exclude the diagnosis. The sensitivity of the procedure is clearly limited by the number of endothelial cells available for examination. CEC often appeared degenerated or necrotic. Those cellular features have been previously described during experimental *R. rickettsii* infection of cultured endothelial cells (19). Interestingly, this detection is achieved even in samples from which cultures cannot be obtained, since only nine of the positive samples with CEC yielded positive cultures. Rickettsial immunofluorescence may of course visualize dead rickettsiae.

Among the treated patients we achieved no positive cultures, probably because of the extreme sensitivity of *R. conorii* to antimicrobial agents (14, 15). Nevertheless, the detection of *R. conorii* in CEC in 28.6% of this group demonstrates the persistence of circulating bacteria (possibly nonviable). Positive cultures of *R. conorii* were obtained for 34 (43%) of the samples from untreated MSF patients. This success rate is better than that achieved for the acute phases of other illnesses. For example, in acute *Streptococcus pneumoniae* pneumonia, the isolation rate ranges from 25 to 35% (2), and in acute Q fever it is only 17% (12). Furthermore, in most cases of the patients studied, only one blood sample was collected.

Histopathologic observations of biopsy specimens and necropsy tissues from patients with rickettsial spotted fevers (7), and of experimental infections of cultured human endothelial cells (20), support the hypothesis that endothelial cells are the primary target cells for infection. The presence of rickettsiae in CEC supports this hypothesis, and the presence of circulating rickettsiae in blood during MSF may be comparable to the circulation of bacteria in infective endocarditis. However, in cases of infective endocarditis, caused by less fastidious organisms, the isolation rate is around 95% (27). This figure is significantly lower (53%) in cases due to *Coxiella burnetii* (12), which like *R. conorii* is strictly intracellular. In order to understand the reasons for the nonisolation of *R. conorii*, we attempted to correlate culture results with other criteria. Firstly, the sensitivity of the method was 59% in the group of early samples (collected from untreated patients with concurrent serum antibody titers of <1:32), whereas it fell dramatically to 7.1% in the group of later samples. Furthermore, comparison of the time of sample collection and the time of inoculation onto shell vials revealed that for 15 of the group of negative cultures, sampling and inoculation were not performed on the same day, suggesting that a long delay is detrimental to rickettsial viability. Among the group from which positive cultures

were achieved, this time delay was usually only a few hours. A sensitivity of 50% for the detection of *R. conorii* in CEC among the group of late-sampled patients demonstrates that this procedure is complementary to the shell vial assay for MSF diagnosis. Although successful isolations may take up to 6 days, a positive result can be obtained within 3 days (28 positive results for 34 isolates in this study). However, successful isolation does not always lead to propagation of the isolate, and in this study only 15 (44%) isolates were successfully established. Isolation and characterization of strains is important for a better knowledge of MSF; for example, the recent isolation of a new spotted fever group rickettsia enabled the demonstration of two distinct species in southern Africa (8). One is boutonneuse fever, caused by *R. conorii* and transmitted by ticks from dogs, and the other is African tick-bite fever, caused by a spotted fever group rickettsia named *Rickettsia africae* and transmitted by *Amblyomma* spp. (9). Our culture conditions are maybe not optimal for some strains of *R. conorii*, and study of improved methods may increase the isolation success rate.

Detection of *R. conorii* by PCR amplification of a 650-bp portion of the 190-kDa antigen gene was positive only for shell vial supernatants of fresh positive cultures. Freezing or freeze-thawing of other samples may have affected the rickettsial DNA; however, *R. rickettsii* and *Rickettsia tsutsugamushi* were previously successfully detected by using similar methodologies (6, 23). Our poor results could be related to the greater size of the fragment amplified: 650 bp in this study compared with 246 bp for *R. rickettsii* and 78 bp for *R. tsutsugamushi*; smaller fragments are more likely to remain intact as PCR templates in pathological specimens. Furthermore, in both the other studies, PCR amplification was performed on blood clots rather than on sera, where bacteria are maybe more numerous. We now carry out PCR amplification on the same supernatant that is inoculated onto shell vials. As endothelial cells are concentrated in the buffy coat, which is included in this supernatant, rather than in serum, this supernatant should be a more suitable specimen for the detection of *R. conorii*. Preliminary results obtained by using this assay are encouraging, but further investigations are still required.

Our study indicates that the evaluated procedures, with the exception of the PCR-based detection method, are helpful in the early diagnosis of rickettsial spotted fevers. Detection of bacteria in CEC is a method suitable for use in all routine laboratories, whereas the isolation of *R. conorii* using the shell vial technique requires cell culture and biohazard facilities. Physicians should be asked to collect samples for these assays prior to antibiotic treatement, as early in the disease as possible, as immediate diagnosis is necessary for optimal therapy in cases of severe forms. Detection of *R. conorii* also remains useful for delayed diagnosis. Samples for culture should be inoculated as soon as possible after collection in order to increase sensitivity of the centrifugation-shell vials procedure.

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