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Serological Cross-Reactions between *Bartonella quintana*, *Bartonella henselae*, and *Coxiella burnetii*

BERNARD LA SCOLA AND DIDIER RAOULT*

Unité des Rickettsies, Faculté de Médecine, Centre National de la Recherche Scientifique, 13385 Marseille Cedex 05, France

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The clinical manifestations of Q fever and bartonelloses can be confused, especially in cases of infectious endocarditis. Differential diagnosis of the diseases is important because the treatments required for Q fever and bartonelloses are different. Laboratory confirmation of a suspected case of either Q fever or bartonelloses is most commonly made by antibody estimation with an indirect immunofluorescence assay. With an indirect immunofluorescence assay, 258 serum samples from patients with Q fever were tested against *Bartonella henselae* and *Bartonella quintana* antigens, and 77 serum samples from patients with infection by *Bartonella* sp. were tested against *Coxiella burnetii* antigen. Cross-reactivity was observed: more than 50% of the chronic Q fever patients tested had antibodies which reacted against *B. henselae* antigen to a significant level. This cross-reaction was confirmed by a cross-adsorption study and protein immunoblotting. However, because the levels of specific antibody titers in cases of *Bartonella* endocarditis are typically extremely high, low-level cross-reaction between *C. burnetii* antibodies and *B. henselae* antigen in cases of Q fever endocarditis should not lead to misdiagnosis, provided serology testing for both agents is performed.

Coxiella burnetii, is the etiologic agent of Q fever. The most common reservoirs of *C. burnetii* are cattle, sheep, and goats (1), but infections after close contact with cats have also been described (18). Human infection occurs after inhalation of contaminated aerosols or ingestion of untreated milk or cheese. In acute cases, when infection is symptomatic, the most common clinical syndromes are self-limited febrile illness, granulomatous hepatitis, pneumonia, and meningoencephalitis (34). In a recent study, 20% of hospitalized Q fever patients presented with maculopapular or purpuric eruptions (34). In fact the clinical picture of Q fever can resemble that of nearly any infectious disease. The main diagnostic indicator for the disease is the epidemiological circumstance under which it is acquired, because most patients report contact with an animal reservoir. The most common form of chronic Q fever is endocarditis (3), which accounts for 60 to 70% of the cases. Chronic cases of endocarditis are usually diagnosed in patients with previous valvular heart disease and/or an immunosuppressive condition, such as cancer, lymphoma, transplantation, or chronic renal insufficiency. The clinical presentation is atypical; infected patients are already known to have valvulopathy and presenting with progressively altering clinical conditions, including unexplained infectious or inflammatory syndrome, hepatomegaly or splenomegaly, or worsening valvular dysfunction. It is only in long-delay-evolution forms that the clinical presentation is of a negative-blood-culture endocarditis. Patients with Q fever endocarditis often have cutaneous signs, and an isolated purpuric rash has been described as the only clinical manifestation in some cases (3). Infection of a vascular aneurysm or prosthesis and osteomyelitis are other common clinical manifestations of chronic Q fever (26).

Bartonella henselae and *Bartonella quintana* are fastidious, slowly growing organisms. *B. quintana* is the etiologic agent of trench fever, a louse-borne disease which was extensively described during World War I and World War II (22) and which is now being reencountered among the inner city homeless and alcoholics. *B. henselae* is responsible for cat scratch disease (CSD), bacillary angiomatosis, and peliosis hepatitis in AIDS patients (4). *B. quintana* has now been associated with bacillary angiomatosis (17, 36), and both species have been associated with other less-specific clinical syndromes, such as self-limited febrile illness (29), bacteremia (20, 30), endocarditis (6, 12, 31, 32), meningitis (20), and neurologic disorders (13, 33), in either immunocompromised or immunocompetent patients. It is likely that *Bartonella* infections are directly transmitted, but arthropod vectors have also been implicated. Indeed, the human body louse (*Pediculus humanus*) has been associated with the transmission of *B. quintana* infections, and the presence of *B. henselae* in cat fleas (*Ctenocephalides felis*) has been demonstrated (16). The domestic cat has been demonstrated to be a reservoir for *B. henselae*. Surveys of domestic cats in the United States have detected the bacterium in up to 40% of animals tested (28). Cats are thought to infect humans either directly through scratches, bites, or licks or indirectly via an arthropod vector. Thus, as well as presenting with indistinguishable clinical manifestations, patients infected by *C. burnetii* or *B. henselae* may have experienced a history of close contacts with animals, especially cats.

Laboratory diagnosis of both diseases is most commonly performed by serology, and cross-reaction between *C. burnetii* antibodies and *B. quintana* antigen was first noted over 20 years ago (5). Our aim was to fully evaluate the cross-reactivity of *B. henselae* and *B. quintana* serology with *C. burnetii* serology in the context of a modern immunofluorescent antibody test. To achieve this aim, we performed all three serological determinations with patients with confirmed acute Q fever or chronic Q fever and with *Bartonella*-related CSD or endocarditis.

* Corresponding author. Mailing address: Unité des Rickettsies, CNRS: EP J 0054, Faculté de Médecine, 27 Boulevard Jean Moulin, 13385 Marseille Cedex 05, France. Phone: 33.91.38.55.17. Fax: 33.91 .83.03.90. Electronic mail address: RAOULT@pacwan.mm-soft.fr.

MATERIALS AND METHODS

Serum selection. In its role as the French National Reference Center for Rickettsial Diseases, our laboratory routinely performs serological diagnostic tests for *Bartonella* and *C. burnetii* infections with sera received from all regions

of France and often from abroad. All of the 335 serum samples positive for *C. burnetii*, *B. henselae*, or *B. quintana* used in this study had been submitted to our center for routine testing.

The criteria used for serological diagnosis of Q fever were those established in a previous large-scale study (35). An immunoglobulin G (IgG) anti-phase I titer of ≥1:800 and an IgA anti-phase I titer of ≥1:25 were considered diagnostic for chronic Q fever. An IgG anti-phase II titer of ≥1:200 and an IgM anti-phase II titer of ≥1:50 were considered indicative of acute Q fever. The criteria for serological diagnosis of acute Q fever had a specificity and positive predictive value of 100%. The criteria for serological diagnosis of chronic Q fever had a specificity of 100% and a positive predictive value of 98.1%. A diagnosis of CSD was considered for patients with titers of ≥1:100 of antibody against *B. henselae* antigen. This cutoff had been previously determined and had a positive predictive value of 68.2% (27). A diagnosis of *Bartonella* endocarditis was considered for patients with titers of ≥1:1,600 of antibody against *B. henselae* or *B. quintana* antigens. Among 9,800 serum samples prospectively tested in our laboratory, 125 from 26 patients yielded titers of ≥1:1,600; of these patients, 22 suffered *Bartonella* endocarditis, according to the Duke endocarditis service criteria (8), with positive culture or PCR amplification with blood or valvular tissue (unpublished data). This assay had a positive predictive value of 88.4%.

Paired serum samples from 30 patients with chronic Q fever were tested: the first specimen was collected before treatment, and the second was collected 1 year after initiation of treatment. All patients had antibody levels indicative of chronic Q fever. Twenty-eight patients were diagnosed as having infectious endocarditis. *C. burnetii* was isolated from 10 of the 18 patients whose samples were inoculated onto the appropriate media prior to antibiotic therapy. No blood samples collected after 1 year of treatment yielded cultures. An additional 198 convalescent-phase serum samples from 198 patients who had been serologically diagnosed as having acute Q fever were tested. All patients had clinical manifestations compatible with acute Q fever. *C. burnetii* was isolated from 4 of 26 patients whose blood samples were inoculated onto the appropriate media prior to antibiotic therapy. Nineteen serum samples from 19 patients with *Bartonella* endocarditis were tested. The 19 patients were diagnosed as having endocarditis by the Duke endocarditis service criteria and by demonstration of titers of ≥1:1,600 of antibody against *B. henselae* or *B. quintana*. *Bartonella* spp. were isolated from 5 of the 14 patients whose blood samples were inoculated onto the appropriate media prior to antibiotic therapy and from the excised valves of two others. DNA specific for *B. henselae* or *B. quintana* was demonstrated in the blood or excised valves of an additional six patients by PCR amplification, followed by determination of amplicon nucleotide base sequence (15). Fifty-eight serum samples from 58 patients with antibody levels indicative of *B. henselae* CSD and with a local adenopathy associated with a cat scratch were also tested. An additional 258 serum samples from volunteer healthy blood donors, matched to the population of Q fever patients by age, sex, and geographic origin, were randomly selected and tested by indirect immunofluorescence assay (IFA) for anti-*B. henselae* and anti-*B. quintana* antibodies.

Serologic procedure. All procedures for *C. burnetii* (35) and *B. henselae* and *B. quintana* (6, 21) have been described previously. The phase II *C. burnetii* Nine Mile strain (ATCC VR 615) was grown in confluent layers of L929 mouse fibroblasts in 150-cm^2 culture flasks. Infection was monitored by Gimenez staining (10). When 90% of the cells were judged to be infected, cell layers and supernatants were harvested, pelleted by centrifugation (10,000 × *g* for 10 min), and resuspended in 15 ml of phosphate-buffered saline (PBS [pH 7.3]). All further steps were conducted at 4°C. Cells were lysed by sonication, and then debris was removed by two successive centrifugations (100 × *g* for 10 min each). The supernatant was then added to 20 ml of PBS with 25% sucrose and centrifuged (6,000 × *g* for 30 min). The resulting pellet was washed three times in PBS (6,000 × *g* for 10 min), pelleted by centrifugation (10,000 × *g* for 10 min), and then resuspended in 1 ml of PBS with 0.1% formaldehyde. The suspension was repelleted by centrifugation (6,000 × *g* for 10 min), resuspended in the smallest possible volume of sterile distilled water, and adjusted to 2 mg/ml spectrophotometrically. To reactivate phase I *C. burnetii* mice were inoculated with the phase II *C. burnetii* Nine Mile strain. Ten days after infection, spleens were removed, ground in 7.5 ml of minimal essential medium, and inoculated into three 75-cm^2 culture flasks containing L929 cell monolayers (2.5 ml per flask). The infected cells were then harvested, and the bacteria were purified as described above. *B. henselae* Oklahoma, provided by D. F. Welch (University of Oklahoma), and *B. quintana* Houston (ATCC 49882) were propagated in 150-cm^2 culture flasks containing ECV 304 human endothelial cell monolayers. After 2 weeks, the supernatant was pelleted by centrifugation (10,000 × *g* for 10 min) and resuspended in 15 ml of PBS. The bacteria were then purified as described above. All antigens prepared in this manner were frozen at −20°C for further immunofluorescence tests. Each of the four antigens was applied with a drawing nib to the wells of a 30-well microscope slide (Dynatech Laboratories Ltd., Billingshurt, United Kingdom), air dried, and fixed in acetone for 10 min. Sera were serially diluted (twofold dilutions from 1:25) in PBS with 3% nonfat powdered milk and then were applied to the antigen-containing wells. The slides were incubated in a moist chamber for 30 min at 37°C and then were washed three times in PBS (10 min each wash). The slides were dried, and then each well was overlaid with 1:300-diluted fluorescein isothiocyanate-conjugated goat anti-human IgG(γ) (Fluoline G; Biomerieux, Marcy l'Etoile, France). Wells with *C. burnetii* antigens were overlaid with anti-human IgA (Fluoline A; Biomerieux) or anti-human IgM (Fluoline M; Biomerieux). Incubation, washes, and drying were performed as described above. The slides were mounted in buffered glycerol (Fluoprep; Biomerieux) and observed with a Zeiss epifluorescence microscope at ×400 magnification. In order to remove IgG, rheumatoid factor adsorbant (Behring, Marburg, Germany) was used prior to IgM and IgA determination according to the manufacturer's instructions.

Serum cross-adsorption. Four serum samples from chronic Q fever patients with high levels of cross-reacting antibodies against *B. henselae* were studied. The sera were adsorbed with *B. henselae* antigen and with a mixture of phase I and phase II *C. burnetii* antigens. Sera were diluted to 1/10 in an antigen suspension previously adjusted to 2 mg/ml. The mixture was shaken for 24 h at room temperature and then centrifuged at 10,000 × *g* for 10 min. The supernatant was retained and treated a further two times in the same manner. *C. burnetii* and *B. henselae* serology testing and Western blot (immunoblot) analysis were performed for all supernatants. Microimmunofluorescence was performed with total Igs (Fluoline H; Biomerieux).

Enzyme treatment. Proteinic antigens were removed prior to immunoblotting (11) by incubation of a suspension of *B. henselae* cells with solutions of proteinase K (2 mg/ml) (Boehringer, Mannheim, Germany) and 5% sodium dodecyl sulfate (SDS) at a ratio of 4:1:2. The mixture was held for 1 h at 37°C, a further volume of proteinase K solution was added, and the mixture was then reincubated for a further 1 h at 37°C.

Western blot analysis. *C. burnetii* phase I and phase II, *B. henselae*, and *B. quintana* cells were suspended in sterile distilled water and adjusted to 2 mg/ml spectrophotometrically. One volume of antigen was mixed with 1 volume of 1× Laemmli solubilizer (19), and the mixture was boiled for 30 min. Ten-microliter aliquots of this preparation or proteinase K-treated cells were electrophoresed in polyacrylamide gels. SDS-polyacrylamide gel electrophoresis was performed with a 12% polyacrylamide separating gel with a 4% polyacrylamide stacking gel within a Mini-Protean II cell apparatus (Bio-Rad, Richmond, Calif.). The gels were electrophoresed at 20 mA for 1 h. A low-range molecular weight standard (Bio-Rad) was used to estimate the molecular weight of the separated antigens. The resolved antigens were electroblotted in a transblot cell onto a nitrocellulose membrane at 50 V for 3 h at 4°C. The blots were blocked overnight at room temperature with 5% nonfat dry milk in TBS (20 mM Tris-HCl [pH 7.5], 500 mM NaCl, 0.1% thimerosal [Merthiolate]) and then washed with distilled water. Sera, diluted to 1:200 in 3% nonfat dry milk in TBS, were overlaid onto the blots and incubated overnight at room temperature. The blots were then washed in TBS three times for 10 min and incubated for 1 h in peroxidase-conjugated goat anti-human IgG and IgM (Diagnostics Pasteur, Marnes-la-coquette, France) diluted at 1:100 in 3% nonfat dry milk in TBS. Blots were again washed three times in TBS. Bound conjugate was detected by colorimetric reaction (0.015% 4-chloro-1-naphthol–0.015% hydrogen peroxide in 16.7% methanol in TBS) for 15 min. Western blot analysis was performed both before and after cross-adsorption.

RESULTS

Seroprevalence for antibodies against *B. henselae* and *B. quintana* from the pool of 258 healthy blood donors. Of 258 blood donor serum samples tested, 3 (1.2%) demonstrated an anti-*B. quintana* antibody titer of 1:50, and 1 (0.4%) demonstrated an anti-*B. henselae* antibody titer of 1:50.

Correlation between *B. henselae*, *B. quintana*, and *C. burnetii* serology. Of the 198 serum samples tested from patients with acute Q fever, 1 was found positive for *B. henselae* (IgG, 1:100) and 1 was found positive for *B. quintana* (IgG, 1:200). Blood cultures from both of these patients remained sterile. Of the 30 serum samples collected from patients with chronic Q fever before treatment (Fig. 1 and 2), 12 (40%) yielded antibodies to *B. henselae* at significant levels (6 serum samples with IgG titer of 1:100 and 6 with IgG titer of ≥200). Infection in seven of these patients was culture confirmed. Seven (23.3%) serum samples were found positive for *B. quintana* with significant antibody levels (four serum samples with IgG titer of 1:100 and three with IgG titer of ≥200). Infection in six of these patients was culture confirmed. Ten of the 12 patients with cross-reacting antibodies to *B. henselae* antigen had endocarditis, and 6 of the 7 patients with cross-reacting antibodies to *B. quintana* had endocarditis. Of the 30 serum samples collected from patients with chronic Q fever after 1 year of treatment (Fig. 3 and 4), 19 (63.3%) were found positive for *B. henselae* with significant antibody levels (6 serum samples with IgG titer of 1:100 and 13 with IgG titer of ≥200), and seven (23.3%) were found to be positive for *B. quintana* with significant antibody levels (5 se-

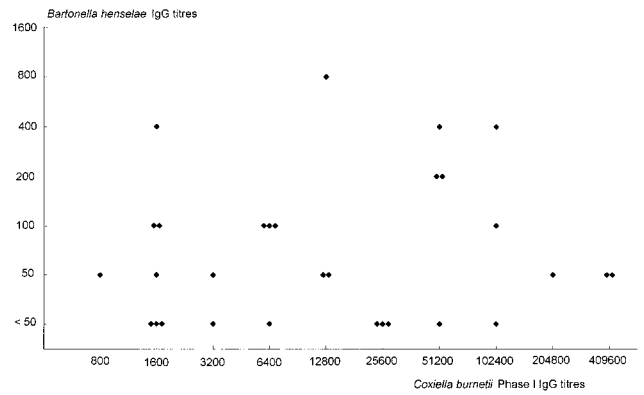

FIG. 1. *C. burnetii* phase I IgG titers and *B. henselae* IgG titers for patients with chronic Q fever before treatment.

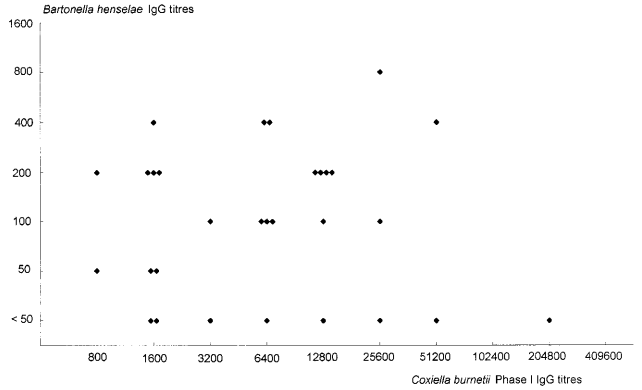

FIG. 3. *C. burnetii* phase I IgG titers and *B. henselae* IgG titers for patients with chronic Q fever after treatment.

rum samples with IgG titer of 1:100 and 2 with IgG titer of ≥200). Within this group, levels of antibodies against *B. henselae* and *B. quintana* were between 1:100 and 1:800 for IgG in all but two serum samples. The two serum samples excepted had an anti-*B. quintana* IgG titer of 1:1,600. One of these serum samples was collected from a patient with culture-confirmed *C. burnetii* infection. Of the 19 serum samples collected from patients with *Bartonella* endocarditis, 1 reacted strongly with *C. burnetii* antigen with significant antibody levels (IgG phase II titer of 1:400 and phase I titer of 1:100). This specimen was collected from a patient whose excised valve contained PCR-detected *B. henselae* DNA. Two other patients yielded lower anti-*C. burnetii* titers (IgG phase II titers of 1:100 and 1:50, respectively). Of the 58 serum samples collected from patients with CSD, none had diagnostic antibody titers for *C. burnetii*, although 8 yielded lower-titer reactions (one with IgG phase II titer of 1:100 and 7 with IgG phase II titer of 1:50).

Serological cross-adsorption. Cross-adsorption of the four serum samples studied resulted in the disappearance of homologous and heterologous antibodies when adsorption was performed with *C. burnetii* antigens, but the disappearance of only homologous antibodies when adsorption was performed with *B. henselae* (Table 1).

Western blot analysis. Western blot analysis performed with the four adsorbed serum samples yielded several common bands (Fig. 5) and confirmed cross-adsorption results with microimmunofluorescence. After treatment with proteinase K, no bands were observed.

DISCUSSION

C. burnetii is a small gram-negative bacterium that multiplies exclusively in eukaryotic cells. In cell cultures or embryonated eggs, outer membrane polysaccharides undergo an antigenic shift called phase variation. When isolated from animals, *C. burnetii* exists in the phase I form, which is extremely infectious; subculture results in a shift to the phase II form, which is not infectious (26). Serological study of patients with *C. burnetii* infections demonstrates antibodies directed against both phase variants.

The most striking characteristic of Q fever is its clinical polymorphism. In most cases, infection is asymptomatic (7); however, when infection is symptomatic, both acute and chronic forms have been described. Although methodologies for the isolation and culture of *C. burnetii* from both acute and chronic Q fever have been developed (24), laboratory diagnosis remains most commonly achieved by antibody estimation assays.

Bartonella spp. are small gram-negative rods which exist in association with or inside eukaryotic cells. As with *C. burnetii*, a broad spectrum of clinical manifestations are associated with *Bartonella* infection. *Bartonella* spp. can be isolated from clinical material with blood-rich media; however, plates may require several weeks of incubation before yielding colonies.

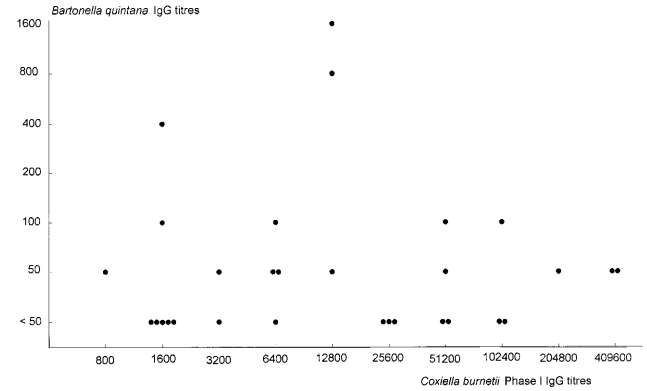

FIG. 2. *C. burnetii* phase I IgG titers and *B. quintana* IgG titers for patients with chronic Q fever before treatment.

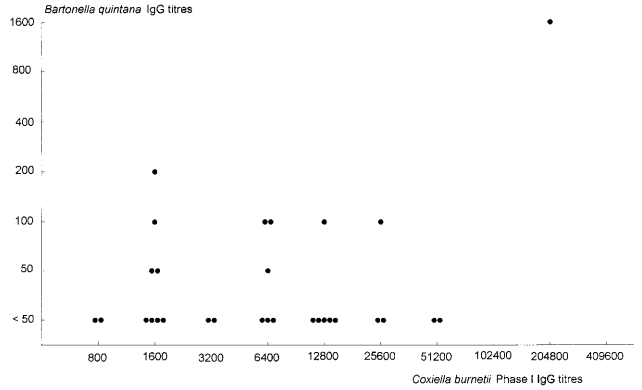

FIG. 4. *C. burnetii* phase I IgG titers and *B. quintana* IgG titers for patients with chronic Q fever before treatment.

TABLE 1. Total Ig levels of four serum samples with antibodies to both *C. burnetii* and *B. henselae* before adsorption, after adsorption with *B. henselae*, and after adsorption with *C. burnetii* phases I and II

Serum and organism	Total Ig level (titer)		
	Before adsorption	After adsorption with:	
		B. henselae	*C. burnetii* phases I and II
1			
C. burnetii	1:40,000	1:40,000	1:80
B. henselae	1:640	<1:20	1:40
2			
C. burnetii	1:40,000	1:40,000	<1:20
B. henselae	1:160	<1:20	<1:20
3			
C. burnetii	1:20,000	1:20,000	<1:20
B. henselae	1:640	<1:20	<1:20
4			
C. burnetii	1:80,000	1:80,000	<1:20
B. henselae	1:40	<1:20	<1:20

Bacteria have been most often isolated from blood samples and, more recently, from cutaneous tissue biopsies of infected patients (17). Although cell culture methods, which are more efficient than the use of axenic media, have been described, the laboratory diagnosis of *Bartonella* infections is still most frequently achieved with antibody estimation assays, most commonly the IFA.

The necessity of specialized culture methods for the isolation of these organisms has resulted in isolation rarely being attempted. Moreover, the required methods are generally only employed when other evidence of infection, usually serological, has been presented. That serological diagnosis is often retrospective, being made when antibiotic regimens have already been implemented, further confounds isolation attempts, the tests for which are most sensitive when specimens are collected early in the course of illness. The rate for both *Bartonella* spp. and *C. burnetii* therefore remains low, and the values obtained in this study are similar to those previously reported (24). Unfortunately, negative cultures are therefore not indicative of the absence of infecting *Bartonella* spp. or *C. burnetii*, and thus other methods of laboratory diagnosis are required. Serological assays are presently the most frequently performed of these methods, usually in an IFA format. This method has long been applied to the diagnosis of *C. burnetii* infections, and its efficacy in the diagnosis of both acute and chronic cases of Q fever has been well evaluated (25, 35). Prior to this study, cross-reactions between *C. burnetii*-specific antibodies and other antigens have not been reported. Although cross-reactions between *Legionella pneumophila* and *C. burnetii* have been suspected, a study in which 154 Q fever serum samples were evaluated for anti-*C. burnetii* and anti-*L. pneumophila* antibodies by cross-adsorption and immunoblotting concluded that no serological cross-reaction between these bacteria occurred (9). Serological cross-reaction between *Bartonella* species and *Chlamydia* species has, however, been noted previously. In a report of three cases of *B. quintana* endocarditis, serological cross-reaction between *Chlamydia pneumoniae* and anti-*B. quintana* antisera was observed in all cases (6). These reactions were removed by cross-adsorption. In a report of a case of *B. henselae* endocarditis, the authors observed high levels of antibodies reacting against *Ehrlichia chaffeensis* and a positive titer of cytoplasmic staining antineutrophil cytoplasmic antibody, but no serological cross-adsorption was performed (14). A cross-reaction between *C. burnetii* and *B. quintana* was in fact noted in one serum sample over 20 years ago with a passive hemagglutination test (5). The author did not specify if this cross-reactivity was observed with serum of an acute or chronic Q fever patient.

In sera from patients with acute Q fever, reactivities with *B. henselae* (0.5%) and *B. quintana* (0.5%) are comparable to those of the seroprevalence of antibodies against these bacteria among matched blood donors, which are 0.4 and 1.2% for *B. henselae* and *B. quintana*, respectively. In contrast, we found that more than 50% of chronic Q fever patients have sera which react to a significant level with *B. henselae*, and more than 20% of these patients have sera which yielded significant titers of antibody against *B. quintana*. Both of these prevalences are higher than that observed in the normal population of the matched blood donors. Cross-adsorption studies indicated that reactivity with *B. henselae* resulted from cross-reac-

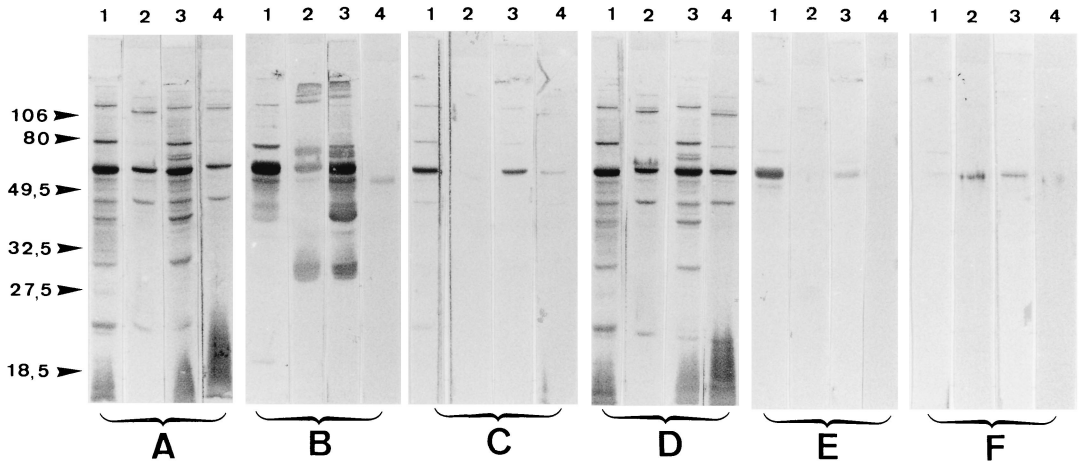

FIG. 5. Western blot of chronic Q fever patients (serum samples 1 to 4 [lanes 1 to 4, respectively]). (A and B) Untreated sera on *C. burnetii* antigens (A) and on *B. henselae* antigens (B). (C and E) Sera adsorbed with a mixture of phase I and II *C. burnetii* antigens on *C. burnetii* antigens (C) and on *B. henselae* antigens (E). (D and F) Sera adsorbed with *B. henselae* antigens on *C. burnetii* antigens (D) and on *B. henselae* antigens (F). Molecular masses (kilodaltons) are given to the left.

tion with *C. burnetii* antibodies, because prior adsorption of these sera with *C. burnetii* removed reactivity. Adsorption of cross-reacting sera with *B. henselae* only removed reactivity against *B. henselae*. These results were confirmed by both microimmunofluorescence and Western blot analysis. In this study, Western blot analysis performed before and after treatment of bacteria by proteinase K indicated that the cross-reaction was probably due to proteinic antigens. The Western blotting method used was similar to that previously described (2).

Serological cross-reactions between *Coxiella* and *Bartonella* species are important to identify, because patients infected by these bacteria may have identical clinical presentations, most notably infectious endocarditis. Because these microorganisms cannot be cultivated by standard blood culture procedures, they must be detected by specific culture methods or serology. These tests should be implemented in all cases of blood culture-negative endocarditis encountered. Although for both infections, doxycycline is the most commonly used antibiotic, the prescription regimens are significantly different. In patients with *Bartonella* endocarditis, this antibiotic regimen should be used for only 4 weeks, and furthermore, recent studies have shown that combination of doxycycline with aminoglycosides may be beneficial (6). Indeed, only aminoglycosides appear to have a bactericidal effect on *B. henselae* (23). For patients with Q fever endocarditis, doxycycline must be prescribed for at least 3 years, in association with fluoroquinolone (aminoglycosides are inactive) (26). IFAs for *C. burnetii* and *B. henselae* or *B. quintana* are all therefore necessary when any of these bacteria are suspected as being etiologic agents. When all serological tests are performed, the very high titers of antibody against *C. burnetii*, compared with the low titers of cross-reacting antibodies against *B. henselae* in patients with Q fever endocarditis, should not present a problem with interpretation.

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