

Serodiagnosis of *Bartonella bacilliformis* Infection by Indirect Fluorescence Antibody Assay: Test Development and Application to a Population in an Area of Bartonellosis Endemicity

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***Bartonella bacilliformis* causes bartonellosis, a potentially life-threatening emerging infectious disease seen in the Andes Mountains of South America. There are no generally accepted serologic tests to confirm the disease. We developed an indirect fluorescence antibody (IFA) test for the detection of antibodies to *B. bacilliformis* and then tested its performance as an aid in the diagnosis of acute bartonellosis. The IFA is 82% sensitive in detecting *B. bacilliformis* antibodies in acute-phase blood samples of laboratory-confirmed bartonellosis patients. When used to examine convalescent-phase sera, the IFA is positive in 93% of bartonellosis cases. The positive predictive value of the test is 89% in an area of Peru where *B. bacilliformis* is endemic and where the point prevalence of infection is 45%.**

Bartonella bacilliformis causes bartonellosis, an illness that is currently limited to high-altitude valleys of the Andes Mountains of Peru, Columbia, and Ecuador. It is one of several members of the genus *Bartonella*, along with *B. elizabethae*, *B. henselae*, and *B. quintana*, that are known to cause severe illness in humans. Bartonellosis is typically characterized by an acute phase of fever and hemolytic anemia followed by a second phase of cutaneous vascular lesions called “verruca peruana” (11). Recently there have been increases in the number of reported cases from areas where bartonellosis is endemic, along with an emergence of the disease in new locations and as a threat to travelers (1, 5, 6, 12). This increasing disease burden, coupled with the recognition of other *Bartonella* spp. as emerging pathogens of animals and humans, makes the study of South American bartonellosis increasingly important (4, 5, 10, 14).

Though bartonellosis has been recognized since pre-Columbian times, diagnosis remains problematic and is usually based on clinical impression and the demonstration of the intraerythrocytic bacilli on a Giemsa- or Wright-stained thin blood smear (3). The sensitivity of the thin-smear procedure has been shown to be only 36% (5). Culture of *B. bacilliformis* is difficult, requiring special media and techniques with up to an 8-week incubation time. There have been no generally accepted serologic assays available to confirm clinical suspicion of the disease or to conduct seroepidemiologic surveys of exposed populations. Crude-extract antigens, whole-cell antigens, and protein antigens have been used for the immunodiagnosis of bartonellosis (7, 9). Knobloch et al. (8) identified and prepared protein antigens of *B. bacilliformis* to overcome problems with nonspe-

cific reactivity associated with the crude-extract and whole-cell antigen preparations. However, data on the sensitivity and specificity of these antigens for diagnostic testing have not been published.

We developed an indirect fluorescence antibody (IFA) test that uses an irradiated whole-cell antigen preparation cocultivated with Vero cells. Using this method of antigen preparation, the same IFA technique was previously found to be 88% sensitive and 95% specific for the serodiagnosis of another member of the genus *Bartonella*, *B. henselae*, which causes cat-scratch disease (15). This paper describes the development of an IFA test for *B. bacilliformis* and the subsequent performance of the test as an aid in the diagnosis of acute bartonellosis and as a diagnostic tool for epidemiologic surveys.

Antigen preparation. Two strains of *B. bacilliformis*, a Peruvian isolate from an area of Peru where bartonellosis is endemic (CON600-01) and an American Type Culture Collection isolate (ATCC 35685), were each cocultivated with Vero cells, to which individual *Bartonella* organisms readily adhere. A T-150 flask of Vero cells was inoculated with approximately 10⁶ to 10⁷ agar-grown *B. bacilliformis* organisms. The medium used was minimum essential medium supplemented with 10% fetal calf serum, 10 mM HEPES, 10 mM nonessential amino acids, and 2 mM L-glutamine. The cells and bacteria were incubated at 28°C in a sealed flask without additional CO₂ and harvested on day 3 postinoculation. At harvest, all but 2 ml of the medium was removed from the flask, and a sample of sterile glass beads was introduced and gently rocked to remove the Vero cell monolayer. The *Bartonella*-infected Vero cells were subsequently inactivated by gamma irradiation and frozen as single-use 0.2-ml aliquots at –70°C. Drops of the bacterial suspension were mounted on slides, air dried, fixed in acetone for 15 min, and, if not used immediately, stored at –70°C. Since the Peruvian isolate demonstrated higher antibody titers than the ATCC 35685 strain, it was used as the antigen in all tests.

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IFA test. The IFA test was performed with twofold serum dilution steps using standard techniques (15). Fluorescein-labeled affinity-purified antibody to human immunoglobulin G (heavy plus light chains) (Kirkegaard & Perry, Gaithersburg, Md.) served as the conjugate in all tests. Incubation periods were for 30 min at 37°C. Slides were read using a 40× objective, 10× oculars, and a UV epifluorescence microscope (Olympus Optical Company LTD, Tokyo, Japan). The IFA test was scored by observing definite fluorescence of intact *Bartonella* bacilli, which is the standard technique for IFA testing.

During test development, sera from 33 confirmed bartonellosis patients were evaluated using the IFA test. Confirmation of diagnosis was based on a positive blood culture or at least 10% of red blood cells being infected with *B. bacilliformis* on a Giemsa-stained thin blood smear. Sera from 101 healthy controls (obtained from Centers for Disease Control and Prevention, Atlanta, Ga.) were also tested. Results of these tests were used to establish the test characteristics (sensitivity and specificity). In addition, sera from patients with diseases other than bartonellosis were assessed for cross-reactive antibodies to *B. bacilliformis*. Serum samples from two patients each with cat-scratch disease, Lyme disease, typhoid fever, brucellosis, leptospirosis, secondary syphilis, dengue, or ehrlichiosis were tested. Serum samples from patients with *Chlamydia* infections were not available for testing. Sera drawn from 14 bartonellosis patients were also tested using antigens from four different *Bartonella* species *B. quintana*, *B. henselae*, *B. elizabethae*, and rodent *Bartonella* Sh7768GA variant C2 (a strain isolated from *Sigmodon hispidus* in Georgia). As determined in previous studies, the 1/64 serum dilution end point was used as a positive cutoff value for testing these antigens (8, 13).

Epidemiologic investigation. Study sites selected by Ministry of Health officials as being representative of areas of long-established bartonellosis endemicity were established in villages near Caraz City, Ancash, Peru (approximately 475 km northeast of Lima) in order to evaluate the usefulness of the IFA test as an aid in diagnosing bartonellosis cases. Community volunteers were asked to participate in a 2-year follow-up study designed to determine disease burden and risk factors for infection. Serum samples were obtained from 387 community volunteers and were used to estimate the point prevalence of infection in February 1998.

In addition, patients at Caraz Hospital between June 1997 and January 2000 presenting with clinical bartonellosis were asked to donate blood for culture or PCR, serology, and a thin blood smear. Sera from 106 bartonellosis patients who met the case definition of having slide-positive, PCR-positive, or culture-confirmed *B. bacilliformis* infections were examined in this prospective application of the test. Blood was cultured in sealed flasks using a modified F-1 medium (agarose with 10% sheep blood) with a liquid overlay of RPMI with 10% fetal bovine serum. Cultures were observed for 8 weeks at 28°C without additional CO₂. PCR was performed on blood and culture isolates to amplify a portion of the citrate synthase gene using standard techniques (13). PCR products from samples yielding positive PCR results were sequenced for identification.

Informed consent was obtained from patients and community volunteers or their guardians, and human experimentation guidelines of the U.S. Department of Health and Human Services and the Uniformed Services University of the Health Sciences were followed.

Preliminary test development. Twenty-eight of 33 patients with laboratory-confirmed bartonellosis (85%) had titers of $\geq 1/256$ (Table 1). Titers ranged from 1/32 to 1/1,024. Paired

TABLE 1. IFA test results

Study group	No. positive/ no. tested	% Positive
Healthy controls	8/101	8
Controls with infections other than bartonellosis	2/16	12.5
Community volunteers from an area of <i>Bartonella</i> endemicity	175/387	45
Bartonellosis patients—test development phase	28/33	85
Bartonellosis patients—application phase	86/106	81

convalescent-phase sera were available for three of the five patients with initial titers of $<1/256$, and there was a fourfold rise in titer in all three. Of the 101 healthy control sera, 93 (92%) had titers of $\leq 1/128$. Figure 1 shows the distribution of *B. bacilliformis*-specific antibodies among healthy controls and patients with bartonellosis. Since the goals of this test are to aid in the diagnosis of patients with suspected bartonellosis and to serologically rule out the disease in epidemiologic surveys, using the 1/256 serum dilution end point as a positive cutoff value was chosen to yield an optimal combination of both sensitivity and specificity.

In the group of controls with infections other than bartonellosis, one of two patients with high IFA titers for cat-scratch disease also had a titer of 1/512 for *B. bacilliformis* antibodies, and one of two patients with secondary syphilis had a titer of 1/256. Sera from patients with Lyme disease, typhoid fever, brucellosis, leptospirosis, dengue, or ehrlichiosis were all seronegative, with titers of $<1/256$.

When other *Bartonella* spp. antigens were tested with sera from bartonellosis patients, positive antibody titers were observed in 1 of 14 sera tested with the *B. henselae* antigen, 2 of 14 sera tested with the *B. quintana* antigen, 7 of 14 sera tested with the *B. elizabethae* antigen, and 5 of 14 sera tested with the rodent *Bartonella* antigen.

Point prevalence of infection. Of 387 volunteers from an area of bartonella endemicity in Peru, 175 (45%) were found to be seropositive for *B. bacilliformis* antibodies by the IFA test. Seventy-four percent of volunteers who had a history of bartonellosis within the last year had a positive IFA test, while 39% of people with a more distant or negative history of bartonellosis had a positive IFA test (prevalence risk ratio = 3.7; 95% confidence interval, 1.9 to 6.9).

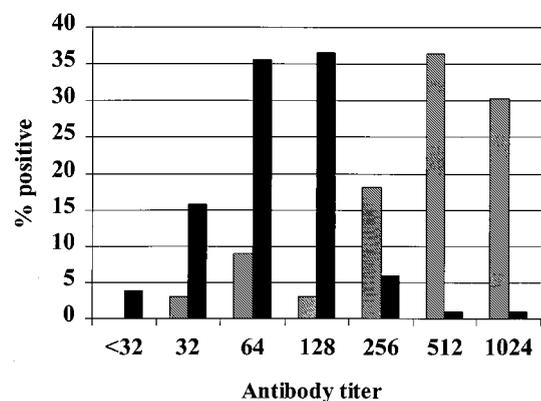


FIG. 1. Distribution of *B. bacilliformis* antibodies among patients with bartonellosis (▨; n = 33) and healthy controls (■; n = 101).

Prospective application of the IFA test in acute bartonellosis patients. Eighty-six of 106 patients (81%) with laboratory-confirmed bartonellosis were initially seropositive for *B. bacilliformis*, with titers of 1/256 or higher. Convalescent-phase sera were available for 11 of the 20 patients whose acute-phase sera tested negative, and there was at least a fourfold rise in titer in 10 of them (91%). One patient's acute-phase serum had a titer of 1/128, and the paired convalescent-phase serum was positive at 1/256. However, since this represented only a twofold rise in titer, it was classified as a negative test.

Bartonellosis is a very common infection in certain populations living in high-altitude valleys of the Andes Mountains. Our finding of a 45% point prevalence of antibodies to *B. bacilliformis* using an IFA test demonstrates the significance of this disease. The IFA test has been used for many years in diagnostic laboratories and provides a relatively simple method to detect antibodies to a wide variety of pathogens. Because only a small amount of antigen is needed for each test, the IFA test provides an economical serologic assay, an important consideration for use in Peru. Furthermore, *B. bacilliformis* is a fastidious, slow-growing bacterium that cannot be cultivated using standard operating protocols found in many clinical laboratories. Thus, the Giemsa-stained peripheral blood smear is the only widely available method for confirming a diagnosis. However, the thin smear has not been found to be a sensitive assay for the presence of *Bartonella* (3). Given the potentially high fatality rate of this illness, serologic assays to provide a more timely diagnosis of bartonellosis are needed. Our study demonstrates the first successful development and application of an IFA test for this disease.

During IFA test development, 85% of patients with laboratory-confirmed bartonellosis had positive titers of antibody to *B. bacilliformis* in serum on initial testing. Similarly, during the prospective application of this test, 81% of acute bartonellosis patients had positive titers in serum. Combining the results of these two groups of confirmed cases, the sensitivity was shown to be 82%. When convalescent-phase specimens were available, this serologic test was positive in 93% (13 of 14) of bartonellosis cases, confirming the value of this IFA test as a clinical diagnostic screening tool.

In our experience, the IFA for *Bartonella* antibodies is genus specific (does not react with patient serum from other well-characterized diseases). In this study, 92% of negative-control sera were seronegative for *B. bacilliformis* antibodies, indicating that the specificity of the IFA is high enough to be useful in epidemiologic community surveys. The significance of one patient with secondary syphilis having a low false-positive antibody titer for *B. bacilliformis* remains to be determined. Likewise, although our anecdotal finding of human antibodies in sera tested by the IFA assay using a rodent *Bartonella* antigen is of uncertain clinical significance, recent studies have shown that rodents are often infected with *Bartonella* species (2, 10). The finding does, however, emphasize the need to interpret any test result in the context of clinical and epidemiologic

information. Our study corroborates the findings that an IFA assay is a useful diagnostic test for selected *Bartonella* spp. Given a 45% prevalence rate, the positive predictive value of the IFA test is 89% in the acute stages of disease. Similarly, a negative test correctly excludes bartonellosis in 86% of patients.

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REFERENCES

1. Amano, Y., J. Rumbea, J. Knobloch, J. Olson, and M. Kron. 1997. Bartonellosis in Ecuador: serosurvey and current status of cutaneous verrucous disease. *Am. J. Trop. Med. Hyg.* **57**:174-179.
2. Birtles, R. J., J. Canales, P. Ventosilla, E. Alvarez, H. Guerra, A. Llanos-Cuentas, D. Raoult, N. Doshi, and T. G. Harrison. 1999. Survey of *Bartonella* species infecting intradomiciliary animals in the Huayllacallan Valley, Ancash, Peru, a region endemic for human bartonellosis. *Am. J. Trop. Med. Hyg.* **60**:799-805.
3. Caceres-Rios, H., J. Rodriguez-Tafur, F. Bravo-Puccio, C. Maguina-Vargas, C. S. Diaz, D. C. Ramos, and R. Patarca. 1995. Verruga peruana: an infectious endemic angiomatosis. *Crit. Rev. Oncog.* **6**:47-56.
4. Ellis, B., R. Regnery, L. Beati, F. Bacella, M. Rood, G. Glass, E. Marston, T. Ksiazek, D. Jones, and J. Childs. 1999. Rats of the genus *Rattus* are reservoir hosts for pathogenic *Bartonella* species: an old world origin for a new world disease? *J. Infect. Dis.* **180**:220-224.
5. Ellis, B., L. Rotz, J. Leake, F. Samalvides, J. Bernable, G. Ventura, C. Padilla, P. Villaseca, L. Beati, R. Regnery, J. Childs, J. Olson, and C. Carrillo. 1999. An outbreak of acute bartonellosis (Oroya fever) in the Urubamba region of Peru, 1998. *Am. J. Trop. Med. Hyg.* **61**:344-349.
6. Gray, G., A. Johnson, S. Thornton, W. Smith, J. Knobloch, P. Kelley, L. Obregon Escudero, M. Arones Huayda, and F. Wignall. 1990. An epidemic of Oroya fever in the Peruvian Andes. *Am. J. Trop. Med. Hyg.* **42**:215-221.
7. Knobloch, J. 1988. Analysis and preparation of *Bartonella bacilliformis* antigens. *Am. J. Trop. Med. Hyg.* **39**:173-178.
8. Knobloch, J., R. Bialek, G. Muller, and P. Asmus. 1988. Common surface epitope of *Bartonella bacilliformis* and *Chlamydia psittaci*. *Am. J. Trop. Med. Hyg.* **39**:427-433.
9. Knobloch, J., L. Solano, O. Alvarez, and E. Delgado. 1985. Antibodies to *Bartonella bacilliformis* as determined by fluorescence antibody test, indirect haemagglutination and ELISA. *Trop. Med. Parasitol.* **36**:183-185.
10. Kosoy, M., R. Regnery, T. Tzianabos, E. Marston, D. Jones, D. Green, G. Maupin, J. Olson, and J. Childs. 1997. Distribution, diversity, and host specificity of *Bartonella* in rodents from the southeastern United States. *Am. J. Trop. Med. Hyg.* **57**:578-588.
11. Laughlin, L. W. 2000. Bartonellosis, p. 394-398. In G. T. Strickland (ed.), *Hunter's tropical medicine and emerging infectious diseases*, 8th ed. W. B. Saunders Company, Philadelphia, Pa.
12. Matteelli, A., F. Castelli, A. Spinetti, F. Bonetti, S. Graifenberghi, and G. Carosi. 1994. Short report: verruga peruana in an Italian traveler from Peru. *Am. J. Trop. Med. Hyg.* **50**:143-144.
13. Norman, A. F., R. Regnery, P. Jameson, C. Greene, and D. C. Krause. 1995. Differentiation of *Bartonella*-like isolates at the species level by PCR-restriction fragment length polymorphism in the citrate synthase gene. *J. Clin. Microbiol.* **33**:1797-1803.
14. Regnery, R., J. Childs, and J. Koehler. 1995. Infections associated with *Bartonella* species in persons infected with human immunodeficiency virus. *Clin. Infect. Dis.* **21**:S94-S98.
15. Regnery, R., J. Olson, A. Bradley, and B. William. 1992. Serologic response to "*Rochalimaea henselae*" antigen in suspected cat-scratch disease. *Lancet* **339**:1443-1446.