JOURNAL OF CLINICAL MICROBIOLOGY, Dec. 1999, p. 4045–4047 Vol. 37, No. 12
0095-1137/99/$04.00+0

Identification of *Bartonella* Species Directly in Clinical Specimens by PCR-Restriction Fragment Length Polymorphism Analysis of a 16S rRNA Gene Fragment

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Received 18 June 1999/Returned for modification 3 August 1999/Accepted 24 August 1999

It is now established that two species of *Bartonella*, namely, *Bartonella henselae* and *B. quintana*, cause bacillary angiomatosis in human immunodeficiency virus-infected patients. In addition, *B. henselae* causes cat scratch disease and *B. quintana*, *B. henselae*, and *B. elizabethae* can cause bacteremia and endocarditis in immunocompetent persons. We have developed a PCR-restriction fragment length polymorphism-based assay for direct detection and identification to species level of *Bartonella* in clinical specimens. This is accomplished by PCR amplification of *Bartonella* DNA using primers derived from conserved regions of the gene carrying the 16S ribosomal DNA, followed by restriction analysis using *Dde*I and *Mse*I restriction endonucleases. We amplified a *Bartonella* genus-specific 296-bp fragment from 25 clinical samples obtained from 25 different individuals. Restriction analysis of amplicons showed that identical patterns were seen from digestion of *B. henselae* and *B. quintana* amplicons with *Dde*I, whereas a different unique pattern was seen by using the same enzyme with *B. vinsonii* and *B. elizabethae*. With *Mse*I digestion, *B. henselae* and *B. vinsonii* gave nearly identical patterns while *B. quintana* and *B. elizabethae* gave a different pattern. By combining the restriction analysis data generated with *Mse*I and *Dde*I, unique "signature" restriction patterns characteristic for each species were obtained. These patterns were useful in identifying the *Bartonella* species associated with each tissue specimen.

Twelve species of *Bartonella* have been identified. Four, *Bartonella henselae*, *B. quintana*, *B. elizabethae*, and *B. bacilliformis*, are recognized as human pathogens, and one, *B. clarridgeiae*, has been implicated as a human pathogen by serology (8, 13, 15). *B. bacilliformis*, the first organism in the genus to be identified, causes bartonellosis (3). Recently, *B. henselae* was detected by PCR (1) and isolated from the lymph nodes of patients with cat scratch disease (CSD) (6), as well as from the blood of pet cats (11, 12), providing strong evidence for *B. henselae* as the causative agent of CSD. Both *B. henselae* and *B. quintana* cause bacteremia and bacillary angiomatosis in both immunocompromised and immunocompetent persons (10, 11, 18). *B. quintana* is also the etiologic agent of trench fever (20, 21), and both *B. quintana* and *B. elizabethae* can cause endocarditis (4, 7, 16, 20).

The implication of these *Bartonella* species in a variety of human diseases and the difficulty in isolating them from clinical specimens underscore an urgent need for better detection and identification methods. Birtles identified *Bartonella* species by PCR-restriction fragment length polymorphism (RFLP) analysis (2). We describe the development of a PCR-RFLP assay for the identification of *Bartonella* to the species level and demonstrate the application of this method directly to clinical diagnostic specimens.

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MATERIALS AND METHODS

Bartonella DNA fragments were amplified from 25 tissue specimens, including fresh or paraffin-embedded clinical specimens (lymph node or lymph node aspirates, skin, subcutaneous nodules, or other tissues), obtained from patients participating in epidemiologic studies of CSD and bacillary angiomatosis (19). Amplifications of control tissues were also run in parallel. Control isolates were *B. henselae* B91-002000, *B. vinsonii* B92-010225, *B. elizabethae* B92-002005, and *B. quintana* ATCC VR-358.

Bartonella species were grown on Trypticase soy agar with 5% sheep blood or on chocolate agar plates for 4 to 7 days at 37°C. A few colonies (8 to 10) were harvested in 1 ml of 0.1 M phosphate-buffered saline, pH 7.0. Template DNA was prepared from suspended cells or from fresh or paraffin-embedded clinical specimens by the method described by Heller et al. (9). Positive and negative controls were processed for DNA preparation and PCR amplification along with the clinical specimens. The negative control was a section of fresh or paraffin-embedded skin free of *Bartonella* infection. The positive control was either *Bartonella* organisms or paraffin slices that had been artificially contaminated with *Bartonella* organisms.

Ten microliters of bacterial or tissue lysates was used to amplify the 16S ribosomal DNA (rDNA) fragment by the method of Relman et al. (14). PCR amplification was carried out in 100-μl reaction mixtures consisting of 10 μl of DNA and 90 μl of the amplification mix, which contained the following components: 20 pmol each of p12B and p24E primers, 0.5 mM $MgCl_2$, 200 μM each deoxynucleoside triphosphate, 10 μl of Gene Amp PCR buffer (Perkin-Elmer, Norwalk, Conn.), and 2.5 U of *Taq* DNA polymerase (Perkin-Elmer).

The PCR amplification was performed in a PC-100 Thermal controller (MJ Research, Watertown, Mass.) for 35 cycles. Each cycle consisted of 120 s at 94°C, 60 s at 60°C, and 90 s at 72°C, and a final extension of 10 min at 72°C was done. The amplified products were detected by electrophoresis on a 1% agarose gel (14 by 14 cm) in 1× Tris-borate-EDTA buffer at 100 V for 60 min. Gels were stained with ethidium bromide and photographed. PCR controls included a known positive DNA extract and a reagent blank.

For digestion of PCR-amplified DNA from cultures and clinical specimens, 10 to 15 μl of PCR-amplified DNA was restricted with 10 U of *Dde*I and *Mse*I in a total volume of 15 to 20 μl, respectively, according to the manufacturer's specifications (New England Biolabs, Inc., Beverly, Mass.). The restriction fragments were separated by electrophoresis on agarose gels (4% NuSieve agarose [3:1] [FMC Bioproducts, Rockland, Maine] at 60 V for 2 h. Gels were photographed and fragment sizes were determined with interpolation by using the BioImage system whole-band software analysis (Millipore Corp., Ann Arbor, Mich.).

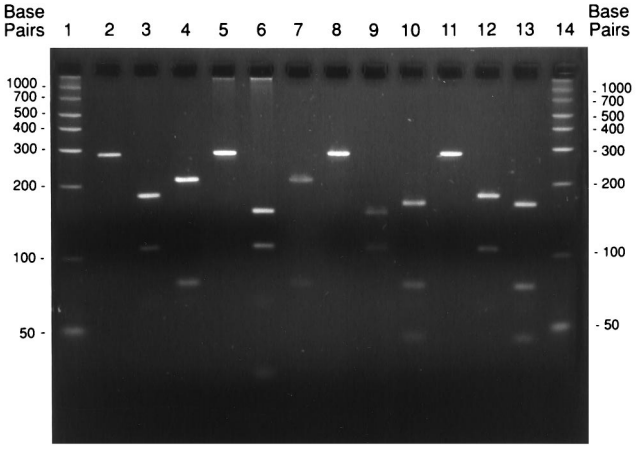

FIG. 1. PCR-RFLP of *B. henselae*, *B. vinsonii*, *B. elizabethae*, and *B. quintana*. Lane 1, 50-bp ladder; lanes 2 to 4, *B. henselae* B91-002000; lanes 5 to 7, *B. vinsonii* B92-010225; lanes 8 to 10, *B. elizabethae* B92-002005; lanes 11 to 13, *B. quintana* ATCC VR-358; lane 14, 50-bp ladder. PCR products were uncut (lanes 2, 5, 8, and 11) or cut with *Dde*I (lanes 3, 6, 9, and 12) or *Mse*I (lanes 4, 7, 10, and 13).

For clinical specimens with PCR-amplified DNA revealing more than one band, the 296-bp DNA in the agarose gel was detected with ethidium bromide, excised, and placed in a 1.5-ml microcentrifuge tube. The agarose slice was washed once with 500 μl of Tris-EDTA buffer and twice with enzyme buffer. The buffer was discarded, and the agarose plug was cut into small pieces. Ten units of the restriction enzymes (*Dde*I and *Mse*I) was added in a 100-μl solution (10× buffer [10 μl], bovine serum albumin [2.5 μl], and deionized water to a volume of 100 μl). DNA was precipitated from the supernatant with 3 M sodium acetate and 2.5 volumes of 95% cold ethanol. The pellet was washed once in cold 70% ethanol, dried in a vacuum dessicator, and dissolved in 20 μl of water. Fragments were detected by electrophoresis on 4% NuSieve agarose gels and were analyzed as described previously.

RESULTS

Figure 1 shows the 296-bp fragment as well as the various RFLP patterns derived from digestion of amplicons of the four species with *Dde*I and *Mse*I, and Table 1 lists the observed and predicted sizes of the fragments from the digested amplicons. *B. henselae* and *B. quintana* gave nearly identical patterns with *Dde*I, whereas *B. vinsonii* and *B. elizabethae* gave a different and unique pattern. With *Mse*I, *B. henselae* and *B. vinsonii* had nearly identical patterns, and *B. quintana* and *B. elizabethae* had patterns different from those of *B. henselae* and *B. vinsonii* but nearly identical to each other. Composite types generated from restriction analysis using *Dde*I and *Mse*I were unique for each species (Table 1).

Table 2 shows the distribution of *Bartonella* species among 25 clinical specimens. Twenty-one of 26 (81%) of the isolates gave the restriction pattern for *B. henselae*, 4 (15.38%) gave that for *B. quintana*, and 1 (4%) gave that for *B. elizabethae*.

TABLE 2. Distribution of *Bartonella* species among clinical diagnostic specimens evaluated

Species	No. of specimens	Tissue	Clinical diagnosis
B. henselae	21	Lymph node	CSD
		Skin	BA[a]
		Blood	Bacteremia
B. quintana	4	Skin	BA
		Bone	BA

[a] BA, bacillary angiomatosis.

The RFLP patterns of *B. henselae* and *B. quintana* obtained from clinical specimens are shown in Fig. 2. All 21 *B. henselae* amplified PCR products have the same composite restriction pattern with *Dde*I and *Mse*I. This pattern is identical to that found for the *B. henselae* type strain (B91-002000). All four *B. quintana* amplified PCR products have the same composite restriction pattern, which is identical to that of the *B. quintana* type strain (ATCC VR-358). It should be noted that minor differences in fragment lengths among isolates within the same species may be encountered (5). The sizes of digested DNA fragments are shown in Table 1.

DISCUSSION

We were able to differentiate among the different *Bartonella* species implicated in human disease by extending the 16S rDNA-based detection method for *Bartonella* species by using restriction analysis of the 296-bp amplicons. This was achieved by generating species-specific composite patterns based on digestion of 16S rDNA amplicons with *Dde*I and *Mse*I restriction enzymes. The RFLP method was applied to amplicons from clinical specimens, allowing direct species identification for these specimens. The predicted and observed fragment lengths for all four species matched each other. Predicted fragments 28 for *B. vinsonii* and *B. elizabethae* cut with *Dde*I were not observed because they were small and were not visualized on gels.

Our method was shown to be more advantageous than a previously described method (1) because only one set of primers was used in this study, followed by restriction analysis, allowing for direct identification in a variety of clinical specimens of the *Bartonella* species implicated in human disease (Table 2). Although the method described by Anderson et al. (1) allows for the detection of *B. quintana* and *B. henselae* in lymph nodes and lymph node aspirates, it requires multiple steps and the use of radioactive probes.

Our PCR-RFLP-based assay may offer the advantage of early diagnosis of suspected *Bartonella* species infections and can be used to differentiate among three species causing human disease in North America. This rapid and specific test is

TABLE 1. Identification of *Bartonella* species by PCR-RFLP analysis of a 16S rDNA fragment

Bartonella sp.	*Dde*I			*Mse*I			PCR-RFLP composite pattern
	Lengths of fragments[a] (bp)		No. of restriction sites	Length of fragments (bp)		No. of restriction sites	
	O	P		O	P		
B. henselae	190, 115	187, 109	1	214, 81	217, 79	1	I
B. vinsonii	158, 114	159, 109, 28	2	218, 82	217, 79	2	II
B. elizabethae	154, 110	159, 109, 28	2	166, 79, 52	173, 79, 44	2	III
B. quintana	180, 106	187, 109	1	168, 75, 47	173, 79, 44	2	IV

[a] O, observed; P, predicted (GenBank sequences).

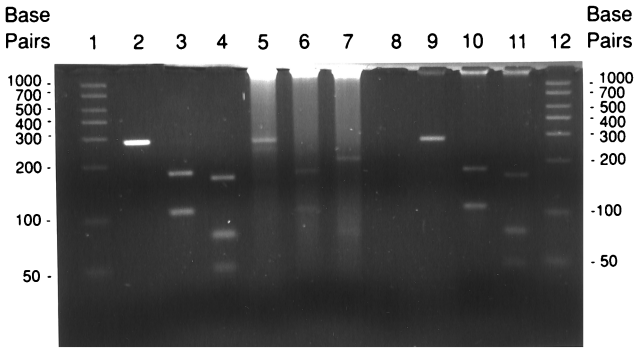

FIG. 2. PCR-RFLP of *B. quintana* and *B. henselae* from clinical specimens. Lane 1, 50-bp ladder, lanes 2 to 4, *B. quintana* H93-176; lanes 5 to 7, *B. henselae* B92-007003; lane 8, blank; lanes 9 to 11, *B. quintana* B93-007356; lane 12, 50-bp ladder. PCR products were left uncut (lanes 2, 5, and 9) or were cut with *Dde*I (lanes 3, 6, and 10) or *Mse*I (lanes 4, 7, and 11).

an alternative to culture that provides more-timely information to clinicians, who can then direct antibiotic therapy and suggest prevention strategies to their patients based on species-specific test results (11).

ACKNOWLEDGMENTS

This work was initiated in 1993 while G.M.M. was a Visiting Associate at the Centers for Disease Control and Prevention (CDC).

This study is supported in part by NIH R29 AI36075 and the California Universitywide AIDS Research Program (to J.E.K.).

REFERENCES

1. **Anderson, B., K. Sims, R. Regnery, L. Robinson, M. J. Schmidt, S. Goral, C. Hager, and K. Edwards.** 1994. Detection of *Rochalimaea henselae* DNA in specimens from cat scratch disease patients by PCR. J. Clin. Microbiol. **32:**942–948.
2. **Birtles, J. B.** 1995. Differentiation of *Bartonella* species using restriction endonuclease analysis of PCR-amplified 16S rRNA genes. FEMS Microbiol. Lett. **129:**261–266.
3. **Brenner, D. J., S. P. O'Connor, H. Winkler, and A. G. Steigart.** 1993. Proposals to unify the genera *Bartonella* and *Rochalimaea*, with descriptions of *Bartonella quintana* comb. nov., *Bartonella vinsonii* comb. nov., *Bartonella henselae* comb. nov., and *Bartonella elizabethae* comb. nov., and to remove the family *Bartonellaceae* from the order *Rickettsialles*. Int. J. Syst. Bacteriol. **43:**777–786.
4. **Daly, J. S., M. G. Worthington, D. J. Brenner, C. W. Moss, D. G. Hollis, R. S. Weyant, A. G. Steigerwalt, R. E. Weaver, M. I. Daneshvar, and S. P. O'Connor.** 1993. *Rochalimaea elizabethae* sp. nov. isolated from a patient with endocarditis. J. Clin. Microbiol. **31:**872–881.
5. **Dauga, C., I. Miras, and P. A. D. Grimont.** 1996. Identification of *Bartonella henselae* and *B. quintana* 16S rDNA sequences by branch-genus and species-specific amplification. J. Med. Microbiol. **45:**192–199.
6. **Dolan, M. J., M. T. Wong, R. L. Regnery, J. H. Jorgensen, M. Garcia, J. Peter, and D. Drehner.** 1993. Syndrome of *Rochalimaea henselae* adenitis suggesting cat scratch disease. Ann. Intern. Med. **118:**331–336.
7. **Drancourt, M., J. L. Mainardi, P. Brouqui, F. Vandenesch, A. Carta, F. Lehnert, J. Etienne, F. Goldstein, J. Acar, and D. Raoult.** 1995. *Bartonella* (*Rochalimaea*) *quintana* endocarditis in three homeless men. N. Engl. J. Med. **332:**419–423.
8. **Ellis, B. A., R. L. Regenry, L. Beati, F. Bacellar, M. Rood, G. G. Glass, E. Marston, T. G. Ksiazek, D. Jones, and J. E. 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.
9. **Heller, M. J., L. J. Burgart, C. J. TenEyck, M. E. Anderson, T. C. Greiner, and R. A. Robinson.** 1991. An efficient method for the extraction of DNA from formalin-fixed, paraffin-embedded tissue by sonication. BioTechniques **11:**372–377.
10. **Koehler, J. E., F. D. Quinn, T. G. Berger, P. E. Leboit, and J. W. Tappero.** 1992. Isolation of *Rochalimaea* species from cutaneous and osseous lesions of bacillary angiomatosis. N. Engl. J. Med. **327:**1625–1631.
11. **Koehler, J. E., M. A. Sanchez, C. S. Carrido, M. J. Whitfeld, T. G. Berger, M. C. Rodriguez-Barradas, P. E. LeBoit, and J. W. Tappero.** 1997. Molecular epidemiology of *Bartonella* infections in patients with bacillary angiomatosis-peliosis. N. Engl. J. Med. **337:**1876–1883.
12. **Koehler, J. E., C. A. Glasser, and J. W. Tappero.** 1994. *Rochalimaea henselae* infection: a new zoonosis with the domestic cat as reservoir. JAMA **271:**531–535.
13. **Kordick, D. L., E. J. Hilyard, T. L. Hadfield, K. H. Wilson, A. G. Steigerwalt, D. J. Brenner, and E. B. Breitschwerdt.** 1997. *Bartonella clarridgeiae*, a newly recognized zoonotic pathogen causing inoculation papules, fever, and lymphadenopathy (cat scratch disease). J. Clin. Microbiol. **35:**1813–1818.
14. **Relman, D. A., J. S. Loutit, T. M. Schmidt, S. Falkow, and L. S. Tompkins.** 1990. The agent of bacillary angiomatosis; an approach to the identification of uncultured pathogens. N. Engl. J. Med. **323:**1573–1580.
15. **Slater, L., D. F. Welch, D. Hensel, and D. W. Coody.** 1990. A newly recognized fastidious gram-negative pathogen as a cause of fever and bacteremia. N. Engl. J. Med. **323:**1587–1593.
16. **Spach, D. H., A. S. Kanter, N. A. Daniels, D. J. Nowowiejski, A. M. Larson, R. A. Schmidt, B. Swaminathan, and D. J. Brenner.** 1995. *Bartonella* (*Rochalimaea*) species as a cause of apparent "culture negative" endocarditis. Clin. Infect. Dis. **20:**1044–1047.
17. **Spach, D. H., A. S. Kanter, M. J. Dougherty, A. M. Larson, M. B. Coyle, D. J. Brenner, B. Swaminathan, G. M. Matar, D. F. Welch, R. K. Root, and W. E. Stamm.** 1995. *Bartonella* (*Rochalimaea*) *quintana* bacteremia in inner-city patients with chronic alcoholism. N. Engl. J. Med. **332:**424–428.
18. **Tappero, J. W., J. E. Koehler, T. G. Berger, et al.** 1993. Bacillary angiomatosis and bacillary splenitis in immunocompetent adults. Ann. Intern. Med. **118:**363–365.
19. **Tappero, J. W., J. C. Mohle-Boetani, J. E. Koehler, et al.** 1993. The epidemiology of bacillary angiomatosis and bacillary peliosis. JAMA **269:**770–775.
20. **Vinson, J. W., and H. S. Fuller.** 1961. Studies on trench fever. 1. Propagation of *Rickettsia*-like organisms from a patient's blood. Pathol. Microbiol. **24:** S152–S166.
21. **Vinson, J. W., G. Varela, and C. Molina-Pasquel.** 1969. Trench fever. 3. Introduction of clinical disease in volunteers inoculated with *Rickettsia quintana* propagated on blood agar. Am. J. Trop. Med. Hyg. **18:**713–722.