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Genomic Fingerprinting of *Bartonella* Species by Repetitive Element PCR for Distinguishing Species and Isolates

MARIA C. RODRIGUEZ-BARRADAS,[1,2]* RICHARD J. HAMILL,[1,2,3] ERIC D. HOUSTON,[1] PAUL R. GEORGHIOU,[1,2]† JILL E. CLARRIDGE,[3,4,5] RUSSELL L. REGNERY,[6] AND JANE E. KOEHLER[7]

Section of Infectious Diseases, Medical Service,[1] and Laboratory Services,[4] Veterans Affairs Medical Center, and Departments of Medicine,[2] Pathology,[5] and Microbiology and Immunology,[3] Baylor College of Medicine, Houston, Texas, Division of Viral and Rickettsial Diseases, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia,[6] and Division of Infectious Diseases, Department of Medicine, University of California, San Francisco, California[7]

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Repetitive-element PCR (rep-PCR) with primers based on repetitive extragenic palindromic (REP) and enterobacterial repetitive intergenic consensus (ERIC) repeated DNA sequences was used for genomic fingerprinting of *Bartonella* species. This technique was applied by using either extracted genomic DNA or preparations of whole bacterial cells directly. PCR fingerprints with either the REP-based primers (REP-PCR) or primers based on the ERIC repeat (ERIC-PCR) revealed species-specific band patterns for the various *Bartonella* isolates. DNA fingerprints obtained from rep-PCR of extracted genomic DNA or from preparations of whole cells yielded comparable patterns. ERIC-PCR banding patterns were less complex than those obtained by REP-PCR but allowed better discrimination between strains within species. By combining results of REP-PCR and ERIC-PCR, five different fingerprint profiles were identified among 17 isolates of *Bartonella henselae*, but only one profile was identified among the five isolates of *Bartonella quintana*. Other *Bartonella* species yielded distinct rep-PCR fingerprints. rep-PCR is a useful technique for identification of *Bartonella* organisms to the species level and offers the advantage of ease of performance, with only small quantities of cells needed for the whole-cell procedure. This technique also appears to be useful for subtyping *B. henselae* isolates.

Bartonella spp. are aerobic, fastidious, gram-negative bacilli that include *Bartonella bacilliformis*, the original member of this genus, and the four species of the former genus *Rochalimaea* (2).

B. bacilliformis, the causative agent of bartonellosis, a disease manifesting both as an acute form (Oroyo fever) and as a cutaneous form (verruga peruana) was initially described in 1913 (21). *Bartonella* (formerly *Rochalimaea*) *quintana* was described in 1917 as the etiologic agent of trench fever (25). In 1991, a new species, *Bartonella henselae*, was identified as the cause of a bacteremic febrile syndrome, bacillary angiomatosis, and peliosis hepatis (18, 20, 29), and in 1993, another species, *Bartonella elizabethae*, was described, having been isolated previously from a patient with endocarditis (4). In addition to the description of new species, the spectrum of illness caused by *Bartonella* spp. has progressively expanded to include cat scratch disease (6, 19), endocarditis (4, 23), brain abscess (24), aseptic meningitis (12, 22), and possibly AIDS-related encephalopathy (16). *Bartonella vinsonii*, isolated from voles, has not yet been associated with human disease.

Until now, definitive identification of *Bartonella* organisms to the species level has been done with molecular biologic techniques that include 16S rRNA sequencing (4, 11, 12, 18, 20), DNA hybridization (4, 23, 29), and PCR-restriction fragment length polymorphism (PCR-RFLP) analysis of the citrate synthase gene (6, 11, 18). These techniques involve lengthy procedures, are available only in research laboratories, and are not useful for subtyping.

Repetitive element PCR (rep-PCR) is a recently described method which generates DNA fingerprints that discriminate between bacterial species and strains (7–9, 17, 26–28, 30, 31). This method involves the application of oligonucleotide primers based on families of short, highly conserved extragenic repetitive sequences, including the repetitive extragenic palindromic (REP) and the enterobacterial repetitive intergenic consensus (ERIC) sequences. REP and ERIC sequences are present in species throughout the eubacterial kingdom (27). By using appropriate outward-facing PCR primers directed at these repeated sequences, multiple-amplification products, which reflect distance polymorphisms between adjacent DNA repeats, may be generated (27).

rep-PCR with oligonucleotide primers based on the REP element (REP-PCR) or the ERIC DNA repeat (ERIC-PCR) has been used for evaluating laboratory and clinical isolates of *Bacillus subtilis* (28), *Citrobacter diversus* (30), *Enterobacter aerogenes* (8), *Rhizobium meliloti* (5), *Streptococcus pneumoniae* (26), *Acinetobacter baumannii* (17), *Burkholderia* (formerly *Pseudomonas*) *cepacia* (9), and *Legionella pneumophila* (7). Fingerprints obtained by this technique have proved to be useful for differentiating between species and for assessment of clonal relationships among bacterial isolates. In addition, modifications of this method allow the use of unprocessed whole cells to generate DNA fingerprints with quality similar to that obtained from purified preparations of genomic DNA (31).

Our objective was to apply the above-described techniques to *Bartonella* species and to determine whether DNA finger-

* Corresponding author. Mailing address: Veterans Affairs Medical Center, Section of Infectious Diseases (111G), 2002 Holcombe Blvd., Houston, TX 77030. Phone: (713) 794-7384. Fax: (713) 794-7045.

† Present address: Wesley Medical Centre, Auchenlower, Queensland 4066, Australia.

TABLE 1. Characteristics and PCR results of *Bartonella* species studied[a]

Sp. and isolate	Geographic area of origin	Clinical source	Specimen cultured	PCR pattern		Strain
				REP	ERIC	
B. henselae						
Houston-1	Texas	HIV	Blood	1	B	II
CA-1	California	HIV	Blood	1	A	I
SA-2	Texas	CSD	Lymph node	1	B	II
GA-1	Georgia	CSD	Lymph node	1	C	III
Goldie-1	Georgia	Cat (CSD)	Blood	1	D	IV
Tiger-2	South Carolina	Cat (CSD)	Blood	1	C	III
Bridges	Georgia	Cat	Blood	2	A	V
Riggs-1	South Carolina	Cat	Blood	1	C	III
Placerville	California	Cat	Blood	1	C	III
Krystal	Florida	Cat	Blood	1	B	II
JK-9	California	HIV	Tissue-BA	1	C	III
JK-13	Georgia	HIV	Tissue-BA	1	C	III
JK-33[b]	California	HIV-A	Tissue-BA	1	B	II
JK-34[b]	California	HIV-A	Blood	1	B	II
JK-38[c]	California	Cat (HIV-A)	Blood	1	C	III
JK-41	Oregon	HIV-B	Lymph node	1	C	III
JK-42[d]	Oregon	Cat (HIV-B)	Blood	1	C	III
B. quintana						
OK-90-268	Oklahoma	HIV	Blood	3	E	VI
D-Perm	Soviet Union	NA	Body lice	3	E	VI
Sh-Perm	Soviet Union	NA	Body lice	3	E	VI
WA-1	Washington	HIV-Endo.	Blood	3	E	VI
JK-35	California	HIV	Tissue-BA	3	E	VI
B. elizabethae						
ATCC 49927	Massachusetts	Endo.	Blood	4	F	VII
B. vinsonii						
ATCC VR-152	Canada	Vole	Spleen	5	G	VIII
B. bacilliformis						
ATCC 35685	Lima, Peru	NA	NA	6	H	IX

[a] Houston-1 = ATCC 49793. HIV, patient with HIV infection; CSD, patient with cat scratch disease; Cat (CSD), cat to which patient with cat scratch disease was exposed; BA, bacillary angiomatosis lesion; Endo., patient with endocarditis; NA, not available.
[b] JK-33 and JK-34 were isolated from the same patient.
[c] JK-38 was isolated from the cat to which patient HIV-A was exposed.
[d] JK-42 was isolated from the cat to which patient HIV-B was exposed.

prints obtained by REP- and ERIC-PCR, from both extracted genomic DNA and whole-cell preparations, would allow discrimination among *Bartonella* species and among different strains within a species.

(This work was presented in part at the 33rd Interscience Conference on Antimicrobial Agents and Chemotherapy, 1993 [20a].)

MATERIALS AND METHODS

Bacterial isolates. Isolates studied are listed in Table 1. *B. henselae* and *B. quintana* were obtained from the Houston Veterans Affairs Medical Center (Houston-1 = ATCC 49793, type strain); from the University of California, San Francisco (all JK isolates); and from the Centers for Disease Control and Prevention (remainder of isolates). *B. elizabethae* (ATCC 49927, type strain) was kindly provided by J. S. Daly (University of Massachusetts Medical School, Worcester). *B. vinsonii* (ATCC VR-152, type strain) and *B. bacilliformis* (ATCC 35685, type strain) were obtained from the American Type Culture Collection. Isolates obtained from the University of California were evaluated without previous knowledge of the specific species. *Bartonella* isolates were incubated on Trypticase soy agar supplemented with 5% sheep blood at 37°C with 5% CO_2, with the exception of *B. bacilliformis*, which was grown in the absence of CO_2 at 27°C.

DNA extraction. Bacterial cells from each isolate were scraped from the agar and transferred to microcentrifuge tubes containing Hanks' balanced salt solution. The cells were washed twice with Hanks' balanced salt solution and harvested by centrifugation at 15,000 × *g*. The resulting pellet was resuspended in lysing buffer (50 mM Tris base, 50 mM EDTA, 8% sucrose, 5% Triton X-100) supplemented with 10 mg of lysozyme (Sigma) per ml and 200 mg of proteinase K per ml and incubated at 37°C for 2 to 16 h. Spheroplasts were extracted twice with phenol:chloroform:isoamyl alcohol (25:24:1), and nucleic acids were precipitated with 3 volumes of ice-cold ethanol and centrifuged at room temperature at 15,000 × *g*. The ethanol was decanted, and the pellet was dried under vacuum at room temperature for 15 min. The dried nucleic acid pellet was redissolved in Tris-EDTA (10 mM Tris, 1 mM EDTA), pH 8.4, with 1.0 mg of RNase A per ml. DNA concentration was measured fluorometrically (TKO Mini-Fluorometer; Hoefer Scientific).

Whole-cell preparations. Colonies were scraped from the plate, suspended, washed once in 1 ml of 1 M NaCl, washed once in distilled water, and resuspended in distilled water to a final concentration of 10^7 to 10^8 CFU/ml.

REP-PCR and ERIC-PCR. The design and synthesis of the REP- and ERIC-based oligonucleotide primers used in this study have been described in detail previously by Versalovic et al. (27). At ambiguous positions in the REP consensus sequence, REP1R-Dt and REP2-Dt primers contained multiple nucleotides (A, C, G, or T). Each 25-μl reaction mixture contained 50 pmol of each of the two opposing primers, 100 ng of genomic DNA or 2 μl of the whole-cell suspension as template bacterial DNA, 1.25 mM (each) 4 deoxynucleoside triphosphates, and 2 U of Amplitaq DNA polymerase in a buffer with 10% dimethyl sulfoxide (vol/vol) (8). PCR amplifications were performed in an automated thermal cycler (Perkin-Elmer Cetus) with an initial denaturation step (95°C, 7 min), followed by 30 cycles of denaturation (90°C, 30 s), annealing (REP primers, 40°C, 1 min; ERIC primers, 51°C, 1 min), and extension (65°C, 8 min), followed by a single final extension (65°C, 16 min). Seven μl of each PCR product was electrophoresed in a 1.0% agarose (Ultrapure; Gibco-BRL, Grand Island, N.Y.) gel at 100 V 2.5 h in 1× Tris-borate-EDTA buffer and stained with 0.5 μg of ethidium bromide per ml. Agarose gels were visualized and photographed under UV transillumination.

Analysis of band patterns. Sizes of bands generated by electrophoresis of the PCR amplifications were assigned by direct comparison with concurrently run 1-kb DNA ladder standards (Gibco-BRL). The presence or absence of bands within a gel lane was determined by two observers (R.J.H. and M.C.R.-B.) with one fully blinded to the source of the isolates. DNA fingerprints of isolates were compared for similarity by visual inspection of band patterns. Fingerprints were considered highly similar when all visible bands represented in each isolate had the same apparent migration distance. Variations in intensity and shape did not represent differences. For complex fingerprints, such as those obtained by REP-PCR, the absence of up to two bands from one isolate when all other visible bands in the fingerprints had matching positions was allowed before isolates were considered different by visual inspection. Previous studies (17, 30) have shown that, by using the above-defined criteria, visual inspection alone is equivalent to more rigorous mathematical comparisons in evaluating fingerprints obtained by rep-PCR and allows identifications of strains that belong to the same clone. For less complex fingerprints (<10 bands), such as those obtained by ERIC-PCR, the presence or absence of a distinct band was considered a difference.

RFLP. RFLP was performed on the REP-PCR product of the *B. quintana* isolates. The restriction enzymes used were *Ava*II, *Hae*III, *Hha*I, *Csp*I, *Not*I and *Sac*II (Gibco-BRL).

RESULTS

PCR amplification of extracted DNA or unprocessed bacteria with REP or ERIC primers revealed species-specific patterns of amplification products for the various *Bartonella* isolates (Fig. 1). As described previously for other bacteria for which this technique has been used, related organisms shared some bands (7–9, 17, 29). DNA fingerprints obtained from rep-PCR of extracted genomic DNA or from preparations of whole cells yielded comparable patterns.

REP-PCR amplification with degenerate primers generated fingerprint patterns having approximately 10 to 15 bands per isolate (Fig. 1A). These bands ranged in size from 0.5 to 4.0 kb. *B. henselae* and *B. quintana* shared a prominent band at 1 kb but differed in other landmark bands at around 0.5, 1.6, and 3 kb. Neither *B. bacilliformis*, *B. elizabethae*, or *B. vinsonii* isolates shared more than one of these landmark bands.

Fingerprints obtained by ERIC-PCR were less complex than those obtained by REP-PCR with degenerate primers (Fig. 1B). Each of the *Bartonella* species yielded very distinct fingerprints with a common band present at 1.3 kb for *B. henselae* isolates and at 1.1 kb for *B. quintana* strains and not apparent

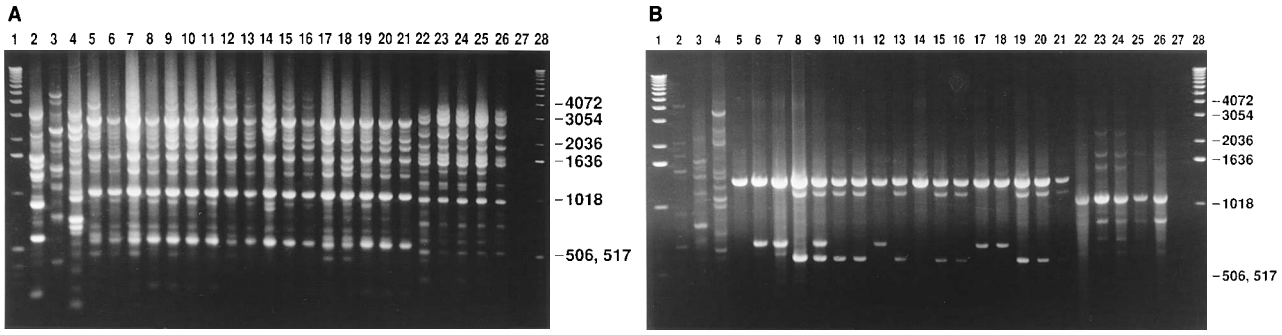

FIG. 1. Whole-cell-based fingerprints generated by REP-PCR (A) and ERIC-PCR (B) of *Bartonella* species. Lanes 1 and 28, 1-kb ladder, marker sizes in base pairs; lane 2, *B. bacilliformis*; lane 3, *B. elizabethae*; lane 4, *B. vinsonii*. Lanes 5 to 21, *B. henselae* isolates: lane 5, CA-1; lane 6, SA-2; lane 7, Houston-1; lane 8, GA; lane 9, Goldie; lane 10, Tiger; lane 11, JK-13; lane 12, Krystal; lane 13, JK-9; lane 14, Bridges; lane 15, Riggs; lane 16, Placerville; lane 17, JK-33; lane 18, JK-34; lane 19, JK-38; lane 20, JK-41; lane 21, JK-42. Lanes 22 to 26, *B. quintana* isolates: lane 22, D-Perm; lane 23, Sh-Perm; lane 24, OK-90; lane 25, WA; lane 26, JK-35; lane 27, negative control.

in the *B. bacilliformis*, *B. elizabethae*, or *B. vinsonii* isolates tested.

JK isolates were evaluated without knowledge of species identity. Of the eight JK isolates, identification of four to species level has been reported (JK-9 [patient 3] [11] and JK-33, JK-34 [patient 4], and JK-38 [cat 4A] [10]). The species and genus of the remaining four isolates were determined by both PCR-RFLP (18) and sequencing of a 300-bp region of the genes coding for 16S rRNA of at least two clones, as previously described (10, 11). All these isolates were correctly identified as *B. henselae* or *B. quintana* on the basis of the REP-PCR and ERIC-PCR fingerprint patterns.

In contrast to previous observations for other bacterial species, REP-PCR fingerprints were not very sensitive in distinguishing among strains within a species. Among the *B. henselae* isolates, only one (Bridges, lane 14, Fig. 1A) met our definition for a different fingerprint. ERIC-PCR of *B. henselae* isolates yielded four different patterns based on the presence of one to four distinct bands (Fig. 1B). A higher discrimination power among strains by ERIC-PCR over REP-PCR has been described also for *R. meliloti* (5). The one isolate differing by REP-PCR fingerprint (Bridges) could not be distinguished from *B. henselae* CA-1 by ERIC-PCR. Five different banding profile patterns of *B. henselae* were identified when results of REP-PCR and ERIC-PCR were combined (Table 1).

The *B. quintana* isolates showed indistinguishable patterns both by REP-PCR and by ERIC-PCR. These results were obtained in duplicate from PCR products from whole-cell organisms harvested at different times. Variations in the intensity of certain bands were observed depending on the concentration of the products and quality of the photograph. Although Fig. 1 suggests that isolate JK-35 (lane 26) is lacking more than two distinct bands, repeated experiments consistently showed that all bands were present and that the same banding pattern existed among all *B. quintana* isolates studied. RFLP analysis of the REP-PCR product of these isolates with seven different restriction enzymes, while providing banding patterns of increased complexity, yielded identical fingerprints (data not shown).

DISCUSSION

rep-PCR, with degenerate primers based on the REP element, appears to be a reliable method for distinguishing among members of the genus *Bartonella*. In this study, characteristic and reproducible fingerprints were obtained that allowed differentiation among species. Furthermore, rep-PCR with whole organisms in place of purified genomic DNA gave patterns comparable with those obtained with extracted DNA, obviating the step of DNA extraction in order to perform the procedure.

rep-PCR has previously been shown to be sensitive in detecting minor differences among strains of the same bacterial genus and species (7–9, 17, 26–28, 30, 31). rep-PCR has been compared with genomic fingerprinting with restriction endonuclease analysis (8), with plasmid profiling (8, 17), with electrophoretic types by multilocus enzyme electrophoresis (26, 30), and with outer membrane protein profiles (30) and yielded discriminatory power comparable with that of those methods. For members of the family *Enterobacteriaceae*, REP-PCR seems to be a more powerful discriminatory technique compared with the corresponding ERIC-PCR patterns (8, 17, 30). This was not the case among the *B. henselae* isolates, for which ERIC-PCR, although yielding less complex fingerprints, proved to be more discriminatory.

When the results of REP-PCR and ERIC-PCR were combined, a total of five different banding profiles were identified among the 17 *B. henselae* isolates. Organisms obtained from the same patient had identical fingerprints with either set of primers. No association could be made between a specific strain and a clinical presentation, suggesting that the broad spectrum of clinical manifestations caused by this species is determined more by host factors than by the organism. No geographic clustering for any given strain could be identified; the most prevalent pattern (strain III) corresponded to isolates obtained from a variety of locations.

Two of the human immunodeficiency virus (HIV)-infected patients with *B. henselae* infection had exposure to cats that were bacteremic with *B. henselae*. A comparison of the strains isolated from these two patients with those from their cats revealed that for one patient the strains were identical (JK-41 from the patient and JK-42 from his cat) but that for the other patient the two isolates obtained from the patient were identical (JK-33 and JK-34) but different from the cat isolate (JK-38). These findings were corroborated by total genomic RFLP, in which JK-33, JK-34, and the Houston-1 isolate all had an identical pattern which differed from that of JK-38 (9a).

B. quintana isolates studied originated from very different locations and time periods. Two strains were originally isolated in the Soviet Union, probably around the 1970s, and the other three are recent isolates from this country (from Oklahoma, Washington, and California). The similarity of rep-PCR fingerprints for these isolates suggests that they share a clonal

origin and that little genetic diversity has occurred over the years. Restricted diversity has been described for other human pathogens (e.g., *Bordetella* spp., *Salmonella* spp., and *Legionella pneumophila*) (1, 7, 15).

Other methods, including cellular fatty acid profiles (4, 12, 18, 29), PCR-RFLP analysis of a fragment of the ribosomal operon (13), and pulsed-field gel electrophoresis (14), have been proposed for identification and subtyping of *Bartonella* species. Using a PCR-based RFLP method, Matar et al. (13) identified seven strains among 11 *B. henselae* isolates obtained from Oklahoma but could not differentiate between the two *B. quintana* isolates studied. However, Maurin et al. (14), using pulsed-field gel electrophoresis, were able to differentiate between the Fuller strain of *B. quintana* and a recent French isolate, suggesting that this technique might be more sensitive for subtyping this species. Cellular fatty acid analysis, although useful for identification of *Bartonella* isolates to the species level (4, 12, 18, 29), seems to be less sensitive for subtyping (13, 29).

In conclusion, rep-PCR appears to be a useful technique for identification of *Bartonella* organisms to the species level. Compared with other conventional molecular biologic methods, rep-PCR offers the advantage of ease of performance, with only small quantities of cells needed for the whole-cell procedure, and availability of results within hours, which makes it a technique that could easily be adopted for use in clinical microbiology laboratories (3). A combination of REP-PCR and ERIC-PCR amplification was able to distinguish among strains of *B. henselae* but not among the *B. quintana* isolates examined. The role of rep-PCR in epidemiologic studies involving *Bartonella* spp. and in comparison with other genomically based fingerprint techniques needs further evaluation.

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