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Polymerase Chain Reaction-Based Restriction Fragment Length Polymorphism Analysis of a Fragment of the Ribosomal Operon from *Rochalimaea* Species for Subtyping

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Restriction endonuclease analysis of a polymerase chain reaction-amplified DNA fragment which included the spacer region between the genes coding for 16S and 23S rRNAs and a portion of the gene coding for 23S rRNA (spacer + 23S) was done on 10 previously characterized clinical isolates of *Rochalimaea henselae*, one clinical isolate of *Rochalimaea quintana*, and the type strains of *R. henselae*, *R. quintana*, *Rochalimaea vinsonii*, and *Bartonella bacilliformis*. *Brucella abortus* DNA was not amplified by the primer set used. The clinical isolates of *Rochalimaea* were obtained from blood or tissue from patients with and without preexisting disease. The amplicon from each strain was digested with five endonucleases (*AluI*, *HaeIII*, *TaqI*, *HinfI*, and *MseI*). *AluI* and *HaeIII* were useful in species differentiation and subtyping of *R. henselae*. *R. henselae* isolates showed six different restriction patterns with *AluI* and four patterns with *HaeIII*. *TaqI*, *HinfI*, and *MseI* were useful only in species differentiation. These observations indicate that PCR amplification of the spacer + 23S region of the ribosomal DNA of *Rochalimaea* spp., along with restriction endonuclease analysis, allows differentiation of *Rochalimaea* spp. from closely related genera, differentiation among the species within *Rochalimaea*, and differentiation of strains within *R. henselae*. The subtyping potential of this method may be useful for further clinical and epidemiologic studies of the spectrum of diseases caused by *R. henselae*.

Rochalimaea henselae is a fastidious, slow-growing, hemotropic, gram-negative bacillus that can cause persistent bacteremia in immunocompetent and immunocompromised persons as well as bacillary angiomatosis (BA) and bacillary peliosis in persons infected with the human immunodeficiency virus (HIV) or who are otherwise immunocompromised (5, 7, 9, 10, 11, 13).

Because of the widespread interest in this newly discovered organism and its implication in a variety of human diseases, it would be useful to have appropriate subtyping methods to study its epidemiology. Until now, only cellular fatty acid (CFA) profiling has been able to subdivide *R. henselae* into different groups (13). A method involving the determination of restriction fragment length polymorphism (RFLP) in an amplified fragment of the gene coding for citrate synthase (7) is useful for the definitive identification of *R. henselae* but has not been reported to be useful for subtyping.

We report on the development of a polymerase chain reaction (PCR)-based RFLP for the differentiation of *Rochalimaea* species and *Bartonella bacilliformis* from one another and for the subtyping of *R. henselae*. The method uses PCR to amplify a fragment of the genomic DNA which includes the spacer region between the genes coding for 16S and 23S rRNAs and a portion of the gene coding for 23S rRNA (hereafter referred to as spacer + 23S). Because the protocol involves PCR amplification of the target sequence, it does not require a large number of cells or lengthy DNA extraction procedures.

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MATERIALS AND METHODS

Source of strains. Eight clinical isolates of *R. henselae* and one isolate of *Rochalimaea quintana*, which were obtained from Oklahoma, were described previously (13). In addition, one *R. henselae* isolate from the blood of an HIV-infected patient in California provided by John R. Scott, Veterans Affairs Medical Center, San Diego, Calif., and one *R. henselae* isolate from a lymph node specimen provided by Michael T. Wong, Wilford Hall U.S. Air Force Medical Center, San Antonio, Tex., were used in the present study. The type strain of *R. henselae* (B91-002000) was provided by Russell Regnery, Centers for Disease Control (CDC). *R. quintana* ATCC VR 358 and *Rochalimaea vinsonii* ATCC VR 152 were obtained from Ted Tzianabos, CDC. *B. bacilliformis* ATCC 35685 and *Brucella* abortus (isolated from the bone marrow of a patient in New York) were obtained from Robert Weaver, CDC.

Extraction of DNA. The *Rochalimaea* strains were grown on Trypticase soy agar (TSA II [BBL, Cockeysville, Md.]; 5% sheep erythrocytes) at 35°C in 5% CO_2 for 3 to 5 days. The bacterial cells were lysed to release the genomic DNA for amplification by the PCR method of Heller et al. (3). The growth from each plate was washed in 1 ml of 0.01 M phosphate-buffered saline (pH 7.2) in 1.5-μl microcentrifuge tubes. The washed pellet was suspended in 100 μl of lysis buffer (50 mM KCl, 10 mM Tris [pH 8.3], 1.5 mM $MgCl_2$, 0.01% gelatin, 0.5% Tween 20, and 0.5 mg of proteinase K [BRL Life Technologies, Inc., Gaithersburg, Md.] per ml). A total of 2 to 5 mg of glass beads (diameter, 106 μm; acid washed; catalog no. G4649; Sigma Chemical Company, St. Louis, Mo.) was added to each tube. Tubes were placed in a sonicating water bath (model 5200; Branson, Shelton, Conn.) for 25 min at 45°C. After sonication, the tubes were

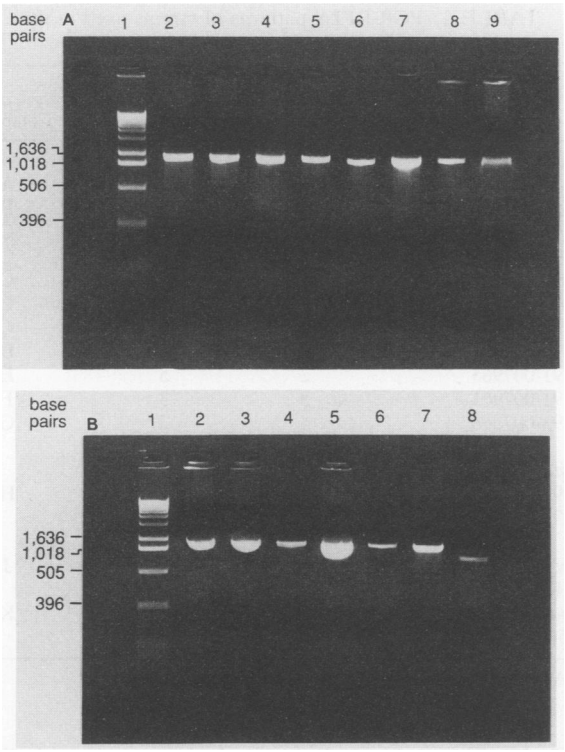

FIG. 1. PCR-amplified spacer + 23S fragment of rDNA from *R. quintana*, *R. vinsonii*, *R. henselae*, and *B. bacilliformis*. (A) Lane 1, 1-kb ladder; lane 2, *R. quintana* ATCC VR 358; lane 3, *R. vinsonii* ATCC VR 152; lane 4, *R. henselae* B91-002000; lanes 5 to 8, *R. henselae* B91-007960 to B91-007963, respectively; lane 9, *R. quintana* B91-007964. (B) Lane 1, 1-kb ladder; lane 2, *R. henselae* B91-007965; lane 3, *R. henselae* B91-007966; lane 4, *R. henselae* B92-008750; lane 5, *R. henselae* B92-008751; lane 6, *R. henselae* B91-007959; lane 7, *R. henselae* B91-007967; lane 8, *B. bacilliformis* ATCC 35685.

placed in a boiling water bath for 15 min to inactivate the proteolytic enzymes and were centrifuged at 12,000 × *g* for 20 s. Supernatants were transferred to new microcentrifuge tubes and were stored at −20°C.

PCR amplification of the spacer + 23S region. Two oligonucleotide primers were used for the PCR amplification of the spacer + 23S region. Primer RPC5 (5′-AAG TCG TAA CAA GGT A-3′) is the reverse complement of the PC5 primer of Wilson et al. (14) and is located at the 3′ end of the gene coding for 16S rRNA. Primer R23S2693 (5′-TAC TGG TTC ACT ATC GGT CA-3′) is located 440 bases from the 5′ end of the gene coding for 23S rRNA in *Escherichia coli* (1) and was selected on the basis of sequence conservation in that region. The primers were synthesized at the Biotechnology Core Facility, CDC.

Ten microliters of the cell lysate was used to amplify the spacer + 23S region. DNA was denatured at 99°C for 3 min and was then quickly cooled in an ice-water bath. PCR amplification was carried out in 100-μl reactions consisting of 10 μl of freshly denatured DNA and 90 μl of the amplification cocktail, which contained the following components: 20 μM (each) primers RPC5 and R23S2693, 200 μM (each) the four deoxyribonucleotides, 10 μl of GeneAmp 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.). Cycles 1 to 3 included the following: denaturation at 94°C for 70 s, annealing at 55°C for 150 s, and extension at 72°C for 180 s. Cycles 4 to 34 included the following: denaturation at 94°C for 30 s, annealing at 55°C for 150 s, and extension at 72°C for 180 s. After the final cycle was complete, the amplicons were allowed to extend at 72°C for 10 min. The amplified products were detected by electrophoresis on a 1% agarose gel (14 by 14 cm) in 1× Tris-borate-EDTA buffer at 120 V for 60 min. Gels were stained with ethidium bromide, placed on a UV transilluminator (Foto/Prep I; Fotodyne, New Berlin, Wis.), and photographed with type 55 Polaroid film (Polaroid, Cambridge, Mass.).

Restriction of the amplified DNA. Five microliters of each PCR-amplified product was used for digestion with the restriction endonucleases according to the manufacturer's specifications (New England BioLabs, Inc., Beverly, Mass.). A panel of five restriction enzymes was used: *Alu*I, *Hae*III, *Taq*I, *Hin*fI, and *Mse*I. RFLP was determined after electrophoresis of the restricted DNA on an agarose gel (1% agarose [GIBCO Bethesda Research Laboratories Life Technologies, Gaithersburg, Md.] plus 1.5% NuSieve agarose [3:1] [NuSieve; FMC BioProducts, Rockland, Maine]). Gels were stained with ethidium bromide and photographed.

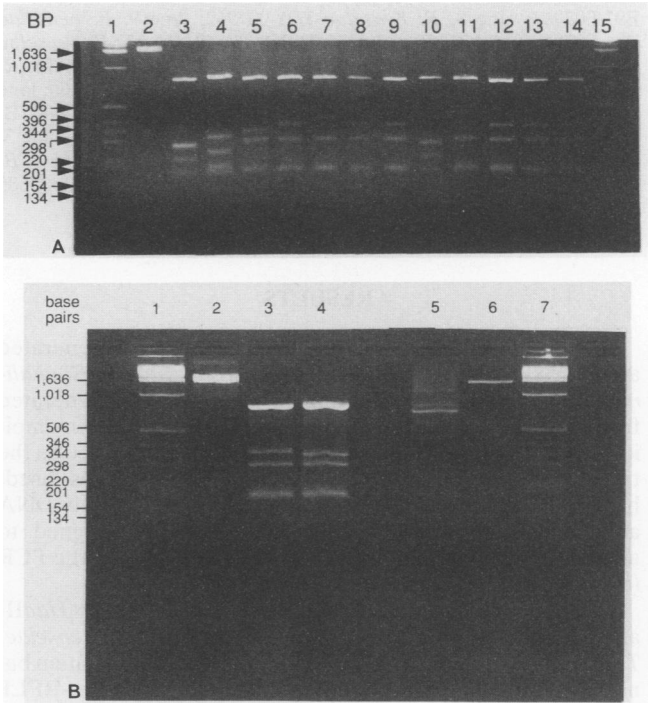

FIG. 2. PCR-based RFLP patterns of PCR-amplified spacer + 23S sequence of rDNA from *Rochalimaea* isolates and *B. bacilliformis* by using the *Hae*III endonuclease. (A) Lane 1, 1-kb ladder; lane 2: *R. henselae* B91-002000 (uncut); lane 3, *R. quintana* ATCC VR 358; lane 4, *R. vinsonii* ATCC VR 152; lane 5, *R. henselae* B91-002000; lane 6, *R. henselae* B91-007960; lane 7, *R. henselae* B91-007961; lane 8, *R. henselae* B91-007962; lane 9, *R. henselae* B91-007963; lane 10, *R. quintana* B91-007964; lane 11, *R. henselae* B91-007965; lane 12, *R. henselae* B91-007966; lane 13, *R. henselae* B92-008750; lane 14, *R. henselae* B92-008751; lane 15, 1-kb ladder. (B) Lane 1, 1-kb ladder; lane 2, *R. henselae* B91-002000 (uncut); lane 3, *R. henselae* B91-007959; lane 4, *R. henselae* B91-007967 (Rh); lane 5, *B. bacilliformis* ATCC 35685 (Bb); lane 6, *B. bacilliformis* ATCC 35685 (uncut); lane 7, 1-kb ladder.

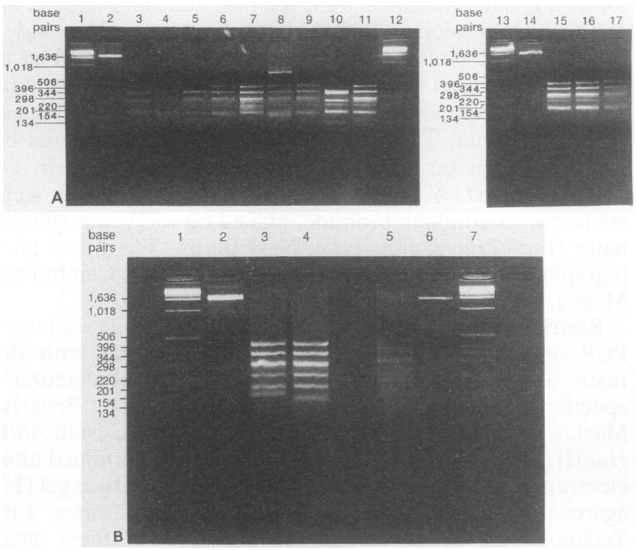

FIG. 3. PCR-based RFLP patterns of PCR-amplified spacer + 23S sequence of rDNA from *Rochalimaea* isolates and *B. bacilliformis* by using the *Alu*I endonuclease. (A) Lane 1, 1-kb ladder; lane 2, *R. henselae* B91-002000 (uncut); lane 3, *R. quintana* ATCC VR 358; lane 4, *R. vinsonii* ATCC VR 152; lane 5, *R. henselae* B91-002000; lane 6, *R. henselae* B91-007960; lane 7, *R. henselae* B91-007961; lane 8, *R. henselae* B91-007962; lane 9, *R. henselae* B91-007963; lane 10, *R. quintana* B91-007964; lane 11, *R. henselae* B91-007965; lane 12, 1 kb-ladder; lane 13, 1 kb-ladder; lane 14, *R. henselae* B91-002000 (uncut); lane 15, *R. henselae* B91-007966; lane 16, *R. henselae* B92-008750; lane 17, B92-008751 *R. henselae*. (B) Lane 1, 1-kb ladder; lane 2: *R. henselae* B91-002000 (uncut); lane 3, *R. henselae* B91-007959; lane 4, *R. henselae* B91-007967; lane 5, *B. bacilliformis* ATCC 35685; lane 6, *B. bacilliformis* ATCC 35685 (uncut); lane 7, 1-kb ladder.

TABLE 1. PCR-RFLP patterns of strains used in the present study

Species and CDC or ATCC[a] no.	PCR-RFLP type		
	*Hae*III	*Alu*I	Composite
R. henselae			
B91-002000	1	1	A
B91-007959	1	6	B
B91-007960	2	2	C
B91-007963	2	2	C
B91-007967	2	2	C
B92-008750	2	2	C
B92-008751	2	2	C
B91-007962	2	4	D
B91-007965	2	5	E
B91-007961	3	3	F
B91-007966	4	2	G
R. quintana			
B91-007964	5	7	H
ATCC VR 358			
R. vinsonii ATCC VR 152	6	8	J
B. bacilliformis 35685	7	9	K

[a] ATCC, American Type Culture Collection.

RESULTS

PCR amplification of the spacer + 23S sequence generated an approximately 1,600-bp fragment from all three *Rochalimaea* species (Fig. 1). The size of the amplicon generated from *B. bacilliformis* was approximately 1,000 bp. An amplicon was not obtained when *B. abortus* DNA was used as the target and the spacer + 23S set of primers was used; however, an amplicon was obtained when *B. abortus* DNA and an universal set of primers (fD1-rD1), designed to amplify 16S rDNA (12) of eubacteria, were used in the PCR (data not shown).

Of the five restriction endonucleases evaluated, only *Hae*III and *Alu*I differentiated between strains within *R. henselae*. *Taq*I, *Hin*fI, and *Mse*I were useful in species differentiation but not for subtyping *R. henselae*. Figures 2 and 3 show the RFLP patterns of 10 clinical isolates of *R. henselae*, 1 isolate of *R. quintana*, and the American Type Culture Collection strains by using *Hae*III (Fig. 2) and *Alu*I (Fig. 3).

The RFLP patterns were different for the three *Rochalimaea* species and for *B. bacilliformis* by using *Hae*III and *Alu*I. The RFLP pattern of *R. quintana* B91-007964 was identical to that of *R. quintana* ATCC VR 358 when *Hae*III and *Alu*I were used. Four distinct RFLP patterns were seen among the *R. henselae* isolates when *Hae*III was used, and six RFLP patterns were seen among the *R. henselae* isolates when *Alu*I was used. The PCR-based RFLP data are summarized in Table 1. A single unique pattern was seen for all *R. henselae* species with each of the following enzymes: *Taq*I, *Hin*fI, and *Mse*I. Four bands were seen with *Taq*I, three bands were seen with *Hin*fI, and seven bands were seen with *Mse*I (data not shown). *R. quintana* DNA does not have restriction sites for *Taq*I, *Hin*fI, or *Mse*I. *R. vinsonii* and *B. bacilliformis* DNAs were not restricted with *Taq*I, *Hin*fI, or *Mse*I. Table 2 shows a comparison of fatty acid groups and PCR-based RFLP types.

DISCUSSION

We developed a PCR-based RFLP method that differentiates between *B. bacilliformis*, *R. henselae*, *R. quintana*, and *R. vinsonii*. The size of the amplicon generated from *B. bacilliformis* by the spacer + 23S PCR was clearly different from that produced from the three *Rochalimaea* species. By DNA-DNA hybridization, *R. henselae*, *R. quintana*, and *R. vinsonii* are 55 to 71% interrelated, and they are only 41 to 55% related to *B. bacilliformis* (13). By 16S rRNA sequencing, *R. quintana* and *R. vinsonii* are 98.7 and 99.3% homologous to *R. henselae*, respectively, while the three *Rochalimaea* species show 95.6% homology to the 16S rRNA sequence of *B. bacilliformis* (6).

Although the primer set used in the present study did not amplify *Brucella* DNA, it produced amplicons of different sizes when genomic DNA from group B streptococci, *Streptococcus pneumoniae*, *Listeria monocytogenes*, *Pseudomonas aeruginosa*, *Vibrio cholerae*, and *Afipia* spp. were used as templates (5a). Therefore, this primer set is not suitable for direct application to clinical specimens. Sequencing of the region bounded by the two primers may provide information that can be used to design *Rochalimaea*-specific primers that amplify the spacer + 23S region for detection, species identification, and subtyping in one step.

Because the PCR-based RFLP method distinguishes between *R. henselae* and *R. quintana*, it will be useful for determining the role of *R. quintana* in patients with BA and peliosis hepatis. Although *R. henselae* appears to be the

TABLE 2. Comparison of fatty acid groups and PCR-based RFLP types

Strain no.[a]	CDC or ATCC[b] no.	Species	Fatty acid group	Composite PCR-RFLP type
	B91-002000	*R. henselae*	3	A
89-675	B91-007963	*R. henselae*	1	C
89-674	B91-007962	*R. henselae*	1	D
90-615	B91-007965	*R. henselae*	1	E
90-782	B91-007966	*R. henselae*	1	G
87-66	B91-007959	*R. henselae*	2	B
88-64	B91-007960	*R. henselae*	2	C
91-148	B91-007967	*R. henselae*	2	C
88-712	B91-007961	*R. henselae*	2	F
90-268	B91-007964	*R. quintana*	3	H
ATCC VR 358				
ATCC VR 152		*R. vinsonii*	4	J
ATCC 35685		*B. bacilliformis*	5	K

[a] Strain numbers are those of Welch et al. (13).
[b] ATCC, American Type Culture Collection.

major etiologic agent of BA, the role of *R. quintana* in infections in HIV-infected persons has not been fully elucidated. Welch et al. (13) isolated *R. quintana* from the blood of a 55-year-old HIV-infected man. The patient had a history of several days of high fever, confusion, and ataxia and had a diagnosis of cryptococcal meningitis. Also, *R. quintana* was isolated from the cutaneous lesions of three patients with BA in San Francisco (4) and was implicated as the cause of endocarditis in a patient with HIV infection (11a). If the PCR-based RFLP of spacer + 23S is optimized for direct application to clinical specimens, this technique could be used to identify the species of the etiologic agent of BA and to delineate the role of *R. quintana* in BA and other diseases. This would not be possible with the primer set of Relman et al. (10) without additional characterization of the amplicons by sequencing or other means.

The ability to subtype *R. henselae* by the PCR-based RFLP method into at least three groups has major implications for future epidemiologic studies. Because *R. henselae* is a fastidious and slow-growing organism, a PCR-based subtyping method for *R. henselae* offers significant advantages over other molecular typing methods (multilocus enzyme electrophoresis, genomic DNA fingerprinting, ribosomal DNA analysis, etc.), which require large numbers of cells for enzyme or DNA extraction. Until now, CFA profiling has allowed the differentiation of strains within *R. henselae* (13). The eight isolates of *R. henselae* were divided into two groups by using CFA profiling. These were further subtyped into seven composite PCR-RFLP types by using *Alu*I and *Hae*III (Table 2). PCR-RFLP type C is found in the two CFA groups. These results show that PCR-RFLP is more discriminatory than CFA, but no epidemiologic correlation can be made at this point. Although CFA analysis is useful for definitive identification at the genus and species levels, it may not be sufficiently sensitive for subtyping within a species. Furthermore, CFA profiles are influenced by even slight variations in the different batches of culture media used for growing the organism (5); this greatly diminishes its utility in subtyping.

In conclusion, we developed a PCR-based RFLP method to subtype isolates of *R. henselae*. The desirable features of the method are that it is simple and rapid and can be performed with very small quantities of *Rochalimaea* cells. Therefore, this method should be useful for future epidemiologic studies of the diseases caused by *R. henselae*. Two recent studies (2, 8) suggest that *R. henselae* may be involved in cat scratch disease. The PCR-based RFLP subtyping method should be useful in epidemiologic studies to determine the role of *R. henselae* in BA and cat scratch disease, the role of cats in transmission of *R. henselae* in BA, cat scratch disease, and other diseases, and the involvement of vectors such as fleas in the transmission of *R. henselae*.

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