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Comparison of Different DNA Fingerprinting Techniques for Molecular Typing of *Bartonella henselae* Isolates

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Seventeen isolates of *Bartonella henselae* from the region of Freiburg, Germany, obtained from blood cultures of domestic cats, were examined for their genetic heterogeneity. On the basis of different DNA fingerprinting methods, including pulsed-field gel electrophoresis (PFGE), enterobacterial repetitive intergenic consensus (ERIC)-PCR, repetitive extragenic palindromic (REP) PCR, and arbitrarily primed (AP)-PCR, three different variants were identified among the isolates (variants I to III). Variant I included 6 strains, variant II included 10 strains, and variant III included only one strain. By all methods used, the isolates could be clearly distinguished from the type strain, Houston-1, which was designated variant IV. A previously published type-specific amplification of 16S rDNA differentiated two types of the *B. henselae* isolates (16S rRNA types 1 and 2). The majority of the isolates (16 of 17), including all variants I and II, were 16S rRNA type 2. Only one isolate (variant III) and the Houston-1 strain (variant IV) comprised the 16S rRNA type 1. Comparison of the 16S rDNA sequences from one representative strain from each of the three variants (I to III) confirmed the results obtained by 16S rRNA type-specific PCR. The sequences from variant I and variant II were identical, whereas the sequence of variant III differed in three positions. All methods applied in this study allowed subtyping of the isolates. PFGE and ERIC-PCR provided the highest discriminatory potential for subtyping *B. henselae* strains, whereas AP-PCR with the M13 primer showed a very clear differentiation between the four variants. Our results suggest that the genetic heterogeneity of *B. henselae* strains is high. The methods applied were found useful for typing *B. henselae* isolates, providing tools for epidemiological and clinical follow-up studies.

Bartonella henselae was identified as a new species in 1990 (12) and is the causative agent of cat-scratch disease (CSD) (2). Furthermore, mostly in immunocompromised patients, *B. henselae* causes bacillary angiomatosis, bacillary peliosis hepatis, osteolytic lesions, relapsing fever with bacteremia, endocarditis, and encephalitis (1, 11, 16, 22). Epidemiological studies have implicated asymptomatic cats as a major reservoir of *B. henselae* (6, 7, 15), but the route of transmission to humans remains unknown. Cat scratches or bites are possible modes of transmission. However, *B. henselae* could not be detected in gingival swabs obtained from domestic cats (15). Several studies demonstrated the presence of *B. henselae* in the bloodstream of healthy cats, and these animals can remain bacteremic from several months to several years (6, 7, 15). Until now, three *Bartonella* species had been detected in the blood of cats: *B. henselae* (6, 7, 15), *Bartonella quintana* (10), and recently *Bartonella clarridgeiae* (5). In the last few years, the number of *Bartonella* species as well as the number of diseases recognized as being caused by *Bartonella* species in humans has increased.

Bartonella bacteria are fastidious, slow-growing microorganisms, and distinguishing between the species is quite difficult because they are phenotypically and genotypically very similar. Definitive identification of the species requires molecular techniques. Recently, discrimination between *Bartonella* species and *B. henselae* isolates was successfully performed by repetitive-element PCR (13, 15, 18). By combining results of repetitive extragenic palindromic (REP)-PCR and enterobacterial repetitive intergenic consensus (ERIC)-PCR, Rodriguez-Barradas et al. identified five different fingerprint profiles among 17 *B. henselae* isolates (13). Using a PCR-based restriction fragment length polymorphism (RFLP) analysis of the 16S-23S rRNA intergenic spacer region and digestion of the amplicons with *Alu*I and *Hae*II, Matar et al. (8) could differentiate between *Bartonella* species and obtained six different restriction patterns among 10 *B. henselae* isolates. Recently, Bergmans et al. (3) described the prevalence of two different *B. henselae* types by partial 16S rRNA gene sequence analysis. These types differ from each other in three nucleotides located at positions 172 to 175 of the 16S rRNA gene.

In a recent study (15), we investigated blood cultures of 100 domestic cats, and *B. henselae* was isolated from 13% of these animals. Genomic fingerprinting of nine of these isolates by ERIC-PCR identified two variants of *B. henselae* which were clearly distinguishable by specific patterns of PCR products. In order to investigate their genetic heterogeneity, we applied different DNA fingerprinting techniques to a total of 18 isolates of *B. henselae*, including the type strain Houston-1 and the 12 strains published previously (15).

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MATERIALS AND METHODS

Bacterial strains and growth conditions. A total of 17 strains of *B. henselae* were obtained from domestic cats in the region of Freiburg, Germany (Table 1). Five of these isolates were obtained from cats in households with children suffering from CSD. All strains were cultured on chocolate agar plates containing 10% defibrinated sheep blood. Columbia blood agar was used as base medium for preparing the chocolate agar plates. *B. henselae* 103737 (which is identical to

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TABLE 1. Characteristics of the *B. henselae* isolates studied

Isolate	Position in the gel[d]	Source	No. of CFU/ml of blood	Growth time (days)	Reference or source
Houston-1[a]	C	HIV[b]			11
FR96/BK3	D	Cat	200	10	15
FR96/BK8	E	Cat	120	12	15
FR96/BK26II	F	Cat	30	6	15
FR96/BK36	G	Cat	100	7	15
FR96/BK36II	H	Cat	100	8	15
FR96/BK38	I	Cat	600	6	15
FR96/BK74	K	Cat	200	6	15
FR96/BK75	L	Cat	120	8	15
FR96/BK75II	M	Cat	100	8	15
FR96/BK77	N	Cat	400	7	15
FR96/BK78	O	Cat	240	7	15
FR96/BK79	P	Cat	800	7	15
FR96/K2	Q	Cat (CSD)[c]	400	10	This study
FR96/K4	R	Cat (CSD)	1,200	6	This study
FR96/K5	S	Cat (CSD)	2,000	6	This study
FR96/K6	T	Cat (CSD)	4,000	7	This study
FR97/K7	U	Cat (CSD)	10,000	10	This study

[a] Houston-1 = ATCC 94793 = CIP 103737.
[b] HIV, patient infected with human immunodeficiency virus.
[c] Cat (CSD), cat to which patient with CSD was exposed.
[d] Letters correspond to lanes in Fig. 1 to 6.

B. henselae ATTC 49882 type strain Houston-1) was obtained from the Collection de L'Institut Pasteur, Paris, France, and was used as a control for further testing.

DNA purification. The strains were grown on chocolate agar plates at 37°C in 5% CO_2 for 4 to 5 days. Cultures from plates were harvested in 1 ml of 0.01 M phosphate-buffered saline. The DNA was extracted with a commercially available kit (Qiagen GmbH, Hilden, Germany) and used as template for the PCR.

PFGE. For pulsed-field gel electrophoresis (PFGE), bacteria were harvested from chocolate agar plates after incubation at 37°C for 7 days. Bacteria were suspended in 0.9% NaCl, adjusted to an optical density at 600 nm (OD_{600}) of 0.85, and washed three times with 0.9% NaCl. Agarose blocks were prepared by adding 500 μl of cell suspension to 700 μl of 2% PFGE agarose (Sigma, Deisenhofen, Germany). The solidified blocks were incubated in lysis buffer (0.5 mM EDTA, 1% lauroyl sarcosine, 1.8 mg of proteinase K per ml [pH 9.5]) at 56°C overnight. After a thorough washing with TE buffer (10 mM Tris, 10 mM EDTA [pH 7.5]), the blocks were incubated with 20 U of *Sma*I for 6 h under the conditions recommended by the enzyme manufacturer (New England BioLabs, Schwalbach, Germany). PFGE was done with a CHEF DRIII electrophoresis unit (BioRad, Munich, Germany). Agarose gel (2%) was prepared in 0.5× TBE running buffer (90 mM Tris, 90 mM boric acid, 2 mM EDTA [pH 8.5]). Electrophoresis was performed for 30 h at 5.9 V/cm with pulse times from 3 to 12 s at a constant temperature of 14°C. Agarose gels were stained with ethidium bromide and photographed under UV illumination with an EasyImage documentation system (Herolab, Wiesloch, Germany). Low-range PFG marker (New England BioLabs) was used as a molecular weight standard.

ERIC-PCR. The primers ERIC1R (5′-ATGTAAGCTCCTGGGGATTCAC-3′) and ERIC2 (5′-AAGTAAGTGACTGGGGTGAG-AGCG-3′) used in this study have been described previously by Versalovic et al. (21). The reaction mixture contained 8 ng of bovine serum albumin (Sigma) per ml, 200 μM (each) the four deoxynucleoside triphosphates (dNTPs), primers (117 nM each), 2 U of *Taq* polymerase (Pharmacia Biotech, Freiburg, Germany), and 100 ng of genomic DNA in 50.0 μl of TBE buffer. The mixture was overlaid with 2 drops of light mineral oil, and PCR amplification was performed with an automated thermal cycler (Robocycler 40; Stratagene) with initial denaturation (95°C, 7 min), followed by 30 cycles of denaturation (94°C, 1 min), annealing (40°C, 1 min), and extension (65°C, 8 min), with a single final extension (65°C, 16 min). The amplified products (20 μl) were electrophoretically separated in a 1% agarose gel at 120 V for 2 h in 0.5× TBE buffer, stained with ethidium bromide, visualized on a UV transilluminator, and photographed with Polaroid 665 film.

REP-PCR. The primers Rep1R-Dt, 5′-(AGCT)CG(AGCT)CG(AGCT)CA TC(AGCT)GGC-3′, and Rep2-D, 5′-(GA)CG(CT)CTTA-TC(CA)GGCCTAC-3′, used have been described previously by Versalovic et al. (21). Each 30-μl reaction mixture contained 2.5 μl of 10× reaction buffer (Pharmacia Biotech), 50 pmol of each of the primers, 100 ng of genomic DNA, 6.6 ng of bovine serum albumin (Sigma) per ml, 160 μM (each) dNTP, 2 U of *Taq* polymerase (Pharmacia Biotech), and 2.5 μl of dimethyl sulfoxide (Merck, Darmstadt, Germany). The amplification cycles were as follows: 1 cycle at 95°C for 7 min; 30 cycles at 90°C for 30 s, 43°C for 1 min, and 65°C for 8 min; and 1 cycle at 65°C for 16 min.

AP-PCR. The core sequence of phage M13 (5′-GAGGGTGGCGGTTCT-3′) was used for arbitrarily primed (AP)-PCR as a single primer (4). Amplification reactions were carried out in a final volume of 50 μl containing 5 μl of 10× reaction buffer (Pharmacia Biotech), 10 ng of bovine serum albumin (Sigma) per ml, 250 pmol of the M13 primer, 100 ng of genomic DNA, 200 μM (each) dNTP, and 2 U of *Taq* polymerase (Pharmacia Biotech). The amplification cycles were as follows: 1 cycle at 94°C for 5 min, 4 cycles at 94°C for 30 s, 43°C for 2 min, and 72°C for 2 min; 30 cycles at 94°C for 20 s, 50°C for 1 min, and 72°C for 30 s; and 1 cycle at 72°C for 5 min.

16S rRNA type-specific amplification of *B. henselae* DNA. The primers BH1, 5′-AATCCCTCTTTCTAAATAGCC-3′, and BH2, 5′-TAAACCTCTTTCTAA ATAGCC-3′, in combination with the broad-host-range primer 16SF, 5′-AGAG TTTGATCCTGG(CT)TCAG-3′, were used for the type-specific amplification of *B. henselae* DNA as described by Bergmans et al. (3).

DNA amplification was carried out in 50-μl reaction volumes containing 5 μl of 10× reaction buffer (Pharmacia Biotech), 8 ng of bovine serum albumin (Sigma) per ml, 200 μM (each) dNTP, 20 pmol of each primer, 100 ng of genomic DNA, and 2 U of *Taq* polymerase (Pharmacia Biotech). PCR cycling consisted of 30 cycles of 20 s at 95°C, 30 s at 56°C, and 1 min at 72°C preceded by an initial denaturation of 3 min at 95°C and followed by a final extension of 5 min at 72°C. PCR products were separated on a 1.5% agarose gel and visualized by staining with ethidium bromide.

The DNA molecular weight marker VI (Boehringer GmbH, Mannheim, Germany) and a 1-kb ladder (ΦX174 replicative-form DNA *Hin*cII digest; Pharmacia Biotech) were used as standards for electrophoresis of DNA.

PCR amplification and restriction analysis of the spacer region between the 16S and 23S DNAs. Oligonucleotide primers RPC5 (5′-AAGTCGTAACAAG GTA-3′) and R23S2693 (5′-TACTGGTTCACTATCGGTCA-3′) described by Matar et al. (8) were used for the amplification of the spacer region separating the 16S and 23S regions. The amplification was performed in 100-μl reaction volumes containing 10 μl of 10× reaction buffer (Pharmacia Biotech), 8 ng of bovine serum albumin (Sigma) per ml, 200 μM (each) dATP, dCTP, dGTP, and dTTP, 20 pmol of each primer, 100 ng of DNA, and 2.5 U of *Taq* polymerase (Pharmacia Biotech). Amplifications were performed with an automated thermal cycler (Robocycler 40; Stratagene) with initial denaturation (3 cycles of 70 s at 94°C, 150 s at 55°C, and 3 min at 72°C), followed by 30 cycles of denaturation (94°C, 30 s), annealing (55°C, 150 s), and extension (72°C, 180 s), with a single final extension step (72°C, 10 min). Five microliters of each PCR product was digested with different restriction endonucleases (*Hin*dIII, *Taq*I, *Hae*III, *Hin*fI, *Pvu*II, and *Alu*I) according to the manufacturer's instructions (New England BioLabs). The resulting DNA fragments were analyzed after electrophoresis on an ethidium-bromide-stained 2% agarose gel (Biozym).

Amplification and sequencing of 16S rRNA genes. Universal prokaryotic primers TPU1 and RTU8 (Table 2) were used to amplify a 1,500-bp fragment of the 16S rRNA gene from the purified genomic DNA of the cultured isolates FR96/BK3, FR96/BK38, and FR97/K7. The PCR was carried out in a final volume of 100 μl containing 10 μl of 10× reaction buffer (Perkin-Elmer Cetus; Norwalk, Conn.), 200 μM (each) dNTP, 0.3 μM (each) primer, 2 U of *Taq* polymerase (Ampli*Taq* Gold; Perkin-Elmer Cetus), and 50 ng of DNA with an automated thermal cycler (model 2400; Perkin-Elmer Cetus) under the following conditions: an initial step at 94°C for 9 min; followed by 30 cycles at 94°C for 30 s, 50°C for 30 s, and 72°C for 90 s; and a final elongation step at 72°C for 10 min. The purity of the amplified product was determined by electrophoresis in a 1.5% agarose gel (FMC Bioproducts; Rockland, Maine). After the PCR products were purified with the QIAquick PCR purification kit (Qiagen), both strands were sequenced with an automated thermal cycler (model 2400; Perkin-Elmer Cetus) with the Prism DyeDeoxy terminator cycle sequencing kit (Perkin-Elmer, Applied Biosystems Division, Foster City, Calif.). Sequencing products were purified with MicroSpin S-200 HR columns (Amersham Pharmacia Biotech,

TABLE 2. Oligonucleotide primers used for amplification and sequencing of 16S rDNA

Primer	Sequence (5′ to 3′)	Position[a]
TPU1	AGA GTT TGA TCM TGG CTC AG	EC8–27
TPU2	CCA RAC TCC TAC GGG AGG CA	EC334–353
TPU3	CAG CMG CCG CGG TAA TWC	EC519–536
TPU4	GGA TTA GAT ACC CTG GTA GTC C	EC785–806
TPU5	AAA CTY AAA KGA ATT GAC GG	EC907–926
TPU6	GGG CKA CAC ACG TGC TAC AAT	EC1220–1240
RTU2	TGC CTC CCG TAG GAG TYT GG	EC334–353
RTU3	GWA TTA CCG CGG CKG CTG	EC519–536
RTU4	TAC CAG GGT ATC TAA TCC TGT T	EC781–802
RTU5	CCG TCA ATT CMT TTR AGT TT	EC907–926
RTU6	ATT GTA GCA CGT GTG TMG CCC	EC1220–1240
RTU8	AAG GAG GTG ATC CAK CCR CA	EC1525–1544

[a] Numbering is based upon *Escherichia coli* sequence.

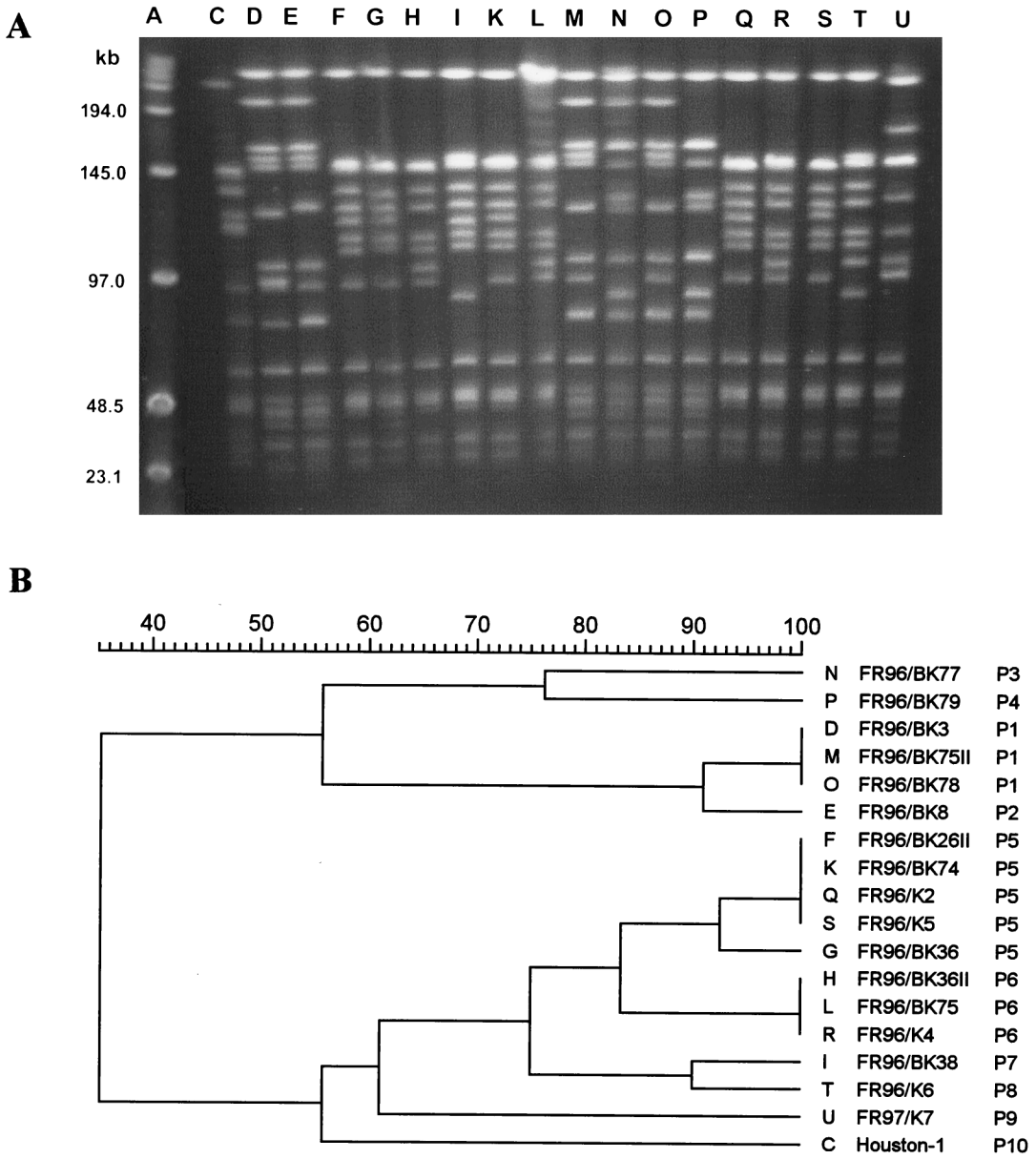

FIG. 1. DNA fingerprint analysis of the 18 *B. henselae* strains by PFGE (A) and dendrogram of the fingerprints as determined by the Dice method (B). Lane A, molecular size markers; lane C, pattern P10 (variant IV); lanes D, E, M, N, O, and P, patterns P1, P2, P3, and P4 (variant I); lanes F, G, H, I, K, L, Q, R, S, and T, patterns P5, P6, P7, and P8 (variant II); lane U, pattern P9 (variant III).

Uppsala, Sweden) and analyzed with an ABI Prism 377 DNA sequencer (Perkin-Elmer, Applied Biosystems Division).

16S rRNA sequence analysis. Comparison of 16S rDNA sequences with databases was done as previously described (19).

Visual analysis of band patterns. The fingerprints obtained were compared for similarity by visual inspection of band patterns. Sizes of DNA fragments amplified by PCR were determined by direct comparison with the DNA marker. Fingerprints were considered highly similar when all visible bands obtained had the same migration distance for each isolate. Variations in intensity and shape of bands among isolates were not considered differences. The presence or absence of one distinct band was considered a difference.

Computer-assisted analysis of the DNA fingerprints. All fingerprints were analyzed with the Windows version of GelCompar software version 4.0 (Applied Maths, Kortrijk, Belgium). The patterns produced by ERIC-, REP-, and AP-PCR were compared with the Pearson correlation coefficient, which considers both number of bands and band intensity. PFGE fingerprints were analyzed by applying the Dice coefficient to peaks. For clustering, the unweighted pair group method with arithmetic means (UPGMA method) was used. A tolerance in the band positions of 1.2% was applied for comparison of the fingerprint patterns. Fingerprint analysis and the methods and algorithms used in this study were performed according to the instructions of the manufacturer.

Nucleotide sequence accession numbers. The 16S rDNA nucleotide sequence data reported in this study have been deposited in the EMBL, GenBank, and DDBJ nucleotide sequence databases. Accession numbers are as follows: FR96/BK3 to AJ223778, FR96/BK38 to AJ223779, and FR97/K7 to AJ223780.

RESULTS

PFGE. Digestion of DNA from the *B. henselae* strains with *Sma*I (CCCGGG) created between 10 and 15 chromosomal fragments for each isolate. The molecular sizes of the *Sma*I fragments ranged from 20 to more than 200 kb (Fig. 1A). Among the 18 strains, 10 different patterns were observed by visual inspection of band patterns (Table 3).

With the Dice coefficient, these 10 types could be discrimi-

TABLE 3. Summary of results obtained by different typing methods for *B. henselae* variants

Gel position[c]	Isolate	Pattern or group obtained by indicated method					16S rRNA type-specific PCR[a]	Proposed variant[b]
		PFGE	ERIC-PCR	REP-PCR	AP-PCR	*Alu*I RFLP		
C	Houston-1	P10	E4	R4	M4	A1	1	IV
D	FR96/BK3	P1	E1.1	R1	M1	A2	2	I
E	FR96/BK8	P2	E1	R1	M1	A2	2	I
M	FR96/BK75II	P1	E1	R1	M1	A2	2	I
N	FR96/BK78	P	E1	R1	M1	A2	2	I
O	FR96/BK78	P1	E1	R1	M1	A2	2	I
P	FR96/BK79	P4	E1	R1	M1	A2	2	I
F	FR96/BK26II	P5	E2	R2	M2	A2	2	II
G	FR96/BK36	P5	E2	R2	M2	A2	2	II
H	FR96/BK36II	P6	E2	R2	M2	A2	2	II
I	FR96/BK38	P7	E2	R2	M2	A2	2	II
K	FR96/BK74	P5	E2	R2	M2	A2	2	II
L	FR96/BK75	P6	E2	R2	M2	A2	2	II
Q	FR96/K2	P5	E2	R2	M2	A2	2	II
R	FR96/K4	P6	E2	R2	M2	A2	2	II
S	FR96/K5	P5	E2	R2	M2	A2	2	II
T	FR96/K6	P8	E2	R2	M2	A2	2	II
U	FR97/K7	P9	E3	R3	M3	A1	1	III

[a] According to Bergmans et al. (3).
[b] Variants I and II are according to the method of Sander et al. (15).
[c] Letters correspond to lanes in Fig. 1 to 6.

nated at a cutoff level of 92% similarity. The four variants (I to IV) could be differentiated at a similarity level of 74% in the dendrogram. Variant I could be subdivided into two groups (Fig. 1B). The average sum of fragment sizes was at least 1.5 Mbp for all *B. henselae* strains investigated.

ERIC-PCR. Investigation of the 18 strains of *B. henselae* by ERIC-PCR resulted in banding patterns, which allowed the definition of subtypes. The patterns consisted of approximately 7 to 11 bands per isolate (Fig. 2A). The molecular sizes of the fragments ranged from 200 to 1,500 bp. The patterns obtained allowed a visual differentiation of four major fingerprint patterns (E1, E2, E3, and E4). Comparison of the banding patterns within strains was done by the method of Pearson (Fig. 2B). The four variants (E1 to E4) could be identified at 88% similarity of PCR fingerprints in the dendrogram. The first variant (E1) contained 6 isolates, the second (E2) contained 10 isolates, and the third (E3) contained only 1 isolate (Table 3). The type strain of *B. henselae* (Houston-1) was different from all of our isolates, indicating an additional variant, E4. The isolate FR96/BK3 (lane D) had an additional band of approximately 500 bp by visual inspection (E1.1) compared with the other strains of variant I. This was also recognized in the computer-assisted analysis, in which this isolate was clearly discriminated from all other strains within variant E1. The fingerprint patterns of the four isolates from cats to which patients with CSD were exposed (FR96/K2, FR96/K4, FR96/K5, and FR96/K6) formed a single closely related group within the cluster E2 and showed a similarity of 95% between the isolates.

REP-PCR. Fingerprints created by REP-PCR were more complex than the patterns generated by ERIC- or AP-PCR but less complex than those obtained by PFGE. They consisted of 17 to 19 bands per isolate, showing a molecular size of up to 2,200 bp (Fig. 3A). Visual inspection of the band patterns and computer-assisted analysis of the DNA fingerprints determined by the Pearson method (Fig. 3B), with a cutoff level of 97% correlation on the dendrogram, revealed four major fingerprint profiles (R1 to R4). The reference strain (pattern R4) and the isolate FR97/K7 (pattern R3) were different from all other strains. The first and second clusters (patterns R1 and R2, respectively) contained the same isolates as those seen by ERIC-PCR (Table 3). By REP-PCR, the distinction between the isolate FR96/BK3 (lane D) and the other strains within group E1 as shown by ERIC-PCR could not be detected. Within the groups R1 and R2 no differences were observed between the patterns by visual inspection.

AP-PCR. The M13 core sequence primer (Fig. 4) generated the lowest number of amplification products. Only three to seven fragments between 0.2 and 1.7 kbp in size could be amplified. However, the patterns obtained with the M13 primer showed a very clear differentiation between the four fingerprint patterns seen by ERIC- and REP-PCR at a cutoff level of 88% correlation on the dendrogram. The patterns were sufficiently distinct to allow division of the strains into four subgroups, M1, M2, M3, and M4 (Table 3).

16S rRNA type-specific amplification of *B. henselae* DNA. With the primers BH1 and BH2 and the broad-host-range primer 16SF, the two types described by Bergmans et al. (3) could be determined for all strains (Table 3). Interestingly, Bergmans type 1 was detected in only one isolate (FR97/K7) and in the reference strain (Fig. 5A) corresponding to variants III and IV, respectively, observed in this study. The 16 isolates of variants I and II were Bergmans type 2 (Fig. 5B).

PCR amplification and restriction analysis of the spacer region between the 16S and 23S DNAs. PCR with primers described earlier by Matar et al. (8) generated DNA fragments of approximately 1,600 bp in size from all strains investigated. From the restriction endonucleases evaluated, only *Alu*I created a fragment pattern which allowed differentiation between the strains (Fig. 6). The reference strain and isolate FR97/K7 (resembling Bergmans type 1) showed six bands (pattern A1, Table 3), whereas all other *B. henselae* strains showed seven bands (pattern A2, Table 3) corresponding to Bergmans type 2.

16S rRNA sequences. The 16S rRNA sequences were determined for one representative strain from each of the three

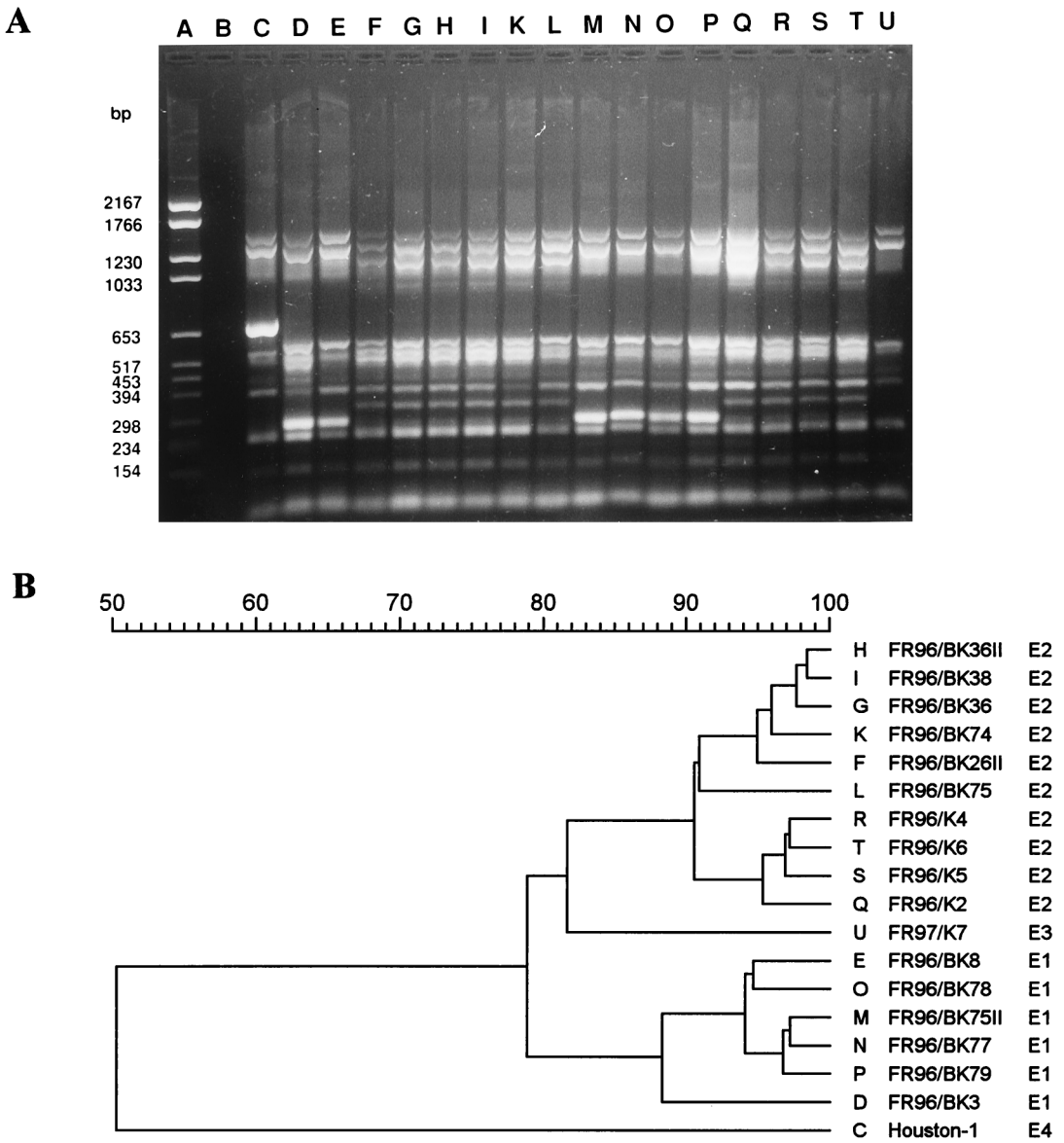

FIG. 2. DNA fingerprint analysis of the 18 *B. henselae* strains by ERIC-PCR (A) and dendrogram of the fingerprints as determined by the Pearson method (B). Lane A, molecular size marker; lane B, negative control; lane C, pattern E4 (variant IV); lanes D, E, M, N, O, and P, pattern E1 (variant I); lanes F, G, H, I, K, L, Q, R, S, and T, pattern E2 (variant II); lane U, pattern E3 (variant III).

variant groups of the German isolates (FR96/BK3, corresponding to variant I; FR96/BK38, corresponding to variant II; and FR97/K7, corresponding to variant III). Sequencing of 16S rRNA genes from isolates FR96/BK3, FR96/BK38, and FR97/K7 revealed that FR96/BK3 and FR96/BK38 were identical and contained the type 2 sequence pattern described by Bergmans et al. (3). The sequence from isolate FR97/K7 differed in 3 bp located at positions 172 to 175 of the 16S rRNA gene, showing the Bergmans type 1 sequence pattern (data not shown). Comparison of isolate sequences with EMBL and GenBank databases with the FASTA-opt algorithm of the Heidelberg Unix Sequence Analysis Resources program package (version 4.0; Deutsches Krebsforschungszentrum, Heidelberg, Germany) showed that the isolate sequences showed highest similarity (>99%) to the reference sequences M73229 (Bergmans type 1) and Z11684 from *B. henselae* (Bergmans type 2).

DISCUSSION

The aim of this study was to compare the usefulness of various molecular methods for typing *B. henselae* isolates. Our results should allow further analysis of strain relatedness and may provide a useful basis for further studies on the epidemiology and the pathogenicity of this bacterium. Six different subtyping techniques, including PFGE, ERIC-PCR, REP-PCR, AP-PCR, 16S rRNA type-specific PCR, and PCR amplification and restriction analysis of the spacer region between the 16S and the 23S rDNAs, were established to investigate the genetic relatedness of 17 *B. henselae* strains isolated from blood cultures of domestic cats in the region of Freiburg, Germany, and the Houston-1 reference strain. By all methods, four different major variants of the *B. henselae* isolates could be distinguished. The cat isolates showed three variants (I to III)

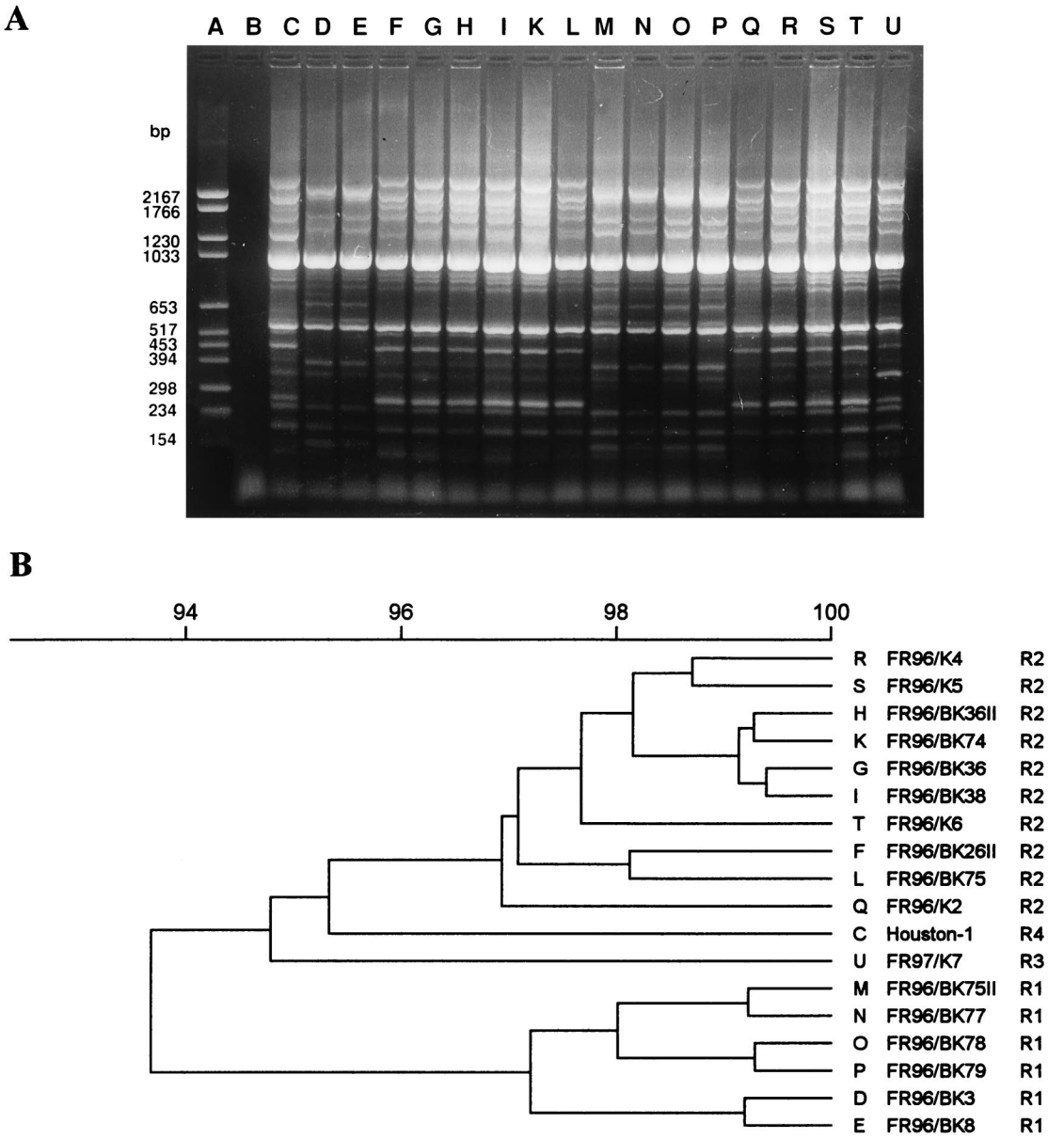

FIG. 3. DNA fingerprint analysis of the 18 *B. henselae* strains by REP-PCR (A) and dendrogram of the fingerprints as determined by the Pearson method (B). Lane A, molecular size marker; lane B, negative control; lane C, pattern R4 (variant IV); lanes D, E, M, N, O, and P, pattern R1 (variant I); lanes F, G, H, I, K, L, Q, R, S, and T, pattern R2 (variant II); lane U, pattern R3 (variant III).

which differed from the Houston-1 reference strain (variant IV).

PFGE has been previously shown to be a suitable method for differentiation of *Bartonella* strains at the species level (9, 14) and for isolate identification (14). Genomic DNA analysis by PFGE with *Sma*I revealed 10 different subgroups (P1 to P10). Analysis of PFGE fingerprints was performed initially with the Pearson correlation coefficient according to the other typing techniques. This method considers not only bands but also the densitogram of a whole pattern, thus being advantageous in the analysis of fingerprints containing bands of different brightness (e.g., AP-PCR). On the other hand, this method is strongly affected by differences in the amount of DNA from lane to lane or by artifacts, such as incomplete restriction. Lane L of the pulsed-field gel (strain FR96/BK75) exhibits such an artifact, disarranging the correspondent dendrogram as calculated with the Pearson coefficient (dendrogram not shown). Therefore, PFGE fingerprints were analyzed by the UPGMA method, with the Dice coefficient applied to peak positions.

The molecular weight sums of the DNA fragments revealed a genome size of approximately >1.5 Mbp in all strains investigated. This result is in good agreement with the genome size of 2.0 Mbp determined earlier by Roux and Raoult (14). The fact that the sum of the fragments was not exactly 2.0 Mbp can be explained by the eventual presence of double bands, especially in the high-molecular-weight range above 194 kbp.

Analysis by REP- and ERIC-PCR generates species- and strain-specific DNA fingerprints of gram-negative enteric bacteria (21). Repetitive sequences of the bacterial genome are useful targets for DNA-based typing due to their restricted length

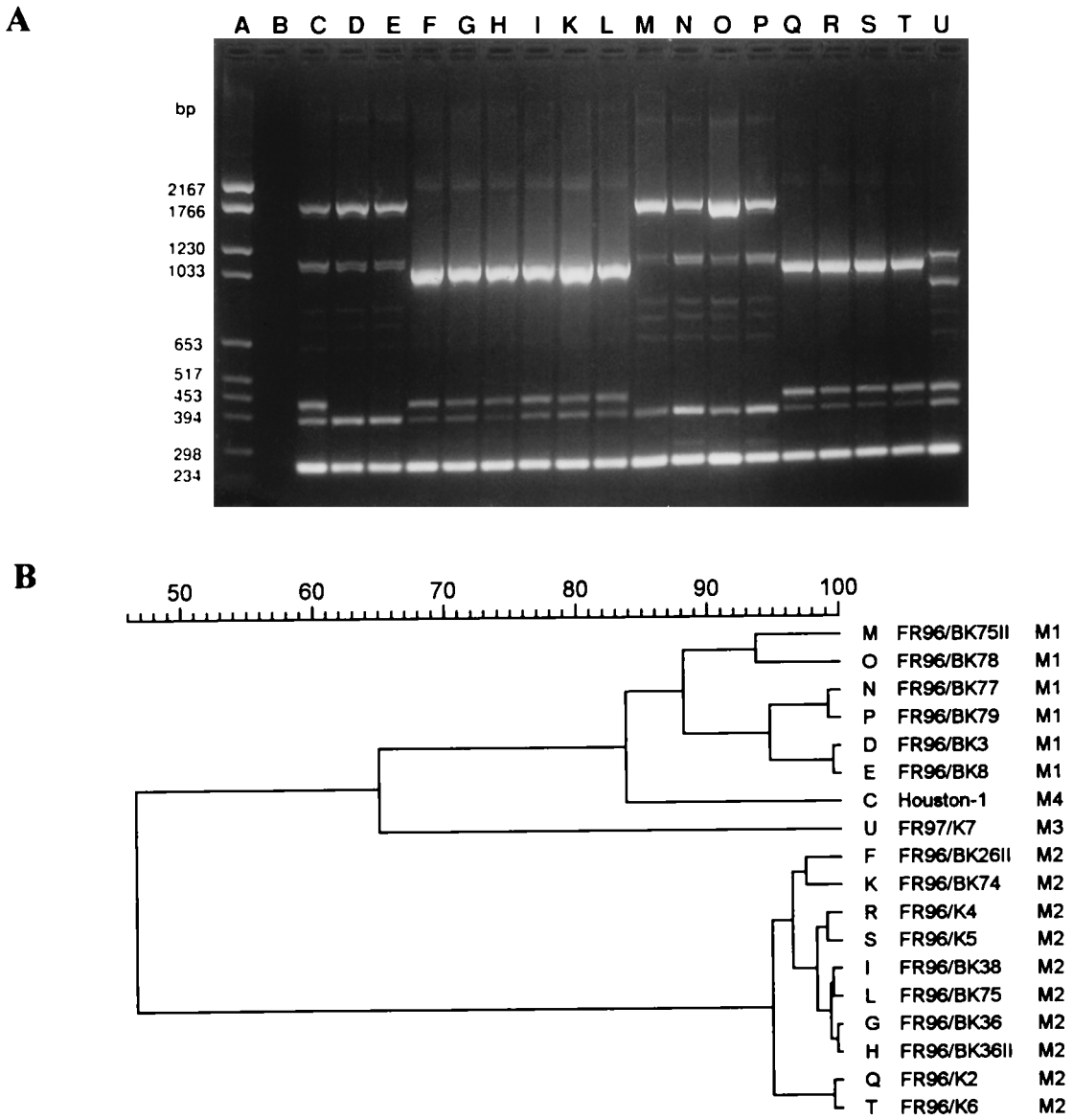

FIG. 4. DNA fingerprint analysis of the 18 *B. henselae* strains by AP-PCR with the M13 core sequence primer (A) and dendrogram of the fingerprints as determined by the Pearson method (B). Lane A, molecular size marker; lane B, negative control; lane C, pattern M4 (variant IV); lanes D, E, M, N, O, and P, pattern M1 (variant I); lanes F, G, H, I, K, L, Q, R, S, and T, pattern M2 (variant II); lane U, pattern M3 (variant III).

and their widespread occurrence, although little is known about their function (20, 21). However, it is not known whether such sequences exist in *Bartonella* spp. Arbitrary binding of primers cannot be excluded. Both methods have been applied earlier to differentiate *Bartonella* organisms on the species level and to subtype various *B. henselae* isolates (13). The 17 isolates of *B. henselae* (7 cultured from tissue and blood of human immunodeficiency virus-infected patients, 2 from patients suffering from CSD, 8 from the blood of cats) investigated by Rodriguez-Barradas et al. (13) comprised five different fingerprint profiles with the combined results of REP-PCR and ERIC-PCR. We could confirm the applicability of ERIC-PCR for distinguishing between *B. henselae* and *B. quintana* isolates in a recent study (15). Concordant with the results of Rodriguez-Barradas et al. (13), ERIC-PCR banding patterns (7 to 11 bands per isolate) were less complex than those obtained by REP-PCR (17 to 19 bands per isolate). Variants I to IV were detected by both methods, although the divergence within the clusters was higher by ERIC-PCR (pattern E1.1 within cluster E1).

In addition to DNA repeat-based genetic variation, DNA polymorphisms can be detected by the selective amplification of sequences with arbitrarily chosen primers. The core sequence of phage M13 was used successfully for molecular typing of other bacteria, such as *Acinetobacter baumanii* (4). When this primer was used with AP-PCR, only four to seven fragments could be amplified, but the patterns were highly discriminative for the *Bartonella* strains.

RFLP analysis of the 16S-23S rDNA spacer region with *Alu*I allowed us to differentiate only two groups (A1 and A2). In contrast, Matar et al. (8) found six distinct *Alu*I RFLP patterns in 11 culture-grown *B. henselae* strains. The correlation in our

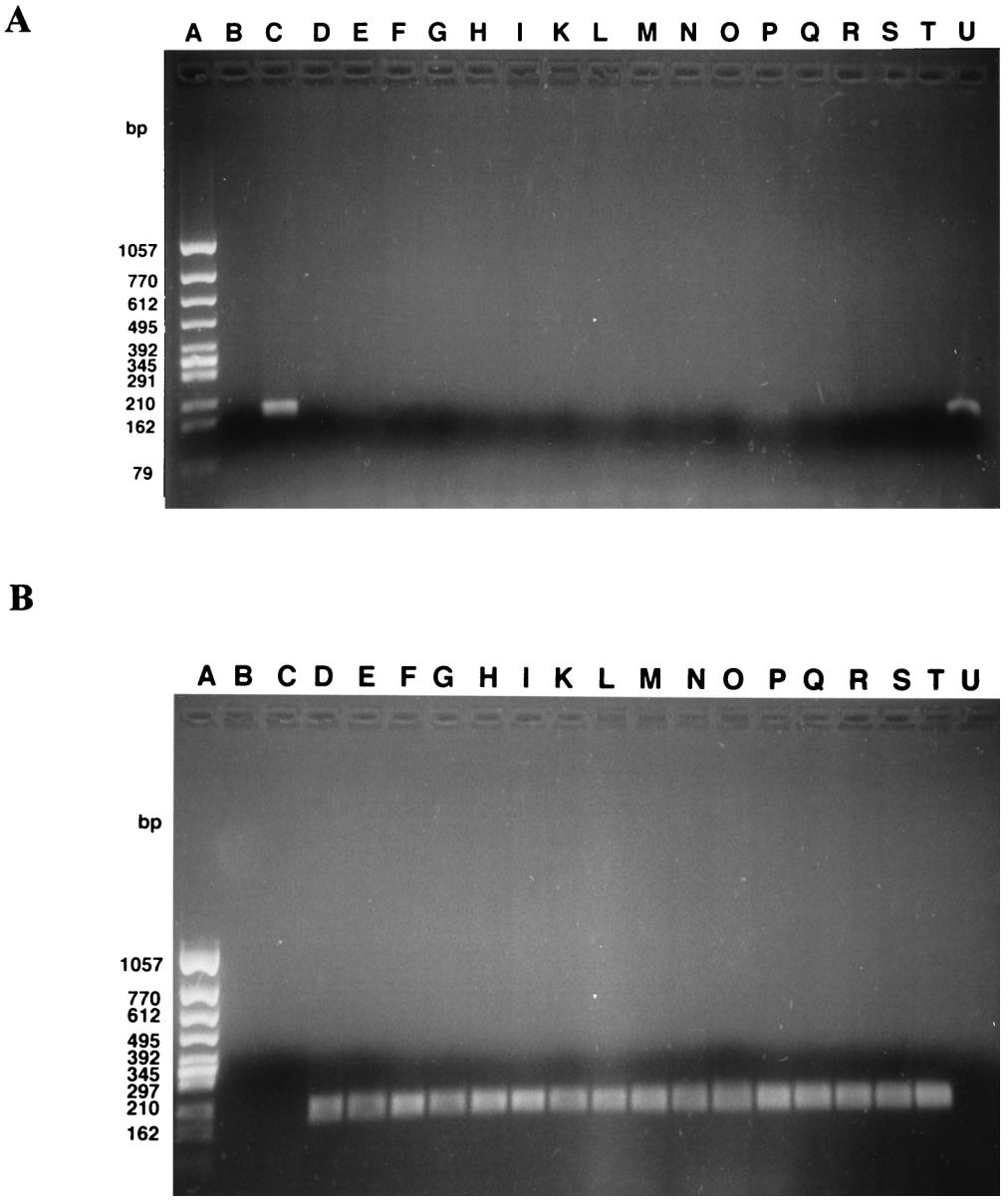

FIG. 5. Type-specific PCR products of the 18 *B. henselae* strains. PCR products were obtained with primer BH1 (A) and primer BH2 (B), which detect *B. henselae* Bergmans type 1 and Bergmans type 2, respectively. Lanes A, molecular size markers; lanes B, negative controls; lanes C and U, Bergmans type 1; lanes D, E, F, G, H, I, K, L, M, N, O, P, Q, R, S, and T, Bergmans type 2. Bergmans type 1 includes variants III and IV. Bergmans type 2 includes variants I and II.

isolates between the *Alu*I RFLP pattern and the 16S rRNA type was 100%. By the 16S rRNA type-specific PCR described by Bergmans et al. (3), the 18 *B. henselae* strains yielded two different types: 16 were Bergmans type 2, whereas strain FR97/K7 and the reference strain were Bergmans type 1. The majority (82%; 32 of 41 samples) of the lymph nodes from patients with CSD investigated by Bergmans et al. (3) contained type 1 *B. henselae*. In contrast, 16 of 17 of our isolates from cats contained type 2 *B. henselae*. In contrast, 16 of 17 of our isolates from cats contained type 2 *B. henselae*. These results suggest that *B. henselae* type 1 might be more pathogenic than *B. henselae* type 2 in humans. The predominance of different types in various geographic regions (The Netherlands and Germany) may also explain the discrepancies between our results and those of Bergmans et al. (3). Whereas *B. henselae* type 1 seems to be predominant in CSD patients in The Netherlands, *B. henselae* type 2 is predominant in bacteremic German domestic cats.

On the basis of the typing results obtained by different methods, the cat isolates were divided into three variant groups. Comparison of the 16S rDNA sequences/ of one member of each group revealed that isolates FR96/BK3 (variant I) and FR96/BK38 (variant II) showed no differences in their 16S rRNA gene sequences, while the sequence of FR97/K7 (variant III) differed at three positions. These intraspecies sequence variations were previously reported by Bergmanns et al. (3). The findings that FR96/BK3 and FR96/BK38 contained the *B. henselae* type 2 16S rRNA, whereas FR97/K7 contained the *B. henselae* type 1, confirmed the results of the 16S rRNA type-specific PCR with the respective isolates.

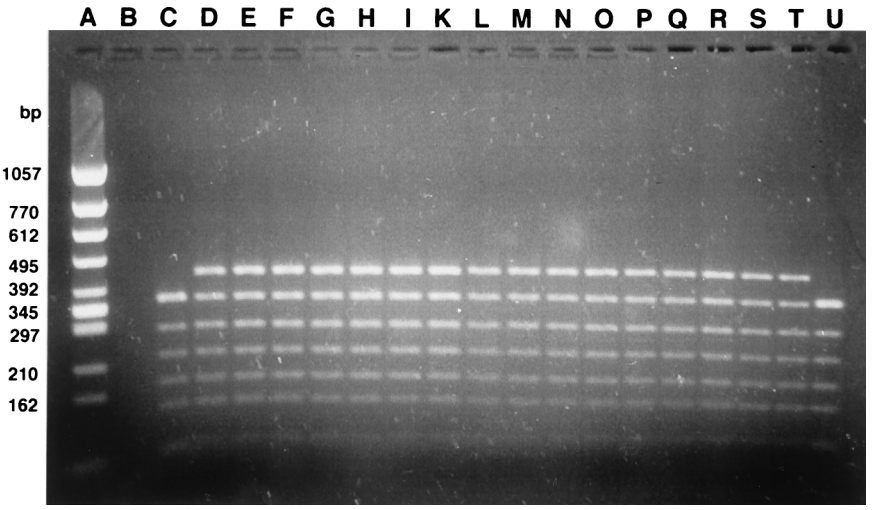

FIG. 6. *Alu*I RFLP patterns of PCR-amplified 16S-23S spacer regions from the 18 *B. henselae* strains. Lane A, molecular size marker; lane B, negative control; lanes C and U, pattern A1; lanes D, E, F, G, H, I, K, L, M, N, O, P, Q, R, S, and T, pattern A2. Pattern A1 corresponds to Bergmans type 1 or to variants IV and III. Pattern A2 corresponds to Bergmans type 2 or to variants I and II.

In conclusion, all fingerprinting methods applied were found to be useful for subtyping *B. henselae*. All methods allowed differentiation of four major variants (I to IV). However, PFGE showed the highest discriminatory power, followed by ERIC-PCR. Lower banding patterns, such as that obtained by AP-PCR with the M13 primer, showed the clearest differentiation of the four variants. Sequence analysis of the 16S rRNA demonstrated that the differences between isolates of variants I and II are not localized within the 16S rRNA sequence. Therefore, PFGE and the PCR-based fingerprint methods (ERIC-PCR, REP-PCR, and AP-PCR) have more discriminatory potency than the 16S rRNA type-specific PCR and the PCR-based *Alu*I RFLP analysis of the 16S rRNA. Major advantages of the PCR-based fingerprint methods (ERIC-PCR, REP-PCR, and AP-PCR) are technical simplicity, wide availability of equipment and reagents, and rapid feasibility. However, macrorestriction endonuclease analysis of genomic DNA by PFGE is more discriminatory but also more expensive and time-consuming.

Our results suggest that the genetic heterogeneity of *B. henselae* strains is high, providing tools for epidemiological and clinical follow-up studies.

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