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Predominance of Two *Bartonella henselae* Variants among Cat-Scratch Disease Patients in The Netherlands

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Restriction endonuclease analysis of the PCR-amplified 16S-23S rRNA gene spacer region was used to investigate the prevalence of *Bartonella henselae* variants in samples from cat-scratch disease (CSD) patients. Analysis of spacer PCR fragments from 27 *Bartonella* DNA-positive samples from Dutch patients with CSD with *Alu*I revealed two restriction fragment length polymorphism (RFLP) patterns, patterns A and B. Twenty samples yielded *B. henselae* pattern A, and 7 samples yielded *B. henselae* pattern B. Three samples from North American patients with CSD were shown to contain *B. henselae* with RFLP pattern B. To be able to detect and differentiate *Bartonella* DNA in clinical material more sensitively and faster, two *B. henselae* PCRs which amplify part of the 16S rRNA gene and which can discriminate between two *B. henselae* variants were developed. Thirty-two of 41 *Bartonella* DNA-positive samples from Dutch patients with CSD contained type I *B. henselae*, 7 samples contained type II *B. henselae*, and two samples were negative in both type-specific PCRs. Two samples from North American patients with CSD both contained type II *B. henselae*. A 100% correlation was found between the *Alu*I spacer RFLP pattern and the 16S rRNA PCR type. We have shown that Dutch patients with CSD contain a limited number of *B. henselae* variants, suggesting that, in contrast to systemic bartonellosis, CSD in immunocompetent patients is caused by a limited number of *B. henselae* variants.

Bartonella henselae is a fastidious, slowly growing microorganism that has been cultured from the blood and tissue of individuals that suffer from human immunodeficiency virus-related syndromes, including bacillary angiomatosis (BA) and bacillary peliosis hepatis (16). Koehler et al. (9) cultured *B. henselae* from 7 pet cats of patients with BA, from fleas taken from an infected cat, and from 23 of 61 cats in the greater San Francisco Bay region. Two of the 61 cats were infected with bartonellas which were different from all other known *Bartonella* species, as determined by citrate synthase PCR-restriction fragment length polymorphism (PCR-RFLP) analysis and 16S rRNA gene sequence analysis (9). The results of Koehler et al. (9) may implicate the cat as a major reservoir for *B. henselae* and may indicate that fleas are involved in the transmission of the organism. Later studies showed that this organism is also involved in cat-scratch disease (CSD) in immunocompetent patients (1, 3, 6, 13). Culture of *Bartonella* species from the lymph nodes of patients with CSD has proven to be extremely difficult in contrast to the culture of *Bartonella* species from tissue and blood of patients with BA and bacillary peliosis hepatis. Therefore, classical characterization and differentiation techniques which require cultivation in vitro have not yet been applied to bartonellas found in patients with CSD. As a consequence, little is known about the prevalence of different *B. henselae* variants in CSD.

Regnery et al. (12) described RFLP analysis of PCR fragments of the citrate synthase gene as a useful method for species identification of cultured *B. henselae*, *Bartonella quintana*, and *Bartonella vinsonii* organisms, but attempts to differentiate within the *B. henselae* species have not been successful by this method (11). Using a PCR-based RFLP analysis of the 16S-23S rRNA intergenic spacer region, Matar et al. (10) demonstrated seven composite (*Alu*I and *Hae*III) RFLP types in 11 *B. henselae* strains cultured from patients with BA, septicemia, and parenchymal bacillary peliosis. Furthermore, they showed that *B. quintana* has a characteristic RFLP pattern which is distinct from those of *B. henselae* isolates. Recently, Rodriguez-Barradas et al. (14) examined 17 isolates of *B. henselae* cultured from tissue and blood of human immunodeficiency virus-infected patients, patients with CSD, and cats by REP (repetitive extragenic palindromic)-PCR and ERIC (enterobacterial repetitive intergenic consensus)-PCR. By combining the results of both PCRs, they identified five different fingerprint profiles (14). Until now, these subtyping techniques have been applied only to cultured bartonellas and not directly to clinical material.

In a previous study we showed the prevalence of two *B. henselae* variants in eight patients with CSD by partial 16S rRNA gene sequence analysis. The partial 16S rRNA gene sequences differ from each other in 3 bp located at positions 172 to 175 of the 16S rRNA gene (3). To investigate the prevalence of the latter and of other *B. henselae* variants in patients with CSD, we applied the 16S-23S rRNA intergenic spacer RFLP described by Matar et al. (10) directly to clinical material from patients with CSD without cultivation of the bacteria that were present. Because the intergenic region spacing the 16S and 23S rRNA genes of bacteria is not entirely transcribed into RNA, there is little selective pressure in this region, and thus, intraspecies sequence divergence in the spacer region is expected to be higher than the divergence in the 16S rRNA gene. In addition, we developed two type-specific PCRs that can discriminate between the two *B. henselae* variants found so far in Dutch patients with CSD.

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TABLE 1. Sequences and positions of oligonucleotides used for 16S-23S rRNA spacer and 16S rRNA PCR and hybridization

Oligonucleotide name	Oligonucleotide sequence[a]	Target organism	Target rRNA gene	Nucleotide position (direction)[b]	Reference
Primers					
RPC5	AAGTCGTAACAAGGT	Eubacteria	16S	1,439 (→)	10
R23S2693	TACTGGTTCACTATCGGTCA	Eubacteria	23S	440 (←)	10
16SF	AGAGTTTGATCCTGG(CT)TCAG	Eubacteria	16S	10 (→)	This study
16SR	CTTTACGCCCA(AG)TAA(AT)TCCG	Eubacteria	16S	521 (←)	This study
BH1	CCGATAAATCTTTCTCCCTAA	*B. henselae* type I	16S	192 (←)	This study
BH2	CCGATAAATCTTTCTCCAAAT	*B. henselae* type II	16S	192 (←)	This study
BP probe	B-TCTCTACGGAATAACACAGAGA	Bartonella	16S	124 (→)	This study

[a] B-, 5′ biotinylation. Bases between parentheses are mixed at one position.
[b] Position in the *B. henselae* rRNA gene. Arrows indicate direction of primers and probe (→, forward; ←, reverse).

MATERIALS AND METHODS

Bacterial strains. *B. henselae* ATCC 49882 and ATCC 49793 were obtained from the Centers for Disease Control and Prevention, Atlanta, Ga. *B. henselae* 90-782, 91-100, 91-148, and 92-967, *B. quintana* 90-268 and 92-263, and *Bartonella bacilliformis* KC583 (ATCC 35685) were kindly provided by D. F. Welch, University of Oklahoma Health Sciences Center, Oklahoma City (16). *B. henselae* CAT5, which was isolated from the blood of a cat whose owner suffered from CSD, was kindly provided by M. F. Peeters, St. Elisabeth Hospital, Tilburg, The Netherlands.

Clinical samples. One hundred eight pus aspirates and lymph node biopsy specimens obtained from Dutch patients with clinically diagnosed CSD and six lymph node biopsy specimens from North American patients with CSD (obtained from B. Swaminathan, Centers for Disease Control and Prevention) were used for the spacer RFLP analysis. All samples contained *Bartonella* DNA, as determined by a *Bartonella*-specific PCR-hybridization assay described earlier (3). Forty-two of the 108 Dutch samples and 2 of the 6 North American samples were used in the *B. henselae* type-specific PCRs. Additionally, one blood sample from a cat, which was positive in the *Bartonella*-specific PCR (3), was available for spacer RFLP analysis and for the *B. henselae* type-specific PCRs.

Extraction of DNA from bacterial strains. Freeze-dried bacterial cells of *B. henselae* 91-148 and 92-967, *B. quintana* 90-268 and 92-263, and *B. bacilliformis* KC583 were suspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]), and total DNA was extracted as described previously (2). *B. henselae* ATCC 49882 and ATCC 49793 were grown for 1 week at 35°C with 5% CO_2. Colonies were scraped from the agar plate and were suspended and washed in 0.01 M phosphate-buffered saline (pH 7.2). The washed pellet was resuspended in TE buffer, and the cells were lysed by incubating them for 10 min at 95°C. All other bacterial strains used in the study were lysed in this way. These crude lysates were centrifuged at 13,000 × *g* in a microcentrifuge for 30 s, and the supernatants were used directly for PCR.

Extraction of DNA from clinical material. Clinical samples, consisting of 0.1 to 1 g of fresh material, were incubated in 3 ml of digestion buffer (500 mM Tris [pH 9], 20 mM EDTA, 10 mM NaCl, 1% sodium dodecyl sulfate [SDS], 0.5 mg of proteinase K per ml) for 15 to 24 h at 60°C to release total DNA. The digested material was extracted twice with Tris-buffered phenol (pH 7.4), precipitated with ethanol, and washed with 70% ethanol. The DNA pellet was resuspended in 20 to 250 μl of sterile water, depending on the viscosity of the DNA extract.

Oligonucleotide primers. All primers used in the study are summarized in Table 1 and Fig. 1. To amplify the 16S-23S rRNA spacer region of the bacterial DNA present in the clinical samples and in lysates of the bacterial strains, the broad-host-range PCR primers RPC5 and R23S2693 described by Matar et al. (10) were used. The expected size of the product resulting from PCR with these primers on genomic DNA from *B. henselae* is approximately 1,700 bp.

For the type-specific amplification of *B. henselae* DNA, reverse primers were developed on the basis of two 16S rRNA gene sequences found in clinical samples from patients with CSD (3). Primer BH1 is derived from the type I *B. henselae* 16S rRNA sequence and has one, two, and three base differences with the *B. bacilliformis*, the *B. quintana*, and the type II 16S rRNA sequences, respectively. Primer BH2 is derived from the type II *B. henselae* 16S rRNA sequence and has three base differences with the *B. quintana* and the *B. bacilliformis* sequences. Primer 16SF is a broad-host-range forward primer derived from primer 16S1F described by our group (3). The expected size of the product resulting from PCR with these primers on genomic DNA from *B. henselae* is approximately 185 bp.

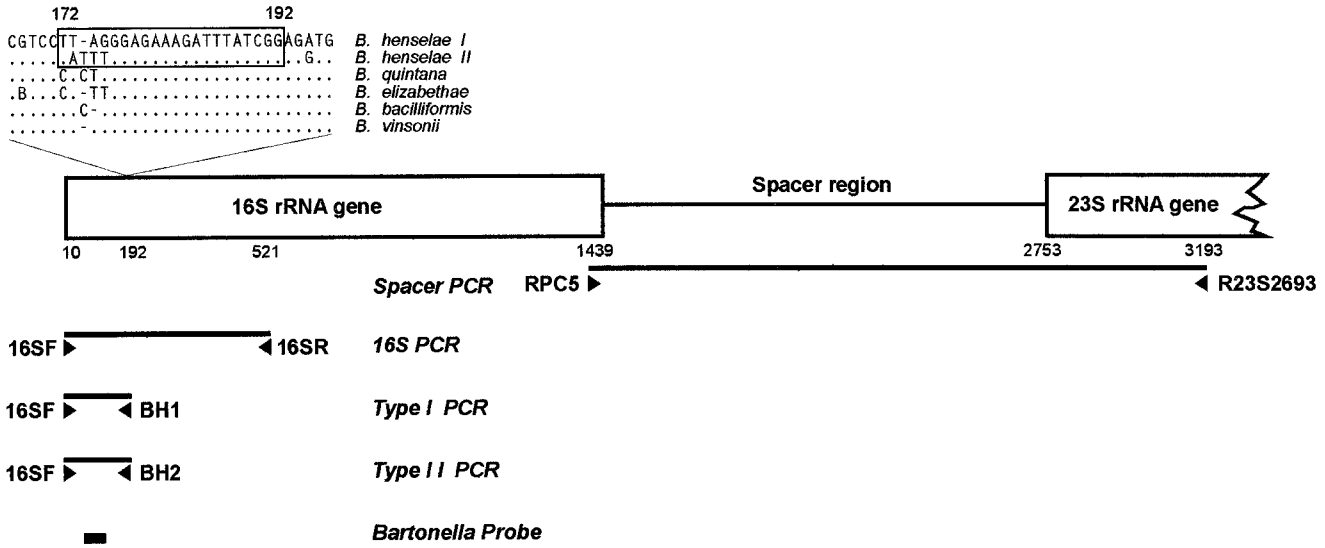


FIG. 1. Schematic presentation of the 16S rRNA gene, intergenic spacer region, and part of the 23S rRNA gene of *B. henselae* and positions of the primers and probe used for eubacterial and *B. henselae*-specific 16S rRNA gene PCR, eubacterial 16S-23S rRNA spacer PCR, and hybridization. The sequence of the type-specific part of the *B. henselae* 16S rRNA gene is shown and is aligned with homologous sequences of relevant *Bartonella* species. The boxed regions in the *B. henselae* type I and type II sequences represent the target sequences for primers BH1 and BH2, respectively. ▶, primer.

To amplify a 500- to 600-bp 16S rRNA gene sequence of any bacterial species present in a sample, PCR primer 16SF was used together with broad-host-range primer 16SR, which is derived from primer 16S1RR described by our group (3).

All primers were purchased from Perkin-Elmer Cetus, Gouda, The Netherlands.

PCR amplification. DNA amplification with primers RPC5 and R23S2693 was carried out in 50-μl reaction volumes. Each reaction mixture contained 20 pmol of each primer, 0.5 U of SuperTth DNA polymerase (HT Biotechnology Ltd., Cambridge, United Kingdom), and standard amounts of amplification reagents (200 μM [each] deoxynucleoside triphosphate, 50 mM Tris-HCl [pH 9.0], 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 0.1% Triton X-100). A 50-μl overlay of sterile mineral oil was added to the tubes; this was followed by the addition of 1 μl of a clinical DNA extract or bacterial lysate. To minimize nonspecific amplification, a so-called touchdown PCR program (7) was used: 3 min at 95°C; this was followed by two cycles of 20 s at 95°C, 1 min at 50°C, and 1.5 min at 72°C and then two cycles identical to the previous two cycles, but with an annealing temperature of 48°C; after every following two cycles, the annealing temperature was lowered by 2°C until it reached 42°C. Then, an additional 40 cycles of 20 s at 95°C, 1 min at 40°C, and 1.5 min at 72°C were followed by the touchdown PCR program. All PCRs were carried out in an OmniGene Thermal Cycler (Hybaid Ltd., Teddington, United Kingdom).

Type-specific amplification of *Bartonella* DNA with primer 16SF and either primer BH1 or BH2 was carried out under reaction conditions similar to those described above, with the following modifications: amplification was carried out in 25-μl reaction volumes and each reaction mixture contained 10 pmol of each primer and 137.5 ng of TaqStart antibody (Clontech Laboratories, Palo Alto, Calif.). The TaqStart antibody blocks the action of the DNA polymerase in the mixture until it has been heated at 70°C, thus providing a hot start. The touchdown amplification program for both primers BH1 and BH2 was as described above for primers RPC5 and R23S2693, with the following modifications: the extension time was 1 min, and annealing was performed for 30 s by using a touchdown amplification program from 64 to 56°C; this was followed by 40 cycles at 54°C.

Amplification of eubacterial DNA with primers 16SF and 16SR was carried out under conditions similar to those for the amplification with primers RPC5 and R23S2693, with the following modifications: 10 pmol of primer 16SF, 20 pmol of primer 16SR, 0.25 U of SuperTth DNA polymerase, and 5 μl of a clinical DNA extract diluted 1/10 were used in reaction volumes of 25 μl. The touchdown amplification program was as described above for primers RPC5 and R23S2693, with the following modifications: the extension time was 1 min and annealing was performed for 1 min by using a touchdown amplification program from 66 to 58°C; this was followed by 40 cycles at 56°C. The program finished with 7 min at 72°C.

To test the PCR mixture for contamination, a negative control consisting of the reaction mixture without the DNA template was included in each experiment.

Gel electrophoresis and Southern blotting of PCR products. Spacer PCR products were checked by electrophoresis of 5 μl of the reaction mixture in an ethidium bromide-stained, 2% agarose gel. Ten microliters of the type-specific PCR products was also checked under similar conditions. A 1-kb DNA ladder (Gibco BRL, Life Technologies B.V., Breda, The Netherlands) was used as a DNA size standard. The DNA was visualized on a UV transilluminator and photographed. The type-specific PCR products were subsequently transferred from the gel to a Hybond N^+ nylon membrane by a vacuum blotting system (Millipore B.V., Etten-Leur, The Netherlands).

***Bartonella*-specific oligonucleotide probe.** The 5′-biotinylated oligonucleotide probe BP, specific for the genus *Bartonella*, was derived from positions 124 to 145 of the *B. henselae* 16S rRNA sequence (Table 1 and Fig. 1). The BP sequence is identical to those of the 16S rRNA sequences of the seven other *Bartonella* species present in the 16S rRNA prokaryotic nucleotide sequences database of EMBL (release 41): *B. bacilliformis*, *B. quintana*, *B. vinsonii*, *B. elizabethae*, *B. doshiae*, *B. grahamii*, and *B. taylorii*. The sequence of probe BP differs by at least 2 bases from that of any other 16S rRNA sequence present in the database. The probe was used to detect the type-specific *Bartonella* PCR products.

Hybridization of PCR products. The nylon membranes (approximately 320 cm^2), containing PCR products of both type-specific PCRs, were prehybridized for 5 min at 55°C in 20 ml of 2× SSPE (360 mM NaCl, 20 mM Na_2HPO_4, 2 mM EDTA) with 0.1% SDS. Thirty picomoles of the *Bartonella*-specific, biotinylated oligonucleotide BP was added and was hybridized for 1 h at 55°C; this was followed by two washing steps of 10 min each at 55°C with 50 ml of 2× SSPE–0.1% SDS. The membranes were incubated with 1:4,000-diluted streptavidin-peroxidase conjugate (Boehringer, Mannheim, Germany) for 45 min at 42°C in 20 ml of 2× SSPE–0.5% SDS and were subsequently washed three times for 10 min each time with 50 ml of 2× SSPE containing 0.5%, 0.1%, and no SDS, respectively. The membrane was incubated with enhanced chemiluminescence (ECL) detection reagents (Amersham B.V., 's-Hertogenbosch, The Netherlands) for 1 min and was used to expose an ECL hyperfilm (Amersham B.V.) for 5 s to 10 min to visualize hybridization.

Restriction of amplified spacer DNA. Seven to 15 μl of each PCR-amplified spacer product was digested for 1.5 h with 5 U of restriction enzyme *Alu*I or 2 U of *Sau*3A. The resulting DNA fragments were analyzed after electrophoresis on an ethidium bromide-stained, 3% NuSieve low-melting-point agarose gel (FMC BioProducts, Rockland, Maine). Marker V (Boehringer) was used as a DNA size standard. The DNA was visualized on a UV transilluminator and photographed.

Purification and DNA sequence analysis of PCR products. The PCR products generated with the broad-range 16S rRNA primers 16SF and 16SR were extracted with TE-buffered phenol and were subsequently purified on a QiaQuick PCR product purification spin column (Qiagen, Hilden, Germany). These products were sequenced directly by using fluorescent dye terminators in the cycling sequencing system (Perkin-Elmer Cetus).

RESULTS

Strain differentiation of *Bartonella* species by RFLP analysis of the 16S-23S RNA spacer region. PCR amplification with the broad-host-range PCR primers RPC5 and R23S2693 resulted in an approximately 1,700-bp 16S-23S rRNA spacer PCR fragment in 69 of the 108 clinical samples from Dutch patients with CSD, in 3 of the 6 clinical samples from North American patients with CSD, in the blood sample from a cat, and in lysates of all *B. henselae* and *B. quintana* strains used in the study. In the remaining samples no PCR product was obtained except in the case of *B. bacilliformis* KC583, from which a PCR product of approximately 1,400 bp was generated.

The six *B. henselae* strains isolated from North American patients with BA and one from a Dutch cat (CAT5) yielded four different *Alu*I RFLP patterns: pattern A was generated from strain ATCC 49882, pattern B was generated from strains 91-148 and CAT5, pattern C was generated from strain ATCC 49793, and pattern D was generated from strains 90-782, 91-100, and 92-967 (Fig. 2A). The four patterns differed from each other in only one or two bands. The difference between pattern A and pattern B is caused by the presence of one additional *Alu*I restriction site in the PCR fragment. As a result, a 430-bp fragment of pattern B is cleaved into two fragments of approximately 350 and 80 bp in pattern A. Digestion of the spacer product obtained from two *B. quintana* strains resulted in identical patterns (pattern E), which were clearly distinct from all *B. henselae* patterns, which is in accordance with the results described earlier by Matar et al. (10). Additionally, the *Alu*I RFLP pattern (pattern F) obtained from *B. bacilliformis* KC583 DNA differed clearly from the patterns obtained from all *B. henselae* and *B. quintana* isolates (Fig. 2A). The six *B. henselae* strains yielded three very similar *Sau*3A RFLP patterns which could be distinguished from each other by very small differences in the sizes of the upper band. *Sau*3A pattern A was generated from strains ATCC 49882, 91-148, and CAT5, pattern B was generated from strain ATCC 49793, and pattern C was generated from strains 92-967, 91-100, and 90-782 (Fig. 2B). *Sau*3A does not give any information in addition to that obtained from the *Alu*I typing results of the *B. henselae* strains used in the study, because all strains with *Alu*I RFLP patterns A and B gave *Sau*3A RFLP pattern A, all strains with *Alu*I pattern C gave *Sau*3A pattern B, and all strains with *Alu*I pattern D gave *Sau*3A pattern C. As in the *Alu*I analysis, both *B. quintana* strains yielded identical *Sau*3A RFLP patterns (pattern D) which were clearly distinct from the *B. henselae* patterns. The *Sau*3A RFLP pattern (pattern E) obtained from *B. bacilliformis* KC583 was unique and differed completely from the patterns obtained from all other *Bartonella* isolates (Fig. 2B). The *Alu*I and *Sau*3A spacer RFLP results obtained from all *Bartonella* strains are summarized in Table 2.

Of the 69 spacer PCR fragments obtained from clinical samples from Dutch patients with CSD, 27 were sufficient to obtain visible digestion patterns on an agarose gel. Digestion of spacer PCR fragments from clinical samples from patients with CSD with the restriction endonuclease *Alu*I resulted in two RFLP patterns, patterns A and B (Fig. 2A). Of the 27 samples tested, 20 yielded *Alu*I RFLP pattern A, whereas 7 samples

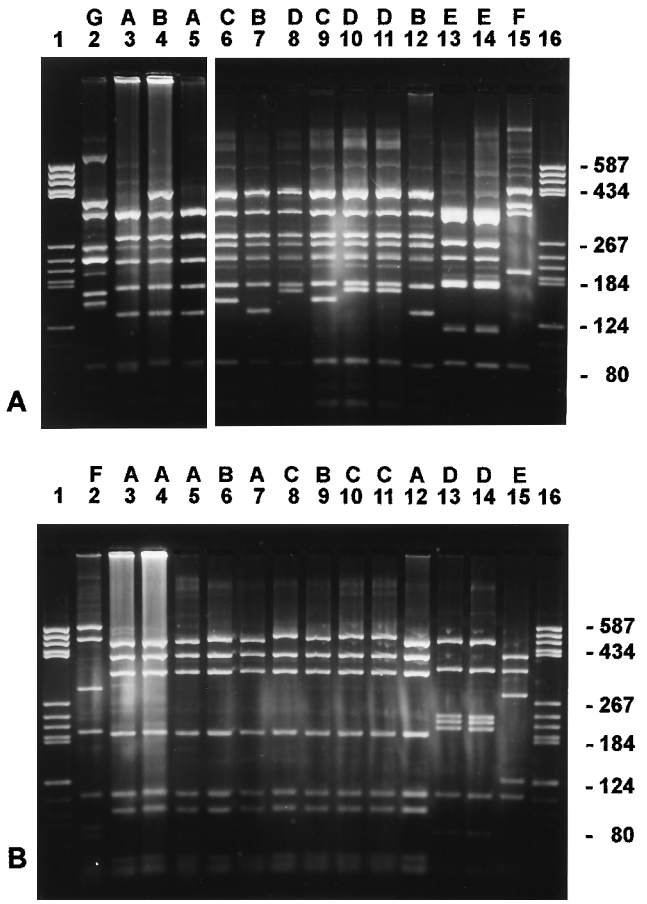


FIG. 2. *Alu*I (A) and *Sau*3A (B) RFLP patterns of PCR-amplified 16S-23S spacer regions from *Bartonella* species present in clinical material from patients with CSD and from *B. henselae*, *B. quintana*, and *B. bacilliformis* strains. Lanes 1 and 16, molecular size standards (in base pairs); lanes 2, blood sample from a cat; lanes 3 and 4, clinical samples from Dutch patients with CSD; lanes 5 to 12, *B. henselae* strains; lane 5, ATCC 49882; lane 6, ATCC 49793; lane 7, 91-148; lane 8, 92-967; lane 9, ATCC 49793; lane 10, 91-100; lane 11, 90-782; lane 12, CAT5; lane 13, *B. quintana* 90-268; lane 14, *B. quintana* 92-263; lane 15, *B. bacilliformis* KC583.

TABLE 2. *Alu*I and *Sau*3A 16S-23S spacer RFLP patterns and results of *B. henselae* type-specific PCR of *B. henselae*, *B. quintana*, and *B. bacilliformis* strains and samples from patients with CSD

Strain and isolate or sample	*Alu*I RFLP pattern	*Sau*3A RFLP pattern	16S rRNA PCR type
B. henselae			
ATCC 49882	A	A	I
91-148	B	A	II
CAT5	B	A	II
ATCC 49793	C	B	I
90-782	D	C	I
91-100	D	C	I
92-967	D	C	I
B. quintana			
90-268	E	D	I
92-263	E	D	I
B. bacilliformis KC583	F	E	I
Clinical samples (n = 40)			
Group 1 (n = 20)	A	A	I
Group 2 (n = 6)	B	A	II
Group 3 (n = 1)	B	A	—[a]
Group 4 (n = 12)	ND[b]	ND	I
Group 5 (n = 1)	ND	ND	II
Cat blood	G	F	—

[a] —, no PCR product was obtained with either primer set.
[b] ND, not done.

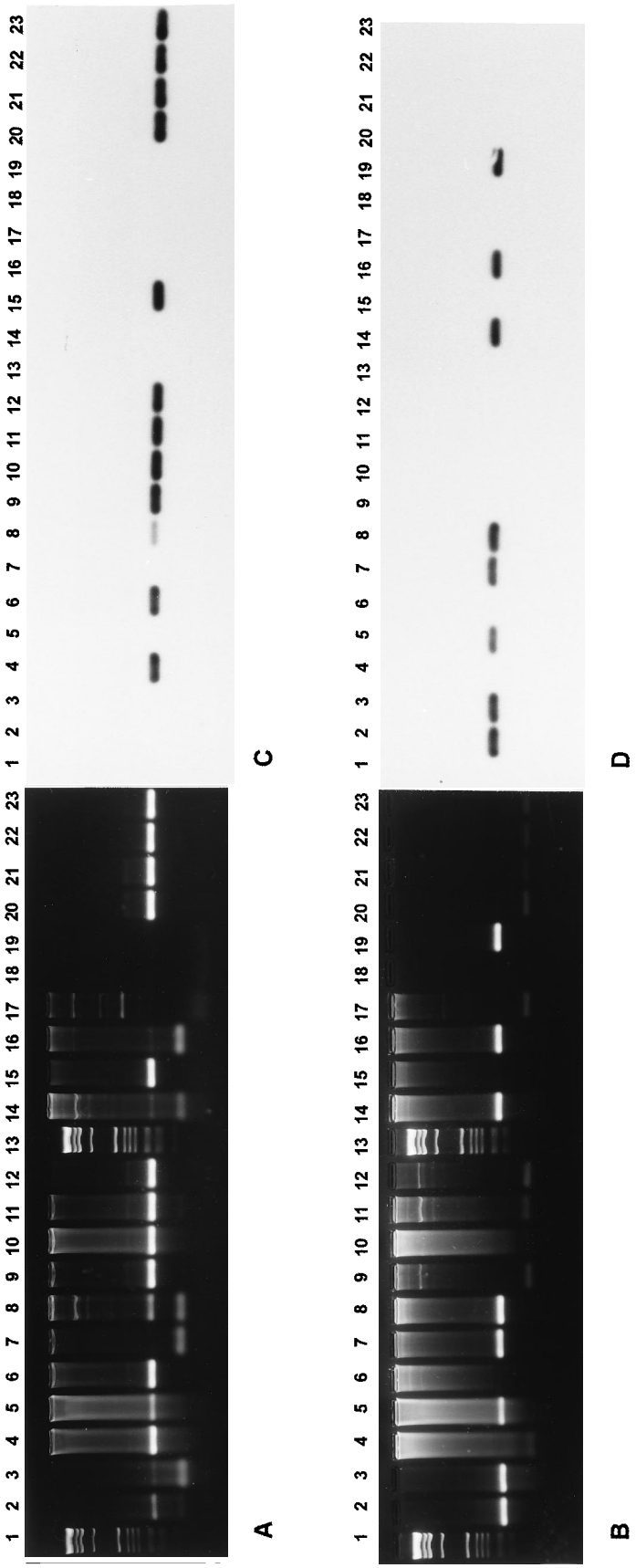
yielded pattern B. Analysis of the three samples from North American patients with CSD showed that these samples contained *B. henselae* RFLP pattern B. RFLP analysis of the spacer PCR product with the restriction endonuclease *Sau*3A resulted in RFLP pattern A in all 30 clinical samples from patients with CSD (Dutch and North American) (Fig. 2B). Thus, *Alu*I has more potency than *Sau*3A for discriminating *B. henselae* isolates present in clinical samples from patients with CSD.

The *Bartonella* species present in the blood sample from a cat showed totally different *Alu*I and *Sau*3A RFLP patterns that had few bands in common with those of the RFLP patterns described above; two of eight bands with *Alu*I and one of five bands with *Sau*3A, respectively, were identical in size to those of *Alu*I and *Sau*3A RFLP patterns A (Fig. 2).

The *Alu*I and *Sau*3A RFLP results for all of the clinical samples are summarized in Table 2.

Differentiation of *B. henselae* by type-specific PCRs. Only 69 of the 108 *Bartonella* DNA-positive samples from Dutch patients with CSD yielded a 16S-23S rRNA spacer PCR product, of which only 27 samples yielded sufficient PCR product to be analyzed by digestion with restriction endonucleases. This low degree of sensitivity of the spacer PCR might be due to the size of the PCR product to be formed. Because the chromosomal DNA in clinical samples is often fragmented, a 1,700-bp fragment may be too large to be amplified in clinical material. Therefore, we needed PCR primers that yielded smaller PCR fragments and that could discriminate between different *B. henselae* variants. Partial sequence analysis of the 16S rRNA gene PCR products from Dutch patients with CSD had revealed two sequences differing from each other in 3 bp located at positions 172 to 175 of the 16S rRNA gene (3). On the basis of these differences we designed two type-specific primers, BH1 and BH2, to analyze a number of clinical CSD samples for the presence of both *B. henselae* 16S rRNA gene types by PCR (Fig. 1 and Table 1).

All *Bartonella* strains were subjected to PCRs with the broad-host-range primer 16SF and either of the type-specific primers BH1 or BH2. The PCRs were designated the type I PCR and the type II PCR, respectively. At an annealing temperature of 54°C, *B. henselae* ATCC 49882, ATCC 49793, 90-782, 91-100, and 92-967 (*Alu*I spacer RFLP patterns A, C, and D) were positive in the type I PCR, while *B. henselae* 91-148 and CAT5, which had *Alu*I spacer RFLP pattern B, yielded a PCR product only in the type II PCR. *B. henselae* 92-967 (*Alu*I RFLP pattern D) also yielded a small amount of PCR product in the type II PCR. All type-specific PCR products hybridized with the *Bartonella*-specific oligonucleotide probe BP. Amplification of DNA from *B. bacilliformis* and from two *B. quintana* strains yielded PCR products in the type I PCR when annealing was performed at 54°C, although their 16S rRNA sequences have one and two mismatches with the BH1 primer sequence, respectively (Fig. 1). When the annealing temperature of the type I PCR was raised to 56°C, the amount of PCR product from both *B. quintana* strains decreased. At 58°C no type I PCR product was obtained with the *B. quintana* DNA, while a small amount of type I PCR product


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23
A
B
C
D

was still visible when *B. bacilliformis* DNA was amplified at an annealing temperature of 58°C. Type-specific PCR products of some strains are depicted in Fig. 3.

Forty-two of the 108 samples from Dutch patients with CSD and 2 of the 6 samples from North American patients with CSD were subjected to both type-specific PCRs. The 42 Dutch samples comprised the 27 samples that were analyzed by spacer RFLP, 4 samples that were positive in the spacer PCR but that yielded insufficient PCR product for RFLP analysis, and 11 samples that were negative in the spacer PCR. When the type I PCR was performed at an annealing temperature of 54°C, PCR fragments were observed in 32 of the 42 samples from Dutch patients with CSD tested (Fig. 3A). Seven samples from Dutch patients with CSD and the two samples from North American patients with CSD yielded a type II PCR fragment. Three of the 42 Dutch clinical samples were negative in both the type I PCR and the type II PCR. All type I and type II PCR products hybridized with the *Bartonella*-specific oligonucleotide probe BP (Fig. 3). Seven of the nine type II-positive samples also yielded a small amount of PCR product in the type I PCR, and two of these PCR products gave a weak hybridization signal with the *Bartonella*-specific probe BP. No PCR product was observed in the other seven samples when the type I PCR was repeated with an annealing temperature of 56°C (data not shown). Remarkably, all 20 Dutch samples that had spacer *Alu*I RFLP pattern A were positive in the type I PCR. Eight of the 9 type II-positive samples were analyzed by the spacer RFLP method, and all yielded *Alu*I RFLP pattern B.

Several clinical samples from patients with CSD did not yield a PCR product in the type I PCR when an annealing temperature of 58°C instead of 54°C was used, while other samples with *B. henselae* type I still did. To determine whether differences in the 16S rRNA gene of the *B. henselae* type present in the former samples were responsible for this apparent differential behavior, partial 16S rRNA gene sequences were determined. Four clinical samples from patients with CSD that yielded less type I PCR product at 58°C and three clinical samples from patients with CSD that still yielded ample type I PCR product at 58°C were used. Additionally, DNA from *B. henselae* 92-967, which yielded a large amount of type I PCR product together with a small amount of type II PCR product, was amplified in order to determine its partial 16S rRNA gene sequence. PCR products were obtained by amplification with broad-host-range primers 16SF (positions 10 to 29 of the *B. henselae* 16S rRNA gene) and 16SR (positions 521 to 502) to ensure that the sequence of the BH1 primer site (positions 192 to 172) could be determined. The sequences of the PCR fragments obtained were compared with the two *B. henselae* sequences found in clinical samples from patients with CSD (3). This comparison revealed that the first 350 bp of the 16S rRNA gene sequences from all seven clinical samples and of *B. henselae* 92-967 were identical to the type I 16S rRNA gene sequence.

Neither the type I PCR nor the type II PCR yielded a PCR fragment from the blood sample from the cat, corroborating the RFLP result showing that the *Bartonella* species in this sample differs significantly from those found in human samples (Fig. 3). Further details on this organism will be described elsewhere.

All RFLP and type-specific PCR results were combined and are summarized in Table 2.

DISCUSSION

Subtyping of *B. henselae* may be of critical importance for epidemiological studies of CSD and BA to determine the involvement of the cat as a reservoir and of fleas and other vectors in the transmission of *B. henselae*. Because culture of *B. henselae* from pus aspirates and lymph node biopsy specimens from patients with CSD is extremely difficult, typing of the strains from those samples cannot easily be performed. The study described here is the first one in which a PCR-based typing technique has been used directly on clinical samples from patients with CSD. We have applied a PCR-based RFLP method described by Matar et al. (10) to clinical samples from patients with CSD that contained *Bartonella* DNA as determined by a PCR-hybridization assay (3). The PCR-RFLP method has been shown to be quite useful in discriminating *B. henselae* strains in clinical material. Although the primer set used in the study produces 16S-23S rRNA spacer PCR fragments with most eubacterial species, we have shown that these broad-host-range primers can also be used to type bacteria present in clinical material from patients with CSD without the need to culture clinical samples for these organisms. The rationale for using these primers came from a previous study in which we showed by PCR and sequence analysis that pus aspirates and lymph nodes from patients with CSD did not contain any detectable bacterial DNA other than *Bartonella* DNA (3).

RFLP analysis of the spacer PCR products revealed only two *B. henselae* RFLP variants among 27 samples from Dutch patients with CSD. Matar et al. (10) found six distinct *Alu*I RFLP types in 11 culture-grown *B. henselae* strains. However, virtually all of the strains used in the latter study originated from patients with BA. This would suggest that *B. henselae* strains causing disease in immunocompromised hosts are more heterogeneous than strains causing CSD in immunocompetent individuals. It is possible that cats carry a variety of different *B. henselae* types and that only a limited number of these types can cause disease in immunocompetent human hosts. However, if the natural defense mechanisms of the host are affected, such as in human immunodeficiency virus-infected individuals, less virulent *B. henselae* types may cause disease. Differences in the geographic origins of the strains may also explain the differences between our results and those of Matar et al. (10). However, we analyzed three clinical samples originating from North American patients with CSD and found that these belong to one of the two RFLP types that we found in Dutch patients with CSD. More samples from CSD patients from various countries will have to be analyzed to determine the extent to which the variations correlate with the geographic spread of the organism.

In a previous report (3) we described that the partial sequencing of the 16S rRNA genes of *B. henselae* present in eight samples from patients with CSD revealed the presence of two

FIG. 3. Analysis of products of type-specific PCRs with DNA from clinical material from patients with CSD and from cultures of *B. henselae*, *B. quintana*, and *B. bacilliformis* as targets. Agarose gel electrophoresis (A and B) and Southern blots hybridized with *Bartonella*-specific oligonucleotide probe BP (C and D) of PCR fragments obtained by type I-specific PCR (A and C) and type II-specific PCR (B and D), both at an annealing temperature of 54°C. Lanes 1 and 13, molecular size standards (in base pairs); lanes 2 and 3, clinical samples from North American patients with CSD; lanes 4 to 12, 14 and 16, clinical samples from Dutch patients with CSD; lanes 17, blood sample from a cat; lanes 18, empty; lanes 19 to 21, *B. henselae* 91-148, ATCC 49882, and ATCC 49793, respectively; lanes 22, *B. quintana* 90-268; lanes 23, *B. bacilliformis* KC583.

B. henselae variants. On the basis of the sequence differences, two variant-specific 16S rRNA PCRs were developed and used to analyze 44 of the 114 samples from Dutch and North American patients with CSD used in the spacer RFLP experiments. The majority of the samples from Dutch patients with CSD (82%) contained *B. henselae* type I, suggesting that this type is predominant in CSD in The Netherlands. All *B. henselae* isolates that were identified as the A type in the spacer *Alu*I RFLP were shown to yield a fragment in the type I 16S rRNA PCR. All samples that had yielded a B type in the spacer *Alu*I RFLP were positive in the type II PCR except for one sample which remained negative in both type-specific PCRs. This 100% correlation between 16S rRNA PCR type and *Alu*I spacer RFLP type is striking, because each typing method uses polymorphism in different parts of the bacterial genome. A possible explanation for this finding is that two mutational events could have occurred at the same time, one in the 16S rRNA gene and one in the 16S-23S rRNA spacer region. As a result, both the 16S sequence type and the *Alu*I RFLP pattern would have changed by this double mutation. Another possibility is the occurrence of two consecutive mutations, one in the 16S rRNA gene and one in the 16S-23S rRNA spacer region, within a short period. In that case, it is to be expected that the chance of detecting descendants with one mutation is very small.

Virtually all of the type II-positive clinical samples also yielded a small amount of product in the type I PCR that did not hybridize with the *Bartonella*-specific probe. The type I PCR signal of these dual reactive samples was lost under more stringent PCR conditions. A likely explanation for this finding is the occurrence of false priming of the type I primer set. Several samples that contained *B. henselae* DNA of RFLP type A yielded significantly smaller amounts or even no PCR product in the type I PCR under stringent PCR conditions. Partial sequence analysis of the 16S rRNA gene of *B. henselae* present in four of the latter samples revealed that the region amplified in the type I PCR was identical to the type I *B. henselae* 16S rRNA sequence (3). Thus, the absence of a signal in the type I PCR under more stringent PCR conditions with some clinical samples is not due to mismatches in the BH1 primer sequence but is probably the result of differences in the target DNA and/or inhibitor concentration between samples. Because PCR with the BH1 primer cannot discriminate between *B. henselae* type I, *B. quintana*, and *B. bacilliformis*, primer BH1 is unsuitable for species or subspecies identification. In contrast, primer BH2 has proven to be highly specific for *B. henselae* type II RFLP type B. PCR-based *Alu*I RFLP analysis of the 16S-23S rRNA spacer region was shown to have more discriminatory potency than the type-specific PCRs. Sequence analysis of the 16S-23S spacer region has shown a frequency of mutation of 0.5 to 4% within the *B. henselae* species (15). This degree of divergence is probably too low to obtain much restriction site polymorphism. Thus, sequencing of spacer regions will be necessary to establish a greater degree of differentiation within the *B. henselae* species. Because sequence analysis is rather laborious, other methods should be optimized for use in epidemiological studies. By using combinations of restriction enzymes in the spacer RFLP method, more variable RFLP patterns could be obtained. End labeling of digested spacer PCR fragments will lower the detection level of the RFLP method and increase the range of fragment sizes to be used for analysis, and thus, smaller amounts of *B. henselae* DNA could also be analyzed by spacer RFLP.

In conclusion, we have shown that samples from Dutch patients with CSD contain a limited number of *B. henselae* variants, suggesting that only a few of the extant *B. henselae* types can cause CSD. In immunocompromised individuals and in persons living under unhygienic conditions, not only *B. henselae* variants but even other *Bartonella* species such as *B. quintana* are found (8). With the rapidly expanding repertoire of the genus *Bartonella*, even more species may appear to be associated with human disease. Possible candidates are *B. grahamii*, *B. taylorii*, and *B. doshiae*, which were isolated from small woodland mammals (4, 5). Yet, pet animals may also carry a variety of bartonellas. Therefore, we are studying the spectrum of *Bartonella* species and subspecies found in cats and arthropods.

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