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Molecular Characterization of First Human *Bartonella* Strain Isolated in Italy

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The aim of this study was to characterize a *Bartonella* strain (BA-1) isolated from a blood culture of an Italian, human immunodeficiency virus-positive patient with bacillary angiomatosis. We analyzed the isolate using molecular biology methods such as whole-cell fatty acid analysis, PCR-restriction fragment length polymorphism analysis, type-specific 16S rRNA PCRs, sequence analysis of the 16S rRNA, pulsed-field gel electrophoresis, and arbitrarily primed PCR. The BA-1 isolate turned out to be a *Bartonella quintana* strain, similar but not identical to *B. quintana* Oklahoma, which was used as a control strain.

Among the 14 species now included in the genus *Bartonella*, at least 7 are human pathogens. *Bartonella henselae*, the most common cause of cat scratch disease, and *B. quintana*, the trench fever bacillus, are related species that can also cause a common spectrum of clinical diseases such as bacillary angiomatosis (BA), peliosis hepatis, relapsing bacteremia with fever, chronic lymphadenopathy, and endocarditis. The isolation of *Bartonella* strains is not a straightforward task but is extremely important for both etiological diagnosis and genetic investigation of the isolates (21). We report molecular characterization of BA-1, a *Bartonella* strain isolated from a patient with BA.

In June 1997, a 37-year-old male who was a heroin user and had been infected with human immunodeficiency virus (HIV) since 1985 was admitted to the Arcispedale S. Maria Nuova (Reggio Emilia, Italy), presenting with high recurrent fever and multiple nodular papules over his whole integument. Histologic examination of a skin biopsy specimen showed features typical of BA. Warthin-Starry staining revealed a cluster of small bacilli. Echography showed that the liver was involved. Patient serum yielded a high diagnostic titer (1:512) when it was tested in an indirect immunofluorescence assay using *B. henselae* as an antigen. Three blood samples showed bacterial growth after 35 days of incubation in BACTEC bottles (BACTEC NR730; Becton Dickinson, Cockeysville, Md.). The organisms were subcultured in 5% CO_2 at 37°C on brain heart infusion agar (BBL, Becton Dickinson) plates supplemented with 5% rabbit blood. After 4 to 7 days, 0.5- to 2-mm-diameter colonies were visible. The isolated organisms were small, gram-negative, oxidase- and catalase-negative, nonfermentative, nonmotile rods.

In this study, the following strains of three different species of *Bartonella* were used as positive controls: *B. henselae* Houston-1 (ATCC 49882, type I strain); *B. henselae* 269608 (type II strain) isolated at the University of California, Davis, from the blood of a 20-year-old pet cat from northern California; *B. quintana* Oklahoma, a blood culture strain isolated from an HIV-positive patient in Oklahoma; and *B. clarridgeiae* (ATCC 51734). *B. henselae* Pavia-1, isolated from a cat in Italy, was also included in the analysis (9).

Fatty acid methyl ester derivatives were prepared for gas chromatographic analysis by using the following four-phase procedure: saponification, methylation, extraction, and a base wash as described elsewhere (5). The fatty acid composition observed for the new isolate was characterized by a relatively simple profile: large amounts of $C_{16:0}$ (hexadecanoic acid) (27.5%), $C_{18:1\omega7}$ (*cis*-11-octadecenoic acid) (39.5%), and $C_{18:0}$ (octadecenoic acid) (31.8%) and minor amounts of $C_{18:2\omega6}$ (*cis*-9,12-octadecadienoic acid) (traces) and $C_{18:1\omega9}$ (*cis*-9-octadecanoic acid) (1.2%). The high similarity between the whole-cell fatty acid (CFA) composition of BA-1 and those of *B. quintana*, *B. henselae*, and *B. clarridgeiae* (data not shown) identified the isolate as a member of the genus *Bartonella* but did not allow identification of the species.

The primers BhCS.781p (5′-GGGGACCAGCTCATGGTG G-3′) and BhCS.1137n (5′-AATGCAAAAAGAACAGTAAA CA-3′) were used to amplify a fragment of the *Bartonella* citrate synthase gene by PCR as previously described (15). The amplicons (379 bp) were subjected to restriction fragment length polymorphism (RFLP) analysis with *Taq*I restriction endonuclease (Fig. 1). We found identical profiles for *B. henselae* Houston-1, *B. henselae* 269608, and *B. henselae* Pavia-1 (bands of 171, 137, and 71 bp). Patterns were also identical in *B. quintana* Oklahoma and BA-1 (bands of 316 and 63 bp). The profile of the *B. clarridgeiae* reference strain was different from those of *B. henselae* and *B. quintana*.

Amplification of the 16S rRNA gene with the universal primer 16SF [5′-AGAGTTTGATCCTGG(CT)TCAG-3′] and with either of the type-specific primers BH1 (5′-CCGATAAA TCTTTCTCCCTAA-3′) and BH2 (5′-CCGATAAATCTTTC

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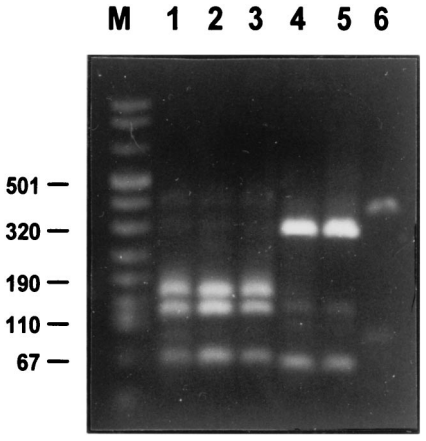

FIG. 1. PCR-RFLP analysis with *Taq*I restriction enzyme of the citrate synthase gene. Lanes: M, molecular marker VIII (Boehringer Mannheim, Mannheim, Germany); 1, *B. henselae* Houston-1 (ATCC 49882, type I strain); 2, *B. henselae* 269608 (type II strain); 3, *B. henselae* Pavia-1; 4, BA-1; 5, *B. quintana* Oklahoma; 6, *B. clarridgeiae* (ATCC 51734). Values at the left are molecular sizes (in base pairs).

TCCAAAT-3′) was carried out as described elsewhere (1, 22). Two annealing temperatures were applied for PCR amplification: 56 and 58°C. As expected, the *B. henselae* Houston-1 and *B. henselae* Pavia-1 isolates yielded a PCR product in the type I PCR while *B. henselae* 269608 was positive in the type II PCR. Surprisingly, the BA-1 isolate and *B. clarridgeiae* yielded a PCR product when the primers specific for type I were used. Similarly to strain *B. henselae* Houston-1 and the reference strain of *B. clarridgeiae*, the new isolate was positive in the type I PCR when the DNA was amplified at 58°C. In contrast, *B. quintana* Oklahoma DNA was negative in both PCRs at both annealing temperatures (Fig. 2).

The 16S rRNA of the BA-1 isolate was amplified by using two universal eubacterial primers, 27f (5′-GAGAGTTTGAT CCTGGCTCAG-3′) and 1495r (5′-CTACGGCTACCTTGTT ACGA-3′) (24). The amplicon, a 1,455-bp product, was cloned in the pGEM-T easy vector (Promega, Madison, Wis.). Sequencing was performed with a commercial T7 sequencing kit (Pharmacia Biotech) with M13 forward and reverse primers, and results were analyzed on a Pharmacia Biotech ALF*Express* automated DNA sequencer. The sequence obtained was compared to others by using the BLAST search tool (http://www.ncbi.n/m.nib.gov/BLAST/). The 16S rRNA gene sequence obtained (GenBank accession number AJ250247) revealed a 99% identity with the sequence described for *B. quintana* Fuller (GenBank accession number M11927), confirming the identification of BA-1 as *B. quintana*.

To further characterize the BA-1 isolate, pulsed-field gel electrophoresis (PFGE) and arbitrarily primed PCR (AP-PCR) were carried out. Genomic DNAs were prepared according to the procedure described by Maurin et al. (14) and were digested with *Sma*I (Fig. 3A). Among the five strains used as controls, *B. quintana* Oklahoma and *B. clarridgeiae* were easily distinguishable from one another and from the three *B. henselae* isolates by visual inspection. In contrast, very similar restriction patterns were obtained for *B. henselae* Houston-1, *B. henselae* 269608, and *B. henselae* Pavia-1. Very similar restriction patterns were also found for *B. quintana* Oklahoma and the BA-1 isolate. The restriction pattern of BA-1 showed 23 bands, 20 of which were common to the pattern found for *B. quintana* Oklahoma. With the Dice coefficient, the patterns of these two strains could be discriminated, having a cutoff of 94% similarity.

AP-PCR was performed with the core sequence of phage M13 (5′-GAGGGTGGCGGTTCT-3′) as described previously (11, 22). The fingerprints were analyzed with GelCompar software, Windows version 4.0 (Applied Maths, Kortrijk, Belgium) (Fig. 3B). The patterns produced were compared by using the Dice coefficient. For clustering, the unweighted pair group method with arithmetic means was used. Three groups were identified on the basis of AP-PCR fingerprints. Group I consisted solely of *B. henselae* strains. Group II contained *B. quintana* Oklahoma and the new isolate. Group III contained only the reference strain of *B. clarridgeiae*. The *B. quintana* Okla-

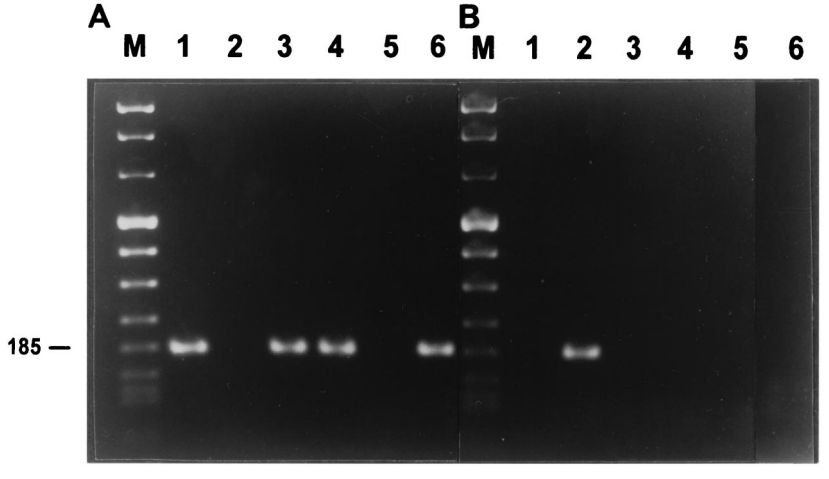

FIG. 2. Type-specific 16S rRNA PCRs. PCR products were obtained at an annealing temperature of 58°C with primers BH1 (A) and BH2 (B). Lanes: M, molecular size marker VIII (Boehringer Mannheim); 1, *B. henselae* Houston-1 (ATCC 49882, type I strain); 2, *B. henselae* 269608 (type II strain); 3, *B. henselae* Pavia-1; 4, BA-1; 5, *B. quintana* Oklahoma; 6, *B. clarridgeiae* (ATCC 51734). The value at the left is a molecular size (in base pairs).

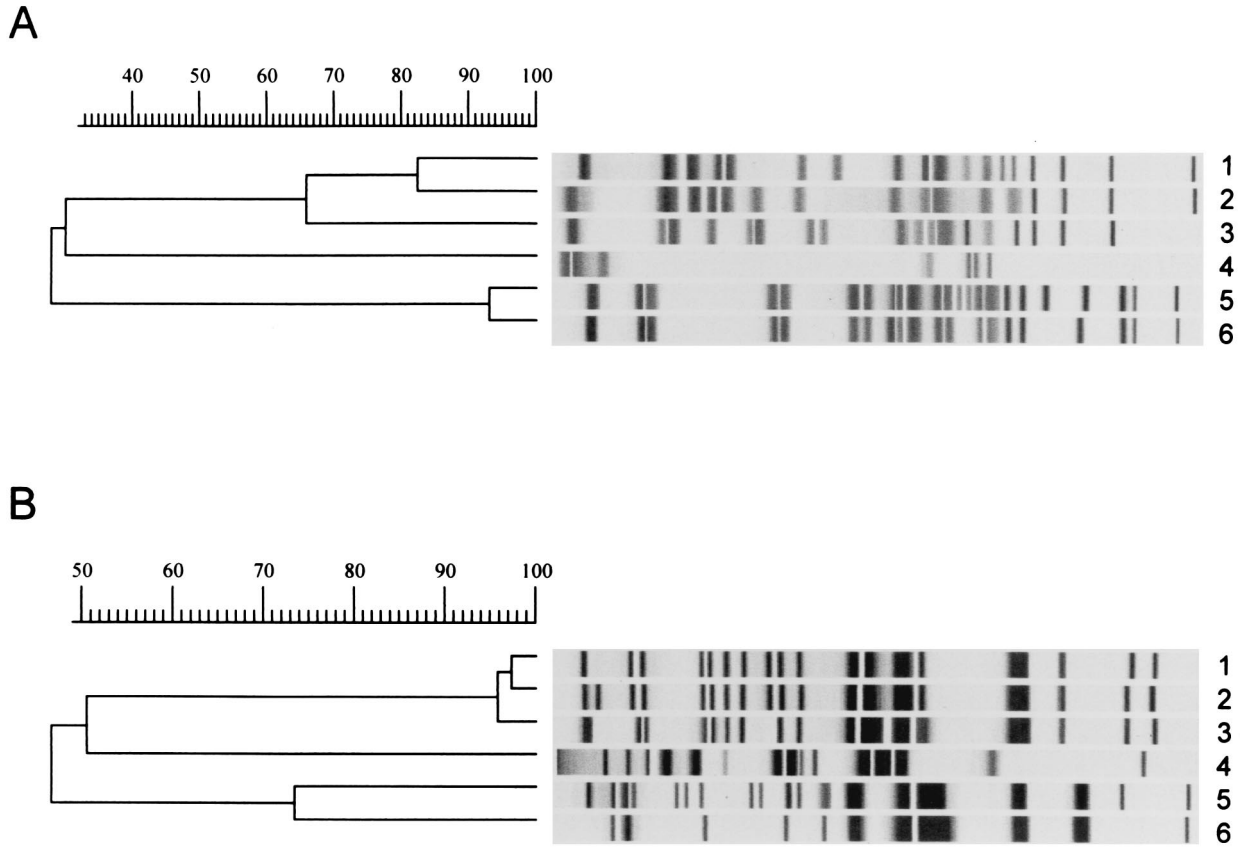

FIG. 3. (A) PFGE analysis with *Sma*I and dendrogram of fingerprints determined by the Dice coefficient. The digestion products were separated at 190 V for 19 h in 1% agarose–0.25× Tris-borate-EDTA with a pulse time increasing from 3 to 10 s. (B) AP-PCR and dendogram of fingerprints determined by the Dice coefficient. Lanes: 1, *B. henselae* Houston-1 (ATCC 49882, type I strain); 2, *B. henselae* 269608 (type II strain); 3, *B. henselae* Pavia-1; 4, *B. clarridgeiae* (ATCC 51734); 5, BA-1; 6, *B. quintana* Oklahoma.

homa and BA-1 isolates had a similarity level of 80% in the dendrogram and could thus be differentiated.

For further in-depth molecular studies, six biological methods were used to characterize the BA-1 isolate. All methods applied were found to be useful. The phenotypic characterization by CFA allowed for the identification of the isolate at the genus level, while the genotypic characterization by PCR-RFLP analysis of a fragment of the citrate synthase gene discriminated among *B. henselae*, *B. quintana*, and *B. clarridgeiae* and identified the BA-1 isolate as *B. quintana*. Amplification and sequencing of the complete 16S rRNA gene were also performed for the BA-1 isolate. The sequence obtained was almost identical to that of *B. quintana* Fuller.

Surprisingly, the type-specific PCRs with BH1 and BH2 were not as strictly species specific for *B. henselae* as expected. In fact, with the exception of *B. quintana* Oklahoma, all strains, including the type strain of *B. clarridgeiae* and the BA-1 isolate, yielded PCR products with these primers.

Compared with CFA and PCR-RFLP analysis, AP-PCR and PFGE showed a higher discriminatory power. As expected, both methods proved to be suitable for strain differentiation at the species level and sensitive in detecting minor differences among strains of the same *Bartonella* genus and species. Strains BA-1 and *B. quintana* Oklahoma produced similar but not identical PFGE and AP-PCR profiles. The genetic variation of *B. quintana* isolates was less pronounced than the variation of the three *B. henselae* isolates. The level of similarity among AP-PCR and PFGE fingerprints for *B. quintana* Oklahoma and BA-1 suggests that these two strains derive from different clones.

B. quintana is an emerging human pathogen that causes a wide spectrum of clinical diseases (urban trench fever, BA, peliosis hepatis, peliosis spleniitis, lymphadenopathy, and endocarditis) in both immunocompetent and immunocompromised hosts (16). Little is known about the geographical distribution of *B. quintana* in Europe. Although many *B. quintana* strains have been isolated in France (3, 4, 7, 8, 13, 14, 17, 18), the organism has rarely been recovered in other European countries (10, 23). In 1995, Drancourt et al. (7) reported the isolation of *B. quintana* from three homeless patients with endocarditis in France. Brouqui et al. (3) investigated the prevalence of *Bartonella* species in blood samples from all of the homeless people who availed themselves of the emergency department of the University Hospital, Marseille, France, between October 1993 and October 1994. For 10 out of 71 homeless patients (14%), blood cultures were positive for *B. quintana*, and in 21 of the patients (30%), high titers of antibodies against this organism were detected. No such epidemiological study has yet been carried out in Italy.

In conclusion, our study has shown that (i) *B. quintana* is an

etiological agent of BA in Italy, (ii) the BA-1 isolate can be discerned from *B. quintana* Oklahoma, and (iii) contrarily to what we had expected, the type-specific primers BH1 and BH2 produced species-specific PCR products not only for *B. henselae* but for *B. clarridgeiae* and *B. quintana* BA-1 as well. Our results suggest that, in addition to *B. henselae*, *B. quintana* should be included as an antigen in the immunofluorescence test for the serological diagnosis of some *Bartonella* infections, even in those countries where trench fever and other *B. quintana* infections are not known to occur. Further investigations, however, are necessary (i) to determine the incidence of *B. quintana* infections, (ii) to establish whether more than one *B. quintana* clone exists in Italy, and (iii) to assess the degree of cross-reactivity between our isolate and the *B. quintana* Fuller strain. Urban trench fever has emerged among homeless persons in France (2), homeless, alcoholic, HIV-negative persons in Seattle, Wash. (12), and intravenous-drug abusers in Baltimore, Md. (6), and it has reemerged in Burundi after an absence of 12 years (19). *B. quintana* has been molecularly demonstrated to exist in body lice collected from homeless persons in Russia (20). All of this emphasizes the need for establishing regional surveillance laboratories for the detection and characterization of fastidious microbial pathogens as well as active surveillance programs. Outbreaks of louse-borne infections can occur in populations that live under squalid conditions (e.g., refugees or those left homeless as a consequence of war or natural disaster), where louse infestations are frequent. Even if the factors leading to outbreaks remain poorly understood, all methods for reducing louse infestation and avoiding crowding under poor hygienic conditions have been advocated as strategies for the prevention of *B. quintana* infection.

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