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Strategy To Detect and Identify *Bartonella* Species in Routine Clinical Laboratory Yields *Bartonella henselae* from Human Immunodeficiency Virus-Positive Patient and Unique *Bartonella* Strain from His Cat

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We wished to develop a cost-effective, rapid strategy to detect and identify *Bartonella* species in the clinical laboratory and to determine the prevalence of *Bartonella* infection in the Houston veteran population. *Bartonella* colonies were identified by colony morphology, Gram stain, RapID ANA, repetitive extragenic palindromic-PCR (REP-PCR) and whole-cell fatty acid (CFA) analysis, and these methods were compared for their usefulness. A new test order for "*Rochalimaea* culture" (the genus *Bartonella* was previously known as the genus *Rochalimaea*) was instituted, and in addition, all blood specimens submitted for fungal culture (obtained in an isolator tube) were processed for *Bartonella* culture. Over a 16-month period we isolated *Bartonella henselae* from only 0.4% (2 of 533) of total cultures but from 1% (2 of 204) of human immunodeficiency virus-positive patients. After sufficient growth, identification of the *Bartonella* isolates to the species level could be obtained in 2 days. The REP-PCR allowed discrimination of all known species, whereas CFA analysis distinguished all except *B. henselae* and *Bartonella quintana*. The RapID ANA results failed to differentiate between *B. henselae* and *B. quintana*, and results for other species differed by only one or two tests. Blood obtained from a kitten which had been introduced into the household of one patient 2 months before the onset of fever yielded a *Bartonella* strain which was shown to be different from the strain from the patient and distinct from other *Bartonella* species by a combination of REP-PCR, CFA, and growth characteristics. Subsequent analysis of the citrate synthase gene sequence showed only an 86% similarity with any of the other known *Bartonella* species, suggesting that this isolate represents a distinct, previously uncharacterized species of *Bartonella*.

Recently, all the species of the two genera *Rochalimaea* and *Bartonella* were combined into the family *Bartonellaceae* and the genus *Bartonella* (2), giving five well-described species, *B. bacilliformis*, *B. elizabethae*, *B. vinsonii*, *B. quintana*, and *B. henselae*. In general, the *Bartonella* species grow on most enriched blood-containing media but are difficult to isolate in the clinical laboratory because they can take from 5 to 28 days on original culture to form visible colonies (4, 9, 18, 22, 26, 27). Although there are reports of isolations worldwide, the conditions for culture are not standard. In addition, many cases are diagnosed not by culture but only on the basis of serology, detection of specific DNA, or observation of distinctive pathology and organisms (12, 17). Identification after initial isolation is difficult because slow growth or lack of growth in liquid media limits the usefulness of commercial identification strips and some standard biochemical methods. Measurement of preformed enzymes has shown distinctions between species that sometimes rest on only one or a few biochemicals (6, 26). Whole-cell fatty acid (CFA) analysis has proved to be useful for identification because *Bartonella* species as a group have a unique and characteristic CFA profile (4, 16, 25). PCR amplification directly from the specimen has been important for detecting *Bartonella* infections (1, 12, 18). Sequencing of the 16S rRNA gene after PCR amplification both directly from the specimen and from purified genomic DNA has been fundamental in classifying *Bartonella* strains at the species level (12, 13, 18). Recently, a PCR-based technique to amplify random, repetitive extragenic palindromic (REP) sequences has been used to distinguish strains of gram-negative rods (25), and we have tested it in the research laboratory to distinguish among species of *Bartonella* (19). Other characterization techniques have included immunofluorescence, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and pulsed-field gel electrophoresis (14).

Bartonella species are associated with several distinct diseases; however, the frequency of occurrence in an adult veteran population is unknown. Historically, *B. bacilliformis* has been associated with verruga peruana and Oroya fever and *B. quintana* has been associated with trench fever. More recently, both *B. henselae* and *B. quintana* have been associated with bacillary angiomatosis, bacteremia, and prolonged febrile illness (8, 9, 11, 14, 15, 16, 20, 21, 26, 27). Isolation of the organism is usually from blood, but isolation can also be achieved from tissue, although with more difficulty (1, 5, 9, 10, 13). Although it has been proposed that *Afipia felis* is the etiologic agent of cat scratch disease, more recent studies by PCR, culture, and serology strongly indicate that *B. henselae* is the infecting species (1, 5, 22). Domestic cats have been demonstrated to be a reservoir for *B. henselae* (8, 17). *B. elizabethae*, *B. henselae*, and *B. quintana* have all been isolated from

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TABLE 1. Sources and characteristics of the *Bartonella* strains examined in the study

Species and strain	Clinical source[a]	Geographic origin	Growth rate (SBHI)[b]	Colony description	RapID ANA profile no.
B. henselae Houston-1A[c]	Blood, HIV+ patient	Texas	Slow-rapid	Rough/smooth	000671
B. henselae Houston-1B	Blood, HIV+ patient	Texas	Slow-rapid	Rough/smooth	000671
B. henselae Houston-2	Blood, HIV+ patient	Texas	Slow	Rough	000671
New strain, Houston-2 cat[d]	Blood, cat	Texas	Rapid	Rough	000671
B. henselae Houston-3	Blood, HIV+ patient	Texas	Slow	Rough	000671
B. henselae SA-2	Lymph node, CSD patient	Texas	Rapid	Smooth	000671
B. henselae GA-1	Lymph node, CSD patient	Georgia	Slow	Smooth	000671
B. henselae CA-1	Blood, HIV+ patient	California	Rapid	Smooth	000671
B. henselae Tiger-2	Blood, cat	South Carolina	Slow	Rough	000671
B. henselae Goldie-1	Blood, cat	Georgia	Slow	Rough	000671
B. quintana OK-90-268	Blood, HIV+ patient	Oklahoma	Slow	Smooth	000671
B. quintana WA-1	Blood, HIV+ patient	Washington State	Slow	Rough	000671
B. quintana Sh. perm	NA	Soviet Union	Rapid	Smooth	000671
B. quintana D-perm	NA	Soviet Union	Rapid	Smooth	000671
B. vinsonii ATCC VR-152	Spleen, vole	Canada	Rapid	Smooth	000661
B. elizabethae ATCC 49927	Blood, endocarditis patient	Massachusetts	Rapid	Smooth	00067/61
B. bacilliformis-1 ATCC 35685	NA	Peru	Rapid	Smooth	000641
B. bacilliformis-2	Gift from C. Krueger	Texas	Rapid	Smooth	000641

[a] HIV+, HIV positive; CSD, cat scratch disease; NA, not available.
[b] Growth rates: slow, 7 to 10 days for easily visible colonies; slow-rapid, colonies mixed; rapid, 4 days for easily visible colonies.
[c] The Houston-1A and Houston-1B strains were isolated from the same patient 3 months apart.
[d] The Houston-2 cat strain was isolated from Houston-2 patient's pet cat.

the blood of patients with endocarditis (5, 7, 23). *B. vinsonii* has only been isolated from a vole.

In 1990, we isolated on mycology media from blood drawn and concentrated in an isolator tube what was originally thought to be a *Rochalimaea quintana*-like organism, and we later identified it as *R. henselae* (3, 16). We report here the isolation of two more strains of *B. henselae* from human immunodeficiency virus (HIV)-positive patients with prolonged febrile illness and the isolation of an unique strain from one patient's cat. We also report a methodology for the biochemical, morphological, CFA, and REP-PCR characterization of *Bartonella* species which can be routinely deployed in the clinical laboratory to rapidly identify the organisms to species.

CASE REPORTS

Patient 1. A 47-year-old, HIV-antibody-positive male with a CD4 count of 26 cells per mm^3 presented in February 1994 with a 2-month history of fever, chills, malaise, myalgias, and arthralgias and 1 week of severe headaches. His temperature (40.5°C), pulse (102 beats per min), and respiration rate (18/min) were elevated. Physical examination was unremarkable. Results of laboratory studies were normal except for moderate hypoxemia. Examination of cerebrospinal fluid revealed that it was normal, and a chest roentgenogram revealed bilateral interstitial infiltrates. The patient was started on trimethoprim-sulfamethoxazole for presumptive *Pneumocystis carinii* pneumonia. Bronchoalveolar lavage examination did not yield any pathogens, and all cultures (blood, sputum, urine, cerebrospinal fluid, and bronchoalveolar lavage) remained negative except for one culture of blood collected on February 1994 in an Isolator 10 microbial tube (Wampole Laboratories, Cranbury, N.J.) which grew *B. henselae* (Houston-2 isolate) (approximately five colonies per plate) by March 1994. Doxycycline was instituted, with rapid resolution of fever and other symptoms. On further questioning, the patient reported that he had acquired a house kitten in October 1993. In April 1994 two cultures of blood from the cat (1.5 ml each) were obtained in a pediatric Isolator 1.5 microbial tube (Wampole Laboratories). After mixing of the contents of the tube, 0.35 ml was plated onto each medium (two plates at 30°C and two plates at 36°C). On each plate about 20 colonies of a novel *Bartonella* species (Houston-2 cat isolate) distinctly different from the patient's isolate were detected.

Patient 2. A 42-year-old male with a diagnosis of AIDS and a CD4 count of 20 cells per mm^3 presented to the clinic on 19 April 1994 with a 1-month history of fever and weight loss. His physical examination was unremarkable, and a chest roentgenogram was normal. He was started on clarithromycin and ethambutol for presumptive *Mycobacterium avium* infection. He initially improved, but 1 month later he presented with recurrence of symptoms, pancytopenia, and increased lung markings on chest roentgenogram. Computed tomography scan of the abdomen showed hepatosplenomegaly and prominent retroperitoneal lymph nodes. Biopsy yielded a normocellular bone marrow. Inclusions suggestive of yeasts were visualized on examination of the buffy coat, and the patient was started on itraconazole for presumptive histoplasmosis. The fevers continued, and at that point they were associated with shortness of breath. A chest roentgenogram showed diffuse interstitial-nodular parenchymal abnormalities and enlarged hili; however, the patient refused any further evaluations or treatments. He progressively deteriorated and died at home. Cultures of blood obtained on 3 February, and 11 March, and 11 April 1994 and cultures of bone marrow obtained on 11 March and 14 April 1994, as well as repeated sputum samples were all negative for mycobacteria and fungii. Cultures of blood obtained on 21 April 1994 in an Isolator tube grew *B. henselae* (Houston-3 isolate).

MATERIALS AND METHODS

Strains. To ensure that *Bartonella* would grow on our media and that we would recognize the colonies, 13 known strains (five species) of *Bartonella* were examined (Table 1). The strains were characterized by Gram stain morphology, biochemical substrate degradation with a RapID ANA II panel, CFA analysis, and REP-PCR.

Determination of growth conditions. Representative strains of each species were tested for growth characteristics with various commercially available media.

TABLE 2. Average sizes of largest colonies at 10 and 17 days

Strain	Colony size (mm) at 10, 17 days under the following growth conditions			
	35°C, humidity, CO_2 (8%)	35°C, humidity, no CO_2	35°C (candle jar), lower humidity, CO_2 (3%)	30°C, humidity, no CO_2
Houston-2 cat	0.6, 0.7	1.2, 1.9	0.7, 1.5	0.8, ND
B. henselae Houston-3	0.5, 1.0	0.6, 1.0	0.7, 0.4	0.8, 1.1
B. quintana WA-1	0.5, 0.6	Contaminated	0.6, 0.6	0.7, 1.5
B. vinsonii	1.2, 1.8	1.2, 2.0	1.2, 2.0	No growth
B. elizabethae	2.0, 3.7	0.6, 1.8	1.0, 1.5	0.6, 1.2

A turbid suspension corresponding to a no. 1.0 McFarland standard in 0.85% saline was made. Twenty microliters placed onto each medium to be tested was streaked for quantitation in the first quadrant and for isolation in the other areas. The incubation conditions were 35°C with added humidity and CO_2. The media tested were brain heart infusion agar with 10% sheep blood (SBHI), Brucella agar, chocolate agar, Columbia agar, Schaedler's agar, and Trypticase soy agar (the last five media also contained 5% sheep blood) (all media were from BBL, Becton-Dickinson Microbiology Systems, Cockeysville, Md.). In a similar experiment, SBHI plates were inoculated and incubated at 30 and 35°C with and without added humidity and/or CO_2. Quantitation of growth and the sizes of the isolated colonies (measured with a calibrated ocular micrometer at ×40) were recorded.

Strategy for isolation and identification. To prevent bias in the recognition of *Bartonella* species, we became familiar with the growth characteristics of the five species. Our strategy to recover *Bartonella* species was based on the observations that (i) most isolations of *Bartonella* have been from blood, (ii) in our 950-bed tertiary-care hospital, patients who are at risk for fungal disease are those also reportedly at risk for *Bartonella* disease, and (iii) for fungal cultures, blood is routinely submitted in Isolator tubes, which are also recommended for use in *Bartonella* isolation. We thus modified our routine fungal culture procedure to allow also the recovery of *Bartonella* species and added a test "*Rochalimaea* culture" to the laboratory test menu for the submission of tissue, blood, or other specimens from patients in whom any form of *Bartonella*-caused disease was suspected. When *Rochalimaea* culture was ordered, SBHI plates and closed Mycoflasks (Remel, Lenexa, Kans.) were inoculated and incubated at both 35°C in a CO_2 incubator (7 to 9% CO_2) and at 30°C (no CO_2), both with added humidity (90 to 95%). Plates were carefully examined with a stereoscopic microscope (Nikon SMZ-2T with MKII Fiber Optic Light) weekly for 4 weeks. Specimens submitted for fungal culture were plated onto the media described above (and other mycology media) but were incubated only at 30°C and were examined as described above. Plates were sealed with Shrink Seal (Scientific Device Laboratory, Glenview, Ill.). Each plate received about one-fifth of the total specimen. Every colony type or colony-like bump was examined by Gram and acid-fast stains; plates were examined for mixed growth.

The following three methods of identification were used after sufficient growth was obtained. Identification required several heavily inoculated plates harvested after 3 to 11 days; the fresh isolates took longer than the subcultures to grow.

Biochemical analyses. For each strain, a morphological description and a biochemical characterization with a RapID ANA II System panel (Innovative Diagnostic Systems, Inc., Norcross, Ga.) according to the manufacturer's instructions were performed. This system is a qualitative micromethod based on the microbial degradation of specific substrates detected by various indicator systems. The reactions used are a combination of conventional tests and single-substrate chromogenic tests usually for the identification of medically important anaerobic bacteria but were used in the present study for the identification of *Bartonella* species.

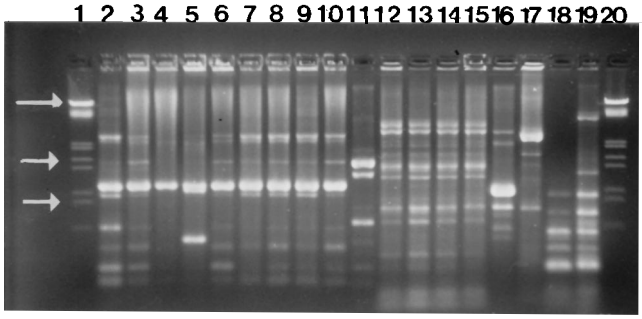

FIG. 1. Patterns of PCR products from *Bartonella* species generated by using REP primers. DNA molecular mass standards (bacteriophages lambda DNA *Eco*RI and *Hin*dIII digests) are shown in lanes 1 and 20. The top, middle, and bottom arrows designate bands of 5.148, 1.584, and 0.831 kb, respectively. Lanes 2 to 10, *B. henselae* Goldie-1, Tiger-2, SA-2, CA-1, GA-1, Houston-1A, Houston-1B, Houston-3, and Houston-2, respectively; lanes 12 to 15, *B. quintana* Sh-perm, D-perm, OK-90-268, and WA-1, respectively; lane 11, Houston-2 cat; lane 16, *B. vinsonii*; lane 17, *B. elizabethae*; lane 18, *B. bacilliformis*-1; lane 19, *B. bacilliformis*-2.

REP-PCR. A simplified version of a method with conserved primers corresponding to REP sequences in a PCR was developed to generate distinguishable patterns of DNA segments. For our usual protocol, organisms were harvested after 3 to 10 days with a sterile swab and were resuspended in 0.9% sterile saline to a concentration equivalent to that of a no. 1.0 McFarland standard. This preparation could be stored at 4°C for up to 1 year for use as control organisms. The REP1R-1 and REP2-1 PCR primers used in the reaction have been described previously (19, 24). The reaction mixture consisted of 5.0 μl of 5× polymerase buffer, 2.5 μl of dimethyl sulfoxide, 0.5 μl of deoxynucleoside triphosphates (25 mM), 1 μl (50 pmol) of each primer, 4 μl of sample organism suspension, 0.4 μl of *Taq* polymerase (Perkin-Elmer Cetus), and 10.1 μl of water; the mixture was overlaid with 50 μl of light mineral oil. The amplifications were performed on a Coy thermocycler, and the cycles were as follows: 1 cycle for 95°C for 6 min; 35 cycles at 94°C for 1 min, 40°C for 1 min, and 65°C for 8 min; 1 cycle at 65°C for 16 min; and a final soaking at 4°C until analysis. After the reaction, 20 μl of the product was separated on a 1.4% agarose gel, stained with ethidium bromide, visualized on a UV transilluminator, and photographed with Polaroid 667 film. For analysis of the band patterns, the bands were compared with a DNA molecular weight marker III (bacteriophage lambda DNA digested with *Eco*RI and *Hin*dIII) (Sigma, St. Louis, Mo.) by using DNA ProScan's restriction fragment length polymorphism molecular weight software (DNA ProScan, Nashville, Tenn.). After identifying standard bands with known molecular weights, the program calculates the molecular weight of any unknown band. The program also compares and identifies similar patterns.

CFA analysis. Cultures were grown on SBHI with incubation at 35°C in CO_2 (*B. bacilliformis*, however, was incubated at 30°C without CO_2) and were harvested after sufficient growth was obtained (4 to 10 days). The fatty acid extraction procedure for each culture consisted of saponification, methylation, extraction, and a base wash. Fatty acid methyl ester derivatives were separated on a Hewlett-Packard series II 5890 gas chromatograph (16) and were identified by using a computer-assisted comparison of the retention times of the sample with that of a standard mixture (Microbiol-ID, Newark, Del.). Peaks were automatically integrated, and fatty acid identities and percentages were calculated. Numerical analysis of the CFA data resulted in a dendrogram (unweighted pair matching) showing the Euclidean distance (degree of dissimilarity) among species and strains. Care was taken to harvest the cultures at times of comparable growth and to analyze the results from one run and from all runs separately.

Sequencing. The restriction fragment length polymorphism analysis and sequencing of the citrate synthase gene were performed as described previously (16).

Susceptibility testing. The E-test strip (AB Biodisk, Solne, Sweden) was used to measure the MICs. It is a quantitative technique for determination of antimicrobial susceptibility. The system comprises a predefined antibiotic gradient on a paper strip which is used to determine the MICs (in micrograms per milliliter) of the individual antibiotics. It was used as suggested by the manufacturer, except that we used a chocolate Mueller-Hinton agar plate and a longer incubation time. The plate was incubated at 35°C with CO_2 for 3 days, when bacterial growth became visible.

RESULTS

For the first case patient, blood from an Isolator tube was inoculated onto mycology media and one SBHI plate (30°C). A few colonies (about 30 CFU/ml) of *B. henselae* (Houston-2) were recovered at 1 month on the SBHI plate. Blood from the cat (1.5 ml each) was obtained in a pediatric Isolator 1.5 mi-

crobial tube (Wampole Laboratories). After mixing of the contents of the tube, 0.1 ml was plated onto each medium (two plates at 30°C and two at 36°C). At 8 days, there appeared to be more than 1,000 colonies on all four plates. However, subsequently only about 20 to 30 larger colonies per plate (250 CFU/ml) were apparent, leading us to believe that our original colony count of the small colonies included collections of dried cat blood or that some colonies lysed. This *Bartonella* species (Houston-2 cat isolate) was distinctly different from the patient's isolate. For the second case patient, a few colonies of *B. henselae* (Houston-3 isolate) were isolated from blood collected in an Isolator tube after 1 month on both SBHI and the Mycoflask incubated at 35°C.

Gram stains and growth characteristics of the organisms were compared. All of the *B. quintana* and *B. henselae* strains appeared to be gram-negative coccobacilli or slightly curved rods. *B. elizabethae*, *B. bacilliformis*, and *B. vinsonii* were larger and more regularly shaped. Most *B. henselae* colonies, particularly from cats or fresh isolates, were dry, indurated, and deeply embedded in the medium substrate. After subculture, some *B. henselae* colonies became smooth and round. The strains of all of the other test species were smooth and nonadherent. The Houston-2 cat strain differed from the rest of the isolates in that it was indurated yet did not embed in the agar. In addition, only the Houston-2 cat strain and *B. bacilliformis* possessed flagella, as seen by electron microscopy. All six media supported the growth of all strains tested. The best growth was obtained on SBHI and Columbia agars, with slightly less growth found on the other media (data not shown). The average diameters of the largest isolated colonies on SBHI at 10 days varied considerably among species, with *B. vinsonii* and *B. elizabethae* being two to four times larger than *B. henselae*, the Houston-2 cat isolate, or *B. quintana* (Table 2). The relative growth of representative strains of different species on SBHI in different environments is also given in Table 2. Although, CO_2 had no obvious stimulatory effect on most strains except possibly *B. elizabethae*, it would probably be prudent to incubate *Bartonella* cultures in atmospheres with elevated levels of CO_2 and humidity. Attempts to add CO_2 to a candle jar without high humidity failed because evaporation from the plates in the closed environment inadvertently increased the humidity. The data for the walk-in incubator (no CO_2, no added humidity) are not shown, because although it seemed that colonies began to grow, the plates dried out before 10 days. *B. bacilliformis* grew well on SBHI at 30°C with added humidity.

After growing the organisms for 3 to 5 days, we were able to generate a reproducible biochemical profile in 4 h using the RapID ANA II panel. Biochemically, the organisms were inert except for the production of peptidases. Negative reactions included those for catalase, urease, esculin hydrolysis, motility, nitrate reduction, and oxidase. The Houston-2 cat strain and all *B. quintana* and *B. henselae* isolates gave the profile number 000671, indicating reactions negative for carbohydrate hydrolysis of aryl-substituted arabinoside, galactoside, glucoside, and fucoside and positive for the cleavage of leucyl-glycine, glycine, proline, phenylalanine, arginine, and serine. *B. elizabethae* and *B. vinsonii* with profiles of 00066/71 and 000661, respectively, differed in that some did not hydrolyze proline. *B. bacilliformis* additionally did not hydrolyze phenylalanine (profile 000641).

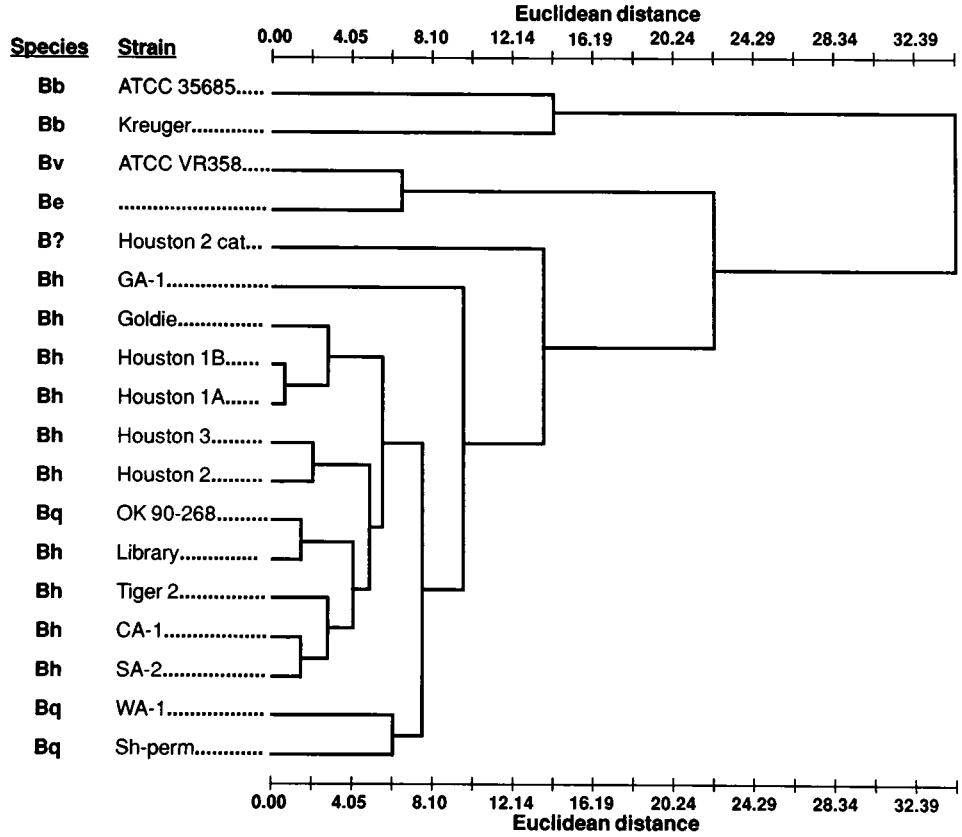

FIG. 2. Dendrogram generated from CFA data from a single run. Strains are as indicated in Table 1. Bb, *B. bacilliformis*; Bv, *B. vinsonii*; Be, *B. elizabethae*; Bh, *B. henselae*; Bq, *B. quintana.*

TABLE 3. Percent CFA methyl ester in the *Bartonella* strains tested

Species and strain	% (avg [range]) CFA methyl ester						
	15:0	16:1ω7c	16:0	17:1ω6c	17:0	Summed feature 7	18:0
B. henselae Houston-1A	0	0.47	21.49	0	0.20	48.29	27.79
B. henselae Houston-1B	0	0.48	21.69	0	0.21	47.78	28.15
B. henselae Houston-2	0	0.29	20.53	0	0.15	44.80	32.46
B. henselae Houston-3	0	0.41	20.52	0	0.24	46.10	31.01
B. henselae SA-2	0	0.38	19.71	0	0.26	48.53	29.83
B. henselae GA-1	0	0	16.29	0	0	45.00	37.32
B. henselae CA-1	0	0.36	18.66	0	0.99	48.16	30.39
B. henselae Tiger-2	0	0.35	17.43	0	0	48.63	31.84
B. henselae Goldie-1	0	0.41	19.83	0	0	49.80	27.12
B. quintana OK-90-268	0.10	0.30	15.00	0.22	2.24	48.32	31.78
B. quintana WA-1	0	0.38	16.07	0.32	2.07	51.43	27.78
B. quintana Sh. perm	0.13	0.48	20.00	0.27	2.42	52.06	23.35
B. quintana D-perm	0	0.59	20.99	0	0.81	49.64	25.29
B. vinsonii ATCC VR-152	1.05	1.09	23.23	2.01	10.58	45.92	13.21
B. elizabethae ATCC 49927	1.32	0.92	24.69	3.34	14.40	40.96	12.38
B. bacilliformis-1 ATCC 35685	0	21.20	28.31	0	0	39.92	1.52
B. bacilliformis-2	0	17.46	21.97	0	0	51.80	1.98
New strain, Houston-2 cat	0.22	0.46	12.32	0.51	2.90	56.94	23.31
B. henselae	0	0 (0–1)	20 (17–26)	0 (0–1)	1 (0–3)	47 (32–58)	32 (18–38)
B. quintana	0	0 (0–1)	18 (15–23)	0 (0–1)	2 (0–5)	50 (48–56)	28 (16–32)
Houston-2 cat	0	0 (0–1)	16 (12–19)	0 (0–1)	2 (2–3)	48 (39–57)	25 (23–26)
B. vinsonii	1 (1–2)	1 (1–2)	23 (23–24)	2 (2–3)	13 (11–16)	43 (41–46)	12 (10–13)
B. elizabethae	1	1	24 (24–25)	3	16 (14–19)	40 (38–41)	12 (11–12)
B. bacilliformis	0	19 (17–21)	25 (22–28)	0	0	46 (40–52)	2

The REP-PCR showed six distinct characteristic patterns distinguishing *B. henselae* (Fig. 1, lanes 2 to 10), *B. quintana* (Fig. 1, lanes 12 to 15), *B. vinsonii*, *B. elizabethae*, *B. bacilliformis*, and the novel cat *Bartonella* strain. The pattern for *B. henselae* isolates was defined as one major band at 1.1 kb and any two of six minor bands with molecular sizes of 2.36, 1.59, 0.91, 0.61, 0.48, or 0.37 kb. The *B. quintana* pattern was six bands with molecular sizes of 3.26, 2.75, 1.47, 1.20, 0.80, and 0.67 kb. The *B. vinsonii* pattern consisted of four bands with molecular sizes of 2.72, 2.21, 1.06, and 0.82 kb. The *B. elizabethae* pattern consisted of three bands with molecular sizes of 2.56, 1.78, and 0.80 kb. The *B. bacilliformis* pattern consisted of five bands with molecular sizes of 0.96, 0.76, 0.61, 0.47, and 0.37 kb. The pattern for the novel cat isolate consisted of three bands with molecular sizes of 1.56, 1.31, and 0.66 kb, none of which were common with bands in the patterns of any of the other *Bartonella* species. These results are reproducible in our laboratory under the specified conditions and allow for the identification of known isolates to the species level. However, since banding patterns can vary because of different cycling conditions, it is important to run control organisms with clinically isolated strains.

The results of the CFA analysis are given in Fig. 2 and Table 3. The results of a single run of all strains (Fig. 2 and the top 18 rows of Table 3) showed that the Houston-2 cat isolate was clearly different from the other *Bartonella* isolates. However, when the results from all runs of all strains are combined (bottom 6 rows of Table 3), the cat isolate cannot be distinguished from *B. quintana* or *B. henselae*. In Fig. 2, the Houston-2 cat isolate is closely related to *B. quintana* and *B. henselae*. The major fatty acids observed for this group are octadecanoic acid (18:1), octadecanoic acid (18:0), and hexadecanoic acid (16:0). *B. vinsonii* and *B. elizabethae* form a separate cluster with less 18:0 and more 17:0 CFAs. *B. bacilliformis* is more distantly related and has a large amount of 16:1ω7c CFA. The fact that essentially no other fatty acids were present excludes the presence of almost all other bacteria except *Brucella* and *Methylobacterium* species.

The restriction fragment length polymorphism pattern of the citrate synthase gene of the Houston-2 cat isolate was distinctly different from that of previously analyzed *B. henselae* and other *Bartonella* species (16), including the new subspecies *B. vinsonii* subsp. *burkhoffii*. Sequence analysis of the citrate synthase gene of the Houston-2 cat isolate showed only an 86% similarity with the citrate synthase genes of any of the other *Bartonella* species (data not shown), a level consistent with being a separate species.

Although the methods were nonstandard, we were able to generate MIC results for *B. quintana* and *B. henselae* with the E-Test strips. By using a 3-day nonstandard incubation period, the organisms were all apparently susceptible to penicillin G, ampicillin, erythromycin, and tetracycline. The extremely large zones of inhibition for these antibiotics (MICs <0.016 μg/ml) prevented use of more than three antibiotic strips on standard plates. Variable susceptibilities were shown to trimethoprim-sulfamethoxazole and vancomycin (Table 4).

Over a 16-month period we received 487 blood specimens in Isolator tubes for fungal culture and 46 specimens for "*Rochalimaea* culture" (23 blood specimens in Isolator tubes, 15 lymph node specimens as either fine needle aspirates or tissue specimens, 5 bone marrow specimens, 1 pericardial fluid specimen, and 2 skin specimens), all of which were cultured on SBHI and were observed for 28 days. Of the 510 blood specimens, 23 were positive for mycobacteria (2 *M. tuberculosis*, 2 *M. kansasii*, and 19 *M. avium-M. intracellulare* complex) and two were positive for *B. henselae*. All of these isolates were from HIV-positive patients. Thus, *B. henselae* was isolated from the blood of 1% (2 of 205) of the HIV-positive patients. The *Mycobacterium* isolates were notable because we did not expect mycobacteria to grow so well on SBHI. The *Mycobacterium* colonies were about the size of *B. henselae* colonies, although they were morphologically distinct (thus mixed cul-

TABLE 4. MIC by E-test

Antibiotic	*B. henselae*		*B. quintana*	
	MIC range (mg/ml)	No. of strains tested	Range (mg/ml)	No. of strains tested
Penicillin	0.004–0.016	5	0.002–0.004	3
Ampicillin	<0.016–0.023	3	<0.016	2
Erythromycin	<0.016–0.094	5	<0.016	1
Tetracycline	<0.016	2	<0.016	3
Trimethoprim-sulfamethoxazole	0.012–32	5	1.0–32	3
Vancomycin	4–12	5	8–48	4

tures of *M. avium-M. intracellulare* complex and *Bartonella* could have been detected). The Gram stain yielded a pleomorphic, curved, gram-variable rod which might be considered a possible *Bartonella* sp. (until acid-fast staining is performed). In 23 attempts, *Bartonella* species were not isolated from tissue.

DISCUSSION

Cultural characteristics proved to be a rapid and inexpensive aid to species identification. The *B. elizabethae* and *B. vinsonii* strains grew faster and formed larger colonies. Both *B. henselae* and *B. quintana* grew more slowly and formed smaller colonies, but they were distinguishable by colony morphology. The Houston-2 cat strain grew faster than either *B. henselae* and *B. quintana* on initial isolation and was unique in its morphology in being indurated but not adherent. *B. bacilliformis* was the only strain that grew substantially better at 30°C.

We compared three rapid systems for strain and species identification, REP-PCR, RapID ANA, and CFA analysis, for 18 strains of *Bartonella* species. CFA and the RapID ANA panel were useful for genus identification. However, the RapID ANA results did not differentiate between Houston-2 cat, *B. henselae*, and *B. quintana* isolates, and the results for other species differed by only one or two tests. CFA analysis distinguished most species but could not consistently discriminate between *B. quintana* and *B. henselae*. REP-PCR was the most useful test, because we found that it distinguished all known species of *Bartonella*. Although REP-PCR has been used for strain identification, the technique has also been found to yield species-specific patterns for *Bartonella* and *Legionella* species and mycobacteria (7, 15a, 19, 24, 28). Thus, in this case, in conjunction with either the morphology and biochemical data or the CFA results which put the isolate into the *Bartonella* genus, REP-PCR allowed the identification of a unique strain (i.e., not one of the five tested species) of *Bartonella* (Houston-2 cat) in the clinical laboratory within a few days of isolation.

Biochemical tests have, in general, been reported to be nondiscriminatory for *Bartonella* species. However, we found some consistent differences in the profiles obtained with the RapID ANA panel. Our RapID ANA results differed from those of Welch et al. (26) in that we found that in addition to leucyl-glycine, glycine, arginine, and serine *B. henselae* hydrolyzed proline and *B. quintana* hydrolyzed proline and phenylalanine. We also found *B. vinsonii* to be slightly more active, hydrolyzing leucyl-glycine and phenylalanine. The results agreed for *B. bacilliformis*. Variation in inoculation or reading could account for differences. With the addition of hemin, Drancourt and Raoult (6) noted several additional positive tests. However, they examined only a few extensively subcultured organisms.

The CFA patterns found in the present study are similar to those published previously (22). Our work confirms the usefulness of CFA in identifying *Bartonella* species. However, in contrast to some reports, we found extensive overlapping of the strains of Houston-2 cat, *B. henselae*, and *B. quintana* after multiple runs, whereas when results from a single-day analysis were compared, the strains could be distinguished. The overall relationships in the dendrogram (Fig. 2) are similar to those developed from 16S rRNA sequences (4).

The observed frequency of isolation of *Bartonella* species in our population of veterans was not high. Some have recommended the use of specialized, commercially unavailable media for isolation such as heart-infusion rabbit blood agar; however, we found all tested strains gave easily observable colonies on fresh commercial SBHI. In addition, the recovery rate of two isolates per 510 blood specimens (0.4%) from an at-risk population or from 2 of 205 HIV-positive patients (1%) is higher than that found by Welch et al. (26) when screening all blood cultures. In Washington State the rate of isolation seems to be higher; 34 isolates of *B. quintana* (nine patients) were isolated from blood cultured in liquid medium over 6 months (1). Our recovery of only *B. henselae* from patients extends the observation of a regional bias in the occurrence of *Bartonella* spp., with more *B. henselae* isolated in the Oklahoma-Texas area and more *B. quintana* isolated in the western United States (9, 11, 26, 27). In our study, no *Bartonella* isolates were recovered from tissue in 23 attempts, even though for one tissue sample Warthin-Starry-positive organisms were reported. Recovery from tissue seems to be difficult, although several laboratories have isolated organisms from cutaneous lesions of patients with bacillary angiomatosis (9) or the lymph nodes of patients with cat scratch disease (27). These cultures may require a different protocol, i.e., that described by Koehler (7a) with an enriched liquid broth to grind and inoculate the tissue. The protocol instituted to culture *Bartonella* sp. from blood on SBHI yielded *M. tuberculosis*, *M. kansasii*, and *M. avium* complex in addition to the two new *B. henselae* isolates reported here.

The isolation of a different strain of *Bartonella* from the cat of a patient from whom *B. henselae* was isolated was unexpected. Several investigators (1, 8, 17, 23, 27) have shown a clear association between cat ownership, cat scratches, and *Bartonella* disease, with the assumption that the cat passes the organisms to the owner. However, since Koehler et al. (8) have documented that as many as 41% of cats can be infected, it may be that *Bartonella* bacteremia is sufficiently prevalent in cats that one cannot assume passage of a particular isolate from pet to owner. We have previously suggested there may be strain differences in isolates of *B. henselae* from a patient and his cat (19). Studies to examine the serologic similarities of the Houston-2 cat isolate to *B. henselae* and *B. quintana* are in progress and could cast a new light on the serological data on the prevalence of *B. henselae* exposure in the cat.

The Houston-2 cat strain fits the description of members of the genus *Bartonella* in being a small, pleomorphic, gram-negative rod which grows slowly on routine media, is proteolytic but not saccharolytic, possesses $C_{18:0}$, $C_{18:1\omega7}$, and $C_{16:0}$ as the predominant CFAs, and is negative for oxidase, urease, and nitrate reductase. It is distinguished from other *Bartonella* species by a citrate synthase gene sequence homology of only 86% and a unique REP-PCR pattern as well as the possession of flagella. Although it is generally accepted that DNA-DNA hybridization is the "gold standard" used to define new species, we have found that within the *Bartonella* genus, we have been able to distinguish all known species by both their REP-PCR patterns and their citrate synthase gene sequence (15b, 19). On

this basis we believe that the Houston-2 cat strain represents a new species.

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