JOURNAL OF CLINICAL MICROBIOLOGY, Aug. 2004, p. 3462–3468 Vol. 42, No. 8
0095-1137/04/$08.00+0 DOI: 10.1128/JCM.42.8.3462–3468.2004

Bartonella koehlerae, a New Cat-Associated Agent of Culture-Negative Human Endocarditis

Boaz Avidor,[1]* Merav Graidy,[1] Gabi Efrat,[1] Cecilia Leibowitz,[1] Gregory Shapira,[1] Ami Schattner,[2] Oren Zimhony,[3] and Michael Giladi[1,4]

The Bernard Pridan Laboratory for Molecular Biology of Infectious Diseases[1] and Infectious Disease Unit,[4] Tel-Aviv Sourasky Medical Center, Tel-Aviv, and Department of Medicine[2] and Infectious Disease Unit,[3] Kaplan Medical Center, Rehovot, Israel

Received 16 February 2004/Returned for modification 26 March 2004/Accepted 14 May 2004

***Bartonella koehlerae* is reported for the first time to be a human pathogen that causes culture-negative endocarditis. It is also shown that this species, isolated twice before from domestic cats, can be recovered as well from a stray cat population in Israel. This work follows a recent report of the same case in which the causative agent was misidentified as *B. henselae*, based on serology and PCR-restriction fragment length polymorphism (RFLP) analysis (A. Schattner, O. Zimhony, B. Avidor, and M. Gilad, Lancet 361:1786, 2003). *B. koehlerae* was identified in the valvular tissue of an endocarditis patient by DNA sequencing of the PCR products of two *Bartonella* genes: the genes for citrate synthase (*gltA*) and riboflavin synthase (*ribC*). The commonly used PCR-RFLP analysis of the TaqI-digested *gltA* PCR product did not distinguish between *B. koehlerae* and *B. quintana* or between *B. elizabethae* and *B. clarridgeiae*. PmlI digestion of the *gltA* amplification product failed to differentiate between *B. quintana*, *B. clarridgeiae*, and *B. elizabethae*. RFLP analysis of the heat shock protein (*htrA*) gene by TaqI digestion misidentified *B. koehlerae* as *B. henselae*. However, RFLP analysis of the *ribC* PCR product, digested with TaqI, was able to distinguish between the human endocarditis-associated *Bartonella* species tested, *B. henselae*, *B. quintana*, *B. elizabethae*, and *B. koehlerae*, as well as between the cat-associated *Bartonella* species, *B. henselae* and *B. clarridgeiae*. Given the expanding number of *Bartonella* species emerging as human pathogens, it is suggested that PCR-RFLP analysis for the diagnosis of *Bartonella* infections target several genes and be coupled with DNA sequencing to avoid species identification.**

Bartonella species are emerging zoonotic bacterial pathogens in humans (2, 5). A common feature of these species includes transmission by an arthropod vector and persistence within mammalian reservoir hosts. Twenty-one species or subspecies are recognized at present, and eight of these have been shown to cause human diseases: *Bartonella bacilliformis*, *B. quintana*, *B. henselae*, *B. elizabethae*, *B. vinsonii* subsp. *berkhoffii*, *B. vinsonii* subsp. *arupensis*, *B. grahamii*, and *B. washoensis* (2, 7, 18, 19, 22, 25, 32, 35). In addition, on the basis of serological evidence it has been suggested that *B. clarridgeiae* causes cat scratch disease in humans (21, 26, 33). Four of these species, *B. quintana*, *B. henselae*, *B. elizabethae*, and *B. vinsonii* subsp. *berkhoffii*, have been implicated in infective endocarditis in humans, with approximately 113 cases reported to date (37). The majority of these cases are due to *B. quintana* and *B. henselae* infections (9, 12, 29), with only one case each caused by *B. elizabethae* and *B. vinsonii* subsp. *berkhoffii* (7, 32). Humans constitute the only vertebrate reservoir of *B. quintana*, which is transmitted from person to person by the human body louse, whereas bacteremic cats are the major reservoir of *B. henselae* (14, 20). *B. elizabethae* has been found in rats, whereas *B. vinsonii* subsp. *berkhoffii* has been found in dogs and coyotes (5, 6, 11).

Bartonella endocarditis is associated with risk factors already identified for *B. quintana* and *B. henselae* infections. *B. quintana* infection is associated with homelessness, alcoholism, and body louse infestation and *B. quintana* endocarditis often occurs in patients without preexisting valvular disease, while *B. henselae* infection is associated with cat contact and cat flea exposure and *B. henselae* endocarditis usually occurs in patients with known valvular disease (9, 12, 28, 29). The detection and identification of *Bartonella* species can be met with difficulties. Cultures of clinical material obtained from endocarditis patients are usually negative for bacteria due to the fastidious nature of *Bartonella* species, especially when samples for culture have been obtained from patients already treated with antimicrobial agents (24). Serology, which is the most widely used method for diagnosis, is hampered considerably by the cross-reactivity among the *Bartonella* spp. and between *Bartonella* and *Chlamydia* or *Coxiella burnetii* (13, 23). PCR amplification of *Bartonella* DNA from valvular biopsy specimens or vegetations is the most accurate tool for diagnosis, provided that tissue is available (12). Recently, a 60-year-old patient with a history of cat contact and 8 months of low-grade fever and fatigue who was diagnosed with *B. henselae* endocarditis by serology and 60-kDa heat shock protein gene (*htrA*) PCR analysis of aortic valve tissue was described (34). Given the expanding number of *Bartonella* species emerging as human pathogens, we decided to reinvestigate this case by applying additional molecular biology-based methods. We present here a reevaluation of the diagnosis for the patient on the basis of DNA sequencing of the PCR products of two *Bartonella* genes and conclude that the true causative agent was in fact *B. koehlerae*, which, as of to date, has rarely been isolated and has

* Corresponding author. Mailing address: The Bernard Pridan Laboratory for Molecular Biology of Infectious Diseases. Tel-Aviv Sourasky Medical Center, 6 Weizmann St., Tel-Aviv 64239, Israel. Phone: 972-3-697-3851. Fax: 972-3-697-3850. E-mail: bavidor@tasmc.health.gov.il.

TABLE 1. Primers used for PCR and sequencing

Oligonucleotide name	Oligonucleotide sequence (5′→3′)	Target organism	Target gene (protein)	Nucleotide positions	PCR product size (bp)	Reference or source
BhCs.781p	GGGGACCAGCTCATGGTGG	*B. henselae*	*gltA* (citrate synthase)	782–800[a]	379	27
BhCs.1137N	AATGCAAAAGAACAGTAAACA	*B. henselae*	*gltA* (citrate synthase)	1160–1139[a]		
CAT-1	GATTCAATTGGTTTGAAGGAGGCT	*B. henselae*	*htrA* (60-kDa heat shock protein)	1181–1204[b]	418	3
CAT-2	TCACATCACCAGGACGTATTC	*B. henselae*	*htrA* (60-kDa heat shock protein)	1598–1578[b]		
Koeh48	ATCCTTAAAGCAGGGGATGC	*B. koehlerae*	*ribC* (riboflavin synthase)	48–67[c]	538	This study
Koeh585	AGCATAACGGGCAAATTGAT	*B. koehlerae*	*ribC* (riboflavin synthase)	585–566[c]		
BARTON-1	TAACCGATATTGGTTGTGTTGAAG	*Bartonella* species	*ribC* (riboflavin synthase)	17–40[d]	588	17
BARTON-2	TAAAGCTAGAAAGTCTGGCAACATAACG	*Bartonella* species	*ribC* (riboflavin synthase)	604–577[d]		

[a] Positions within the *B. henselae gltA* sequence (GenBank accession number L38987).
[b] Positions within the *B. henselae htrA* sequence (GenBank accession number L20127).
[c] Positions within the *B. koehlerae ribC* sequence (GenBank accession number AY116634).
[d] Positions within the *B. koehlerae ribC* sequence (GenBank accession number AY116634).

been isolated only from domestic cats. We also show that *B. koehlerae* can be isolated from stray cats in Israel. Finally, use of a molecular biology-based diagnostic approach is suggested to avoid similar misidentifications.

MATERIALS AND METHODS

Patient clinical specimens. Surgically removed cardiac valve specimens from patients suspected of having *Bartonella* endocarditis were forwarded to The Bernard Pridan Laboratory for Molecular Biology of Infectious Diseases for diagnosis by PCR. Specimen 260197A was aortic valve tissue infected with a *Bartonella* sp. originally identified by restriction fragment length polymorphism (RFLP)-PCR analysis as *B. henselae* (34) and subsequently identified by DNA sequencing of the *gltA* and *ribC* genes as *B. koehlerae*. Specimens 264044M and 264271A were *B. henselae*-infected mitral and aortic valve biopsy specimens, respectively. Specimen 264327A was an aortic valve specimen infected with *B. quintana*. The identities of the *B. henselae* and *B. quintana* isolates from the infected specimens were confirmed by DNA sequencing of the *gltA* gene.

Bacterial strains and culture conditions. *B. henselae* (strain 87-66 [ATCC 49793]) was kindly provided by D. F. Welch, University of Oklahoma Health Sciences Center, Oklahoma City. DNA prepared from this strain was used as a positive control in PCR assays. *B. clarridgeiae* (strain NCSU 94-F40 [ATCC 700095]) and *B. elizabethae* (strain F9251 [ATCC 49927]) were kindly provided by D. L. Kordick, College of Veterinary Medicine, North Carolina State University, Raleigh, and were used in the PCR-RFLP experiments. Bacterial isolates C-508 (*B. koehlerae*), C-792 (*B. clarridgeiae*), and C-594 (*B. henselae*) were recovered from cultures of blood from street cats in Tel-Aviv, Israel, and were identified by DNA sequencing. *Bartonella* species were cultured on chocolate blood agar plates (Hy Laboratories Ltd., Rehovot, Israel) at 37°C with 5% CO_2 in a humid incubator, and the cultures were checked regularly for bacterial growth. Isolates were stored at −80°C by scraping bacterial growth into 0.75 ml of a sterile solution containing 0.74% BBL Fildes enrichment medium (Becton Dickinson and Company, Sparks, Md.), 18.5% glycerol, and 0.3% brain heart infusion powder.

Isolation of *Bartonella* species from cat blood. *Bartonella* species were recovered from cat blood as part of an epidemiological study of cat scratch disease in the Tel-Aviv area of Israel. Cat blood was obtained aseptically from the radial vein of stray cats anesthetized for castration, inoculated into a 2-ml tube containing EDTA, and frozen at −80°C until it was plated. One hundred-microliter aliquots of the thawed blood were cultured on chocolate blood agar plates and incubated for up to 8 weeks at 37°C with 5% CO_2 in a humid incubator. The bacterial growth was scraped into 4 ml of phosphate-buffered saline and washed by centrifugation, and the bacterial pellet was stored at −80°C until it was used for DNA extraction.

Extraction of DNA. DNA was prepared from clinical specimens of patients and from bacterial cultures. Valvular tissue was homogenized in hypotonic buffer (10 mM Tris-HCl buffer [pH 8.0], 10 mM NaCl, 10 mM EDTA). A 200-μl aliquot of this suspension, to which 5 μl of 5 M NaCl (to reach a final concentration of 150 mM NaCl) was added, was purified on a spin column (QIAamp blood kit; Qiagen, Hilden, Germany), and the DNA was eluted with 50 μl of the QIAamp blood kit elution buffer. For bacterial cultures, frozen bacteria were thawed and DNA was extracted by use of the EZ-DNA kit (Biological Industries Co., Kibbutz Beit Haemek, Israel), according to the instructions of the manufacturer. Briefly, the bacteria were mixed with 50 to 100 μl of EZ-DNA solution, and the mixture was incubated at 60°C for 30 to 45 min, until bacterial lysis was apparent. Bacterial DNA was precipitated by addition of an equal volume of ethanol and centrifugation at 12,000 × *g* for 10 min at room temperature. The supernatant was discarded, and the pelleted DNA was dried at 55°C for 2 min and solubilized by addition of 50 μl of freshly prepared 8 mM NaOH. DNA was also prepared from freshly grown bacterial cultures by the sodium dodecyl sulfate-proteinase K method, as described previously (3). All DNA preparations were stored at 4°C until they were used for PCR amplification.

Oligonucleotides. The primers used in this study for PCR amplification and sequencing are presented in Table 1. Oligonucleotides were synthesized commercially (Danyel Biotech Ltd., Rehovot, Israel) and were based on published sequences. Primers Koeh48 and Koeh585 were designed for this study to specifically sequence the *ribC* amplicon of *B. koehlerae*.

PCR. PCR assays for the 60-kDa heat shock protein and the citrate synthase genes (*htrA* and *gltA*, respectively) were performed as described previously (1, 3). Amplification of the riboflavin synthase gene (*ribC*) with primers BARTON-1 and BARTON-2 was carried out as reported previously (17). The PCR conditions for *ribC* amplification with primers Koeh48 and Koeh585 were as follows: denaturation for 10 min at 95°C, followed by 35 cycles of 1 min at 95°C, 1 min at 55°C, and 1 min at 72°C, with a final extension step of 6 min at 72°C. All the PCRs described above were carried out with 50-μl reaction mixtures and a programmable thermal cycler with a heat bonnet (PTC-100; MJ Research, Inc., Watertown, Mass.). A standard PCR mixture consisted of the following: 1 to 5 μl of the appropriate DNA template, 0.5 U of *Taq* DNA polymerase and 5 μl of 10× *Taq* buffer (both from Qiagen), 200 μM (each) deoxynucleoside triphosphate, and 15 to 20 pmol of each primer. Each of the PCR experiments included a positive control of 1 pg of *B. henselae* DNA (strain ATCC 49793) and an additional control consisting of *B. henselae* DNA extracted from the pus or a biopsy specimen from a patient with cat scratch disease. The PCR products were visualized with UV light on a 2% agarose gel that had been prestained with ethidium bromide. Preparation of the amplification mixtures, DNA extractions, and analyses of PCR products were each carried out in separate rooms to avoid contamination.

PCR-RFLP analysis. PCR-RFLP analysis of the *htrA*, *gltA*, and *ribC* amplicons was performed by digestion of 30 μl of the PCR products with 10 U of TaqI restriction enzyme (New England Biolabs, Beverly, Mass.), as described previously for the *gltA* amplicon (3). Digestion of the *gltA* amplicon with the PmlI restriction enzyme (*ECO72I* Fermentas enzyme; MBI Fermentas, Vilnius, Lithuania) was carried out in a mixture consisting of 10 to 20 μl of the PCR product, 5 μl of the appropriate enzyme buffer, 2 μl (20 U) of enzyme, and double-distilled water to a final volume of 50 μl. The mixture was incubated at 37°C for 3 h. The digested PCR products were electrophoresed on a 5% polyacrylamide gel, stained with ethidium bromide, and viewed under a UV light. A 50-bp DNA ladder (MBI Fermentas) was used as a size marker in most experiments. A 1-kb ladder (Gibco BRL Life Technologies, Gaithersburg, Md.) was used in some experiments.

DNA sequencing. Bidirectional sequencing of the *htrA*, *gltA*, and *ribC* amplicons was performed with the primers listed in Table 1. Sequencing of the *ribC* amplicon of *B. koehlerae* was performed only with primers Koeh48 and Koeh585. Purification of the PCR fragments and the sequencing reactions were all performed by a commercial company (Danyel Biotech), using the ExoSAP-IT kit and the MegaBACE 1000 Sequencing system, respectively (both from Amersham Biosciences, Little Chalfont, United Kingdom). Database searches and sequence comparisons were performed with the BLAST search engines provided on the Internet by the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/) and by the BioEdit Sequence Alignment Editor for Windows 95/98NT (version 5.09; www.mbio.ncsu.edu/BioEdit/bioedit.html).

Nucleotide sequence accession numbers. The *htrA* sequences which we determined for *B. koehlerae* and *B. quintana* have been assigned GenBank accession numbers AY548752 and AY548753, respectively. The DNA sequences of the *Bartonella htrA*, *gltA*, and *ribC* genes, which were obtained from the GenBank database sequence for use in this study, were as follows: *htrA*, *gltA*, and *ribC* of *B. henselae*, GenBank accession numbers, L20127, L38987, and AJ 132928, respectively; *gltA* of *B. clarridgeiae*, GenBank accession no. U84386; *gltA* and *ribC* of *B. quintana*, GenBank accession numbers Z70014 and AJ236917, respectively; *gltA* and *ribC* of *B. koehlerae*, GenBank accession numbers AF176091 and AY116634, respectively; *gltA* and *ribC* of *B. elizabethae*, GenBank accession numbers Z70009 and AY116633, respectively; and *gltA* and *ribC* of *B. vinsonii* subp. *berkhoffii*, GenBank accession numbers U28075 and AY116629, respectively.

RESULTS

PCR detection of *Bartonella* DNA in valves from endocarditis patients. The previously reported diagnosis of *B. henselae* infection in specimen 260197A was essentially based on *htrA* PCR analysis (34). Figure 1A presents the results of polyacrylamide gel electrophoresis of the TaqI-digested *htrA* PCR product which was amplified from this specimen. The DNA band pattern of specimen 260197A (lane 1) was characteristic of that for *B. henselae*, as might be predicted from the GenBank database sequence (GenBank accession number L38987). It was identical to the band pattern for DNA extracted from the *B. henselae* isolate (lane 3) and clearly different from that of the TaqI-digested *htrA* PCR product which was amplified from specimen 264327A, which was infected with *B. quintana* (lane 2). However, further analysis of these specimens by *gltA* amplification and TaqI digestion of the PCR products (Fig. 1B) contradicted these results. The DNA pattern of specimen 260197A (lane 3) was clearly different from those of *B. henselae* (lanes 1 and 2), *B. elizabethae* (lane 5), and *B. clarridgeiae* (lane 6) species, whereas it was similar to that of *B. quintana* (lane 4). These results suggest that specimen 260197A was most likely infected by a *Bartonella* species different from that of *B. henselae* and the other *Bartonella* species tested.

DNA sequence analysis. To accurately define the infecting *Bartonella* species in specimen 260197A, a *gltA* PCR product amplified from this specimen was sequenced and compared with the corresponding sequences in the GenBank database. The sequence of a 333-bp fragment of the *gltA* amplicon was found to be identical to the *B. koehlerae gltA* sequence (GenBank accession number AF176091; nucleotide positions 664 to 996). A 450-bp fragment of the *ribC* PCR product amplified from this specimen was sequenced as well to confirm this result and was found to be identical to the *ribC* sequence of *B. koehlerae* (GenBank accession number AY11634; nucleotide positions 91 to 540). These results strongly support the conclusion that *B. koehlerae* was indeed the agent infecting specimen 260197A.

The *htrA* PCR product amplified from this specimen was sequenced, and the resulting sequence data were compared

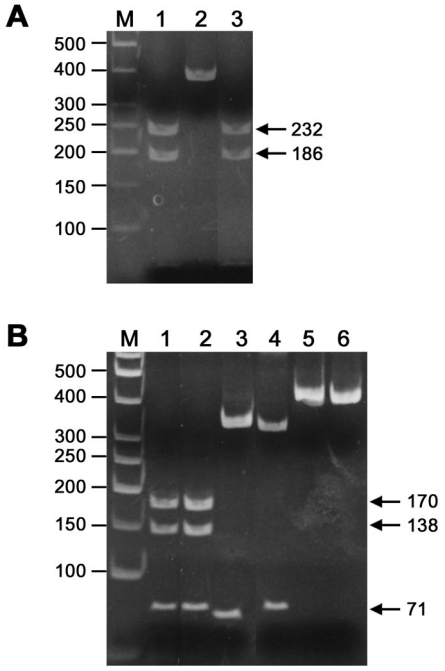

FIG. 1. PCR detection of *Bartonella* infection in endocarditis specimens. The *htrA* and *gltA* PCR products were digested with the TaqI restriction enzyme and analyzed by polyacrylamide gel electrophoresis on a 5% polyacrylamide gel. (A) DNA band pattern generated from *htrA* PCR products. Lane 1, endocarditis specimen 260197A, which was the focus of this report; lane 2, endocarditis specimen 264327A, which was infected with *B. quintana*; lane 3, DNA of a *B. henselae* strain from the American Type Culture Collection. (B) *gltA* PCR products. Lane 1, *B. henselae*-infected specimen from a patient with cat scratch disease; lane 2, endocarditis specimen 264044, which was infected with *B. henselae*; lane 3, endocarditis specimen 260197A; lane 4, *B. quintana*-infected specimen 264237A; lanes 5 and 6, DNA of *B. clarridgeiae* and *B. elizabethae* strains from the American Type Culture Collection, respectively. (A and B) Lanes M, 50-bp molecular size standard (in base pairs). The arrows and numbers indicate the sizes (in base pairs) of the DNA bands.

with the sequences of the *htrA* PCR products of three other specimens from patients with endocarditis, of which two (specimens 264271A and 264044) were infected with *B. henselae* and one (specimen 264327A) was infected with *B. quintana*. All of these sequences were compared with the GenBank *htrA* sequence of *B. henselae* (GenBank accession number L2012), which is the only *Bartonella* species for which an *htrA* sequence is available in the GenBank database. The multisequence alignments of 332-bp fragments of these sequences are presented in Fig. 2. The TaqI restriction site, TCGA, at nucleotide positions 165 to 168, which is present in both *B. koehlerae* and *B. henselae*, but not in *B. quintana*, explains why *B. koehlerae* was initially misidentified as *B. henselae* by the TaqI *htrA* PCR-RFLP analysis, the results of which are presented in Fig. 1A. An additional TaqI restriction site at nucleotide positions 302 to 305, which is present only in *B. quintana*, was predicted to result in an additional DNA fragment of approximately 50 bp, but it was not visualized in this run (Fig. 1A). However, it was clearly visualized in later experiments when more careful electrophoresis conditions were used (data not shown). Of note, the sequences from the two *B. henselae*-infected specimens (specimens 264271A and 264044) were identical to the *B.*

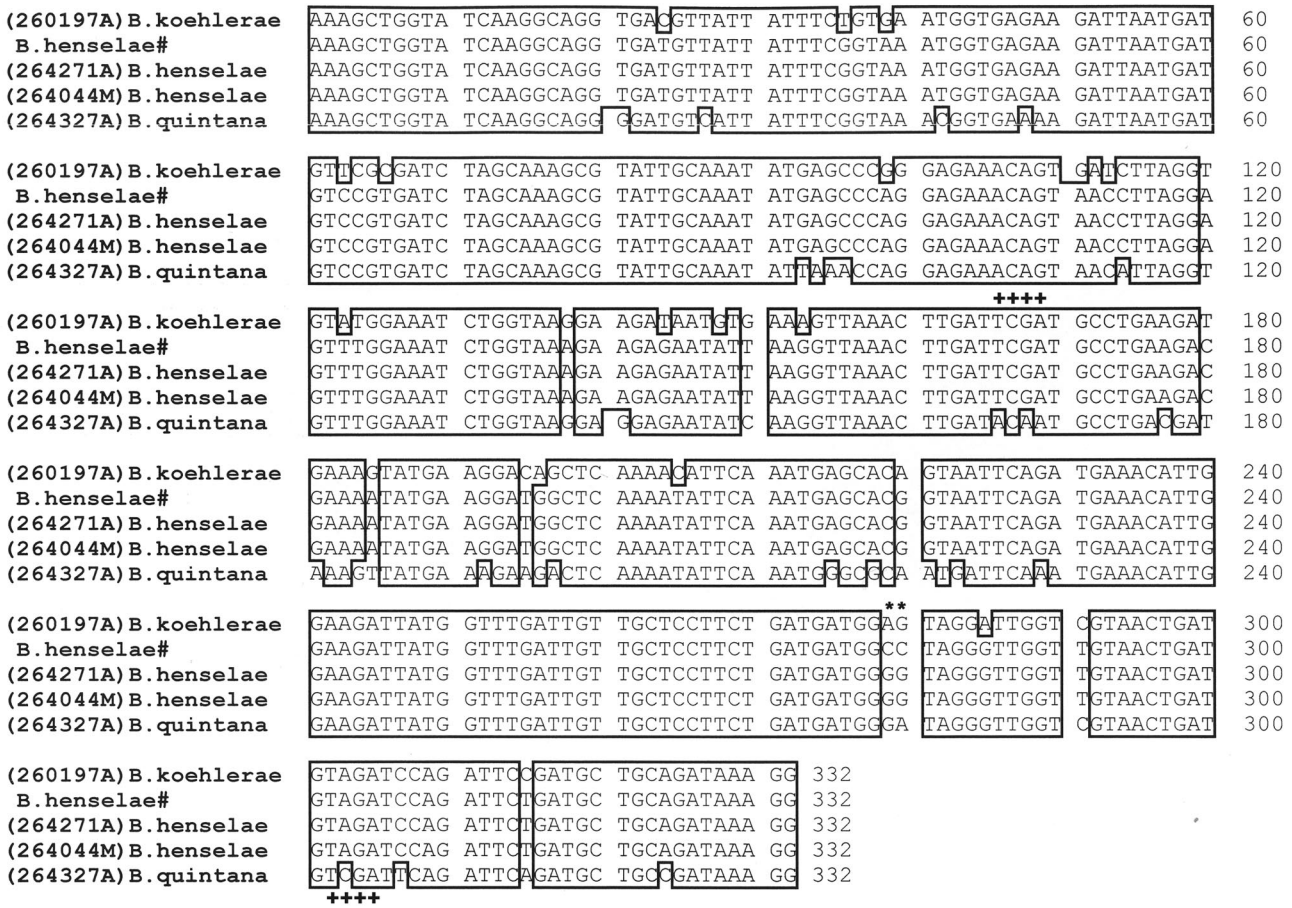

FIG. 2. Nucleotide sequence alignment of a sequenced portion of the *htrA* amplicon amplified from specimens from patients with endocarditis infected with *B. koehlerae*, *B. henselae*, and *B. quintana* with the *B. henselae* GenBank sequence. Specimen numbers are in parentheses. ++++, a TaqI recognition site; **, site where the sequences of the *B. henselae*-infected specimens differed from the corresponding *B. henselae* sequence from GenBank; #, GenBank accession number L20127 (nucleotide positions 1247 to 1578).

henselae sequence in GenBank, except for a two-base difference (GG instead of CC) at nucleotide positions 278 and 279.

A summary of the sequence homology between the *htrA*, *gltA*, and *ribC* fragments of *B. koehlerae* that were sequenced with the corresponding GenBank sequences of *B. henselae*, *B. quintana*, *B. elizabethae*, and *B. vinsonii* subsp. *berkhoffii*, which, as of to date, are the only *Bartonella* species known to cause endocarditis in humans, is presented in Table 2. On the basis of this comparison, *B. henselae* was the species that was the most closely related to *B. koehlerae*, with 91 to 92% homology, followed by *B. quintana*, with 86 to 89% similarity.

Isolation of *B. koehlerae* from cultures of blood of stray cats. DNA was extracted from *Bartonella* isolates recovered from blood samples of 48 stray cats. *Bartonella* identification and subtyping were performed by *gltA* PCR amplification and TaqI digestion of the PCR products. Altogether, three RFLP patterns were identified in these samples, as presented in Fig. 3. Of the 48 bacterial isolates, 40 (83%) had DNA patterns iden-

TABLE 2. Homology of *B. koehlerae* sequence compared with the sequences of other *Bartonella* species known to cause human endocarditis

Gene	Size of PCR-sequenced fragment of *B. koehlerae* (bp)	Nucleotide position[a]	% Sequence similarity of *B. koehlerae* to[b]:			
			B. henselae	*B. quintana*	*B. elizabethae*	*B. vinsonii* subsp. *berkhoffii*
htrA	334	1247–1580	92	86	NA[c]	NA
gltA	333	806–1138	91	89	86	87
ribC	450	1166–1615	92	88	81	85

[a] Nucleotide positions correspond to the *B. henselae htrA*, *gltA*, and *ribC* sequences in GenBank (GenBank accession numbers L20127, L38987, and AJ13298, respectively).

[b] Percent sequence similarity of *B. koehlerae htrA*, *gltA*, and *ribC* sequenced PCR fragments to the corresponding *Bartonella* sequences in GenBank, calculated by use of the BioEdit Sequence Alignment Editor software.

[c] NA, not available.

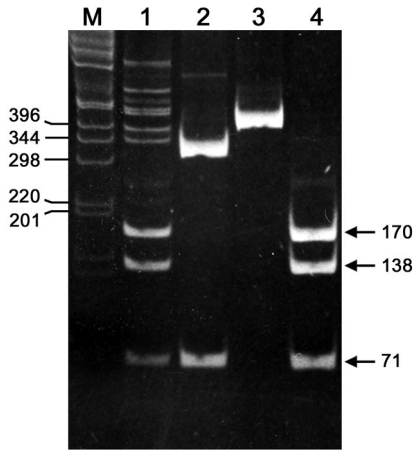

FIG. 3. Analysis of *Bartonella* species recovered from cultures of blood of stray cats by polyacrylamide gel electrophoresis analysis of TaqI-digested *gltA* PCR products amplified from bacterial isolates. Three RFLP patterns corresponding to three different *Bartonella* species were identified. Lane 4, *B. henselae* (isolate C-594); lane 3, *B. clarridgeiae* (isolate C-792); lane 2, *B. koehlerae* (isolate C-508); lane 1, a *B. henselae*-positive control which included the TaqI-digested *gltA* PCR product amplified from the pus of a lymph node of a patient with cat scratch disease; lane M, molecular size standard (in base pairs). The numbers next to the gels are in base pairs.

tical to that of isolate C-594 (lane 4), 7 isolates (15%) had patterns identical to that of isolate C-792 (lane 3), and 1 isolate (2%), isolate C-508, had a distinct pattern (lane 2). The DNA sequences of the *gltA* PCR fragments amplified from isolates C-594, C-792, and C-508 were found to be identical to the *gltA* sequences of *B. henselae* (GenBank accession number L38987; nucleotide positions 820 to 1142), *B. clarridgeiae* (GenBank accession number U84386; nucleotide positions 34 to 338), and *B. koehlerae* (GenBank accession number AF176091; nucleotide positions 647 to 996), respectively. Identification of isolate C-508 as *B. koehlerae* was also confirmed by determination of the sequence of a 461-bp fragment of a *ribC* PCR product, which was found to be identical to that of the *B. koehlerae* *ribC* sequence in the GenBank database (accession number AY116634; nucleotide positions 83 to 543).

PCR-RFLP assays to distinguish *B. koehlerae* from other *Bartonella* species. We studied two PCR-RFLP assays with the aim of distinguishing *B. koehlerae* from *B. henselae*, *B. quintana*, and *B. elizabethae*, all three species of which are known to cause endocarditis in humans, and from *B. clarridgeiae*, which, *B. koehlerae*, can be found in bacteremic cats. Figure 4A presents the results of polyacrylamide gel electrophoresis analysis of the *gltA* PCR products of the different *Bartonella* species digested with the PmlI restriction enzyme. Figure 4A shows a distinct band pattern for *B. koehlerae* (lane 4) that is different from those of *B. henselae* (lanes 1 and 2), *B. quintana* (lane 3), *B. clarridgeiae* (lane 5), and *B. elizabethae* (lane 6). This assay, however, could not distinguish between *B. quintana*, *B. clarridgeiae*, and *B. elizabethae*, all of which had similar band patterns. The assay whose results are presented in Fig. 4B was able to unambiguously identify all of the *Bartonella* species tested. Figure 4B shows the results of polyacrylamide gel electrophoresis analysis of the TaqI-digested *ribC* PCR products of the various *Bartonella* species. *B. koehlerae* (lane 4), *B. henselae* (lanes 1 and 2), and *B. quintana*, *B. clarridgeiae*, and *B. elizabethae* (lanes 3, 5 and 6, respectively) each had distinct RFLP patterns.

DISCUSSION

This report demonstrates for the first time the direct involvement of *B. koehlerae* in culture-negative endocarditis, and this case of endocarditis is the first one in which this new cat-associated species has been recognized as a human pathogen. Isolation of *B. koehlerae* from the blood of domestic cats has been reported previously, but only twice. The first report was from the United States, in which in a study of the prevalence of *Bartonella* species in domestic cats, two isolates were recovered from two kittens living in the same household (10). The second report was a recent one from France, where an isolate was recovered from the blood of a domestic kitten suspected of causing cat scratch-like disease in its owner (31). Nevertheless, the presence of *B. koehlerae* infection in this patient was not confirmed either by PCR or by serology. We report here on the amplification and sequencing of *B. koehlerae* DNA from the aortic valve tissue of a patient with infective endocarditis, which strongly implicates this bacterium as the causative agent of this disease, as has been repeatedly demonstrated in other cases of human endocarditis caused by *B. quintana* and *B. hen-*

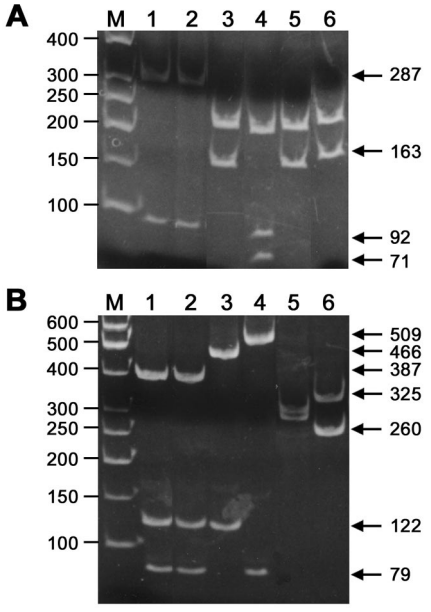

FIG. 4. PCR-RFLP assays used to distinguish *B. koehlerae* infections from those caused by other *Bartonella* species. Restriction enzyme-digested PCR products of *Bartonella* species were analyzed by polyacrylamide gel electrophoresis. (A) DNA band patterns obtained by PmlI (*ECO72I* Fermentas enzyme) digestion of *gltA* amplification products; (B) DNA band patterns obtained by TaqI digestion of *ribC* PCR products (amplified with primers BARTON-1 and BARTON-2). (A and B) Lanes 1, DNA of a *B. henselae* from the American Type Culture Collection; lanes 2, 3, and 4, *B. henselae*, *B. quintana*, and *B. koehlerae*-infected specimens from patients with endocarditis, respectively; lanes 5 and 6, DNA from *B. clarridgeiae* and *B. elizabethae* strains from the American Type Culture Collection, respectively; lanes M, molecular size standard (in base pairs). The arrows and numbers indicate sizes (in base pairs) of DNA bands.

selae (8, 12, 15, 16, 32), as well as in a single case caused by *B. vinsonii* subsp. *berkhoffii* (32).

The prevalence of *B. koehlerae* and its route of transmission are still to be determined. We report here for the first time the isolation of these bacteria from a stray cat population, suggesting that it is not confined only to domestic cats, as reported previously. The fact that our patient did not own a cat but often fed street cats in his backyard supports the assumption that a *B. koehlerae*-infected stray cat may have been the source of his infection (34). We have isolated *B. koehlerae* from only 1 (2%) of 48 cats. It is not clear, though, whether this low rate of recovery was due to a low prevalence of this species or its more fastidious nature compared with those of *B. henselae* and *B. clarridgeiae*, or both (10). Rolain et al. (30) recently reported on the detection of *B. koehlerae* DNA in 3 (3.7%) of 81 cat fleas, collected from various locations in France. In addition, a recent report showed that a persistent (78-day), asymptomatic, *B. koehlerae* bacteremia can be experimentally established in domestic cats following intradermal inoculation of *B. koehlerae* (36). In view of these findings, it is likely that *B. koehlerae*, similar to *B. henselae*, persistently infects cats and that the cat flea is most likely responsible for its transmission from cat to cat.

The PCR-RFLP patterns of the TaqI-digested *gltA* PCR products of the *B. koehlerae* strains amplified from the aortic valve specimen (Fig. 1B) and from the feline isolate (Fig. 3), although identical, differed from the PCR-RFLP pattern previously reported by Droz et al. (10). However, in contrast to the report of Droz et al. (10), our sequence data are consistent with the sequence of the *B. koehlerae* type strain published in the GenBank database (accession number AF176091). Furthermore, Droz et al. (10) reported that the *gltA* PCR product of *B. koehlerae* consists of two amplicons of approximately 400 and 550 bp, respectively, whereas our *gltA* amplification resulted in a single amplicon of approximately 400 bp, which is the expected size of the *B. koehlerae* amplification product on the basis of the published DNA sequence. The reason for these discrepancies is not clear, but it may be derived from their use of *gltA* PCR primers specific for *Rickettsia prowazekii*, which, from our experience, may result in nonspecific amplification.

Our study shows that the molecular biology-based diagnosis of *Bartonella* endocarditis by the PCR-RFLP-based approach may lead to an incorrect species identification. *gltA* analysis by TaqI digestion, which was historically shown to accurately discriminate between *Bartonella* species (4, 27), was not able to distinguish between *B. koehlerae* and *B. quintana* (Fig. 1B) or between *B. elizabethae* and *B. clarridgeiae* (Fig. 1B) in our study. Although digestion of the *gltA* PCR product with the PmlI restriction enzyme successfully discriminated between *B. koehlerae* and *B. henselae* and between *B. koehlerae* and *B. quintana*, it could not differentiate between *B. clarridgeiae* and *B. elizabethae* (Fig. 4A). However, the recently described RFLP assay with TacI-digested *ribC* amplification products (17) was successful in discriminating between the endocarditis-associated *Bartonella* species tested (*B. henselae*, *B. quintana, B. elizabethae*, and *B. koehlerae*) as well as between the cat-associated *Bartonella* pathogens, *B. henselae*, *B. clarridgeiae*, and *B. koehlerae*. Nevertheless, the emergence of new *Bartonella* species as pathogens in humans and in nonhuman mammals calls for prudence. Since DNA sequencing is not commonly practiced in most clinical microbiology laboratories, we suggest the use of the multigene PCR-RFLP approach as a preliminary diagnostic step for patients with suspected *Bartonella* endocarditis, followed by DNA sequencing for accurate species identification.

REFERENCES

1. **Anderson, B., K. Sims, R. Regnery, L. Robinson, M. J. Schmidt, S. Goral, C. Hager, and K. Edwards.** 1994. Detection of *Rochalimaea henselae* DNA in specimens from cat scratch disease patients by PCR. J. Clin. Microbiol. **32:**942–948.
2. **Anderson, B., and M. A. Neuman.** 1977. *Bartonella* spp. as emerging human pathogens. Clin. Microbiol. Rev. **10:**203–219.
3. **Avidor, B., Y. Kletter, S. Abulafia, Y. Golan, M. Ephros, and M. Giladi.** 1997. Molecular diagnosis of cat scratch disease: a two-step approach. J. Clin. Microbiol. **35:**1924–1930.
4. **Birtles, R. J., and D. Raoult.** 1996. Comparison of partial citrate synthase gene (*gltA*) sequences for phylogenetic analysis of *Bartonella* species. Int. J. Syst. Bacteriol. **46:**891–897.
5. **Breitschwerdt, E. B., and D. Kordick.** 2000. *Bartonella* infection in animals: carriership, reservoir potential, pathogenicity, and zoonotic potential for human infection. Clin. Microbiol. Rev. **13:**428–438.
6. **Chang, C. C., R. W. Kasten, B. B. Chomel, D. C. Simpson, C. M. Hew, D. L. Kordick, R. Heller, Y. Piemont, and E. B. Breitschwerdt.** 2000. Coyotes (*Canis latrans*) as the reservoir for human-pathogenic *Bartonella* sp.: molecular epidemiology of *Bartonella vinsonii* subsp. *berkhoffii* infection in coyotes from central coastal California. J. Clin. Microbiol. **38:**4193–4200.
7. **Daly, J. S., M. G. Worthington, D. J. Brenner, C. W. Moss, D. G. Hollis, R. S. Weyant, A. G. Steigerwalt, R. E. Weaver, M. I. Daneshvar, and S. P. O'Connor.** 1993. *Rochalimaea elizabethae* sp. nov. isolated from a patient with endocarditis. J. Clin. Microbiol. **31:**872–881.
8. **De La Rosa, G. R., B. J. Barnett, C. D. Ericsson, and J. F. Turk.** 2001. Native valve endocarditis due to *Bartonella henselae* in a middle-aged human immunodeficiency virus-negative woman. J. Clin. Microbiol. **39:**3417–3419.
9. **Drancourt, M., J. I. Mainardi, P. Brouqui, F. Vandenesch, A. Carta, F. Lehnert, J. Etienne, F. Goldstein, J. Acar, and D. Raoult.** 1995. *Bartonella* (*Rochalimaea*) *quintana* endocarditis in three homeless men. N. Engl. J. Med. **332:**419–423.
10. **Droz, S., B. Chi, E. Horn, A. G. Steingerwalt, A. M. Whitney, and D. J. Brenner.** 1999. *Bartonella koehlerae* sp. nov., isolated from cats. J. Clin. Microbiol. **37:**1117–1122.
11. **Ellis, B. A., L. Regnery, L. Beati, F. Bacellar, M. Rood, G. G. Glass, E. Marston, T. G. Ksiazek, D. Jones, and J. E. Childs.** 1999. Rats of the genus *Rattus* are reservoir hosts for pathogenic *Bartonella* species: an Old World origin for a New World Disease? J. Infect. Dis. **180:**220–224.
12. **Fournier, P. E., H. Lelievre, S. J. Eykyn, J. I. Mainardi, T. J. Marrie, F. Bruneel, C. Roure, J. Nash, D. Clave, E. James, C. Benoit-Lemercier, L. Deforges, H. Tissot-Dupont, and D. Raoult.** 2001. Epidemiologic and clinical characteristics of *Bartonella quintana* and *Bartonella henselae* endocarditis—a study of 48 patients. Medicine **80:**245–251.
13. **Fournier, P. E., J. L. Mainradi, and D. Raoult.** 2002. Value of microimmunofluorescence for diagnosis and follow-up of *Bartonella* endocarditis. Clin. Diagn. Lab. Immunol. **9:**795–801.
14. **Heller, R., M. Artois, V. Xemar, D. De Breil, H. Gehin, B. Jaulhac, H. Monteil, and Y. Piemont.** 1997. Prevelance of *Bartonella henselae* and *Bartonella clarridgeiae* in stray cats. J. Clin. Microbiol. **35:**1327–1331.
15. **Holmes, A. H., T. C. Grenough, G. J. Balady, R. L. Regnery, B. E. Anderson, J. C. O'Keane, J. D. Fonger, and E. L. McCrone.** 1995. *Bartonella henselae* endocarditis in an immunocompetent adult. Clin. Infect. Dis. **21:**1004–1007.
16. **Jalava, J., P. Kotilainen, S. Nikkari, M. Skurnik, E. Vanttinen, O.-P. Lehtonen, E. Eerola, and P. Toivanen.** 1995. Use of the polymerase chain reaction and DNA sequencing for detection of *Bartonella quintana* in the aortic valve of a patient with culture-negative infective endocarditis. Clin. Infect. Dis. **21:**891–896.
17. **Johnson, G., M. Ayers, S. C. C. McClure, S. E. Richardson, and R. Tellier.** 2003. Detection and identification of *Bartonella* species pathogenic for humans by PCR amplification targeting the riboflavin synthase gene (*ribC*). J. Clin. Microbiol. **41:**1069–1072.
18. **Karem, K. L., C. D. Paddock, and R. L. Regnery.** 2000. *Bartonella henselae*, *B. quintana*, and *B. bacilliformis*: historical pathogens of emerging significance. Microb. Infect. **2:**1193–1205.
19. **Kerkhoff, F. T., A. M. C. Bergmans, A. Van Der Zee, and A. Rothova.** 1999. Demonstration of *Bartonella grahamii* DNA in ocular fluids of a patient with neuroretinitis. J. Clin. Microbiol. **37:**4034–4038.
20. **Kordick, D. L., T. T. Brown, K. Shin, and E. B. Breitschwerdt.** 1999. Clinical and pathological evaluation of chronic *Bartonella henselae* or *Bartonella clarridgeiae* infection in cats. J. Clin. Microbiol. **37:**1536–1547.
21. **Kordick, D. L., E. J. Hilyard, D. L. Hadfield, K. H. Wilson, A. G. Steigerwalt, D. J. Brenner, and E. B. Breitschwerdt.** 1997. *Bartonella clarridgeiae*, a newly recognized zoonotic pathogen causing inoculation papules, fever, and lymphadenopathy (cat scratch disease). J. Clin. Microbiol. **35:**1813–1818.

22. **Kosoy, M., M. Murray, R. D. Gillmore, Jr., Y. Bal, and K. I. Gage.** 2003. *Bartonella* strains from ground squirrels are identical to *Bartonella washoensis* isolated from a human patient. J. Clin. Microbiol. **41:**645–650.
23. **La Scola, B., and D. Raoult.** 1996. Serological cross-reactions between *Bartonella quintana*, *Bartonella henselae*, and *Coxiella burnetii*. J. Clin. Microbiol. **34:**2270–2274.
24. **La Scola, B., and D. Raoult.** 1999. Culture of *Bartonella quintana* and *Bartonella henselae* from human samples: a 5-year experience (1993 to 1998). J. Clin. Microbiol. **37:**1899–1905.
25. **Maillard, R., P. Riegel, F. Barrat, C. Bouillin, D. Thibault, C. Gandoin, L. Halos, C. Dema, A. Alliot, J. Guillot, Y. Piemont, H. J. Boulouis, and M. Vayssier-Taussant.** 2004. *Bartonella chomelii* sp. nov., isolated from French domestic cattle (*B. taurus*). Int. J. Syst. Evol. Microbiol. **54:**215–220.
26. **Margileth, A. M., and D. F. Baehren.** 1998. Chest-wall abscess due to cat scratch disease (CSD) in an adult with antibodies to *Bartonella clarridgeiae*: case report and review of the thoracopulmonary manifestations of CSD. Clin. Infect. Dis. **27:**353–357.
27. **Norman, A. F., R. Regnery, P. Jameson, C. Greene, and D. C. Krause.** 1995. Differentiation of *Bartonella*-like isolates at the species level by PCR-restriction fragment length polymorphism in the citrate synthase gene. J. Clin. Microbiol. **33:**1797–1803.
28. **Raoult, D., P. E. Fournier, F. Vandenesch, J. I. Mainradi, S. J. Eykyn, J. Nash, E. James, C. Benoit-Lemercier, and T. J. Marrie.** 2003. Outcome and treatment of *Bartonella* endocarditis. Arch. Intern. Med. **163:**226–230.
29. **Raoult, D., P. E. Fournier, M. Drancourt, T. J. Marie, J. Etienne, J. Cosserat, P. Cacoub, Y. Poinsignon, P. Leclercq, and A. M. Sefton.** 1996. Diagnosis of 22 cases of *Bartonella* endocarditis. Ann. Intern. Med. **125:**646–652.
30. **Rolain, J.-M., M. Franc, B. Davous, and D. Raoult.** 2003. Molecular detection of *Bartonella quintana*, *B. koehlerae*, *B. henselae*, *B. clarridgeiae*, *Rickettsia felis*, and *Wolbachia pipientis* in cat fleas, France. Emerg. Infect. Dis. **9:**338–342.
31. **Rolain, J.-M., P. E. Fournier, D. Raoult, and J. J. Bonerandi.** 2003. First isolation and detection by immunofluorescence assay of *Bartonella koehlerae* in erythrocytes from a French cat. J. Clin. Microbiol. **41:**4001–4002.
32. **Roux, V., S. J. Eykyn, S. Wyllie, and D. Raoult.** 2000. *Bartonella vinsonii* subsp. *berkhoffii* as an agent of afebrile blood culture-negative endocarditis in a human. J. Clin. Microbiol. **38:**1698–1700.
33. **Sander, A., A. Zagrosek, W. Bredt, E. Schiltz, Y. Pie'mont, C. Lanz, and C. Dehio.** 2000. Characterization of *Bartonella clarridgeiae* flagellin (*flaA*) and detection of antiflagellin antibodies in patients with lymphadenopathy. J. Clin. Microbiol. **38:**2943–2948.
34. **Schattner, A., O. Zimhony, B. Avidor, and M. Giladi.** 2003. Asking the right question. Lancet **361:**1786.
35. **Welch, D. F., K. C. Carroll, E. K. Hofmeister, D. H. Persing, D. A. Robison, A. G. Steigerwalt, and D. J. Brenner.** 1999. Isolation of a new subspecies, *Bartonella vinsonii* subsp. *arupensis*, from a cattle rancher: identity with isolates found in conjunction with *Borrelia burgdorferi* and *Babesia microti* among naturally infected mice. J. Clin. Microbiol. **37:**2598–2601.
36. **Yamamoto, K., B. B. Chomel, R. W. Kasten, C. M. Hew, D. K. Weber, W. I. Lee, S. Droz, and J. K. Koehler.** 2002. Experimental infection of domestic cats with *Bartonella koehlerae* and comparison of protein and DNA profiles with those of other *Bartonella* species infecting felines. J. Clin. Microbiol. **40:**466–474.
37. **Zeaiter, Z., P.-E. Fournier, G. Greub, and D. Raoult.** 2003. Diagnosis of *Bartonella* endocarditis by real-time nested PCR assay using serum. J. Clin. Microbiol. **41:**919–925.