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

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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 361:1760, 2003). B. koehlerae was identified in the valvular tissue of an endocarditis patient by DNA sequencing of the PCR products of two Bartonella species: 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. claridgeiae. PmlI digestion of the gltA amplification product failed to differentiate between B. quintana, B. claridgeiae, 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. claridgeiae. 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. berkholffii, B. vinsonii subsp. arupensis, B. grahamii, and B. washoenii (2, 7, 18, 19, 22, 25, 32, 35). In addition, on the basis of serological evidence it has been suggested that B. claridgeiae causes cat scratch disease in humans (21, 26, 33). Four of these species, B. quintana, B. henselae, B. elizabethae, and B. vinsonii subsp. berkholffii, 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. berkholffii (7, 32). Humans constitute the only vertebrate reservoir of B. quintana, which is transmitted from person to person by the 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. berkholffii 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 burnetti (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

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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 264372A 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. claridgeiae* (strain NCUS 94-409 [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. claridgeiae*), 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% CO2 in a humid incubator, and the cultures were checked regularly for bacterial growth. Isolates were stored at −80°C by freezing bacterial growth into 0.75 ml of a sterile solution containing 0.74% BBL Filides 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% CO2 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. Valuuer tissue was homogenized in hypertonic 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 pelletted 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 PCR products described above were carried out with 50-μl reaction mixtures and a programmable thermal cycler with a heat block (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 (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 (EC0727 Fermentas enzyme; MB Fermentas, Vilnius, Lithuania) was carried out in a mixture containing 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 amplions was performed with the primers listed in Table 1. Sequencing of the ribC amplicon of B. henselae was performed only with primers Koch68 and Koch585. 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).

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) is 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) is clearly different from those of B. henselae (lanes 1 and 2), B. elizabethae (lane 5), and B. claridgeiae (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 1096). 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 AF116634; 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 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.
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-

| Gene  | Size of PCR-sequenced fragment of B. koehlerae (bp) | Nucleotide position | % Sequence similarity of B. koehlerae to:
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<tr>
<td>htrA</td>
<td>334</td>
<td>1247–1580</td>
<td>B. henselae: 92</td>
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<td></td>
<td></td>
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<td>B. quintana: 86</td>
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<td>B. elizabethae: NA</td>
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<td>B. vinsonii subsp. berkhoffii: NA</td>
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<tr>
<td>gltA</td>
<td>333</td>
<td>806–1138</td>
<td>B. henselae: 91</td>
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<td>B. quintana: 89</td>
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<td>B. elizabethae: 86</td>
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<td>B. vinsonii subsp. berkhoffii: 87</td>
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<tr>
<td>ribC</td>
<td>450</td>
<td>1166–1615</td>
<td>B. henselae: 92</td>
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<td></td>
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<td>B. quintana: 88</td>
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<td>B. elizabethae: 81</td>
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<td></td>
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<td>B. vinsonii subsp. berkhoffii: 85</td>
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* Nucleotide positions correspond to the B. henselae htrA, gltA, and ribC sequences in GenBank (GenBank accession numbers L20127, L38987, and AJ13298, respectively).
* 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.
* NA, not available.

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).
PCR product amplification of the (2%) sequences of isolate C-508, had a distinct pattern (lane 2). The DNA patterns identical to that of isolate C-792 (lane 3), and 1 isolate identical to that of isolate C-594 (lane 4), 7 isolates (15%) had gltA sequences of Bartonella clarridgeiae (GenBank accession number U84386; nucleotide positions 34 to 338), and Bartonella koehlerae. 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-

![Image](http://jcm.asm.org/DownloadedFromhttp://jcm.asm.org)
selae (8, 12, 15, 16, 32), as well as in a single case caused by B. vinsonii subsp. berkholffii (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). Rolaín 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 (7). In our study, 1 (2%) of 48 cats was B. koehlerae-positive, with the sequence of the type strain published in 2000. However, in contrast to the report of Droz et al. (10), our sequence data are consistent 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, 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 PmII 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 TaqI-digested rbc 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