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Use of *rpoB* Gene Analysis for Detection and Identification of *Bartonella* Species

PATRICIA RENESTO,[1] JOANNY GOUVERNET,[2] MICHEL DRANCOURT,[1] VERONIQUE ROUX,[1] AND DIDIER RAOULT[1]*

Unité des Rickettsies, CNRS UPRES-A 6020, Faculté de Médecine, Université de la Méditerranée,[1] and Service de l'Information Médicale, Hôpital de la Timone,[2] 13385 Marseille, France

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Identification of *Bartonella* species is of increasing importance as the number of infections in which these bacteria are involved increases. To date, these gram-negative bacilli have been identified by various serological, biochemical, and genotypic methods. However, the development of alternative tools is required, principally to circumvent a major risk of contamination during sample manipulation. The aim of our study was to investigate the possible identification of various *Bartonella* species by comparison of RNA polymerase beta-subunit gene (*rpoB*) sequences. This approach has previously been shown to be useful for the identification of members of the family *Enterobacteriaceae* (C. M. Mollet, M. Drancourt, and D. Raoult, Mol. Microbiol. 26:1005–1011, 1997). Following PCR amplification with specific oligonucleotides, a 825-bp region of the *rpoB* gene was sequenced from 13 distinct *Bartonella* strains. Analysis of these sequences allowed selection of three restriction enzymes (*Apo*I, *Alu*I, and *Afl*III) useful for discerning the different strains by PCR-restriction fragment length polymorphism (PCR-RFLP) analysis. To confirm the potential value of such an approach for identification of *Bartonella*, the *rpoB* PCR was then applied to 94 clinical samples, and the results obtained were identical to those obtained by our reference PCR method. Twenty-four isolates were also adequately identified by PCR-RFLP analysis. In all cases, our results were in accordance with those of the reference method. Moreover, conserved regions of DNA were chosen as suitable primer targets for PCR amplification of a 439-bp fragment which can be easily sequenced.

The bacterial genus *Bartonella* is a recently restructured taxon (3) which contains 14 species, among which 7 have been identified as human pathogens (12, 13, 22). These bacteria have been implicated in a large spectrum of infections. *Bartonella quintana* is the etiologic agent of trench fever, endocarditis, bacillary angiomatosis (22), and chronic bacteremia in homeless patients (4). *Bartonella henselae* can also cause both bacillary angiomatosis (17, 32) and endocarditis (8, 11) but is most commonly associated with cat scratch disease (7, 28) an illness which has also recently been attributed to *Bartonella clarridgeiae* (18). Only one isolate of *Bartonella elizabethae* has been obtained from the heart valve of a patient with endocarditis (5). Finally, *Bartonella bacilliformis* is responsible for bartonellosis (15).

Among the most profound clinical manifestation of *Bartonella* infection is endocarditis, a disease which often requires a surgical intervention with heart valve replacement and which is mainly caused by either *B. quintana* or *B. henselae* (22, 23). Early diagnosis of the infectious agent is important. Presently, serological techniques are most widely used, but shortcomings linked to cross-reactions with *Chlamydia* species (9, 19, 21) and variable sensitivities (22) have been reported. Histological examination of organ biopsy specimens is useful for the diagnosis of bacillary angiomatosis and peliosis hepatis, for example, but is not suitable for other clinical manifestations of *Bartonella* infections (22). Finally, biochemical procedures, such as cell wall fatty acid analysis, failed to dicriminate *Bartonella* spp. (5, 9, 33). Diagnosis can also be achieved by restriction fragment length polymorphism (RFLP) analysis of amplified DNA fragments such as the 16S-23S intergenic spacer region (ITS) (20, 30) or the citrate synthase gene (14, 26). Comparison of PCR-amplified genomic fragment sequences also leads to molecular identification of different bacterial species. Of these sequences, that of the 16S rRNA-encoding gene is by far the most widely used (35), and this approach has now become a standard method for the detection of pathogens (6, 34). However, the 16S rRNA genes of *Bartonella* species share more than 97.8% similarity (2, 3, 5). Thus, differences between them are not sufficient for confident discrimination of species (10, 31). Identification of *Bartonella* can, however, be reliably achieved by sequencing, for example, a portion of the citrate synthase gene (27). Indeed, DNA similarities of this 379-bp fragment are approximately 80 to 90% (9, 14). However, while PCR-based protocols offer several advantages over standard culture techniques, the risk of cross-contamination represents a major drawback. This is particularly true when numerous samples are manipulated in parallel. As a consequence, the finding of new PCR primers must be considered.

In the present study we assessed the usefulness of RNA polymerase beta-subunit-encoding gene (*rpoB*) sequence comparison as an alternative tool for identification of *Bartonella* spp. Comparison of *rpoB* sequences has been used for phylogenetic analyses among some members of the domains *Archae*

* Corresponding author. Mailing address: Unité des Rickettsies, CNRS UPRES-A 6020, Faculté de Médecine, Université de la Mediterranée, 13385 Marseille, France. Phone: 33 4 91 83 43 75. Fax: 33 4 91 83 03 90. E-mail: Didier.Raoult@medecine.univ-mrs.fr.

TABLE 1. Bacterial strains used in this study

Species	Strain	Source (reference)[a]	GenBank accession no.
B. alsatica	IBS 382T	Gift from Pr. PIÉMONT, Strasbourg, France	AF165987
B. bacilliformis	KC583	ATCC 35685	AF195988
B. berkhoffii	NCSU 93-CO1	ATCC 51672	AF165989
B. clarridgeiae	Houston-2 cat	ATCC 51734	AF165990
B. doshiae	R18^T	NCTC 12862	AF165991
B. elizabethae	F9251^T	ATCC 49927	AF165992
B. henselae	Houston-1^T	ATCC 49882	AF171070
B. henselae	Serotype Marseille	Endocarditis isolate (8)	AF171071
B. grahamii	V2^T	NCTC 12860	AF165993
B. quintana	Fuller^T	ATCC VR-358	AF165994
B. taylorii	M6^T	NTCC 12861	AF165995
B. tribocorum	CIP 105476^T	Gift from Pr. PIEMONT, Strasbourg, France	AF165996
B. vinsonii	Baker^T	ATCC VR-152	AF165997

[a] ATCC, American Type Culture Collection, Manassas, Va.; NCTC, National Collection of Type Cultures, Central Public Health Laboratory, London, United Kingdom.

(16, 25) and *Bacteria* (29). Recent work clearly illustrates that *rpoB* sequence analysis is a powerful tool for the identification of members of the family *Enterobacteriaceae* (24). In order to investigate the possible characterization of the genus *Bartonella* through *rpoB* gene sequencing, a 825-bp portion of this gene from 13 distinct strains was determined and further analyzed. A new PCR-RFLP method of *Bartonella* identification proposed from the present work was validated by analysis of clinical samples previously characterized by PCR sequencing of the ITS or the 16S rRNA gene.

MATERIALS AND METHODS

Bacterial strains and DNA sequencing. By using a QIAamp tissue kit (Qiagen, Hilden, Germany), DNA was purified from the *Bartonella* strains listed in Table 1 and from other bacterial strains used in this study, which included *Chlamydia trachomatis, Chlamydia pneumoniae, Borrelia burgdorferi, Leptospira interrogans, Treponema pallidum, Serpulina pibscicot, Rickettsia prowazekii, Rickettsia rhipicephali, Staphylococcus aureus, Staphylococcus haemolyticus, Streptococcus* sp., *Francisela tularensis*, the agent of human granulocytic ehrlichiosis, *Listeria ivanovii, Campylobacter jejuni, Corynebacterium jeikeium, Escherichia coli, Pseudomonas aeruginosa, Legionella pneumophila, Mycobacterium tuberculosis*, and *Coxiella burnetii*. PCR amplifications of *rpoB* fragments were performed with primers 1400F and 2300R (Table 2). These oligonucleotide sequences were deduced from a partial *rpoB* gene sequence of *B. quintana* obtained in the laboratory (unpublished data). For the reported experiments all primers used were either synthesized in the laboratory (392 DNA/RNA Synthesizer; Perkin-Elmer, Warrington, United Kingdom) or purchased from Eurogentec (Seraing, Belgium). Following a first denaturation step (94°C for 2 min), a three-step cycle of 94°C for 30 s, 53°C for 30 s, and 72°C for 1 min was repeated 35 times. The PCR program was ended by a single 2-min extension step at 72°C (Peltier thermal cycler model PTC 200; MJ Research Inc., Watertown, Mass.). Amplicons were then resolved by 1% agarose gel electrophoresis and visualized by staining with ethidium bromide. The QIAquick PCR purification kit (Qiagen) was used to prepare amplicons for automated sequencing, which was performed on an Applied Biosystems model ABI 310 automatic DNA sequencer (Perkin-Elmer) with dRhodamine Terminator Cycle Sequencing Ready Reaction Buffer (DNA sequencing kit; Perkin-Elmer) and primers 1400F and 2300R. In order to sequence the extremities of the 1400F-2300R region, two other primers, deduced from newly obtained sequence data, were used. The sequences of these oligonucleotides, designated 1596R and 2028F, respectively, are indicated in Table 2.

TABLE 2. Oligonucleotide primer sequences

Primers[a]	Oligonucleotide direction[b] and sequence
1400F[a]	CGCATTGGCTTACTTCGTATG
2300R	GTAGACTGATTAGAACGCTG
1596F	GGACAAATACGACCATAATGCG
2028D	GGAAAATGATGATGCGAATCGTGC
1873R	TCYTCCATMGCWGAMAGATAAA

[a] F, forward; R, reverse.
[b] Y, C or T; M, A or C; W, A or T.

For each bacterial sample, a 16S rRNA PCR was also carried out in order to ensure the quality of DNA extraction. Such a PCR was performed by using the same PCR program used for *rpoB* gene amplification (see above), but with a hybridization temperature of 52°C and oligonucleotide primers FD4 and RD1 for chlamydiae, primers FD3 and RD1 for spirochetes, and primers FD1 and RP2 for the other strains (24, 35).

Clinical samples. Lymph node or pus aspirate samples from patients suspected of having cat scratch disease are sent to the Unité des Rickettsies for diagnosis. Detection of *Bartonella* DNA in these specimens was carried out by a previously described procedure (20). Briefly, a PCR was performed with primer pair QVE1 (TTCAGATGATGATCCCAAGC) and QVE3 (AACATGTCTGAATATATC TTC), which amplified a fragment of the 16S-23S rRNA intergenic spacer region. The amplified fragments were then sequenced (ABI 310 automatic DNA sequencer; Perkin-Elmer), and the resulting nucleotide alignments obtained were compared with those for sequences in both the public domain (GenBank) and our own laboratory database for final identification. Of the 94 samples received in the last 12 months, PCR amplification of the *rpoB* gene was also performed by using the conditions described above with primers 1400D and 2300R. The 21 positive amplicons, all previously identified as *B. henselae*, were then digested with *Apo*I (50°C overnight in the presence of bovine serum albumin). Finally, the DNAs from some of these samples were also sequenced.

During 1999, several *Bartonella* strains were isolated on blood-enriched agar plates from the blood of patients and cats. These isolates were initially identified by ITS PCR coupled with sequencing, as described above. A PCR-RFLP analysis of the isolates collected and identified by the Unité des Rickettsies in 1999 was performed from the *rpoB* amplicons obtained with primers 1400D and 2300R. Finally, the results obtained by ITS PCR sequencing and *rpoB* PCR-RFLP analysis were compared.

PCR-RFLP analysis of *Bartonella* amplification products. Enzymatic digestion was performed by incubation of 5 μl of the purified PCR products obtained with primers 1400F and 2300R with appropriate buffer and 10 U of endonuclease. Following overnight incubation at the optimal temperature recommended by the manufacturer, the digestion products were separated on 1% agarose gels.

Data analysis. The nucleotide sequences of the *rpoB* gene fragments obtained were compiled and analyzed by computer with the autoassembler program of the ABI PRISM 310 Genetic Analyzer (Perkin-Elmer). Comparison of the sequences was performed with PC gene programs (Intelligenetics) by using the NALIGN program, and endonuclease sites were identified by using an in-house program (J. Gouvernet, unpublished data).

Nucleotide sequence accession number. The accession numbers of the sequences submitted to GenBank are given in Table 1.

RESULTS

Specific amplification of the *Bartonella rpoB* gene. By using primers 1400F and 2300R, an amplification product of 825 bp

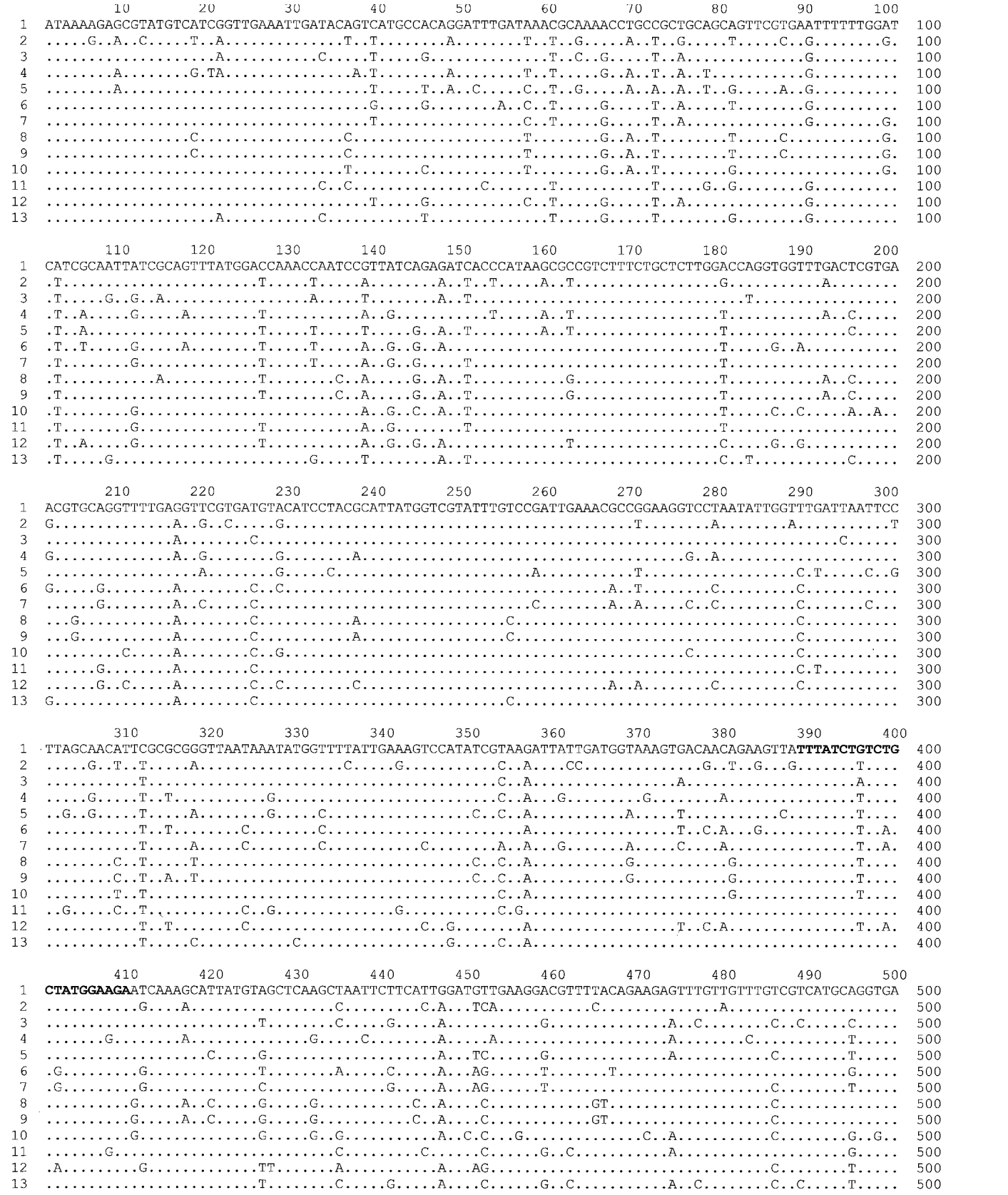

FIG. 1. Alignment of nucleotide sequences of the *Bartonella rpoB* genes amplified by PCR. The primers used were 1400F and 2300R. Homologies are indicated by dots. 1, *B. alsatica*; 2, *B. bacilliformis*; 3, *B. berkhoffii*; 4, *B. clarridgeiae*; 5, *B. doshiae*; 6, *B. elizabethae*; 7, *B. grahamii*; 8, *B. henselae* Houston; 9, *B. henselae* Marseille; 10, *B. quintana*; 11, *B. taylorii*; 12, *B. tribocorum*; 13, *B. vinsonii*.

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             510       520       530       540       550       560       570       580       590       600
 1  AGTTTTAATGGCGCCGCGGGATCATGTAGATTTGATGGATGTTTCACCCAAACAGTTGGTCTCGGTAGCAGCAGCCCTTATTCCATTTTTAGAAAATGAT  600
 2  ......G....T...A..T.....CA.......A..............A...........T..T.....T..TT.T..............G.........  600
 3  ......G.....A......A.......G.....................A..G...........T...........T..............G.........  600
 4  ............A..A..T......A.............C........A...........T..T.....T..T..A..............G.........  600
 5  ......G.....A..A...........T.................G..A......C.C..T..A.....T..T..G..............G.........  600
 6  ............A.....C........T....................A...........T..A..C.....C..A........G...............  600
 7  ..................T........T....................A...........T..A..T........G........T.....G.........  600
 8  ......G.....T.....C........T....................A...........T..A.....T..T..T........G.....G.........  600
 9  ......G.....T.....C........T....................A...........T..A.....T..T..T........G.....G.........  600
10  ......G...........A........C....................A..G.....A..T..A.....T..T..T..C...........G.........  600
11  ......G........A..C........G....................A........A..T........T.....T..............G.........  600
12  ..................C........T....................A...........T..A..T.....T..G........C.....G.........  600
13  ......G.....A..............G....................A....................G.....T..............G.........  600

             610       620       630       640       650       660       670       680       690       700
 1  GATGCGAATCGTGCGTTGATGGGATCAAACATGCAGCGTCAGGCAGTTCCGCTTATACGTGCTGAAGCACCATTTGTTGGTACGGGAATGGAATCAATTG  700
 2  .................A.................A.....A........AT.G..C...T......................A..T.....GG....A.  700
 3  ..........................C.................G.........G................C..............C........CG...  700
 4  .....A..C.....TC..........C..T...........A............T....C..A.....G...C..........A..T......G....A.  700
 5  .....A........A..............T.....A........G.....A...G..........G.....G...........T........GG....A.  700
 6  ...............C.T........G....................G..T...G.G..C........G...........G.....C........CG...  700
 7  ...............C.T........G.......................T...G.............G...........G............G.C....  700
 8  ..........................T..T....................A...G.....................................G.....A.  700
 9  ..........................T..T....................A...G.....................................G.....A.  700
10  .....A....................G..T..............C.........G................................T...........A.  700
11  ..............A...........G.................G.....A...G....C........G..............A..............A.  700
12  ..............AC..................................T...G......................................G.TG...  700
13  .....A......................................G.........G................C..............T........TG...  700

             710       720       730       740       750       760       770       780       790       800
 1  TTGCTCGCGATTCAGGTGCAGCCGTTAGTGCAAAGCGTGGCGGTATTGTTGATCAAGTTGATGCAACCCGTATTGTTATTCGTGCGACAGAAGATTTAGA  800
 2  .G.....T...........T..TA..........A.....T..........................A.................A..............  800
 3  .......T........C.......................T..............G.......................C....................  800
 4  .....T.T...........C..TA..G....G..A.....T.........................TG.................A.....G........  800
 5  ....A...........A..T..TA..G.......A.....T..............G...........G....................G......C....  800
 6  ....A...........A..T..G..G..C.....A..CA.T.................C.....G.....................A..............  800
 7  ....G...........A..T..T..G........A...A.T...G.............C.....T..............C......A..............  800
 8  .A..............A..T..T...GC.....G....A.T..........................A................................  800
 9  .A..............A..T..T...GC.....G....A.T..........................A................................  800
10  .A..............G..T..T.................T...........................................................  800
11  ....C.................G.................T.................G....................C.....A..............  800
12  ....A..A........A..T..T..A........A..CA.T.......................G..............C.....A..............  800
13  .......T........C.....T.................T.................G....................C....................  800

             810       820
 1  TCCTTCAAAATCTGGTGTTGACATT      825
 2  .....................T...      825
 3  ...C.....G...........T...      825
 5  ...A.................T...      825
 6  ............A..C.....T...      825
 7  ............A..C.....T...      825
 8  .....................T...      825
 9  .....................T...      825
10  ...............G.....T...      825
11  .........G..G........T...      825
12  ............A........T...      825
13  .........G...........T..C      825
```

FIG. 1—*Continued.*

	BhensM	*BhensH*	*Bquin*	*Bals*	*Bvin*	*Btayl*	*Bdosh*	*Bberk*	*Bgra*	*Btrib*	*Bbac*	*Bclar*
B. hens. H	99.76											
B. quintana	92.85	92.85										
B. alsatica	90.79	90.79	91.27									
B. vinsonii	89.94	89.94	91.15	92.48								
B. taylorii	89.82	89.82	91.15	92.24	92.12							
B. doshiae	89.33	89.33	88.48	87.88	88.24	88.48						
B. berkhoffii	88.85	89.09	90.55	92.48	96.00	91.27	87.88					
B. grahamii	88.85	88.85	89.09	89.70	88.85	89.7	88.00	89.33				
B. tribochorum	88.85	88.73	89.09	89.94	89.45	89.33	87.88	89.58	93.94			
B. bacilliformis	88.61	88.61	87.64	87.88	86.91	87.52	88.48	86.18	85.94	85.94		
B. clarridgeiae	87.76	87.64	87.88	88.36	87.39	88.00	87.88	87.27	87.15	87.52	88.36	
B. elizabethae	87.15	87.76	88.12	88.73	87.88	88.73	85.94	88.48	84.85	84.18	84.85	86.67

FIG. 2. Similarity values between *rpoB* sequences of various *Bartonella* species. Values were deduced from the data presented in Fig. 1 by comparison of 825 nucleotides. *BhensH, B. henselae* Houston; *BhensM, B. henselae* Marseille; the remaining abbreviations across the top correspond to the species on the left, from top to bottom, respectively.

was obtained for all *Bartonella* strains analyzed. The PCR carried out with this pair of oligonucleotide primers was shown to be highly specific. Indeed, among the 21 other bacterial strains tested, no amplification products were observed. In contrast, and as expected, a positive response was obtained for all these strains by using 16S rRNA primers (data not shown).

Sequencing of the *Bartonella rpoB* amplified fragment. All PCR fragments were then sequenced at least in duplicate. The lengths of the fragments sequenced were always 825 bp, and neither insertions nor deletions were observed among the species analyzed (Fig. 1). The percentages of DNA similarity between pairs of strains are presented in Fig. 2. The comparison of the nucleotide alignments of the *rpoB* gene fragments from the *Bartonella* strains sequenced revealed levels of similarity between 84.9 and 99.8%. Two serotypes of *B. henselae*, namely, Houston and Marseille (8), exhibited a strong homology (99.8%), with only 2 bases among the 825 bases sequenced being different.

PCR-RFLP identification of *Bartonella*. The suitabilities of a large number of endonucleases were then assessed for all the *Bartonella* strains sequenced by using a homemade program (Gouvernet, unpublished data). From the resulting analysis it appeared that the combination of both successive digestions allowed easy discrimination of these strains. Thus, *Apo*I digestion led to four different patterns. *B. quintana* was the only

FIG. 3. Restriction profiles obtained after digestion of a portion of the *rpoB* gene with *Apo*I. Ethidium bromide-stained agarose gels of *Apo*I restriction endonuclease digests of DNA amplified by using primers 1400F and 2300R are shown. Lane A, molecular mass markers (marker IV; Boehringer). *Ba, B. alsatica; Bba, B. bacilliformis; Bbe, B. berkhoffii; Bc, B; clarridgeiae; Bd, B. doshiae; Be, B. elizabethae; Bg, B. grahamii; Bh1, B. henselae* Houston; *Bh2, B. henselae* Marseille; *Bq, B. quintana; Bta, B. taylorii; Btr, B. tribocorum; Bv, B. vinsonii*. Numbers on the left are in base pairs.

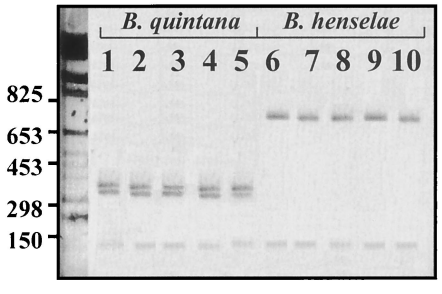

FIG. 4. *Apo*I digestion profiles of *rpoB* amplicons from either *B. henselae* or *B. quintana*. Ethidium bromide-stained agarose gels of *Apo*I restriction endonuclease digests of DNA amplified by using primers 1400F and 2300R are shown. Lane A, molecular mass (marker IV; Boehringer); lanes 1 to 5, DNA extracts from blood of patients infected with *B. quintana*; lanes 6 to 10, DNA extracts from lymph node or pus aspirate samples from patients suspected of having cat scratch disease and identified as *B. henselae*-positive samples. Numbers on the left are in base pairs.

TABLE 3. Endonucleases available for identification of *Bartonella* group 2 species by PCR-RFLP analysis[a]

Group 2 species	Sizes of fragments (bp) after digestion with:				
	*Aci*I	*Alu*I	*Hin*6I	*Mae*II	*Mnl*I
B. bacilliformis	72, 750	9, 69, 141, 255, 348	94, 218, 510	33, 55, 57, 99, 120, 158, 300	139, 309, 374
B. grahamii	105, 285, 432	2, 36, 39, 54, 63, 118, 154, 158, 198	23, 78, 88, 200, 433	23, 78, 88, 200, 433	164, 658

[a] Computer analysis was performed from the same partial *rpoB* gene sequences shown in Fig. 2.

strain for which four predicted fragments were obtained, thus allowing its direct identification and its placement in the first group. At the opposite extreme, neither *B. bacilliformis* nor *Bartonella grahamii* was shown to have an *Apo*I restriction site, and both species were classified in the second group. In the third group, we found *B. henselae* (serotypes Marseille and Houston) as well as *Bartonella alsatica*, for which the pattern was two bands of 735 and 87 bp, respectively. Finally, the seven other strains were all hydrolyzed near the middle of the inititial 825-bp PCR fragment, leading to two bands of approximately 400 bp each. In fact, two subgroups were obtained. In the first subgroup, the *Apo*I site was located at position 434 (*B. elizabethae* and *Bartonella tribochorum*) and in the second subgroup it was located at position 471 (*Bartonella berkhoffii, B. clarridgeiae, Bartonella doshiae, Bartonella taylorii*, and *Bartonella vinsonii*). However, the RFLP patterns of both subgroups were too close to permit their differentiation in agarose gels. This analysis was validated by the experimental data presented in Fig. 3 and 4. Under our experimental conditions, the small 37-bp fragment expected for the *B. quintana* strain was not detected, probably because of the sensitivity of the method. However, the profiles obtained allowed differentiation of the four patterns. Moreover, and as illustrated in Fig. 4, the digestion profiles obtained with *Apo*I allow easy differentiation of *B. henselae* and *B. quintana* strains. This is of importance when considering the fact that both of these bacterial species are implicated in endocarditis, which is the most common clinical manisfestation of *Bartonella* infection (13). By using a second endonuclease, all the *Bartonella* strains from the four previously determined groups can be definitively identified. Such a differentiation can be reached with the restriction enzymes listed in Tables 3 to 5, among which were included *Alu*I and *Afl*III. Indeed, *Alu*I digestion of the *rpoB* amplicons of the group 2 *Bartonella* yielded either five or three fragments, and thus allowed easy differentiation of *B. bacilliformis* and *B. grahamii*, respectively. This enzyme was also shown to be efficient in the differentiation of the bacteria included in group 4 since a specific profile was obtained for each of the seven strains included in this group. Finally, the differentiation of *B. alsatica* and both serotypes of *B. henselae* (group 3) can be achieved by using *Afl*III. While the *B. alsatica rpoB* fragment was devoid of such a restriction site and consequently was not hydrolyzed by *Afl*III, it was not the case concerning *B. henselae*, which was digested into three and four fragments for serotypes Houston-1 and Marseille, respectively. In fact, when combined with a first digestion step with *Apo*I, all of the restriction enzymes presented in Tables 3 to 5 allowed identification of all the *Bartonella* species by RFLP analysis.

Confirmation of diagnosis obtained with clinical samples. Among the 94 samples tested, 21 were found to be positive for *Bartonella*. This result was deduced from PCR assays performed both with ITS primers used under established conditions and with *rpoB* primers specific for *Bartonella* and designed in this study, namely, primers 1400D and 2300R. Thus, each positive or negative result obtained by the ITS PCR methodology was confirmed by *rpoB* PCR (Table 6). All amplification products were identified by sequencing of the ITS amplicon as being derived from strains of *B. henselae*. This result was confirmed by digestion of all fragments obtained with *Apo*I and by sequencing of some of the *rpoB* amplicons. During the same period, 24 isolates were collected in the laboratory from the blood of either patients or cats. PCR sequencing of ITS identified these strains as follows: 10 *B. quintana* isolates, 4 *B. clarridgeiae* isolates, and 10 *B. henselae* isolates. All these isolates were also analyzed by PCR-RFLP analysis of the *rpoB* gene. In all cases, the patterns of digestion obtained with *Apo*I and *Alu*I corroborated the diagnosis previously deduced from ITS sequence analysis.

DISCUSSION

Several techniques for detection of *Bartonella* species in clinical material have been described elsewhere (23). Among these, PCR-based protocols present several advantages over culture. For example, they allow the detection of slowly growing pathogens or bacteria in fixed biopsy material. Nevertheless, some limitations must be considered, in particular, the potential for contamination of samples to lead to false-positive results. This may be remedied in part by use of different rooms for procedures upstream and downstream of the amplification step. Currently, the 16S rRNA gene has been the focus for

TABLE 4. Endonucleases available for identification of *Bartonella* group 3 species by PCR-RFLP analysis [a]

Group 3 species	Sizes of fragments (bp) after digestion with:					
	*Afl*III	*Bsa*A1	*Fnu*DII	*Hha*I	*Hin*6I	*Mae*II
B. alsatica	822	165, 657	2, 116, 191, 201, 312	154, 160, 198, 310	154, 158, 198, 312	166, 198, 200, 258
B. henselae Houston	57, 765	54, 111, 657	2, 85, 116, 189, 201, 229	203, 205, 314	203, 307, 312	55, 111, 198, 223, 235
B. henselae Marseille	57, 314, 451	54, 111, 342, 315	2, 85, 116, 189, 430	305, 517	307, 515	55, 91, 111, 144, 198, 223

[a] See footnote *a* of Table 3.

TABLE 5. Endonucleases available for identification of *Bartonella* group 4 species by PCR-RFLP analysis[a]

Group 4 species	Sizes of fragments (bp) after digestion with:				
	*Alu*I	*Hha*I	*Hin*6I	*Mae*II	*Mnl*I
B. berkhoffii	249, 573	105, 154, 160, 403	107, 154, 158, 403	23, 166, 200, 433	10, 812
B. clarridgeiae	6, 69, 141, 255, 351	8, 154, 660	8, 156, 658	8, 23, 57, 88, 150, 196, 300	40, 384, 398
B. doshiae	13, 75, 105, 122, 150, 357	247, 261, 314	249, 261, 312	8, 18, 21, 42, 78, 88, 150, 417	27, 52, 139, 154, 450
B. elizabethae	78, 105, 639	2, 8, 44, 61, 93, 97, 160, 357	2, 8, 44, 61, 95, 97, 158, 357	223, 599	75, 164, 209, 374
B. taylorii	6, 141, 249, 426	8, 52, 146, 148, 154, 154, 160	8, 52, 146, 148, 154, 156, 158	23, 200, 599	398, 424
B. tribocorum	24, 105, 123, 570	5, 58, 247, 512	5, 58, 249, 510	23, 166, 198, 200, 235	154, 658
B. vinsonii	102, 147, 573	2, 105, 144, 154, 160, 257	2, 107, 144, 154, 158, 257	166, 223, 433	52, 770

[a] See footnote *a* of Table 3.

most PCR methods because it is one of the most conserved genes (32, 34, 35). However, for the identification of *Bartonella*, this approach is not considered satisfactory due to the high percent similarity of the sequence of this gene among different *Bartonella* species (10, 31). Conversly, identification of these bacteria can be more reliably achieved by either ITS (20, 30) or citrate synthase gene (9, 14, 27) PCR assays. However, because of the potential risk for contamination discussed above, there remains a need for the development of alternative assays. We thus attempted to develop such an assay based on the *rpoB* gene. This gene has previously been used for phylogenetic analysis among some members of the domains *Archae* (16, 25) and *Bacteria* (29) and has been demonstrated to be a powerful tool for the identification of members of the family *Enterobacteriaceae* (24).

Initially, different pairs of primers used to amplify the *rpoB* gene fragments of members of the family *Enterobacteriaceae* were tested with *Bartonella*. From the preliminary sequences obtained, two primers which were observed to be specific for *Bartonella* species were deduced. The specificities of these primers, designated primers 1400F and 2300R, were then demonstrated by a PCR assay involving 21 bacterial strains unrelated to members of the family *Bartonellaceae*. In a second step, the *rpoB* gene fragments of 13 distinct species were amplified and automatically sequenced. Analysis of the resulting nucleotide sequences demonstrated that *Apo*I digestion of the initial 825-bp PCR fragment allowed differentiation of *B. quintana* from all other species. This point is of importance when one considers the importance of this strain in human infections (22). The other species each yielded one of three *Apo*I profiles but could be distinguished from one another if those profiles were combined with the results of a second digestion with either *Alu*I (group 2 and 4) or *Afl*III (group 3). This *rpoB* sequencing methodology was validated from experiments performed with DNAs from clinical isolates as the DNA template. Indeed, all *B. henselae*-positive samples identified by routine methodology from lymph nodes or pus aspirates were, without exception, shown to be positive by the *rpoB* PCR done with our *Bartonella*-specific primers. These data were validated by the digestion of all the amplicons obtained with *Apo*I, for which the pattern was two bands of 735 and 87 bp, respectively. As indicated in the Results section, the same profile of digestion can be achieved with *B. alsatica*. However, this bacterial species has been isolated from the blood of rabbits and has never been demonstrated to be responsible for infection in humans (13). While there was no doubt, the initial diagnosis was firmly confirmed for some samples by the sequencing of the *rpoB* amplicons. Similarly, the identifications obtained by PCR-RFLP analysis with clinical or animal isolates were also in accordance with previous identifications. Finally, alignment of *Bartonella rpoB* sequences allowed the design of a new internal primer chosen from the most conserved regions of the *rpoB* gene region sequenced (1873R, underlined part of the sequence in Fig. 1; Table 2). When used in combination with primer 1400F, a 439-bp PCR product was obtained (data not shown). Sequencing of such an amplified portion of the gene could be an alternative for identification of these bacteria in properly equipped molecular biology laboratories.

TABLE 6. Identification of *Bartonella* from clinical samples

Clinical material	No. of samples	No. of samples positive by:		Identification (no. of isolates) by:	
		ITS PCR	*rpoB* PCR	ITS and 16S rRNA sequencing	*rpoB* PCR-RFLP analysis and *rpoB* sequencing
Lymph node or pus aspirate	94	21	21	*B. henselae* (21)	*B. henselae*[a] (21)
Blood of patients	10	10	10	*B. quintana* (10)	*B. quintana* (10)
Cat blood	14	14	14	*B. clarridgeiae* (4), *B. henselae* (10)	*B. clarridgeiae* (4), *B. henselae* (10)

[a] DNAs from 5 to 21 samples were positive for *Bartonella*.

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