

Molecular Analysis of Riboflavin Synthesis Genes in *Bartonella henselae* and Use of the *ribC* Gene for Differentiation of *Bartonella* Species by PCR

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The biosynthesis pathway for riboflavin (vitamin B₂), the precursor of the essential cofactors flavin mononucleotide and flavin adenine dinucleotide, is present in bacteria and plants but is absent in vertebrates. Due to their conservation in bacterial species and their absence in humans, the riboflavin synthesis genes should be well suited either for detection of bacterial DNA in human specimens or for the differentiation of pathogenic bacteria by molecular techniques. A DNA fragment carrying the genes *ribD*, *ribC*, and *ribE*, which encode homologues of riboflavin deaminase (RibD) and subunits of riboflavin synthetase (RibC and RibE), respectively, was isolated from a plasmid-based DNA library of the human pathogen *Bartonella henselae* by complementation of a *ribC* mutation in *Escherichia coli*. Sequence analysis of the *ribC* gene region in strains of *B. henselae*, which were previously shown to be genetically different, revealed that the *ribC* gene is highly conserved at the species level. PCR amplification with primers derived from the *ribC* locus of *B. henselae* was used to isolate the corresponding DNA regions in *B. bacilliformis*, *B. clarridgeiae*, and *B. quintana*. Sequence analysis indicated that the riboflavin synthesis genes are conserved and show the same operon-like genetic organization in all four *Bartonella* species. Primer oligonucleotides designed on the basis of localized differences within the *ribC* DNA region were successfully used to develop species-specific PCR assays for the differentiation of *B. henselae*, *B. clarridgeiae*, *B. quintana*, and *B. bacilliformis*. The results obtained indicate that the riboflavin synthesis genes are excellent targets for PCR-directed differentiation of these emerging pathogens. The PCR assays developed should increase our diagnostic potential to differentiate *Bartonella* species, especially *B. henselae* and the newly recognized species *B. clarridgeiae*.

Bacteria of the genus *Bartonella* are fastidious, gram-negative, slow-growing microorganisms. During recent years, the number of *Bartonella* species isolated increased remarkably (7, 20), and the number of recognized diseases caused by *Bartonella* species increased as well (2, 40). Five species are known to cause human diseases. *Bartonella bacilliformis* is the causative agent of bartonellosis, a biphasic disease which is endemic in regions of the South American Andes. Up to now, *B. elizabethae* has been isolated only once, from the blood of a patient with endocarditis (12). The two species most often involved in human infections worldwide are *B. henselae* and *B. quintana*. The latter species is the causative agent of trench fever and of bacillary angiomatosis in human immunodeficiency virus (HIV)-infected patients (46). A large number of clinical manifestations, especially cases of endocarditis in homeless people, have also been related to this agent (31). *B. henselae*, which was first isolated in 1992 from the blood of an HIV-infected patient (36), is the main causative agent of cat scratch disease (CSD) and is known to be involved in different clinical disorders in immunocompromised as well as in immunocompetent patients.

The newly recognized species *B. clarridgeiae* was first isolated from the cat of a patient with *B. henselae* septicemia (25)

and was later detected in cat populations in India, the United States, and France (17, 19, 23, 29). Recently, two cases of CSD caused by *B. clarridgeiae* were described, although both cases were confirmed only serologically (24, 28).

The *Bartonella* species *B. henselae*, *B. quintana*, and *B. clarridgeiae* are phenotypically and genotypically very similar, and differentiation of these species usually requires molecular techniques. The serologic cross-reactivity between *B. henselae* and *B. quintana* in patients with CSD is very high (95%), and the seroprevalence of *B. henselae* in healthy people is up to 30% (33, 41). Therefore, the development of species-specific molecular techniques, especially for the detection and differentiation of infections possibly caused by the newly recognized species *B. clarridgeiae*, seems to be urgent.

Genes encoding enzymes of the riboflavin biosynthetic pathway (3) are evolutionarily conserved in bacteria and plants and absent in humans. They are, therefore, excellent target candidates for the detection and differentiation of invasive pathogenic bacteria. Riboflavin (vitamin B₂) is the precursor of flavin mononucleotide and flavin adenine dinucleotide, which are both essential cofactors for electron transport functions of proteins involved in the basic energy metabolism of the cell.

Riboflavin is synthesized from GTP, and the corresponding biosynthetic pathway is present in bacteria, fungi, and plants but absent in vertebrates, including humans. In *Escherichia coli*, five enzymes, designated RibA (GTP-cyclohydrolase II), RibB (DHBP synthetase), RibC (riboflavin synthase), RibD (riboflavin deaminase/reductase), and RibE (ribityl-lumazine synthetase), are involved in riboflavin synthesis. The coding

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s) ^a	Reference or source
Strains		
<i>B. bacilliformis</i> ATCC 35685		American Type Culture Collection
<i>B. clarridgeiae</i>		
73	From cat	19
R13762	From cat	29
R8051	From cat	29
<i>B. henselae</i>		
Houston-1	Variant IV, from HIV patient	36
FR96 strains	Variants I to IV, From cat	42
R8015	From cat	E. Marston (29)
R8016	From cat	E. Marston (29)
R8017	From cat	E. Marston (29)
R8018	From cat	E. Marston (29)
<i>B. quintana</i>		
CIP103739		CIP
München		A. Sander (44)
<i>E. coli</i>		
BSV23	<i>thi hsd</i> ($r^- m^-$) <i>ribC</i> ::Tn5 Km	A. Bacher
RR28	<i>thi leu pro lac gal ara xyl endA recA hsd</i> ($r^- m^-$) <i>pheS12 supE44</i>	38
Rib2	RR28 with a point mutation in <i>ribD</i>	A. Bacher
TOP10	F ⁻ <i>mcrAΔ(mrr-hsdRMS-mcrBC) f80lacZΔM15 ΔlacX74 deoR recA1 araD139 Δ(ara-leu)7697 galU galK rpsL endA1 nupG</i>	Invitrogen
Plasmids		
pZERO-2	Cloning vector; <i>ccdB neo ori</i> _{ColEI} Km	Invitrogen
pBH-RIBC1	pZERO-2 with a 2.3-kb <i>Pst</i> I DNA fragment carrying the <i>ribC</i> gene from <i>B. henselae</i> ; Km	This study

^a Km, resistant to kanamycin.

genes, designated *ribA* to *ribE*, have been most extensively investigated in *E. coli* (3, 13, 38) and *Bacillus subtilis* (35).

Homologues of the riboflavin synthesis genes were isolated from many microorganisms, including *Actinobacillus pleuropneumoniae* (15), *Azospirillum brasilense* (47), *Haemophilus influenzae* (14), *Helicobacter pylori* (5), *Photobacterium* spp. (26), *Pichia guilliermondii* (27), and *Saccharomyces cerevisiae* (45), and from the plant *Arabidopsis thaliana* (22).

The functional importance of riboflavin synthesis genes has led to their conservation during evolution, and homology among different genera is significant, as shown, e.g., for the RibA protein of *H. pylori*, which is 40 to 60% similar to homologues in nonrelated bacterial species (5).

This study reports the characterization of the genes *ribC*, *ribD*, and *ribE* from *B. henselae*, *B. quintana*, *B. bacilliformis*, and *B. clarridgeiae*. The function of the *B. henselae ribC* gene has been confirmed, and the sequence of the *ribC* DNA region could be used as a target for the molecular differentiation of *Bartonella* species by PCR analysis.

MATERIALS AND METHODS

Culture conditions. Bacterial strains are listed in Table 1. *B. clarridgeiae* was cultivated on Columbia blood agar plates. Cultures of *B. henselae* and *B. quintana* were propagated on chocolate agar, and *B. bacilliformis* was grown on hemin cysteine blood agar. *B. henselae*, *B. quintana*, and *B. clarridgeiae* were grown at 37°C. *B. bacilliformis* was cultured at 30°C. Cultures were propagated in a humid atmosphere containing 5% carbon dioxide.

E. coli was grown in Luria-Bertani (LB) medium (32) at 37°C. When necessary, the medium was supplemented with kanamycin at a concentration of 20 mg/liter.

The riboflavin-deficient mutant strains of *E. coli*, which are unable to grow on

rich media without addition of riboflavin, were propagated on LB agar supplemented with riboflavin (400 mg/liter).

Isolation and manipulation of bacterial DNA. Isolation, cloning, and manipulation of DNA were performed with *E. coli* TOP10 according to standard protocols (39). Plasmid pBH-RIBC1 (Table 1) was isolated from *E. coli*, previously grown in 100 ml of LB medium with kanamycin (20 mg/liter), by anion-exchange chromatography with a commercial kit (Qiagen).

The genomic DNA library of *B. henselae* Houston-1 (Table 1) was constructed by cloning *Pst*I-fragmented DNA into plasmid pZERO-2 with the Zero Background cloning kit from Invitrogen according to the manufacturer's recommendations. The library was propagated in *E. coli* TOP10. For the isolation of the riboflavin synthesis genes, the DNA library was transferred into the riboflavin-deficient mutant strain *E. coli* BSV23. Clones harboring plasmids which restored riboflavin synthesis were selected by the ability to grow on LB agar without added riboflavin.

PCR amplification. The DNA sequences of the primer oligonucleotides used for the PCR analysis of the *Bartonella* species and the sizes of the corresponding PCR products are listed in Table 2. PCR analysis was performed with 50 μl of a PCR mixture described earlier (4) which contained 1 U of *Taq* DNA polymerase, 25 pmol of each primer, and 100 ng of target DNA. Amplification under standard conditions was performed in a Techne thermocycler with 30 cycles each of 1 min at 94°C, 2 min at 55°C, and 3 min at 72°C, followed by a terminal extension step of 10 min at 72°C.

Optimized annealing temperatures for the species-specific PCR analyses are given in Table 2. The PCR products were electrophoretically separated on 1.2 or 1.6% agarose gels and stained with ethidium bromide.

The sizes of the PCR products were determined by comparison to a 1-kb marker DNA ladder (Gibco-BRL).

DNA sequence analysis. The sequences of the riboflavin synthesis genes were determined on both strands by the dideoxynucleotide chain termination method with the PRISM ready reaction dye cycle sequencing kit (ABI) with fluorescence-labeled deoxynucleoside triphosphates. Products of the sequencing reactions were separated on a polyacrylamide gel under denaturing conditions and analyzed in an ABI sequencing apparatus. Database searches were performed with the BLAST search engines provided via the Internet by the National Center for Biotechnology Information (33a). Nucleotide and protein sequence comparisons

TABLE 2. Primers and conditions used for the differentiation of *Bartonella* species by PCR

Species is specific for PCR assay	Primers	Nucleotide sequence	Annealing temp (°C)	PCR product size (bp) ^a
<i>B. clarridgeiae</i>	PBC5 PBC15	TACATAACGAGCCAATT TAGCTTTAGAACAATATGGT	50	895
<i>B. henselae</i>	PBH-L1 PBH-R1	GATATCGGTTGTGTTGAAGA AATAAAAGGTATAAAACGCT	55	393
<i>B. bacilliformis</i>	PBH-L1 PBB-R1 ^b	Same as for <i>B. henselae</i> AAAGGCGCTAACTGTTC	62	386
<i>B. quintana</i>	PBH-L1 PBQ-R1 ^b	Same as for <i>B. henselae</i> AAAGGGCGTGAATTTTG	60	390
Various species	PBH3 PBH4	CCAAGTGCTACATAACCATC CGGGTTGTTATTGCTCTTAC	55	1,723 ^b

^a The sizes of the PCR products were calculated from the riboflavin synthesis gene cluster of each species.

^b The size of a fragment from *B. henselae* was calculated.

were performed with the BESTFIT and PILEUP algorithms of the University of Wisconsin Genetics Computer Group software.

Nucleotide sequence accession numbers. The DNA sequences of the *ribC* DNA regions of *B. henselae*, *B. clarridgeiae*, *B. quintana*, and *B. bacilliformis* have been assigned EMBL database accession no. AJ132928, AJ236916, AJ236917, and AJ236918, respectively. The sequences of the RibD, RibC, and RibE proteins from *E. coli* were obtained from the SWISSPROT database (accession no. P25539, P29015, and 1786617, respectively).

RESULTS

Cloning of the *ribC* gene from *B. henselae*. A plasmid-based DNA library consisting of *Pst*I-fragmented DNA from *B. henselae* Houston-1 cloned into plasmid pZERO-2 was transferred into the *ribC* mutant *E. coli* BSV23. Plasmids which restored riboflavin synthesis in the mutant were selected by growth on LB agar without addition of riboflavin. Three clones that were able to grow normally on LB agar with kanamycin were obtained, and the corresponding plasmids were isolated and restricted with the enzymes *Pst*I, *Hind*III, and *Eco*RV. This analysis revealed that all three plasmids carried an identical *B. henselae* *Pst*I fragment of 2.3 kb (Fig. 1). One plasmid, designated pBH-RIBC1, was chosen for further analysis. After *E. coli* BSV23 was retransformed and reproducible growth of the transformants on LB agar had confirmed that the restora-

tion of riboflavin synthesis was plasmid mediated, *B. henselae* DNA was completely sequenced on both strands.

Analysis of the DNA sequence indicated that the 2,307 bp contained three open reading frames which are transcribed in identical directions (Fig. 1). Comparison of the deduced amino acid sequences with protein sequences in databases showed that the open reading frames encode homologues of the riboflavin synthesis proteins RibD (riboflavin deaminase/reductase, EC 3.5.4.-), RibC (riboflavin synthase [alpha chain], EC 2.5.1.9), and RibE (ribityl-lumazine synthase, EC 2.5.1.9); these proteins were previously isolated from *E. coli*, and their functions were characterized (3). Consequently, the *B. henselae* genes were designated *ribD*, *ribC*, and *ribE*.

The *ribD* gene, located at the left end of the cloned fragment (Fig. 1), consists of 1,089 bp. A comparison of the 363 amino acids encoded by *ribD* to corresponding proteins from *E. coli* and *B. subtilis* indicated that the part of the gene encoding the first 8 to 10 amino acids of the N terminus, including the start codon, was not on the cloned DNA fragment. The fact that plasmid pBH-RIBC1 did not restore riboflavin synthesis in the *ribD* mutant *E. coli* Rib2 (data not shown) further indicated that the *ribD* gene is partial and not functional in *E. coli*. The deduced *B. henselae* RibD protein has significant homology with RibD from *E. coli* (33% identity and 48% similarity) (Fig. 2A). Among the species for which molecular data of RibD are available, the riboflavin deaminase/reductase from *B. subtilis* (designated RibG) showed the highest degree of similarity (37% identity and 49% similarity).

The *ribC* gene, located in the center of the 2.3-kb *Pst*I fragment, consists of 621 bp (Fig. 1). This gene is complete and functionally active in *E. coli*, as demonstrated by its complementation of the *ribC* mutant strain BSV23. This confirmed the function of the protein as riboflavin synthase (alpha chain). Riboflavin synthase of *E. coli* catalyzes the terminal step of riboflavin synthesis. The protein is a heteropolymer in which RibC is the alpha subunit and RibE is the beta subunit. The 206 amino acids of the deduced *B. henselae* RibC protein are 36% identical and 50% similar to RibC from *E. coli*. A similar degree of homology was found for RibC proteins from *B. subtilis* (34% identity and 55% similarity) and other bacterial species (data not shown).

The *ribE* gene of *B. henselae*, which in other bacterial species encodes the beta subunit of riboflavin synthase, is 468 bp in length. The 155-amino-acid sequence encoded by *ribE* is ho-

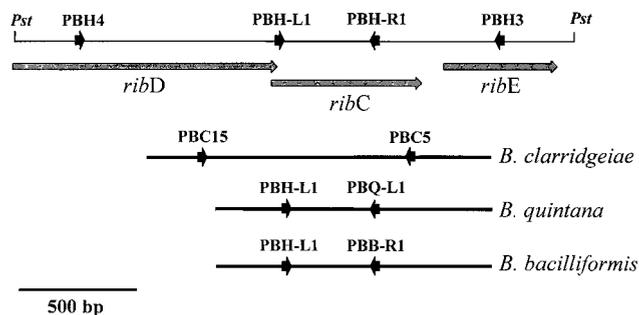


FIG. 1. Schematic representations of the DNA regions comprising the genes *ribC*, *ribD*, and *ribE* in *B. henselae* and other *Bartonella* species. The upper line represents the DNA region of *B. henselae* Houston-1 cloned into plasmid pBH-RIBC1. The binding sites of the oligonucleotide primers used for the differentiation of *Bartonella* species by PCR are marked by the arrowheads. The bars indicate the sequenced DNA regions amplified with primers PBH3 and PBH4 from *B. quintana*, *B. bacilliformis*, and *B. clarridgeiae*. Sequences of primer oligonucleotides are given in Table 2.

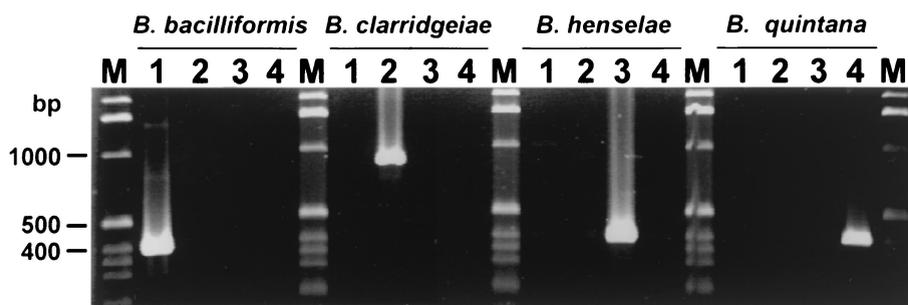


FIG. 5. Species-specific differentiation of *Bartonella* species by PCR analysis with primers designed from the *ribC* DNA region. Primers designed from the *ribC* DNA regions of *B. bacilliformis* (PBH-L1 and PBB-R1), *B. clarridgeiae* (PBH5 and PBH15), *B. henselae* (PBH-L1 and PBH-R1), and *B. quintana* (PBH-L1 and PBQ-R1) (as indicated at the top) were used for PCR analysis of *Bartonella* species under stringent species-specific conditions (Table 2). DNA isolated (100 ng) from *B. bacilliformis* (lanes 1), *B. clarridgeiae* (lanes 2), *B. henselae* (lanes 3), and *B. quintana* (lanes 4) was analyzed. Sizes of PCR products are listed in Table 2. Lanes M, marker DNA fragments. The PCR products were separated on a 1.6% agarose gel and stained with ethidium bromide.

(Table 2), generated products of the expected sizes (Fig. 5 and Table 2). The fact that the PCR products were amplified exclusively from DNA of the species from which the primers were designed indicates that each assay is specific for one species and can be used for differentiation (Fig. 5).

To further examine the specificity and reproducibility of the PCR-directed differentiation system, DNA isolated from all the strains of each *Bartonella* species listed in Table 1 was subjected to PCR analysis with the species-specific primer combinations (results not shown). The fact that the species-specific PCR products of the expected sizes (Fig. 5) were generated exclusively from strains of the species from which the primers were designed indicates that the PCR assays based on the *ribC* DNA region are reproducible, are not strain dependent, and show no interspecies cross-reactions.

The specific signals were also obtained from dilution series of whole cells analyzed by each PCR assay under stringent conditions (Table 2), indicating that the isolation of DNA can be omitted.

DISCUSSION

Species-specific diagnosis of infections caused by bacteria of the genus *Bartonella* is difficult even now. Distinguishing these pathogens from other bacteria in routine cultures is more a fortunate coincidence than a reliable method for identifying these organisms. Serological methods for the detection of *Bartonella* antibodies may be useful for immunocompetent patients with clinical manifestations like CSD, but differentiation between the species *B. henselae* and *B. quintana* is not possible (11, 41). Additionally, no serological tests for the detection of *B. clarridgeiae* antibodies are commercially available, and no serological data concerning infections of immunocompromised and HIV-infected patients exist.

Therefore, the differentiation of *Bartonella* species involved in human infections requires molecular diagnostic procedures. However, most primers used for the differentiation of *Bartonella* species by PCR are only genus specific; identification at the species level requires sequencing of amplified DNA or hybridization with a species-specific probe (1, 37, 43). Restriction fragment length polymorphisms of the 16S rRNA gene and sequence polymorphisms of the citrate synthase gene have been used for the differentiation of *Bartonella* species (8, 9). Recently, the *ftsZ* gene of *Bartonella* species has been successfully used to differentiate *B. henselae*, *B. quintana*, and *B. bacilliformis*, but testing for specificity, strain dependence, or detection in clinical specimens is still in progress (21). However, as the rRNA genes are highly conserved within the genus

Bartonella, the usage of PCR assays based on chromosomal genes, like *ftsZ* and *gltA*, or the riboflavin synthesis genes analyzed in this study, should improve species-specific differentiation.

This study reports the development of species-specific PCR assays for the differentiation of *B. bacilliformis*, *B. clarridgeiae*, *B. henselae*, and *B. quintana* based on sequence information for genes encoding enzymes involved in riboflavin synthesis. The riboflavin synthesis genes were chosen because they are, due to their evolutionary conservation and their absence in humans (3), excellent targets for the diagnosis of invasive pathogens. Their usefulness is further supported by the fact that the genetic organization of riboflavin synthesis genes differs remarkably among bacterial species, which increases the specificity of PCR-based techniques. The *ribC* gene was isolated from *B. henselae*, and the functional complementation of a *ribC*-deficient mutant of *E. coli* confirmed that the encoded protein has the activity of riboflavin synthase (alpha chain), which is involved in the catalysis of the terminal step of riboflavin biosynthesis (13). The *ribC* gene of *B. henselae* is flanked by the genes *ribD* and *ribE*, which encode homologues of the riboflavin synthesis proteins RibD and RibE. In *E. coli*, the RibE and RibC proteins form the multienzyme complex riboflavin synthase, which catalyzes the terminal step in riboflavin synthesis (3). The gene order of *ribD*, *ribC*, and *ribE* is conserved in *B. henselae*, *B. quintana*, *B. clarridgeiae*, and *B. bacilliformis*. The clustering suggests that the genes are organized as an operon, which is also the case for riboflavin synthesis genes in the gram-positive bacterium *B. subtilis* and in gram-negative bacteria, like *Actinobacillus* spp. and *Photobacterium* spp. (15, 35). In the latter species, the *rib* genes are part of the *lux* operon (26). Within these operons, the gene order of *ribD*, *ribC*, and *ribE* homologues is different from that in *Bartonella* species. In other gram-negative organisms, e.g., *E. coli*, *H. pylori*, and *H. influenzae*, the *rib* genes are randomly distributed in the chromosome (3, 5, 14).

The *ribC* gene and the parts of the flanking *ribD* and *ribE* genes corresponding to the C and N termini, respectively, are conserved in the four *Bartonella* species investigated. The amino acid identity of the RibC proteins from *B. henselae* and *B. quintana* (90%) is significantly higher than that of the RibC proteins from *B. bacilliformis* and *B. clarridgeiae* (80%), which are more distantly related. For *B. henselae*, *B. quintana*, and *B. bacilliformis*, this degree of homology is consistent with the relatedness of the *Bartonella* species investigated on the basis of the citrate synthase (*gltA*) and *ftsZ* genes in earlier studies (9, 21, 34). Taken together, these findings indicate that *B.*

henselae and *B. quintana* are more related to each other than to other *Bartonella* species.

For *B. clarridgeiae*, not much sequence data besides those for the riboflavin synthesis genes investigated in this study are available in databases. The gene for 16S rRNA (19, 23), the citrate synthase gene (*gltA*) (10, 34), and the gene for a 60-kDa heat shock protein (30), which is also conserved in other *Bartonella* species (18), have been investigated, but the sequence data have not been used for species-specific PCR assays which allow direct identification of *B. clarridgeiae* without sequencing or restriction fragment length polymorphism analysis. The intermediate level of homology for the *ribC* gene, in the range of 80%, did not indicate a closer evolutionary relationship between *B. clarridgeiae* and any of the other *Bartonella* species investigated in this study.

The comparative analysis of the riboflavin synthesis proteins does not allow us to state any evolutionary relationships between *B. henselae* and another bacterial species for which molecular data on riboflavin synthesis genes are available. The constant degree of homology of the riboflavin synthesis proteins, even to those of unrelated bacterial species, supports the separate evolutionary position of the genus *Bartonella*.

The genetic analysis of the riboflavin synthesis genes *ribD*, *ribC*, and *ribE* in strains of *B. henselae* showed that the *rib* genes are a constant part of the *B. henselae* genome and are highly conserved with respect to the nucleotide sequence. Single nucleotide substitutions were detected exclusively in strains which were characterized earlier as variant II by various other molecular techniques (42). The fact that these substitutions were located at identical positions and concerned identical nucleotides is further evidence for genetic variations within the *B. henselae* population, which supports the assumption that stable subtypes exist within the population. On the other hand, strains harboring 16S rDNA of Bergmans type 1 and type 2 did not show any differences in the *rib* gene DNA sequence, indicating that mutations in these genetic loci are not linked to each other.

PCR analysis with oligonucleotide primers designed from the *ribC* DNA region allowed species-specific differentiation of the *Bartonella* species, as shown by the amplification of DNA from the species from which the primers were designed, but not from the others. The fact that the analysis was not strain dependent might indicate that the approach could be of use for the detection of *Bartonella* species in clinical specimens, which is currently under investigation.

The presence of riboflavin synthesis genes in *Bartonella* species is strong evidence for their ability to produce this essential vitamin, which could be of relevance to their establishment and survival in the host, as shown for the swine pathogen *A. pleuropneumoniae* (16).

In summary, the results indicate that the riboflavin synthesis genes *ribD*, *ribC*, and *ribE* are excellent targets for the differentiation of *Bartonella* species. The species-specific PCR assays developed should increase our diagnostic potential to differentiate among *Bartonella* species of clinical relevance. The PCR assay specific for *B. clarridgeiae* is one of the first systems available for molecular differentiation. It facilitates discrimination of *B. clarridgeiae* and *B. henselae*, which should help to clarify the role of this putative pathogen in human diseases.

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