MOLECULAR IDENTIFICATION

Identification of *Bartonella henselae* and *B. quintana* 16S rDNA sequences by branch-, genus- and species-specific amplification

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Given the controversy surrounding the aetiology of cat scratch disease and the association of both *Bartonella henselae* and *B. quintana* with bacillary angiomatosis, a method for the direct detection in clinical samples of 16S rRNA from the Proteobacteria alpha subgroup was developed. The primary structure of amplified 16S rDNA was determined by cloning and sequencing. Three sequences were identified: one corresponded exactly to GenBank accession number M73229 (*B. henselae*); the second was related to, but distinct from, GenBank accession number Z11684 (referred to as '*B. henselae* variant'); and a third sequence was identical with GenBank accession number M73228 (*B. quintana*). No sequence corresponding to *Afipia* spp. was found. To speed identification and reduce the cost of analysis, a nested amplification method for *B. henselae* and *B. quintana* was devised. These techniques were applied to DNA extracted from 30 unfixed lymph node biopsies, two liver biopsies and 36 node pus samples from patients with suspected cat scratch disease, and from 17 skin biopsies from AIDS patients with suspected bacillary angiomatosis. *B. henselae* or *B. henselae* variant sequences were found in 42 (62%) of 68 samples from suspected cat scratch disease. *B. quintana* was not associated with cat scratch disease, but a *B. quintana* sequence was found in seven (41%) of 17 samples from suspected bacillary angiomatosis patients. *B. henselae* 16S rDNA sequences were not found in bacillary angiomatosis specimens.

Introduction

Cat scratch disease (CSD) has been known for 45 years as an infectious disease transmitted to man by cats [1]. This relatively common cause of subacute regional lymphadenopathy is diagnosed currently on the basis of three of four criteria: contact with or scratch from a cat; negative conventional bacteriological results; positive skin test with heated purulent lymph node material from a patient with CSD; gram-negative bacilli visualised by Warthin Starry silver stain in lymph node tissues [2, 3]. The precise identification of the aetiological agent has been the source of controversy. Various bacteria have been successively incriminated [4, 5], including *Afipia felis* and *Rochalimaea henselae* [6, 7], but at the present time *R. henselae* seems to be the only causal agent of CSD in the USA [8, 9].

The AIDS epidemic has allowed the emergence of infections that were previously unknown or rare. Among these infections is bacillary epithelioid angiomatosis (BA). This debilitating systemic disease is characterised by vascular lesions that are generally cutaneous and less frequently hepatic, splenic, pulmonary or cerebral [10, 11]. Molecular methods have implicated *R. quintana* in this disease, a bacterium described long ago as causing trench fever [9–12]. Both *R. henselae* and *R. quintana* have been isolated from bacillary angiomatosis in the USA [3–7, 13, 14].

Isolation of the causative bacterium from CSD and BA is tedious and often fails. Molecular methods now allow the detection of non-cultivable bacteria, based on 16S rRNA sequence comparisons [15]. Rapidly expanding international data bases now contain c. 3500 bacterial 16S rRNA sequences. Use of these sequences has allowed the detection and identification of bacteria without culture [16].

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The CSD and BA agents belong to the alpha subgroup

of Proteobacteria [17]. In the phylogenetic tree, *Rochalimaea* spp. were found to be so close to *Bartonella bacilliformis*, that *Rochalimaea* spp. were transferred to the genus *Bartonella* [18]. *Grahamella* spp. were also included in the genus *Bartonella* [19]. The alpha subgroup of Proteobacteria also contains *Afipia* spp. [20].

In this work, no attempt was made to isolate the aetiological agents, but the rapidly expanding 16S rRNA sequence databases were used to identify the agents associated with CSD and BA in France. Taking into account the potential diversity of agents causing CSD and BA, primers were designed to amplify selectively, from clinical samples, 16S rRNA genes (16S rDNA) from all members of the alpha subgroup of Proteobacteria. The primary structure of the amplified 16S rDNA was determined by cloning and sequencing. Comparison of the 16S rDNA sequences obtained with the databases allowed the identification of any alpha-Proteobacteria (*Bartonella* spp., *Afipia* spp. or others). To speed identification, a nested amplification method was devised for *B. henselae* and *B. quintana*.

Materials and methods

Bacterial strains

The strains of bacteria used to evaluate the specificity of the PCR tests or as positive controls are listed in Table 1 [21]. *B. elizabethae* and *B. vinsonii* were grown on Chocolate Polyvitex Agar (Diagnostics Pasteur, Marnes-la-Coquette, France). *B. henselae* was grown on Columbia Agar supplemented with horse blood 10% v/v and haemin (Diagnostics Pasteur) 250 mg/L. Plates with *Bartonella* spp. were incubated for 5 days at 37°C in the presence of CO₂ 5%. *A. felis* was grown on charcoal-yeast extract agar for 2–3 days at 30°C without CO₂ [6].

Clinical samples

Unfixed lymph node biopsy specimens (n = 30) and lymph node aspirates (n = 36) were from patients diagnosed clinically as having CSD. Liver biopsies (n = 2) were from hepatic peliosis. Skin biopsies (n = 17) were from AIDS patients with suspected BA. All samples were incubated in a water bath at 65°C for 30 min to inactivate human immunodeficiency virus.

DNA extraction

DNA was extracted from cultures or clinical samples with a modification of a procedure described previously for rickettsiae [22]. Following incubation overnight at 55°C in lysis buffer (0.5 M Tris, pH 9, 20 mM EDTA, 10 mM NaCl, SDS 1% w/v and proteinase K, Sigma, 1 mg/ml), the DNA was purified by phenol-chloroform extraction and ethanol precipitation [23]. A negative control (all the reagents without DNA sample) was included in each experiment to ensure that none of the extraction buffers and reagents was contaminated with target DNA.

16S rDNA amplification

Reaction mixtures were prepared in a laminar flow hood which was irradiated subsequently by UV light. A separate set of pipettes and aerosol-guarded tips were used exclusively for preparation of reaction mixtures to avoid contamination [24]. The areas used for PCR preparation were isolated from electrophoresis units.

PCR amplification was performed with a DNA Thermal Cycler Model 480 (Perkin Elmer). A blank control lacking template DNA was included in each experiment and yielded no amplification product, demonstrating that DNA contamination was not present. DNA extracted from *A. felis* was used as a

Table 1. Strains of bacteria used for evaluation or control purposes

Species	Strain	Status or origin
<i>Bartonella henselae</i>	ATCC 49882, CIP 103737	Type strain
<i>Bartonella vinsonii</i>	ATCC 152-VR, CIP 103738	Type strain
<i>Bartonella elizabethae</i>	ATCC 49927, CIP 103761	Type strain
<i>Bartonella quintana</i>	Clinical isolate	Human skin, France*
<i>Bartonella bacilliformis</i>	CIP 77.27	Human blood, Peru
<i>Afipia felis</i>	ATCC 53690, CIP 103515	Type strain
<i>Agrobacterium tumefaciens</i>	ATCC 23308, CIP 67.1	Type strain
<i>Brucella melitensis</i>	CIP 65.2	Type strain
<i>Caulobacter bacteroides</i>	CIP 101031	Type strain
<i>Rhodobacter sphaeroides</i>	CIP 60.6	Type strain
<i>Sphingomonas paucimobilis</i>	CIP 100752	Type strain
<i>Brevundimonas diminuta</i>	CIP 63.27	Type strain
<i>Corynebacterium xerosis</i>	ATCC 373	Type strain
<i>Escherichia coli</i>	K12 wild type	

*Received from G. Arlet, Hopital Saint-Louis, Paris, France [21].

positive control. If no specific amplification product was detected and when a blur was seen on the agarose gel, the PCR was repeated with a 10- and 100-fold diluted sample to remove inhibitory compounds in the sample.

Two oligonucleotide primers, Alpha and r-Alpha (Table 2) [25], were used for 16S rDNA amplification before sequencing. These primers were selected on the basis of sequence conservation in the alpha subgroup of Proteobacteria after comparison of 87 Proteobacteria alpha subgroup 16S rDNA sequences from GenBank [26]. Bio-Taq polymerase (Bioprobe, Montreuil-sous-bois, France) was used in amplification reactions consisting of 5 min at 94°C; 35 cycles of 94°C for 1 min, 65°C for 1 min and 72°C for 1 min; and a final elongation step at 72°C for 5 min.

Two other primers, A-proteo and r-Alpha-sh (Table 2), were also used in initial sequence experiments. Primer A-proteo was selected on the basis of sequence conservation in the Proteobacteria phylum. Primer r-Alpha-sh is shorter than r-Alpha in order to have an annealing temperature compatible with that of primer A-proteo. Hi-Taq polymerase (Bioprobe) was used in amplification reactions identical to those above except that the primer hybridisation step was at 62°C for 1 min.

Amplified products were detected by electrophoresis on an agarose 0.8% w/v gel in Tris-acetate-EDTA buffer (40 mM Tris-acetate, 2 mM EDTA, pH 8.0). A 1-kb DNA Ladder (Gibco-BRL) was used as a DNA size standard. Gels were stained with ethidium bromide and photographed by standard procedures.

16S rDNA cloning and sequencing

To facilitate cloning, a 12-base dUMP-containing sequence (CUACUACUACUA) was added to the 5' end of the PCR primers [27]. The resulting PCR

product was cloned in pAMP1 plasmid with the CLONEAMP™ System (Gibco-BRL). For each specimen, 12 clones were amplified in *Escherichia coli* strain MC1061. The presence of an insert was confirmed by digestion with *Bam*HI and *Eco*RI [23]. For each specimen, two clones were sequenced by 'cycle sequencing' (Amersham International) with universal primers complementary to plasmid or conserved regions of 16S rDNA labelled with [γ P³³] ATP (Isotopchim, Ganagobie-Peyruis, France) [28, 29].

Nested PCR and RFLP analysis

Proteobacteria alpha subgroup 16S rDNA was amplified with Hi-Taq polymerase and primers A-proteo and r-Alpha-sh (Table 2). The strains listed in Table 1 were used as positive or negative controls. Two samples known to contain different *B. henselae* 16S rDNA sequences were also included as controls.

To detect *Bartonella* spp., PCR was performed with two oligonucleotide primers, Bart and r-BH (Table 2). Primer Bart (position 95) was selected after examination of 87 Proteobacteria alpha subgroup 16S rDNA sequences for sequence signature of the genus *Bartonella*. The target sequence of this primer was identical for *B. henselae*, *B. quintana*, *B. vinsonii*, *B. doshiae* and *B. grahamii*. The corresponding sequence of *B. elizabethae* differed from the other *Bartonella* sequences at six positions (mostly at the 5' end of the oligonucleotide). Primer r-BH (position 1092) was deduced from the 16S rDNA signature sequence of *B. henselae* [14]. Nested PCR was performed with primer Bart, specific for genus *Bartonella* 16S rDNA, and reverse primer r-BH, specific for *B. henselae* 16S rDNA, used at low stringency (65°C). Depending on the amount of alpha 16S rDNA amplified product, the nested PCR was performed with 1 µl of a 1 in 10 or 1 in 100 dilution of PCR product, and one cycle of predenaturation at 94°C for 5 min, followed by 35 cycles comprising denaturation at 94°C for 1 min,

Table 2. Oligonucleotide primers used for 16S rDNA amplification

Name and specificity	Sequence	Position*
Alpha	AGTG TAGAGG TRAAATTCGTAGATATT	681–707
alpha subgroup		
r-Alpha	CCATTGTAGCACGTGTGTAGCCCA	1242–1219
alpha subgroup		
A-proteo	AGAGTTTGATCMTGGCTCAGA	8–28
Proteobacteria		
r-Alpha-sh	GTAGCACGTGTGTAGCCCA	1237–1219
alpha subgroup		
Bart	CACTCTTTTAGAGTGAGCGGCAA	95–107
<i>Bartonella</i> spp.		
r-BH	CCCCCTAGAGTGCCCAACCA [†]	1155–1131
<i>B. henselae</i>		

M = A or C; R = A or G.

**E. coli* numbering system [25].

[†]Base A at the 3' end if this primer hybridises with base T for *B. henselae* and not with A for *B. quintana* or C for *B. vinsonii*, *B. elizabethae* and *B. bacilliformis*.

annealing at 65°C for 1 min, and extension at 72°C for 1 min. The amplicons were then allowed to extend at 72°C for 5 min. Amplified products were detected by electrophoresis as described above.

To identify *B. quintana*, 10- μ l portions of PCR mixes containing amplified *Bartonella* 16S rDNA fragment were digested with *HincII*. This enzyme cuts the *B. quintana* 16S rDNA sequence at position 435, as found on the restriction maps of *B. quintana* sequences (GenBank accession numbers M73228 and M11927). Restriction fragment length polymorphisms (RFLPs) were determined after electrophoresis of the digested DNA on agarose (Bioprobe) 1% w/v plus NuSieve agarose (FMC BioProducts, Rockland, ME, USA) 1% w/v gels. *B. henselae* was detected by performing the PCR with primers Bart and r-BH at high stringency (72°C). Nested PCR was performed under the conditions described above with an annealing temperature of 72°C.

Results

16S rRNA gene sequence analysis

PCR amplification of Proteobacteria alpha subgroup 16S rDNA with primers Alpha and r-Alpha (Table 2) generated a 640-bp fragment for all *Bartonella* spp., *A. felis* and from 22 clinical samples. Included in the 22 clinical samples were cases in which PCR inhibition was suspected and dilution of the original samples yielded amplification products (c. 25% of cases). No amplicon was obtained when *E. coli* or *Corynebacterium xerosis* DNA was used as the target.

No product was observed when the primers were used to amplify a negative control with no DNA template (data not shown).

The 640-bp fragments amplified from the 22 clinical specimens were cloned and sequenced. The 17 fragments amplified from 29 specimens derived from suspected cases of CSD were identical to *B. henselae* 16S rDNA. Of two suspected hepatic peliosis samples, one yielded 16S rDNA of *B. henselae*. Of the 13 samples from suspected cases of BA, four yielded 16S rDNA of *B. quintana*.

A 1210-bp product, including the 5' end of the 16S rDNA, was produced with primers A-Proteo (specific for Proteobacteria) and r-Alpha-sh (specific for the alpha subgroup of Proteobacteria) when *Bartonella* spp., *Brucella abortus*, *Agrobacterium tumefaciens*, *Caulobacter bacteroides*, *Rhodobacter bacteroides*, *Sphingomonas paucimobilis*, *Brevundimonas diminuta* and the positive clinical samples listed above were used as templates for amplification (Fig. 1). The 1210-bp PCR products contained two *B. henselae* 16S rDNA sequences, one of which corresponded exactly to that of *B. henselae* strain ATCC 49882 (accession number: M73229) [7] and was found in five cases. The second sequence differed by three nucleotides (positions 1187–1189) from *B. henselae* accession number Z11684 [17] and was named '*B. henselae* variant'. This sequence was found in 13 cases. The sequence variation was not the result of polymerase-nucleotide incorporation errors because the 'new' sequences were found with two independently cloned PCR products. The *B. quintana* 16S rDNA sequences

Fig. 1. Examples of specific amplification of 16S rDNA fragments from the Proteobacteria alpha subgroup. Lanes: 1, *B. henselae*; 2, specimen from case of CSD (*B. henselae* sequence); 3, *B. quintana*; 4, *B. vinsonii*; 5, *B. elizabethae*; 6, *A. felis*; 7, *Br. melitensis*; 8, *E. coli*; 9, *C. xerosis*; 10–16, specimens from suspected cases of CSD; 17, specimen from suspected case of BA; M, DNA size markers.

found corresponded exactly to the 16S rDNA sequence of *B. quintana* strain ATCC VR-358 (accession number: M73228) [7].

Nested PCR and RFLP analysis

The nested amplification method was tested with 43 additional clinical samples and validated with *Bartonella* type strain DNA and three clinical specimens containing known 16S rDNA sequences of *B. henselae*, *B. henselae* variant and *B. quintana*. A 1210-bp fragment was obtained after amplification with primers A-Proteo and r-Alpha-sh from 24 of 39 samples derived from suspected cases of CSD and from three of four samples derived from suspected cases of BA (Fig. 1).

Nested PCRs at low stringency (65°C) with primers Bart and r-RH yielded a 990-bp amplicon that was specific for the genus *Bartonella*. Fig. 2A shows the PCR products obtained from representative strains of *B. henselae* (lane 1), *B. vinsonii* (lane 4), *B. elizabethae* (lane 5), *B. quintana* (lane 3), specimens from cases of CSD (lanes 2, b, e, f and g) and a case of BA (lane h). No amplification product was observed for *A. felis* (lane 6), *Brucella melitensis* (lane 7), *E. coli* (lane 8), *C. xerosis* (lane 9), some

clinical samples (lanes a, c and d) and negative controls containing no DNA template (data not shown).

Nested PCRs at high stringency (72°C) with the same primers appeared to be specific for *B. henselae*. Fig. 2B shows the PCR products obtained for the type strain of *B. henselae* (lane 1) and specimens from cases of CSD (lanes 2, b, e, f and g). No amplification was observed for *B. quintana* (lane 3), *B. vinsonii* (lane 4), *B. elizabethae* (lane 5), *A. felis* (lane 6), *Br. melitensis* (lane 7), *E. coli* (lane 8), *C. xerosis* (lane 9), some samples from suspected cases of CSD (lanes a, c and d) and BA (lane h), and negative controls containing no DNA template (data not shown).

The *HincII* RFLPs could be used to identify *B. quintana* 16S rDNA in amplicons obtained at 65°C. Two bands of 340 and 650 bp were seen for the *B. quintana* DNA and the sample from a case of BA (Fig. 3; lanes 2 and 5, respectively).

Of 68 samples from suspected cases of CSD, 42 (62%) yielded amplicons characteristic of 16S rDNA from the Proteobacteria alpha subgroup. All of these amplicons corresponded to *B. henselae*.

Fig. 2. A. Examples of specific amplification of 65°C of *Bartonella* 16S rDNA. Lanes: 1, *B. henselae*; 2, specimen from case of CSD (*B. henselae* sequence); 3, *B. quintana*; 4, *B. vinsonii*; 5, *B. elizabethae*; 6, *A. felis*; 7, *Br. melitensis*; 8, *E. coli*; 9, *C. xerosis*; a–g, specimens from suspected cases of CSD; h, specimen from suspected case of BA; M, DNA size markers. B. Examples of specific amplification at 72°C of *B. henselae* 16S rDNA. Lanes as for A.

Fig. 3. Examples of restriction profiles obtained following *HincII* digestion of *Bartonella* 16S rDNA amplicons. Lanes: 1, *B. henselae*; 2, *B. quintana*; 3, *B. vinsonii*; 4, *B. elizabethae*; 5, sample from suspected case of BA; M, molecular size markers.

Of 17 samples of suspected cases of BA, seven (41%) yielded amplicons characteristic of 16S rDNA from the Proteobacteria alpha subgroup. Sequencing or nested PCR indicated that this 16S rDNA corresponded to *B. quintana* (Figs. 2 and 3). No *B. henselae* sequence was associated with cutaneous BA infection in this study.

Discussion

In contrast to previous studies on the molecular diagnosis of *Bartonella* spp. [8, 30], the present study was able to detect members of the Proteobacteria alpha subgroup directly in clinical samples. The 16S rDNA sequencing method was able to identify bacteria accurately to the alpha subgroup of Proteobacteria, including known *Bartonella* and *Afipia* spp. as well as previously unrecognised bacteria. The method is easy to interpret when the sequence found corresponds exactly to a known sequence. However, a variant sequence was found which may correspond to *B. henselae*. This new variant sequence and two published sequences for *B. henselae* (accession numbers M73229 and Z11684) differ mostly in a hypervariable

domain (position 150–170), with nucleotide differences corresponding to 0.2% of the complete 16S rDNA sequence. Sequence variability within a species has not been defined properly. Two 16S rDNA sequences have been described for *B. quintana* (accession numbers M11927 and M73228) [7, 31], but only one type of *B. quintana* sequence was found in this study (M73228). Three different sequences have been reported for two strains of *B. vinsonii* (accession numbers Z31352, M73230, and LO1259) [7, 19, 32]. Three different sequences are also found for *B. bacilliformis* in GenBank (accession numbers X60042 [19], Z11683 [20] and M65249 [17]). Such diversity of sequences for one species may be caused by species heterogeneity [33], heterogeneity of 16S rRNA copies within a strain [34], or sequencing errors. It is accepted that strains with 16S rRNA homology values of < 97.5% are unlikely to belong to a single species [35], but identical or nearly identical sequences are not proof of identity at the species level [36]. The best solution to this problem would be to grow and isolate bacteria and then either perform quantitative DNA-DNA hybridisation or an identification test that correlates with DNA group assignment. However, when bacteria are non-cultivable, or cultivable only at very low efficiency, amplification and sequencing of rDNA may be the method of choice for use in a laboratory which is not specialised in the study of a particular bacterial group.

To reduce the costs and labour associated with DNA sequencing, a set of nested PCR tests was developed. Taking into account the potential diversity of agents causing CSD and BA, the PCR first amplifies a sequence specific for the Proteobacteria alpha subgroup. The second PCR indicates whether the amplified fragment corresponds to the genus *Bartonella*. Any amplified fragment not corresponding to *Bartonella* should be sequenced. The *Bartonella*-specific PCR test can be used for species identification by repeating the PCR at a higher (72°C) temperature (for *B. henselae*) or by *HincII* digestion of the PCR products (for *B. quintana*). No additional hybridisation steps are required for identification of these two species.

In total, 62% of samples from suspected cases of CSD gave positive results in the nested PCR specific for *B. henselae*. This is lower than the figure of 84% reported by Anderson *et al.* [8], but similar to the figure of 60% reported by Bergmans *et al.* [30]. The difference may be related to the type of cases selected. Anderson *et al.* [8] detected *B. henselae* DNA in 86% of patients with *B. henselae* antibody titres of ≥ 64 . The study by Bergmans *et al.* [30], and the present study, reported results from a group of patients who were only suspected as having CSD (lymphadenopathy, cat contact, absence of other obvious diagnosis). In this study, *Bartonella* antibody titres were known in only 19 cases, and only nine of

these 19 patients had positive serology. Of these nine positive patients, eight (88%) yielded a positive PCR test for *Bartonella*.

The major problem in clinical microbiology is to associate a micro-organism with a disease. Although *A. felis* was originally associated with CSD [6], the present study found that *B. henselae* sequences were associated with CSD when the Proteobacteria alpha subgroup amplification was positive. No *Afipia* sequences were found. In the USA, both *B. henselae* and *B. quintana* have been associated with BA [7, 13, 14]. It was surprising to find only the *B. quintana* sequence in skin biopsies when the Proteobacteria alpha subgroup amplification was positive. More specimens need to be studied to verify whether the aetiological agent of BA is limited to *B. quintana* in Europe. The nested PCR described in this paper is now used routinely in our Institute for this purpose.

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