

## Molecular Diagnosis of Cat Scratch Disease: a Two-Step Approach

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**Amplification of *Bartonella henselae* DNA has been proposed as a diagnostic test for cat scratch disease (CSD). The sensitivities of the following three PCR assays were compared. PCR/rRNA with universal primers amplifies part of the 16S rRNA gene, followed by hybridization with a specific *B. henselae* probe; PCR/CS and PCR/HSP amplify portions of the *gltA* and the *htrA* genes, respectively, each followed by restriction fragment length polymorphism analysis. The threshold of detection of *B. henselae* DNA in pus was  $10^{-4}$ ,  $10^{-3}$ , and  $10^{-2}$  ng for PCR/rRNA, PCR/CS, and PCR/HSP, respectively. By these three assays, *B. henselae* DNA was detected in 100, 94, and 69% of 32 pus and lymph node specimens from CSD patients, respectively. The similar sensitivities of the PCR/rRNA and the PCR/CS assays for detecting *B. henselae* DNA in clinical specimens are in contrast to the 10-fold difference in sensitivities in favor of PCR/rRNA demonstrated with purified *B. henselae* DNA in sterile pus, suggesting that in the majority of cases, the bacterial load in clinical specimens is large enough to be identified by the PCR/CS assay. A two-step approach is suggested to achieve maximal sensitivity for detecting *B. henselae* in clinical specimens: initial testing by PCR/CS (which does not require hybridization), followed by PCR/rRNA with PCR/CS-negative specimens when CSD is strongly suspected.**

Cat scratch disease (CSD) is a common cause of infectious subacute regional lymphadenitis. *Bartonella henselae* is considered the principal etiologic agent (6, 20). The disease usually develops following a cat scratch or bite, and in most patients the disease resolves spontaneously within several months. In a minority of cases, however, patients may experience severe systemic disease or may demonstrate other atypical manifestations including Parinaud's oculoglandular syndrome, encephalopathy, neuroretinitis, pneumonia, osteomyelitis, erythema nodosum, arthralgia, arthritis, and thrombocytopenic purpura (1, 7, 8, 13, 14, 17, 26).

The early and accurate diagnosis of CSD in a patient with lymphadenopathy is important to differentiate this benign condition from a neoplastic process. However, this can be difficult due to the limitations of the available confirmatory diagnostic tests. The skin test, which is prepared from pus aspirated from a lymph node of a CSD patient, lacks standardization and has a variable sensitivity that ranges from 79 to 100% (17). Cultures of pus and lymph node specimens require an invasive procedure and are often negative (9). Histopathological examination of lymph nodes also requires an invasive procedure; standard pathologic stains and Warthin Starry silver stain are nonspecific, and the staining procedure with the latter stain is technically difficult. The specificity of staining is improved by the use of an immunohistochemical assay (16). Serological assays have been developed, including an indirect fluorescent antibody (IFA) test and enzyme immunoassays (EIAs). However, these tests are not readily available and are characterized by a relatively low sensitivity (5, 10, 20). Depending either on the technique used (IFA test or EIA) or on the methods for antigen preparation, the sensitivities of serological assays range from 32 to 83% for anti-*B. henselae* immunoglobulin G

(IgG) antibodies and from 16 to 66% for anti-*B. henselae* IgM antibodies (25). In addition, serological cross-reactivity between *B. henselae*, *Bartonella quintana*, and *Coxiella burnetii* might lead to misdiagnosis (24). It has also been suggested that the antigenic variability of *B. henselae* is a possible explanation for the inconsistency of the serodiagnostic test results reported previously (10).

Recently, several PCR assays have been developed for the direct detection of *B. henselae* DNA in clinical samples; however, few clinical studies have been carried out with these assays, and a comparative evaluation of their sensitivities and specificities has not been performed.

In this study, we compared the sensitivities of three PCR assays: one assay is based on the universal approach of amplification of the 16S rRNA gene of all eubacteria followed by identification of the *B. henselae* DNA by a specific probe; the other two assays amplify parts of two *B. henselae* genes encoding for either citrate synthase or a 60-kDa heat shock-like protein. We first determined the threshold of detection of *B. henselae* DNA in pus, and then we evaluated the sensitivities of the three assays using specimens of pus or lymph node tissue from CSD patients. Finally, we suggest a PCR-based approach to achieving maximal sensitivity for detecting *B. henselae* DNA in clinical specimens from patients with CSD.

### MATERIALS AND METHODS

**Bacterial strains and culture.** *B. henselae* 87-66 (ATCC 49793) (27) was provided by D. F. Welch, University of Oklahoma Health Sciences Center, Oklahoma City, and was used to prepare antigen for the EIA. *Afpia felis* AfTA-1 (Tel Aviv-1) was cultured in our laboratory from a lymph node of a CSD patient (11) and was used as a negative control in the dot blot hybridization assay. Isolates BhTA-2 and BhTA-3 were cultured in our laboratory from two patients with typical CSD and were identified by fatty acid analysis and the IFA test as *B. henselae* in the laboratory of D. F. Welch, as well as by PCR of the 16S rRNA and the *gltA* genes as described below. *B. henselae* and *A. felis* isolates were grown on chocolate agar plates (Hy Laboratories Ltd., Rehovot, Israel) and were incubated for 5 to 7 days at 35°C in the presence of 5% CO<sub>2</sub> and at 32°C without CO<sub>2</sub>, respectively. *Staphylococcus aureus* was a blood culture isolate from a patient

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TABLE 1. Descriptions of patients with CSD and laboratory results

Patient no.	Specimen no.	Age (yr)	Source	Regional lymphadenopathy	Location of lymph nodes <sup>a</sup>	Cat contact	Test result				
							IgG EIA	Skin test	PCR/rRNA	PCR/CS	PCR/HSP
1	1	8	Pus	Yes	Axillary	Yes	ND <sup>b</sup>	+	+	-	-
2	2	9	Pus	Yes	Axillary + epi	Yes	+	+	+	+	+
3	3	12	Pus	Yes	Axillary	Yes	+	+	+	+	-
4	4	9	Pus	Yes	Cervical	Yes	-	+	+	+	+
5	5	43	Pus	Yes	Epi	Yes	+	+	+	+	-
5	6	43	Pus	Yes	Epi	Yes	+	+	+	+	-
6	7	17	Pus	Yes	Cervical	Yes	+	ND	+	+	-
6	8	17	Pus	Yes	Cervical	Yes	+	+	+	+	+
7	9	5	Pus	Yes	Not reported	Yes	+	ND	+	+	+
7	10	5	Pus	Yes	Not reported	Yes	+	+	+	+	+
8	11	8	Pus	Yes	Axillary	Yes	ND	+	+	+	-
9	12	47	Pus	Yes	Axillary	Yes	+	+	+	+	+
10	13	9	Pus	Yes	Preauricular	Yes	+	-	+	+	+
11	14	10	Pus	Yes	Axillary	Yes	+	ND	+	+	+
12	15	7	Pus	Yes	Axillary	Yes	+	ND	+	+	+
13	16	16	Pus	Yes	Axillary + epi	Yes	ND	ND	+	+	-
14	17	11	Pus	Yes	Axillary	Yes	ND	+	+	+	+
15	18	11	Pus	Yes	Cervical	Yes	-	ND	+	+	+
16	19	14	Pus <sup>c</sup>	Yes	Axillary + epi	Yes	+	ND	+	+	+
17	20	14	Pus	Yes	Axillary	Yes	+	ND	+	+	+
18	21	Not reported	Pus	Yes	Not reported	Yes	ND	ND	+	+	+
19	22	9	Pus	Yes	Axillary	Yes	+	ND	+	+	+
20	23	8	Pus	Yes	Axillary	Yes	+	ND	+	+	+
21	24	32	FNA	Yes	Axillary	Yes	-	ND	+	+	+
22	25	9	Pus	Yes	Axillary	Yes	+	ND	+	+	+
23	26	6	Pus	Yes	Inguinal	Yes	+	ND	+	+	+
24	27	17	Pus	Yes	Cervical	Yes	+	+	+	-	-
25	28	11	Pus	Yes	Inguinal	Yes	ND	+	+	+	-
26	29	28	Pus	Yes	Inguinal	Yes	+	ND	+	+	+
27	30	11	FNA	Yes	Axillary + epi	Yes	+	ND	+	+	+
28	31	9	LN <sup>d</sup> biopsy	Yes	Preauricular	Yes	+	ND	+	+	+
29	32	6	LN biopsy	Yes	Preauricular	Yes	ND	ND	+	+	-
1 <sup>e</sup>	33	27	Pus	Yes	Axillary	No	-	ND	-	-	-
2 <sup>e</sup>	34	10	LN biopsy	Yes	Cervical	No	-	ND	-	-	-

<sup>a</sup> When both axillary and epitrochlear (epi) nodes were involved, pus was aspirated from the former.

<sup>b</sup> ND, not done.

<sup>c</sup> Pus from a primary cat scratch lesion.

<sup>d</sup> LN, lymph node.

<sup>e</sup> Representative specimens from non-CSD patients.

with endocarditis. DNA from an avirulent *Borrelia burgdorferi* strain, strain B31, was extracted as described previously (12).

**Patients and clinical samples.** Thirty-two samples from 29 patients with clinically diagnosed CSD were used to evaluate the performance of the three PCR assays. The patients' ages ranged from 5 to 47 years (median age, 10.5 years). All patients had a history of cat contact and a typical clinical presentation of CSD, with regional lymphadenopathy in the absence of another obvious diagnosis (Table 1). Specimens included pus (0.5 to 10 ml) aspirated by syringe from 27 lymph nodes, fine-needle aspirates (FNAs) from two nonsuppurative lymph nodes, two lymph node biopsy specimens, and pus from a draining primary cat scratch lesion which was collected directly in a sterile Eppendorf tube. The locations of the affected lymph nodes are indicated in Table 1. One lymph node biopsy specimen showed numerous stellate necrotizing granulomas, and the other showed stellate necrotic areas without granulomas, both consistent with CSD. Ziehl-Neelsen stain was negative for the two biopsy specimens. Patients 5, 6, and 7 underwent two lymph node aspirations each, and these were done 2 to 4 months apart. Negative controls included five lymph node pus aspirates from patients with a clinical diagnosis of bacterial lymphadenitis, four lymph node biopsy specimens, and 2 lymph node FNA specimens from non-CSD patients. Only two of the pus samples were culture positive (one grew *Salmonella* group B and the other grew *Serratia marcescens*); however, the other three patients were treated with antibiotics for prolonged periods prior to pus aspiration. Three biopsy specimens showed nonspecific inflammatory changes, and one lymph node specimen was from a patient with nontuberculous mycobacterial lymphadenitis. None of the control patients was positive for anti-*B. henselae* IgG antibodies, and none had a history of cat contact (clinical information was available for all but one patient). Two additional pus specimens aspirated from abscesses were obtained from the general microbiology laboratory. One sample was sterile,

and *Escherichia coli* grew from the other sample. Specimens were processed immediately after arrival at the laboratory or were stored at -80°C until they were used.

**DNA extraction.** DNA was extracted from bacterial cells, lymph node tissue, or pus as described previously (3). For lymph node tissue, samples were dispersed with a porcelain tissue homogenizer in PCR diluent, and aliquots of 300 µl were used for DNA extraction. Pus (50 to 500 µl) was diluted sixfold with PCR diluent, and 300-µl aliquots were saved for further processing. For DNA extraction, 30 µl of 10% sodium dodecyl sulfate and 3 µl of 10 mg of proteinase K (Boehringer Mannheim Biochemica, Mannheim, Germany) per ml were added, and the mixture was incubated at 55°C for 3 h. The lysates were transferred to 1.5 ml of Phase-Lock Gel (PLG-Heavy) tubes (5 Prime, 3 Prime Inc., Boulder, Colo.) and were extracted three times with a 25:24:1 mixture of buffer-saturated phenol and chloroform-isoamyl alcohol, as recommended by the manufacturer. The extracted DNA was ethanol precipitated and resuspended in 20 to 100 µl of water, depending on the viscosity of the DNA. The concentration of the nucleic acids was determined with a spectrophotometer (Gene Quant II; Pharmacia Biotech, Cambridge, England).

**Oligonucleotides.** The probe and primers used in this study are presented in Table 2. All of the oligonucleotides were synthesized commercially (Tal Ron Scientific Products Ltd., Rehovot, Israel), and the sequences were based on previously published sequences. Primers p93E and p13B were adapted from Relman et al. (22). These are broad-host-range (universal) primers which amplify a portion of the 16S rRNA gene of all eubacteria. *B. henselae* was identified by using Bhens, a previously described *B. henselae*-specific sequence found in the 16S rRNA gene (2). Primers Bhcs.781p and Bhcs.1137n amplify part of the *gltA* gene encoding *B. henselae* citrate synthase, as previously described by Norman et al. (18). Primers CAT-1 and CAT-2 are based on cloned sequences of the *htrA*

TABLE 2. Sequences and positions of oligonucleotides used for the PCR/rRNA, PCR/CS, and PCR/HSP assays

Oligonucleotide name	Oligonucleotide sequence (5'→3')	Target organism	Target gene (protein)	Nucleotide position	Product size (bp)	Band-size (bp) after <i>TaqI</i> digestion	Reference
p93E	CCGCACAAGCGGTGGAGCA	<i>B. henselae</i>	16S rRNA	931-949 <sup>a</sup>	480	ND <sup>b</sup>	22
p13B	AGGCCCGGGAAAGTATTTCAC	Eubacteria	16S rRNA	1390-1371 <sup>a</sup>			
BhCS, 781p	GGGGACCAAGCTCATGGTGG	<i>B. henselae</i>	<i>gltA</i> (citrate synthase)	782-800 <sup>c</sup>	379	169, 137, 73	18
BhCS, 1137h	AATGCAAAAAGAAACAGTAACA	<i>B. henselae</i>	<i>gltA</i> (citrate synthase)	1160-1138 <sup>c</sup>			
CAT-1	GATTCAATTTGGTTTGAAGGAGGCT	<i>B. henselae</i>	<i>htrA</i> (60-kDa heat shock protein)	1083-1106 <sup>d</sup>	418	232, 186	3
CAT-2	TCAACATACCAAGGACGTATTTC	<i>B. henselae</i>	<i>htrA</i> (60-kDa heat shock protein)	1500-1480 <sup>d</sup>			
Bhens probe	TGCGAGCAATTTGGTTGGGCA	<i>B. henselae</i>	16S rRNA	1050-1069 <sup>e</sup>			2

<sup>a</sup> Positions within *E. coli* 16S rRNA gene sequence (GenBank accession no. Eco 16s).

<sup>b</sup> ND, not done.

<sup>c</sup> Positions within *B. henselae gltA* gene sequence (GenBank accession no. L38987).

<sup>d</sup> Positions within *B. henselae htrA* gene sequence (GenBank accession no. L20127).

<sup>e</sup> Positions within *B. henselae* 16S rRNA gene sequence (GenBank accession no. M73229).

gene of *B. henselae* encoding a 60-kDa heat shock-like protein. They were originally used by Anderson et al. (3) as degenerate oligonucleotides to amplify the corresponding genes of either *B. henselae* or *B. quintana*. However, since *B. quintana* has not been reported to cause CSD, we used primers containing only the *B. henselae*-specific sequences.

**PCR.** Three PCR assays were used in this study. (i) PCR/rRNA amplifies a portion of the 16S rRNA gene. Amplification conditions were as described previously by Relman et al. (22), with modifications. The reaction included denaturation for 1 min at 95°C, followed by 35 cycles of 30 s at 94°C, 30 s at 55°C, and 1 min at 72°C, followed by a final extension step of 6 min at 72°C. A 460-bp product was expected. (ii) PCR/CS amplifies a part of the *gltA* gene (encoding for citrate synthase) of *B. henselae*. Conditions were as described previously (18), with minor modifications: 1 min at 95°C, followed by 35 cycles of 20 s at 95°C, 1 min at 51°C, and 2 min at 72°C. This was followed by an extension step of 6 min at 72°C. The expected size of the resultant product is 379 bp. (iii) PCR/HSP amplifies part of the *htrA* gene (60-kDa heat shock-like protein) of *B. henselae*. The PCR/HSP protocol was similar to that of the method of Anderson et al. (3), except that an extension step of 6 min at 72°C was added after the reaction. The product size was expected to be 418 bp.

All PCRs were carried out in a 50- $\mu$ l reaction volume with a programmable thermal cycler with a heat bonnet (PTC-100; MJ Research, Inc., Watertown, Mass.). A standard PCR mixture consisted of the following: 1  $\mu$ l of the appropriate DNA template, 0.5 U of *Taq* DNA polymerase, 5  $\mu$ l of 10 $\times$  *Taq* buffer (both from Advanced Biotechnologies Ltd., Leatherhead, United Kingdom), 200  $\mu$ M (each) deoxynucleoside triphosphate, and 20 pmol of each primer. PCR-negative samples were further diluted to reduce the effect of inhibitory factors. Both positive and negative controls were included in each PCR experiment. The positive controls consisted of reaction mixtures containing 10 pg of DNA of *B. henselae* BhTA-2. Negative controls included DNA extracted from lymph node biopsy specimens or pus from non-CSD patients as well as DNA from *A. felis* AfTA-1, *S. aureus*, and *B. burgdorferi*. A negative control consisting of reaction mixture without DNA template was also included in each experiment. One-tenth of the PCR mixture was electrophoresed on a 2% agarose gel, stained with ethidium bromide, and photographed. A 1-kb DNA ladder (Gibco BRL Life Technologies, Gaithersburg, Md.) was used as a DNA size marker.

**Dot blot hybridization assay.** The dot blot hybridization assay was performed as described previously by Avidor et al. (4). Probe Bhens was used to identify *B. henselae* DNA in the PCR/rRNA products. The probe was labeled by tailing the 3' end of the oligonucleotide with digoxigenin (DIG)-11-dUTP under the conditions recommended by the manufacturer (Boehringer Mannheim). The bound probe was detected by the use of alkaline phosphatase-conjugated anti-DIG antibody and the chemiluminescent substrate CSPD (Boehringer Mannheim) as recommended by the manufacturer. The reaction was visualized by autoradiography, exposing the membranes to X-ray film (Cronex; E. I. Dupont de Nemours & Co., Wilmington, Del.) for 5 to 15 min.

**RFLP analysis of PCR products.** Restriction fragment length polymorphism (RFLP) analysis was applied to the PCR/CS and PCR/HSP products by using *TaqI* restriction enzyme. Thirty microliters of each of the PCR/CS and the PCR/HSP products was incubated with 10 U of *TaqI* (New England Biolabs, Beverly, Mass.) for 3 h at 65°C. Digestion products were electrophoresed on a 12% polyacrylamide gel. The gels were stained with ethidium bromide and viewed under a UV light. A 1-kb ladder (Gibco BRL) was run as a standard size marker. The bands generated by digestion of the PCR/CS product were expected to be 169, 137, and 73 bp according to a previous report (18). Two bands (232 and 186 bp) were predicted to result from digestion of the PCR/HSP product, based on the published DNA sequence of the *htrA* gene (GenBank accession no. L20127).

**Determination of numbers of CFU of *B. henselae* in suspension.** *B. henselae* colonies (5 to 7 days old) grown to confluence were scraped from one chocolate agar plate and were suspended in 5 ml of phosphate-buffered saline. The bacterial suspension was incubated at room temperature for 30 to 60 min and was vortexed vigorously for 1 min, and the optical density (OD) at a wavelength of 600 nm was determined. The suspension was then serially diluted in phosphate-buffered saline and plated onto chocolate agar plates. Colony counting was performed 5 to 7 days after plating. Despite the tendency of *B. henselae* to autoagglutinate, this method was found to be reproducible for a particular *B. henselae* strain, and dilution of the bacterial suspension was proportional to the corresponding CFU count and resulted in colonies of a uniform size. Gram staining of the highly diluted suspension used for plating for determination of the numbers of CFU showed only minimal autoagglutination. BhTA-3 at 1 OD unit at 600 nm yielded 10<sup>8</sup> to 10<sup>9</sup> colonies/ml.

**EIA.** Testing for the presence of anti-*B. henselae* IgG antibodies was performed by EIA as described previously (27). The OD was determined with an automated ELISA reader (Diagnostic Pasteur, France). Serum was considered positive if the mean OD reading was equal to or greater than 3 standard deviations above the mean OD reading for sera from healthy individuals in Israel, as determined in our laboratory (data not shown).

**Skin test.** Pus was aspirated under sterile conditions from a suppurated lymph node of a patient with clinically diagnosed CSD who was seronegative for human immunodeficiency virus and hepatitis B and hepatitis C viruses. The purulent material was checked for sterility by standard bacterial, fungal, and mycobacterial cultures. The aspirated material was diluted 1:4 in saline and was incubated

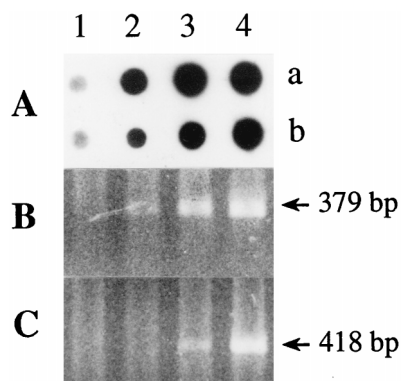


FIG. 1. Threshold of *B. henselae* DNA detection in pus by the three PCR assays. DNA extracted from sterile pus from a non-CSD patient and from *B. henselae* BhTA-2 were mixed, serially diluted, and subjected to gene amplification. Lanes 1 to 4,  $10^{-4}$ ,  $10^{-3}$ ,  $10^{-2}$ , and  $10^{-1}$  ng of *B. henselae* DNA, respectively. (A) PCR/rRNA products dot blotted in duplicate (a and b) and hybridized with a *B. henselae*-specific probe. (B) PCR/CS products on a 2% agarose gel. (C) PCR/HSP products on a 2% agarose gel. The arrows and numbers indicate the sizes of the PCR amplification products.

in a water bath at 60°C for 2 h, then at 4°C overnight, and again at 60°C for 2 h. The inactivated preparation was aliquoted and incubated twice, on 2 successive days, at 56°C for 1 h. This material was stored at 4°C until it was used. A positive skin test for CSD was defined as an induration of  $\geq 5$  mm after 48 to 72 h.

## RESULTS

**Sensitivities of the PCR assays.** The sensitivities of the three PCR assays were compared by testing their thresholds of detection of *B. henselae* DNA in pus. Serial dilutions of purified *B. henselae* DNA were prepared by mixing *B. henselae* DNA with genomic DNA extracted from sterile pus obtained from an abscess from a non-CSD patient. One microliter of each dilution was used as a template in the three PCRs. Products of the PCR/rRNA were dot blotted onto a nylon membrane and hybridized with a *B. henselae*-specific oligonucleotide probe. A total of  $10^{-4}$  ng of bacterial DNA was detected by this method (Fig. 1A). The PCR/CS and PCR/HSP products were analyzed by 2% agarose gel electrophoresis, which detected  $10^{-3}$  and  $10^{-2}$  ng of *B. henselae* DNA, respectively (Fig. 1B and C, respectively).

The finding that PCR/CS was 10-fold more sensitive than PCR/HSP, even though similar techniques were used, was unexpected. To determine whether this difference in sensitivity is due to the use of a particular clinical isolate (BhTA-2) or may result from inhibitory factors in the purulent material, we compared the sensitivities of the two assays using serial dilutions of DNA from a different clinical isolate of *B. henselae* (BhTA-3) and carried out the same experiments in water instead of pus. We also determined the number of CFU present in the *B. henselae* BhTA-3 suspension which was used for the DNA preparation. By taking into account the volumes of bacterial suspensions used for DNA preparation and the CFU counts, we were able to evaluate the sensitivity of the PCR methods on the basis of the number of CFU rather than the total amount of bacterial DNA. Analysis by agarose gel electrophoresis showed that PCR/CS identified 1 *B. henselae* CFU (Fig. 2B) and was 10-fold more sensitive than PCR/HSP, which identified 10 CFU (Fig. 2A).

**PCR detection in clinical samples.** Thirty-two specimens from 29 patients with clinically diagnosed CSD were used to assess the performance of the three PCR assays. In addition, 13 specimens (7 pus, 4 lymph node biopsy, and 2 FNA speci-

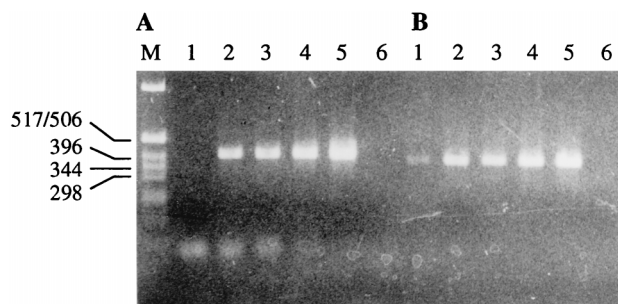


FIG. 2. Threshold of *B. henselae* DNA detection in water by the PCR/CS and the PCR/HSP assays. DNA extracted from *B. henselae* BhTA-3 was serially diluted in water, amplified, and electrophoresed on an agarose gel. Lanes 1 to 5, 1, 10,  $10^2$ ,  $10^3$ , and  $10^4$  CFU, respectively, of the PCR/HSP (A) and PCR/CS (B) products; lane 6, PCR mixture without DNA; lane M, molecular size standards (in base pairs).

mens) from non-CSD patients were used as negative controls. The results are summarized in Table 1. The PCR/rRNA assay detected *B. henselae* DNA in all 32 samples (100%), whereas the PCR/CS and the PCR/HSP assays detected *B. henselae* DNA in 30 of 32 (94%) and 22 of 32 samples (69%), respectively. All specimens positive by the PCR/HSP assay were also positive by the PCR/CS assay. These results indicate that the PCR/CS assay is more sensitive than the PCR/HSP assay and are in accord with experiments which determined the threshold of DNA detection, as described above. None of the PCR assays with the 13 clinical specimens from non-CSD patients was positive, indicating the high specificity of these tests (data for two representative patients are presented in Table 1).

Representative results of the PCR/rRNA assay are presented in Fig. 3. Aliquots of the PCR mixtures were spotted, in duplicate, onto a nylon membrane and hybridized with a DIG-labeled *B. henselae*-specific probe. All of the specimens from patients with CSD were positive for *B. henselae* DNA (samples a1 to a6, b1 to b6, and c1). Controls, which included amplified products from the pus of a non-CSD patient (sample c4) and DNA from *A. felis*, *S. aureus*, and *B. burgdorferi* (samples c3, c5, and c6, respectively), were all negative, indicating the high specificity of the oligonucleotide probe.

Results of the PCR/CS assay are presented in Fig. 4. Figure 4A shows the results of an analysis by agarose gel electrophore-

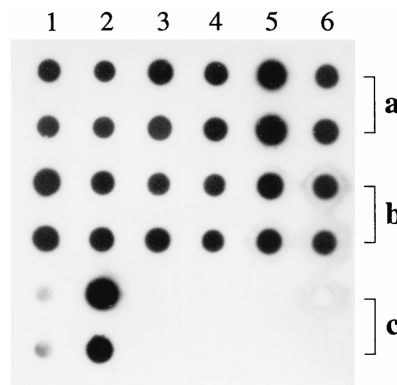


FIG. 3. Dot blot hybridization of PCR/rRNA products. PCR samples were spotted in duplicate on a nylon membrane and hybridized with *B. henselae*-specific probe. a1 to a6, b1 to b6, and c1, samples from CSD patients; c2, *B. henselae* DNA; c3, *A. felis* DNA; c4, pus from a non-CSD patient; c5, *S. aureus* DNA; c6, *B. burgdorferi* DNA.

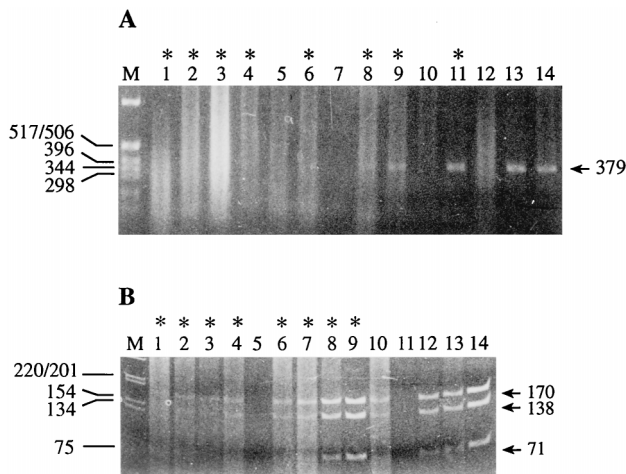


FIG. 4. Analysis of the PCR/CS amplification products. Eight specimens, marked with asterisks, are shown in both panels A and panel B, but not necessarily in the same order. (A) Agarose gel electrophoresis. Lanes 1 to 4, 6, 8, 9, 11, 13, and 14, clinical specimens from CSD patients; lanes 5, 10, and 12, specimens from non-CSD patients; lane 7, PCR mixture without template DNA. A 379-bp product is seen in lanes 8, 9, 11, 13, and 14. (B) RFLP analysis by *TaqI* digestion and polyacrylamide gel electrophoresis. Lanes 1 to 4, 6 to 10, 12, and 13, CSD-positive samples (characteristic bands are seen for all of these samples except that in lane 1); lane 5, PCR mixture without template DNA; lane 11, specimen from a non-CSD patient; lane 14, 10 pg of *B. henselae* DNA. Lanes 2 to 4 and 6 in both panels A and B are CSD samples which were negative by agarose gel analysis but positive by RFLP analysis on polyacrylamide gels. Lane M, molecular size standard (in base pairs). The arrows and numbers indicate the sizes (in base pairs) of the DNA bands.

sis of the PCR products amplified from 10 specimens from CSD patients (lanes 1 to 4, 6, 8, 9, 11, 13, and 14, respectively) and from 3 samples from non-CSD patients (lanes 5, 10, and 12, respectively). Four of the 10 specimens from CSD patients were positive, producing a characteristic 379-bp band (lanes 9, 11, 13, and 14, respectively), and 1 specimen (lane 8) showed a faint, blurry band and the result was interpreted as equivocal. The other five specimens from CSD patients showed a DNA smear along the lane without any distinct bands (lanes 1 to 4 and 6, respectively). Figure 4B shows the PCR/CS products digested with the *TaqI* restriction enzyme and analyzed by polyacrylamide gel electrophoresis. The characteristic bands of the *B. henselae* amplification product were visualized in CSD-positive specimens, including five which were negative or equivocal by agarose gel electrophoresis (Fig. 4B, lanes 2 to 4 and 6 and 8, respectively). One pus sample from a CSD patient (Fig. 4A and B, lanes 1) was negative by the PCR/CS assay but positive by the PCR/rRNA assay.

Figure 5 represents a typical analysis of the PCR/HSP products after *TaqI* digestion and polyacrylamide gel electrophoresis. Two bands of the expected size (232 and 186 bp) were clearly visualized in the CSD-positive samples (lanes 1, 2, 4, 5,

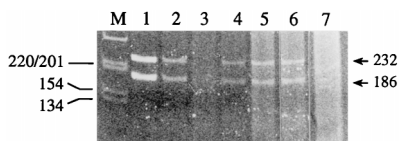


FIG. 5. Analysis of the PCR/HSP products. PCR products were digested with *TaqI* and analyzed by polyacrylamide gel electrophoresis. Lanes 1, 2, and 4 to 6, samples from CSD patients; lanes 3 and 7, pus samples from non-CSD patients; lane M, molecular size standards (in base pairs). Arrows and numbers indicate the sizes (in base pairs) of the digested products.

and 6) and were absent from the non-CSD controls (lanes 3 and 7).

**Comparison of PCR results with serology and skin test results.** Nineteen of the 22 available serum samples (86%) were positive by the EIA for anti-*B. henselae* IgG antibodies; 18 of these (95%) were positive by both PCR/rRNA and PCR/CS assays (Table 1). One patient (patient 24) with anti-*B. henselae* antibodies was positive by PCR/rRNA but negative by the other two PCR assays. This patient was also skin test positive. Skin tests were performed with 11 subjects; 10 were positive and 1 was negative. Eight of the 10 patients with a positive diagnostic skin test result tested positive by both PCR/rRNA and PCR/CS assays, whereas 2 were positive only by the PCR/rRNA assay. Pus aspirated from the lymph node of the skin test-negative patient was positive by all three PCR assays.

## DISCUSSION

The present study was undertaken to compare the sensitivities of three PCR assays for the diagnosis of CSD. We first determined the threshold of *B. henselae* DNA detection in pus. The PCR/rRNA assay, which combines DNA amplification with probe hybridization, was the most sensitive. This finding was not surprising, since hybridization with a *B. henselae*-specific probe has been shown to increase the sensitivity of DNA detection by 10-fold in this assay (6). It was somewhat unexpected, however, to find that the PCR/CS assay was 10-fold more sensitive than the PCR/HSP assay (although similar techniques were used in both assays). A possible explanation for this difference in sensitivity is that factors inhibitory to the PCR/HSP assay are present in the pus or lymph node material. Although a previous report (6) indicated that PCR of the 16S rRNA was not inhibited by pus, Anderson et al. (3) have recently reported that purulent samples from CSD patients can inhibit the PCR/HSP assay and that a 10-fold dilution of the purulent material is necessary to diminish this inhibitory activity. To rule out this possibility, we performed the PCR/CS and the PCR/HSP assay using serial dilutions of purified *B. henselae* DNA both in water and in DNA extracted from sterile pus from a non-CSD patient. The 10 fold difference in sensitivity between the two assays remained unchanged, despite the absence of pus. Tenfold dilutions of the clinical pus specimens in our study also did not alter the results. It can therefore be concluded that the difference in sensitivities between the two assays is not likely due to the presence of inhibitory factors in the clinical specimens.

The low sensitivity of the PCR/HSP assay could also be explained if sequence variations exist in the *htrA* genes (the target of the PCR/HSP assay) from various *B. henselae* isolates. Drancourt et al. (10) recently reported antigenic variability within strains of *B. henselae* which form distinct serogroups. Although there have been no reports of variability in the *htrA* gene of *B. henselae* and the PCR/HSP assay was successful for 12 *B. henselae* isolates tested by Anderson et al. (3), as well as for our 2 isolates (BhTA-2 and BhTA-3), more isolates should be studied before this possibility can be excluded. It is possible that the low sensitivity of the PCR/HSP assay is due to the formation of primer-dimer complexes during the PCR. Such complexes can actually be visualized as faint bands at the bottom of lanes 1 to 3 of Fig. 2A and are absent from the positive reactions of the PCR/CS assay.

We used three PCR assays to study 32 clinical specimens from CSD patients, 28 of which were pus samples aspirated from lymph nodes. The PCR/rRNA assay was the most sensitive, detecting *B. henselae* DNA in 100% of the specimens, while the PCR/CS assay was positive for 94% (30 of 32) of the

samples and the PCR/HSP assay was positive for 69% (22 of 32). The similar sensitivities of the PCR/rRNA and the PCR/CS assays for detecting *B. henselae* DNA in clinical specimens is in contrast to the 10-fold difference in favor of PCR/rRNA which we demonstrated by using purified *B. henselae* DNA in sterile pus. This discrepancy suggests that in the majority of cases, the bacterial load in clinical specimens is large enough to be identified by the less sensitive PCR/CS assay. This bacterial load, however, is probably not large enough to be detected by the least sensitive assay, the PCR/HSP assay. Although we showed that the PCR/CS and PCR/HSP assays were able to identify 1 and 10 *B. henselae* CFU, respectively, we cannot extrapolate this count to the number of bacterial cells in clinical specimens, since *B. henselae* tends to autoagglutinate; thus, a single CFU may contain several or many bacterial cells (20). Also, since PCR has the potential to identify the DNA of dead bacteria as well, the actual number of bacterial cells in the specimens tested might be higher than the number of viable *B. henselae* cells forming the colony.

The 100% sensitivity achieved by the PCR/rRNA assay is very similar to the detection rate recently reported (6). By using *B. henselae*-specific primers (contrary to the universal primers used in our study) to amplify part of the 16S rRNA gene and a *B. henselae*-specific probe, a 96% sensitivity was achieved in pus aspirated from lymph nodes of CSD patients. We obtained a similar sensitivity (94%) by the PCR/CS assay. PCR amplification of the citrate synthase gene of *B. henselae* was first used by Regnery et al. (21). Using primers corresponding to the citrate synthase gene of *Rickettsia prowazekii* and RFLP analysis, those investigators were able to distinguish between *R. prowazekii*, *B. henselae*, and two other *Bartonella* species. We initially applied the *R. prowazekii* citrate synthase primers to our clinical isolates, but the presence of multiple, nonspecific bands interfered with the interpretation of the results (data not shown). In 1995, Norman et al. (18) reported the cloning and sequencing of the *B. henselae* citrate synthase gene (*gltA*). DNA from 28 *Bartonella*-like isolates of feline origin was amplified, but the assay was not tested with clinical specimens (18). We used the corresponding *B. henselae* *gltA* primers, which resulted in specific and sensitive DNA amplification without background. The PCR/HSP assay is the least sensitive. It was positive for only 69% of the specimens tested, a somewhat lower rate than the 84% reported by Anderson et al. (3), who performed this assay with 25 clinical CSD specimens from patients with CSD.

The PCR/rRNA assay has the advantage of using universal, broad-host-range PCR primers which recognize all eubacteria. This broad approach can be used to detect other pathogens that cause infectious lymphadenopathy and that are difficult to culture, such as *A. felis*, mycobacteria, including *Mycobacterium tuberculosis* as well as nontuberculous mycobacteria, and *Chlamydia trachomatis*. However, contamination of PCR reagents, particularly the *Taq* polymerase, by DNA sequences of bacterial 16S rRNA genes has been shown to result in false-positive amplification (15, 19, 23). In our experiments we consistently amplified a DNA fragment of the appropriate size even when no exogenous DNA was added (data not shown). Although it did not interfere with the specific hybridization of the *B. henselae* probe in this study, we have not tested a sufficient number of true-negative clinical specimens to determine the specificity of this test for the diagnosis of CSD.

Our study also indicates that PCR is more sensitive than the anti-*B. henselae* IgG EIA. Of the 22 available serum samples, 19 were positive by EIA; however, 4 of the 19 samples were initially negative (at the time that PCR was performed) and the patients seroconverted later. Three patients, two with paired

serum samples, tested negative by EIA. One of these patients underwent a skin test which was positive, and another patient had a sister who had clinical CSD at the same time and tested positive by both EIA and PCR (patients 15 and 28 in Table 1). Each specimen from the three patients was positive by the three PCR assays. These data indicate that PCR can detect *B. henselae* before the appearance of IgG antibodies and that some patients with CSD do not develop anti-*B. henselae* IgG detectable by the EIA method.

The DNA extraction procedure described here is rather lengthy, but it results in purified DNA free of purulent material. The use of the Phase-Lock Gel tubes for phenol extraction helps to obviate cross contamination between samples. However, the resulting sheared genomic DNA can mask the analysis of the amplified bands by agarose gel electrophoresis, and therefore, polyacrylamide gel electrophoresis is an essential requirement for optimal analysis of the PCR/CS and the PCR/HSP products.

In conclusion, the PCR/rRNA assay in this study was found to be highly sensitive. In addition, this assay can potentially identify other bacterial pathogens causing lymphadenopathy, provided that appropriate probes are available. However, false positivity is a potential problem with this test, and the use of DNA amplification in conjunction with probe hybridization is costly as well as time and labor demanding. The PCR/CS assay, on the other hand, is comparably sensitive, is less laborious, and is less expensive. We propose a two-step approach to achieve maximal sensitivity for detecting *B. henselae* DNA in clinical specimens from patients with CSD: initial testing by the PCR/CS assay followed by the PCR/rRNA assay for PCR/CS-negative specimens, but only for patients in whom CSD is strongly suspected.

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