Cat scratch disease (CSD) has been a recognized entity for approximately a century, since the initial identification of the uncommon manifestation of Parinaud oc- 
uloglandular syndrome. Since that time, the etiologic agent has been under continuous investigation, with various suspected etiologic agents coming briefly to the fore, including fungi, viruses, and atypical mycobacteria. In the 1980s, bacteria were identified in patient samples by Warthin-Starry staining. Bacteria from a patient with CSD were initially cultivated and identified as A felis. However, recent studies using molecular techniques established B henselae as the etiologic agent.

Despite this progress, diagnosis in suspected cases has been problematic because the culture of B henselae is known to be difficult and unreliable. Direct detection of bacteria in tissue specimens by Warthin-Starry stain has traditionally been used; however, this stain is unreliable and lacks species specificity. Therefore, until recently, standard diagnosis of CSD has been based on the combination of multiple clinical features and laboratory information, including a history of animal exposure (often fe-line), lymphadenopathy, skin testing, and antibody testing. Recent efforts to diagnose CSD have relied on polymerase chain reaction (PCR), particularly amplification of the 16S ribosomal RNA (rRNA) and other molecular techniques to demonstrate B henselae infection in these patients, and this approach has significantly increased our ability to diagnose CSD.

Polymerase chain reaction amplification of 16S rRNA is a useful technique for demonstration of B henselae; however, this method has not gained wide clinical application. Recently, PCR using primers against the citrate synthase gene, gltA, was developed for demonstration of B henselae; however, we had limited success using tissue fixed in formalin with this assay. To improve the clinical utility of PCR amplification of B henselae and diagnosis of CSD, we successfully developed a seminested PCR assay using tissue samples from patients with clinical evidence of CSD.

METHODS

Clinical Samples

Clinical samples from 7 patients with clinically suspected CSD were evaluated retrospectively, and fresh tissue samples were an-
alyzed prospectively from 1 patient (case 1). The latter was an 18-year-old man with recent multiple kitten scratches who presented with right inguinal lymphadenopathy. Combined with the clinical history, histologic findings supported the diagnosis of CSD. Both fresh and paraffin-embedded lymph node tissues were available for molecular assays. DNA from this sample and cultured organisms were used for the initial analysis and development of the PCR assay. In addition, 7 archival formalin-fixed, paraffin-embedded samples from 7 patients with the clinical diagnosis of CSD were used for PCR assays. Negative controls were water, normal placenta, and 8 lymph nodes from 6 patients with reactive lymphoid hyperplasia and 2 patients with nonnecrotizing granulomatous lymphadenitis (unknown etiology not related to CSD).

**Bacterial Strains and Cultures**

An isolate of *B. henselae* obtained from the American Tissue Culture Collection (ATCC, Manassas, Va (ATCC 49793) was cultured on blood agar plates at 37°C. An isolate of *A. felis* (ATCC #49714) was grown on buffered charcoal yeast extract agar (bacteroid media) at 30°C.

**DNA Extraction**

DNA was isolated with the Puregene DNA Isolation Kit (Gentra Systems, Minneapolis, Minn). Briefly, the frozen tissue (approximately 30 mg) was homogenized with cold STE (1mM EDTA, pH 8.0; 10mM Tris-hydrochloride, pH 8.0; and 0.1M sodium chloride). The homogenate was centrifuged at 800g for 10 minutes. The pellet was then resuspended in 2 mL of Cell Lysis Solution and 75 mL of 20 μg/mL protease K (International Biotechnologies, Inc, New Haven, Conn). The samples were incubated overnight in a 50°C circulating water bath and treated with RNase (Gentra Systems). DNA was precipitated from the supernatant with isopropanol, washed with 70% ethanol, and resuspended in solubilization solution.

DNA from paraffin-embedded tissue sections was isolated by cutting 5 x 5-μm sections, which were deparaffinized by xylene. These samples were washed with ethanol, followed by air-drying. The tissue sections were digested with 1 μg/mL proteinase K overnight at 37°C in a buffer with 10 mM/L Tris-hydrochloride (pH 8.3), 50 mM/L potassium chloride. The samples were then treated with phenol/chloroform for further purification. DNA quantitation was performed by fluorometry. Tissue sections on 2 samples of CSD were available only on glass slides.22 Tissues from the glass slides were scraped with a clean blade into a small tube and processed for DNA isolation using the same protocol for paraffin-embedded tissue sections.

**PCR Amplification**

*B. henselae* citrate synthase *gltA* gene sequence was accessed by NCI Blast program (accession L39897). Three primers for citrate synthase gene were used, namely, an outer pair of primers consisting of TN-1 and TN-2, and an inner primer (IP) for seminested PCR amplification. The primers (IPs) were designed by a software program available on the Internet (http://www.genome.wi.mit.edu/cgi-bin/primer/primer3www.cgi). We elected to set up primer design conditions as follows: primer size, 18 to 22 base pairs (bp); melting temperature, 55°C to 60°C; GC content, 30% to 60%; and no hairpin formation and minimal self-complementary activity. Primers were synthesized at Oligos Etc Inc (Wilsonville, Ore). The TN-1/TN-2 primer pair amplifies a 181-bp segment of DNA, and the TN-1/IP primer pair for seminested PCR amplified a 139-bp segment of DNA (Table 1).

Polymerase chain reaction amplification was performed with Master Mix (Roche Diagnostics, Indianapolis, Ind) and a standard mix as follows: 25 μL of Master Mix containing 2.5 U of Taq DNA polymerase in 20mM Tris-HCl, 100mM potassium chloride, 3mM magnesium chloride, 0.01% Brij 35, dNTP mix (deoxyadenosine triphosphate, deoxyctydine triphosphate, deoxyguanosine triphosphate, deoxythymidine triphosphate) each 4mM, pH 8.3 (200 pM) each primer; 250 ng DNA, and sterile water to a final volume of 50 μL. Polymerase chain reaction conditions were as follows: initial denaturation for 4 minutes at 95°C, followed by 35 cycles of amplification; denaturation at 94°C for 20 seconds; annealing at 66°C for 30 seconds; extension at 72°C for 2 minutes; and final extension at 72°C for 5 minutes. The second-round PCR amplification by the TN-1/IP primers was identical to the first round, with the exception of annealing temperature, which was set at 59°C. Ten microliters of PCR product was digested with 1 unit of *TaqI* (Promega, Madison, Wis) restriction endonuclease at 37°C for 3 hours in a buffer provided by the manufacturer. Polymerase chain reaction products were evaluated following electrophoresis in polyacrylamide gel (0.5%) at 70 V for 1 to 2 hours, or in metaphor agarose (1%) at 112 V for 1 to 2 hours, and stained with ethidium bromide. Gels were evaluated with UV light and photographed.

**RESULTS**

The 18-year-old man with recent history of multiple kitten scratches presented with lymphadenopathy. He was initially evaluated by fine-needle aspiration biopsy, and the cytologic examination showed a polymorphous lymphoid aggregate. Owing to marked cervical lymphadenopathy, subsequent excisional biopsy of the lymph node was performed, which showed necrotizing granulomatous lymphadenitis with epithelioid histiocytes surrounding central abscesses (Figure 1). Warthin-Starrry stain was attempted; however, this staining failed to show unequivocal evidence of organisms because of high background silver staining. Combined with the clinical history, the histologic findings supported the diagnosis of CSD. Both fresh and paraffin-embedded lymph node tissues were available for molecular assays. DNA from this sample and cultured organisms were used for development of PCR assays. In addition, 7 archival formalin-fixed, paraffin-embedded samples from 7 patients with clinical diagnoses of CSD were used for PCR assays. Clinical features and histologic findings of patients analyzed in this study are shown in Table 2. Negative controls were 8 lymph nodes from 6 patients with reactive lymphoid hyperplasia and 2 patients with granulomatous lymphadenitis, normal placentas, and water.

Initial analysis of the PCR assay was performed with DNA isolated from cultures of *B. henselae* and *A. felis*. Our early attempts to amplify a portion of the 16S rRNA gene or using primers against the citrate synthase gene originally developed by Norman et al26 had inconsistent amplification or nonspecific bands (data not shown).24

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**Table 1. Primers Used for the Polymerase Chain Reaction Assay (the Location and Sequences of Each Primer on Citrate Synthase Gene Are Indicated)**

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Location</th>
<th>Position of Primer</th>
<th>Primer Sequence (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TN-2</td>
<td>Upstream</td>
<td>796–814</td>
<td>TGGTGGAGCTAATGAGATG</td>
</tr>
<tr>
<td>TN-1</td>
<td>Downstream</td>
<td>957–976</td>
<td>GCACAACACCTGCTGATG</td>
</tr>
<tr>
<td>IP</td>
<td>Middle</td>
<td>838–860</td>
<td>GTCCTGTTGAGAAATCTG</td>
</tr>
</tbody>
</table>

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**PCR Confirmation of Cat Scratch Disease—Margolis et al**

Arch Pathol Lab Med—Vol 127, June 2003
Figure 1. Histologic findings for patient with clinicopathologic diagnosis of cat scratch disease. A, Low-power magnification illustrating necrotizing granulomas (hematoxylin-eosin, original magnification ×40). B, Higher magnification showing typical necrotic area with neutrophils surrounded by palisading histiocytes (hematoxylin-eosin, original magnification ×400).

these assays did not produce positive results consistently, we developed a seminested PCR assay. Seminested PCR analysis using DNA isolated from cultures of *B. henselae* and *A. felis* showed specific amplification of *B. henselae* (Figure 2). The primers against the gltA gene did not show amplification of DNA isolated from *A. felis*, while seminested PCR amplification showed clear bands with the DNA isolated from the cultured *B. henselae* organism. The PCR assay described in this study consistently showed positive results in all 8 samples with clinicopathologic diagnosis of CSD (Figure 2). None of the patients with lymphadenopathy unrelated to CSD, including 6 patients with reactive hyperplasia and 2 with granulomatous lymphadenitis, and no DNA isolated from normal placenta or water control samples showed any evidence of PCR amplification. Restriction fragment length polymorphism

<p>| Table 2. Clinical Features of Patients Analyzed by Polymerase Chain Reaction Amplification |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Sex/Age, y</th>
<th>Location of Lymph Node</th>
<th>Cat Exposure</th>
<th>Histology</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M/18</td>
<td>Axillary, cervical</td>
<td>Yes</td>
<td>Necrotizing lymphadenitis</td>
</tr>
<tr>
<td>2</td>
<td>M/23</td>
<td>Epitrochlear, inguinal</td>
<td>Yes</td>
<td>Necrotizing lymphadenitis</td>
</tr>
<tr>
<td>3</td>
<td>F/27</td>
<td>Axillary</td>
<td>Yes</td>
<td>Necrotizing lymphadenitis</td>
</tr>
<tr>
<td>4</td>
<td>M/40</td>
<td>Supraclavicular</td>
<td>Yes</td>
<td>Necrotizing lymphadenitis</td>
</tr>
<tr>
<td>5</td>
<td>M/52</td>
<td>Axillary</td>
<td>Yes</td>
<td>Necrotizing lymphadenitis</td>
</tr>
<tr>
<td>6</td>
<td>F/8</td>
<td>Cervical</td>
<td>Yes</td>
<td>Necrotizing lymphadenitis</td>
</tr>
<tr>
<td>7</td>
<td>F/22</td>
<td>Axillary</td>
<td>Yes</td>
<td>Necrotizing lymphadenitis</td>
</tr>
<tr>
<td>8</td>
<td>M/14</td>
<td>Axillary, supraclavicular</td>
<td>Yes</td>
<td>Necrotizing lymphadenitis</td>
</tr>
</tbody>
</table>

Figure 2. Results of seminested polymerase chain reaction (PCR) using primers specific to Bartonella henselae citrate synthase gene. Lane 1, Molecular size marker. Lane 2, Bartonella henselae DNA control (large prominent band represents expected 194-bp PCR product). Lane 3, Patient with cat scratch disease (CSD) (case 1) lymph node tissue. Lane 4, Alipia felis DNA control. Lane 5, No DNA control. Lanes 6 through 8, Lymph nodes with reactive hyperplasia. Lanes 9 through 12, Four patients with CSD. Lanes 13 through 16, TaqI-digested PCR products of samples shown in lanes 9 through 12, respectively.
(RFLP) analysis with TaqI was used to assure the specific amplification of *B. henselae*. All cases analyzed by this restriction enzyme yielded bands that were consistent with the RFLP profile of *B. henselae* (Figure 2). Following restriction cutting, there are 2 products expected to be generated since the *TaqI* recognition site (TCGA) lies on the gene sequence at base pairs 918–921. Restriction digestion was performed using 1 unit of *TaqI*, which generates 2 fragments with sizes of 82 and 57 bp, respectively.

**COMMENT**

Cat scratch disease is usually a self-limiting disease and does not require therapy.26 The most common manifestation of CSD is lymphadenitis subsequent to scratches from cats or kittens; however, patients may rarely present with Parinaud ocular-lachrymal disease, encephalopathy, osteomyelitis, endocarditis, or hepatosplenic infection.11 Some patients with multisystem involvement may benefit from antibiotic administration; therefore, it is critical that rapid identification of the organism be made by clinical laboratory assays.26,29 Culturing organisms from affected tissue is difficult, and it usually requires 2 to 3 weeks.30,31 Fresh antibiotic administration; therefore, it is critical that rapid identification of the organism be made by clinical laboratory assays.26,29 Culturing organisms from affected tissue is difficult, and it usually requires 2 to 3 weeks.30,31 Fresh material is not usually available because the differential diagnosis of CSD is often brought to the primary physician's attention after the review of histologic material. The skin testing used in the past for diagnosis also has limited clinical utility. Novel assays for detection of CSD in clinical samples is becoming more crucial, especially in light of the difficulties inherent in culturing the organisms that cause CSD.

To improve diagnosis of CSD, recent studies relied on PCR amplification. There have been 2 main molecular approaches to the diagnosis of CSD by PCR. Some of the earlier assays targeted amplification of 16S rRNA gene16–18 or the adjacent intergenic region between the 16S rRNA and 23S rRNA.23 These regions are present in all bacteria with species polymorphism, and conserved segments present within these regions allow specific identification of various organisms. We initially wanted to develop a robust clinical PCR-based detection and used PCR assays based on 16S rRNA amplification. However, as in our past experience and review of previously published data, this amplification was usually associated with presence of nonspecific bands.18

Molecular assays using the 16S rRNA gene generally require further analysis for the specific species of bacteria targeted for amplification. These analyses usually use hybridization with a species-specific probe and subsequent detection by radioactivity, enzyme activity, cloning, or subsequent sequencing.18,19,21–23,32 In the case of the 16S–23S rRNA intergenic region, the product can be identified by size alone. However, this technique has not been pursued in human tissue.23 Recently, the use of a gene specific to *B. henselae*, the citrate synthase gene (gltA),24,26,33 appeared to provide a better opportunity for quick confirmation of *B. henselae*. This gene was originally cloned by Norman et al26 and has been amplified by PCR in cultured clinical isolates from bacteremic cats. Added confirmation of the identity of the PCR product was performed by RFLP analysis using *TaqI* restriction endonuclease.

We initially used primers against the citrate synthase gene originally described by Norman et al26 However, PCR amplification using the original primers against the citrate synthase gene gave inconsistent results on samples isolated from formalin-fixed, paraffin-embedded tissues, similar to our previous experience with the amplification of 16S rRNA gene.18,26 Therefore, we developed a sensitive seminested PCR assay for the diagnosis of CSD. To improve the efficient amplification of DNA that are usually fragmented during fixation in formaldehyde, we targeted amplification fragment size less than 200 bp and designed yet another primer allowing seminested PCR amplification. We used the same target gene, *gltA*, used by Avidor et al;23 however, we used a nested-PCR assay with a shorter amplicon size. With this technique we demonstrated successful amplification of *B. henselae* from fresh tissues, paraffin-embedded archival material, and unstained histologic slide samples. Use of the seminested assay optimized PCR amplification and provided reliable identification of a single, unique band in each CSD patient and in *B. henselae* DNA derived from bacterial culture. Since the amplification product still contains the unique restriction site present in the citrate-synthase gene recognized by the *TaqI* restriction enzyme, it could be used for confirmation of the amplification product by RFLP, eliminating the need for probe hybridization.

We emphasize that precautions are necessary because carryover contamination could be a significant source of false-positive results. Therefore, routine procedures for detection of carryover contamination are essential and should be complemented by prevention with special contamination-control procedures (eg, designated room for PCR amplification and special attention for prevention of microaerosol contamination when small aliquots are taken for the second PCR amplification).

The technique described here lends itself to a rapid timeframe and avoids more cumbersome clinical assays, such as bacterial culture and serology. Seminested PCR as described in this study is reliable for the identification of *B. henselae* and provides laboratory support for diagnosis of CSD in a relatively short amount of time. Thus, both in specificity and rapidity, this technique provides a clear clinical advantage for patients needing a diagnostic evaluation for CSD.

We conclude that the PCR is a reliable assay for identification of *B. henselae*, thus providing useful clinical information in a relatively short amount of time. Given the difficulties of culture and uncertainty in clinical diagnosis, this technique should be useful in arriving at a precise diagnosis of CSD.

**References**


