Immunohistochemical Study of Lymph Nodes in Patients with Cat Scratch Disease

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Background/Purpose: Bartonella henselae is the causative agent of cat scratch disease (CSD), manifesting as fever and acute regional lymphadenopathy. Although serologic testing is the reference method for diagnosis, successful use of immunohistochemical (IHC) stain of regional lymph nodes for the diagnosis of CSD has been reported. To determine the characterization and diagnostic potential of IHC in lymphadenopathy of CSD, lymph nodes were excised from patients with suspected CSD for further evaluation.

Methods: Polyclonal antibody-based IHC studies were performed for the detection of B. henselae. Between January 2001 and December 2004, the reference laboratory of the Center for Disease Control, Taiwan, received a total of 377 sera from 352 reported suspected CSD cases. Twenty-three formalin-fixed paraffin-embedded lymph nodes from 16 patients and two skin biopsies from two patients suspected of having CSD were included in this study. Nine of them were serologically confirmed to have CSD and the others were seronegative but suspected to have CSD by the attending physicians. Seven lymph node specimens were obtained from tuberculosis patients for comparison.

Results: We demonstrated that the microorganisms existed in the cytoplasm of histiocytes within the granulomatous lesions in nine lymph nodes and one skin biopsy. Among the nine lymph nodes with IHC(+) stains, three were seronegative. On the other hand, three cases were IHC(+) and six cases were IHC(−) among nine seronegative patients. In addition, two seronegative patients with skin biopsy showed one IHC(+) and one IHC(−).

Conclusion: IHC can contribute to the etiologic diagnosis of B. henselae lymphadenopathy when serology and molecular techniques are not available. [J Formos Med Assoc 2006;105(11):911–917]

Key Words: Bartonella henselae, cat scratch disease, immunohistochemistry, lymph nodes

Bartonella henselae, a fastidious Gram-negative bacterium, has been known as the principal causative agent of cat scratch disease (CSD) only since 1992.1 Thereafter, a broad spectrum of clinical manifestations, including Parinaud ocu-loglandular syndrome, bacillary angiomatosis, peliosis hepatica, bacteremia, endocarditis, and aseptic meningitis, were recognized as B. henselae infections.2 Immunocompetent hosts usually present with typical CSD, which is characterized by a primary granulomatous skin lesion that develops 3–10 days after contact with an infected cat. Development of this lesion is followed by enlargement of the regional lymph nodes,3 and prolonged fever in children.4 Lymphadenopathy is the most common clinical manifestation of CSD.

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and is described in more than 80% of all cases.\textsuperscript{5} The diagnosis of CSD is reached using pathogen isolation, polymerase chain reaction (PCR) amplification of \textit{B. henselae}, and serologic testing, such as indirect immunofluorescent antibody (IFA) assay and enzyme-linked immunosorbent assay (ELISA). Among these, the development of molecular biology tools, especially the use of PCR, improved the identification of these fastidious bacteria. In humans, serologic testing (mainly IFA) is the reference test for the diagnosis of CSD. An IgG anti-\textit{B. henselae} antibody titer $\geq 1:64$ is considered to be positive for infection when patients are tested at least 2–3 weeks after inoculation. Successful use of immunohistochemical (IHC) stain in cases of bacillary angiomatosis,\textsuperscript{6} \textit{Bartonella} endocarditis,\textsuperscript{7} \textit{Bartonella} uveitis\textsuperscript{8} and splenic rupture caused by \textit{B. henselae}\textsuperscript{9} has been reported. To determine the IHC characteristics of lymphadenopathy and increase the diagnostic potential of IHC in lymph node and skin of CSD patients, we used excised lymph nodes of patients suspected to have CSD for IHC study.

\section*{Methods}

\subsection*{Samples}

Between January 2001 and December 2004, the reference laboratory of the Center for Disease Control, Taiwan, received a total of 377 sera from 352 reported suspected CSD cases. All sera were tested by IFA assay. Twenty-three formalin-fixed paraffin-embedded lymph nodes from 16 patients and two skin biopsies from two patients suspected of having CSD, and seven lymph nodes from patients confirmed to have tuberculosis were included in this study. Half of the 18 cases were serologically confirmed CSD cases and the others were suspected by the attending doctors to be CSD.

\subsection*{Histopathologic investigation}

The lymph node and skin specimens were fixed in 10% buffered formalin, embedded in paraffin, cut at 2–3 $\mu$m, and routinely stained with hematoxylin and eosin. The negative controls consisted of seven paraffin-embedded lymph nodes from patients with positive acid-fast stain or \textit{Mycobacterium tuberculosis} culture. Warthin-Starry staining was also performed in our study.

\subsection*{DNA extraction}

DNA was extracted from the formalin-fixed, paraffin-embedded lymph node and skin biopsies by using a Blood & Tissue Genomic DNA Extraction Miniprep System (Viogene\textsuperscript{®}, Taipei, Taiwan) according to the manufacturer’s instructions. The extracted DNA was used as a template in the PCR assays with the amplified $\beta$-actin gene as internal control.\textsuperscript{10} Purified DNA from a cultured strain of \textit{B. henselae} (Houston-1; ATCC 49882) was used as a positive control.

\subsection*{Amplification of \textit{Bartonella} DNA}

Two-step semi-nested PCR with three primers PAPn1 (5’-TTCTAGAGTTGAAACCGAT-3’), PAPn2 (5’-GAAACACCACGCAACATA-3’) and PAPns2 (5’-GCACCAAGACATTTCCTT-3’) was used to amplify a 275 bp and 209 bp fragment of the \textit{Bartonella} \textit{pap31} gene.\textsuperscript{11} In the first step (PCR1), 10 $\mu$L of extracted DNA was amplified using primers PAPn1 and PAPn2; in the second step (PCR2), 10 $\mu$L of 1:500 diluted PCR1 amplification product was further amplified using primers PAPn1 and PAPns2. The 25 $\mu$L reaction mixture consisted of primer (40 ng/$\mu$L each), bovine serum albumin (0.5 $\mu$g/$\mu$L), MgCl$_2$ (2 mM), dNTP mixture (0.2 mM), 10X buffer, Taq DNA polymerase enzyme (1.25 U), sterile water, and 10 $\mu$L of a DNA sample. PCR amplification was performed under the following conditions: an initial 3 minutes of denaturing at 94°C was followed by 44 cycles of denaturing for 30 seconds at 94°C, annealing for 30 seconds at 56°C, and extension for 45 seconds at 72°C. The amplification was completed by holding the reaction mixture at 72°C for 10 minutes to allow complete extension of the PCR products. PCR products were separated by electrophoresis on 2% agarose gels and visualized by staining with ethidium bromide.
**B. henselae indirect immunofluorescence antibody test**

The antibody titers to *B. henselae* were determined by indirect IFA assay, using *B. henselae* (ATCC 49882) as an antigen. The strain was inoculated to Vero cell (CCRC 60013, Bioresources Collection and Research Center, Taiwan) and incubated at 37°C with 5% CO₂. After incubation, the infected cells were distributed into each well of 12-hole Teflon printed slides (Electron Microscopy Sciences, Fort Washington, PA, USA), and the slides were incubated at 37°C with 5% CO₂ overnight. After incubation, the slides were fixed in acetone and air-dried. The slides were stored at −70°C. For the IFA assay, frozen sera were thawed at room temperature and treated at 56°C for 30 minutes for heat inactivation. The serum was serially diluted from 1:32 to 1:512 by twofold dilutions using phosphate buffered saline (PBS) with 10% skim milk. Thirty microliters of diluted serum was dropped into each well of the previously prepared slides. Slides were incubated at 37°C for 40 minutes and washed with PBS for 10 minutes. Fluorescein-labeled goat anti-human IgG (Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD, USA) was diluted at 1:400 in PBS, and the mixture was applied to each well. The slides were incubated at 37°C for 40 minutes, washed with PBS for 10 minutes, and washed again with double distilled water for 10 minutes prior to examining with a fluorescent microscope (magnification, ×400). The intensity of the bacillus-specific fluorescence was scored subjectively from 1 to 4, and a fluorescence score ≥ 2 at a dilution of 1:64 was considered to be positive.

**Polyclonal antibody preparation**

For the preparation of polyclonal antibodies, bacterial strains, *B. henselae* Houston-1 (ATCC 49882) and *B. henselae* Marseille (strain U4, from Prof. Chomel, UC Davis), were cultivated on chocolate-agar plates at 37°C in 5% CO₂. Bacteria were suspended in adjuvant and injected into New Zealand white rabbits. Serum antibody levels were determined 3 weeks later by IFA assay. The antibody titers were 1:4000 for the Houston-1 strain and 1:3000 for the U4 strain. There was no cross reaction to other bacteria, such as *Streptobacillus moniliformis*.

**Immunohistologic analysis**

Immunohistochemical testing was performed on 5-µm-thick, formalin-fixed, paraffin-embedded tissue sections. After deparaffinization, rehydration, proteinase K digestion and blocking, each tissue section was incubated for 60 minutes at room temperature with polyclonal rabbit anti-*B. henselae* Houston-1 and anti-*B. henselae* Marseille antibodies prepared as described in the preceding section and diluted 1:100 in tris-buffered saline. After incubation with the primary antibody, immunodetection was performed with biotinylated goat anti-rabbit and anti-mouse immunoglobulins (DAKO, Kyoto, Japan), followed by alkaline phosphatase conjugated streptavidin (DAKO). The slides were rinsed, incubated in naphthol phosphate–fast red substrate (Sigma-Aldrich Corp., St. Louis, MO, USA), counterstained with Mayer’s hematoxylin stain, and mounted with the use of aqueous mounting medium.

**Results**

**Demography of suspected CSD cases**

Out of the 352 patients with suspected CSD, 99 were seropositive; of these 99, 51 were male and 48 were female, 37 were children and 62 were adults. There were no statistical differences in gender and age by χ² test. The number of reported CSD cases showed an increasing trend from 2001 to 2004 (37 cases in 2001, 69 in 2002, 126 in 2003, 120 in 2004); 50.9% (179/352) of the cases occurred in summer (Figure 1). The clinical manifestations of the 18 cases for IHC study are shown in the Table. Ten patients had animal contact and half of them (9/18) had fever. Sixteen out of 18 suspected CSD patients had at least one enlarged lymph node, located at 19 sites including the neck (2/19), elbow (3/19), axilla (8/19), groin (4/19) and thigh (2/19).
Histopathology and immunohistochemistry
Histopathologic examination of the 23 lymph nodes showed lesions of necrotizing granulomatous inflammation, microabscess, lymphoid hyperplasia and giant cells (data not shown). IHC staining was used to demonstrate *B. henselae* in human lymph node and skin tissues. Of the 23 lymph node specimens available for immunohistologic study, *B. henselae* was identified in nine specimens (Table). The lymph nodes of seven tuberculosis confirmed cases were all negative by IHC. The positive results showed that the bacteria were identified in the cytoplasm of histiocytes within granulomatous lesions (Figures 2A and 2B), and superimposed on that in the Warthin-Starry stain (Figure 2C). The positive control, antigen slides for IFA, showed the same results with intracytoplasmic distribution of the bacterial colonies in the Vero cells (Figure 2D).

Detection of Bartonella DNA
DNA extraction was performed from only 15 specimens, and 14 of them were positive for *Bartonella* *pap31* gene by PCR (Table). An ethidium bromide-stained 2% agarose gel demonstrating the amplified products of the template

Table. Clinical manifestations, serologic, IHC and PCR results of 18 suspected cat scratch disease cases

<table>
<thead>
<tr>
<th>Case</th>
<th>IFA</th>
<th>IHC</th>
<th>PCR</th>
<th>Cat contact/scratch</th>
<th>Fever</th>
<th>LAP</th>
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<tr>
<td>1</td>
<td>1:512</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Axillary, elbow</td>
</tr>
<tr>
<td>2</td>
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<td>–</td>
<td>–</td>
<td>–</td>
</tr>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>Thigh</td>
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<tr>
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<td>+</td>
<td>+</td>
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</tr>
<tr>
<td>5</td>
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<td>+</td>
<td>+</td>
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<td>6</td>
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</table>

*Skin biopsies. IFA = indirect immunofluorescent antibody assay; IHC = immunohistochemistry stain; PCR = polymerase chain reaction; LAP = lymphadenopathy; ND = not done; + = positive; – = negative.*
DNA derived from lymph node specimens is shown in Figure 3.

**Discussion**

Physicians in Taiwan are increasingly able to recognize the symptoms of CSD, and there are escalating reports of CSD cases year after year. Lymphadenopathy and fever of unknown origin are the two main symptoms, which contribute to the diagnosis of CSD. The location of lymphadenopathy in CSD patients depends on the scratch or bite sites. In a study of 246 CSD patients, the most commonly infected sites were the neck (43% of patients), followed by the axilla (38%) and groin (20%). Thirty-seven percent of patients have enlarged lymph nodes at more than one site. In our study, the axilla was the most commonly infected site (Table). About 51% of the cases occurred in the summer from June to September, suggesting that warm and humid environments favor the growth of potential arthropod vectors, such as fleas. Previous studies from America and Japan have reported high seroprevalence to *B. henselae* infection in domestic cats.

The pathologic findings of lymph node biopsy specimens from immunocompetent patients with CSD varied with the stages of infection. Early in the course of the disease, specimens showed lymphoid hyperplasia, arteriolar proliferation, and
reticulum cell hyperplasia. Subsequently, granulomas appeared, often with central necrosis; at this stage, multinucleated giant cells may become evident. Late in the disease, the specimen characteristically shows multiple stellate microabscesses. In the Warthin-starry stain, rod-shaped bacteria were clustered in cytoplasmic locations such as the vessel walls of lymph node tissue, or in dermal macrophages. The pathogen could also be found at extracellular locations such as in the necrotic debris of lymph nodes or the skin. The silver stain results demonstrated the intracellular location of the bacteria. On IHC study, the extracellular locations of B. henselae were in the cardiac valve, spleen and vitreous fluid. Intracellular B. henselae was also reported in the dermal cells of bacillary angiomatosis in AIDS patients and also demonstrated in histiocytes of lymphoid tissue and Vero cells of IFA coated slide in our study. In contrast to the IHC results, B. henselae were granular in lymph node tissue and rod-shaped on IFA coated slides. The difference may be attributed to the damaging effects of formalin tissue fixation on the antigen of the pathogen. Attempts to amplify the htrA gene and gltA gene of B. henselae with single-step PCR from lymph node tissue were unsuccessful, and the DNA products were 414 bp and 380 bp, respectively. The success of a PCR-based study of fixed, paraffin-embedded material depends on several factors, including the fixative used in tissue processing, the duration of the fixation, the age of the paraffin block, and the length of the DNA fragment to be amplified. Only 60% of 5-year-old specimens were sufficient for amplification of a 536 bp fragment, and the various steps of fixation and embedding of the tissue are known to damage DNA.

We chose nested PCR to increase the sensitivity of the test, and the results indeed showed high sensitivity. However, skillful technique and a well-controlled laboratory environment are required to avoid false-positive results. When serology and molecular techniques are not available, IHC can contribute to the etiologic diagnosis of Bartonella lymphadenopathy.

References


