

## Detection by Immunofluorescence Assay of *Bartonella henselae* in Lymph Nodes from Patients with Cat Scratch Disease

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**Laboratory diagnosis of *Bartonella henselae* infections can be accomplished by serology or PCR assay on biopsy samples. The purpose of our work was to assess immunofluorescence detection (IFD) in lymph node smears using a specific monoclonal antibody directed against *B. henselae* and a commercial serology assay (IFA) compared with PCR detection. Among 200 lymph nodes examined from immunocompetent patients, 54 were positive for *B. henselae* by PCR, of which 43 were also positive by IFD. Among the 146 PCR-negative lymph nodes, 11 were positive by IFD. Based on PCR results, the specificity of this new technique was 92.5%, the sensitivity was 79.6%, and the positive predictive value was 79.6%. At a cutoff titer of 64, the sensitivity of the IFA was 86.8% and the specificity was 74.1%. Diagnosis of cat scratch disease (CSD) may be improved, with a specificity of 100%, when the two tests (IFD and IFA) were negative; the sensitivity was 97.4% if one of the two tests was positive. Since PCR-based detection with biopsy samples is available only in reference laboratories, we suggest using IFD coupled with the commercial serology test for the diagnosis of CSD.**

Sixteen species within the genus *Bartonella* are now characterized, including three that have been extensively characterized as human pathogens, i.e., *B. bacilliformis*, the agent of Carrion's disease (21), *B. quintana* (31), and *B. henselae* (2). *B. henselae*, a species first recognized in 1990 (45), is the main etiological agent of cat scratch disease (CSD) (35) and is also responsible for bacillary angiomatosis and peliosis hepatis in immunocompromised (mainly AIDS) patients (25), bacteremia, and endocarditis (2). Cats are the main reservoir of *B. henselae*, and humans may be contaminated by cat scratches or bites; additionally, the role of the cat flea (*Ctenocephalides felis*) as a vector for human transmission has been proposed (2).

Techniques for diagnosing *Bartonella*-related infections include culturing of the pathogen (7, 24, 27), molecular biology techniques, especially PCR amplification of *Bartonella* spp. genes (1, 19, 23, 37, 38, 48), and serology (32, 43). Isolation of *B. henselae* in CSD patients has rarely been achieved (16, 27). Amplification of *Bartonella* spp. DNA from skin, lymph nodes, granulomatous lesions, osteolytic lesions, or less frequently from other organ biopsies or in leukoclastic vasculitis has been often reported in patients suffering CSD (1, 4, 19, 38, 40, 48) or bacillary angiomatosis (17, 37). PCR-based detection of *Bartonella* species from human specimens by use of various target genes remains the best method for the diagnosis of CSD. Nevertheless, this technique is only available in reference laboratories and contamination may impair its specificity. Serology is the only noninvasive diagnostic technique and has been evaluated for the diagnosis of CSD (18, 32, 36, 39, 43) and other *Bartonella*-related infections, including bacteremia (8–

10, 22) and endocarditis (12, 15, 30, 33). The sensitivity of serology varies from one laboratory to another, ranging from near 100% to less than 30% (43). Commercially prepared antigen slides are now available for *B. henselae* and *B. quintana* serology (18, 20, 34, 39, 44, 46). Accurate diagnosis of CSD is necessary because the presentation and course of the disease may resemble more severe diseases such as malignant tumors or mycobacterial infections.

The purpose of our work was to assess an immunofluorescence assay on lymph node smears using a specific monoclonal antibody (MAb) directed against *B. henselae* and a commercial serology test in comparison to PCR detection using two different target genes as a reference technique to determine the best strategy for the diagnosis of CSD.

### MATERIALS AND METHODS

Clinical specimens from patients suspected to have CSD between October 2001 and October 2002 were included in the study. A case of definite diagnosis of *B. henselae* infection (CSD group) was defined as a case in which a patient had regional lymphadenitis and a history of recent cat contact and where direct identification of a *Bartonella* sp. was made by PCR from lymph node tissue by using two different target genes. A standardized questionnaire (which gathered information about contact with cats or cat fleas, fever, cat scratches or bites, cutaneous lesion at the inoculation site, and treatment) was completed for each patient. When available, a serum sample from each patient was tested for *Bartonella* antibodies (32).

All available sera were examined for the presence of anti-*B. henselae* antibodies by using a commercial immunofluorescence assay (IFA; Focus Technologies, Cypress, Calif.; distributed in France by Eurobio, Paris, France) (32). The slides were prepared with the *B. henselae* Houston-1 and *B. quintana* Oklahoma strains grown in Vero cells for detection of immunoglobulin G (IgG). IgG titers of  $\geq 64$  were used as cutoffs for *B. henselae* antigen.

**DNA extraction and PCR amplification from lymph nodes.** Total genomic DNA was extracted from samples with a QIAamp tissue kit (Qiagen, Hilden, Germany) as previously described (48). Samples were handled under sterile conditions to avoid the risk of cross-contamination. Genomic DNA was stored at 4°C until used as the template in PCR assays. The primers used for amplification and sequencing (*its* and *pap31* genes) have been evaluated previously in our laboratory (Table 1) (48). For amplification of the *Bartonella pap31* gene, a seminested PCR using three primers was applied. In each case, the PCR was

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TABLE 1. Oligonucleotide primers used for PCR amplification and sequencing

Primer (reference gene)	Nucleotide sequence	Detected organism	Reference(s)
URBarto1 ( <i>its</i> )	CTT CGT TTC TCT TTC TTC A	<i>Bartonella</i> species	42
URBarto2 ( <i>its</i> )	CTT CTC TTC ACA ATT TCA AT	<i>Bartonella</i> species	42
PAPn1 ( <i>pap31</i> )	TTC TAG GAG TTG AAA CCG AT	<i>Bartonella</i> species	47, 48
PAPn2 ( <i>pap31</i> )	GAA ACA CCA CCA GCA ACA TA	<i>Bartonella</i> species	47, 48
PAPnS2 ( <i>pap31</i> )	GCA CCA GAC CAT TTT TCC TT		

carried out with PTC-200 automated thermocyclers and by using a *Taq* polymerase kit (Gibco-BRL, Cergy Pontoise, France). PCR amplification was performed under the following conditions: initial 3 min of denaturation at 94°C, followed by 44 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 58°C for both genes, and extension for 45 s at 72°C. The PCR products were separated by electrophoresis on 1.5% agarose gels, visualized by staining with ethidium bromide, and then purified with a QIAquick PCR purification kit (Qiagen). PCR products were sequenced with a d-Rhodamine terminator cycle sequencing reading kit (Perkin-Elmer, Coignieres, France). Sequencing products were resolved with an ABI377 or ABI310 automated sequencer (Perkin-Elmer). Sequences obtained were compared with those in the GenBank DNA database by using the BLAST program (version 2.0; National Center for Biotechnology Information [http://www.ncbi.nlm.nih.gov])

For immunofluorescence detection (IFD), thin smears of all lymph nodes were made before PCR sampling. The IFD test was performed on fresh lymph node specimens. The slides were air dried, fixed with methanol for 10 min at room temperature, and stained for 30 min at 37°C with a mouse MAb specific for *B. henselae* (H2A10; titer of 1/3,200 diluted at 1/800 in phosphate-buffered saline [PBS]) (41). Slides were washed first in PBS (pH 7.2) with Tween and then in PBS (10 min each) and then rinsed with distilled water (5 min). After being air dried, slides were incubated with a fluorescein-isothiocyanate anti-mouse conjugate (Immunotech, Marseille, France) diluted 1:100 in PBS containing 0.2% Evans blue (Biomerieux, Marcy l'Etoile, France) for 30 min. The slides were washed as described above, air dried, mounted with Fluoprep (Biomerieux), and then examined with an epifluorescence microscope (Axioskop 20; Carl Zeiss, Göttingen, Germany) at a ×400 magnification. For each lymph node, a negative control was performed with a mouse MAb directed against *Tropheryma whippelii* (28). A positive control (smear from a *B. henselae* PCR-positive lymph node) was used in each experiment. A positive smear was defined as the presence of specific bacterial fluorescence on the slide stained with the MAb directed against *B. henselae* and an absence of fluorescence on the smear from the same lymph node stained with the control MAb. All smears were examined in two different experiments to confirm the results and to determine interoperator variability. Slides were viewed carefully since bacteria could be seen only in separate foci, and thus the entire smear was read.

Microbiologic cultures of lymph nodes for *Bartonella* isolation were performed either on blood agar plates that were incubated at 37°C with 5% CO<sub>2</sub>, examined weekly, and held for 2 months or by using a shell vial assay with human erythroid leukemia cells at 37°C with 5% CO<sub>2</sub> as previously described (27). When other bacteria or mycobacteria were isolated, they were identified by Gram stain and biochemical reactions using standard bacteriologic methods. Histologic examination of lymph nodes was performed for all samples. Formalin-fixed paraffin-embedded tissue samples were cut and stained using routine methods, including Gram staining, hematoxylin and eosin, and periodic acid Schiff and Warthin-Starry.

**Statistics.** For data comparison, the Student *t* test was performed using Epi Info software.

**RESULTS**

Overall, we used PCR to test 200 lymph node samples (from 189 patients) sent to our laboratory with a suspicion of CSD. The majority of the patients in this study were immunocompetent. Among these, amplicons were obtained for the two target genes in 54 lymph nodes of 52 patients (27.5%) with clinical and epidemiological evidence of *B. henselae* infection (CSD group). The sequences derived from these PCR products were 100% identical to those of *B. henselae* for both genes. In our study, one of these patients was coinfecting with an

atypical mycobacterium. Histologic examination of lymph nodes showed a granulomatous and necrotizing lymphadenitis with characteristic stellate microabscesses surrounded by palisading histiocytes. Of the remaining 137 patients (accounting for 146 lymph node samples), 55 (29%) had lymphadenopathy due to other infectious agents, 17 had malignant tumors (9%), 3 had sarcoidosis, and 62 had lymphadenopathy of unknown cause (Table 2). The mean age ± standard deviation of the 52 patients with proven *B. henselae* lymphadenopathy was 23.4 ± 17.8 years (range, 1 to 56 years) versus 37.8 ± 22.9 years (range, 1 to 78 years) for those in the non-CSD group (*n* = 137). This difference was statistically significant (*P* < 0.05, the Student *t* test). The sex ratio (male to female) was 1.28 for the CSD group versus 1.64 for the non-CSD group. For the 52 patients with CSD, all noted contact with cats, 34 (65.4%) reported scratches or bites, and 30 (57.7%) had a temperature of >38.5°C. One patient was bitten by a mouse. The localization of the lymph node was noted in 37 patients, with the following results: 16 (40.5%) axillary, 12 (32.4%) inguinal, 2 groin, 4 arm, and 3 cervical or jugulocarotid. For the non-CSD group, the localization of the lymph node was noted in 102 patients, with the following results: 21 (20.6%) axillary, 26 (25.5%) cervical, 15 (14.7%) inguinal, 8 (7.8%) mediastinal, and 32 of various sites. No lymph nodes grew *Bartonella henselae* after 3 months of culture.

Serum samples were available for 38 of 52 (73.1%) patients

TABLE 2. Diagnoses for 189 patients with lymphadenopathy

Diagnosis	No. (%) of patients
CSD .....	52 (28)
Bacterial infection	
Coagulase-negative <i>Staphylococcus</i> .....	13
<i>Staphylococcus aureus</i> .....	8
<i>Propionibacterium acnes</i> .....	3
<i>Streptococcus agalactiae</i> .....	2
<i>Pseudomonas aeruginosa</i> .....	1
<i>Enterococcus faecium</i> .....	1
<i>Methylobacterium</i> sp. ....	1
<i>Micrococcus</i> sp. ....	1
<i>Pasteurella</i> sp. ....	1
Tuberculosis .....	31 (16)
Lymphoma .....	18 (10)
Cancer .....	12 (6)
Sarcoidosis .....	5 (3)
Q fever .....	2 (1)
Tularemia .....	1 (1)
<i>B. quintana</i> bacteremia .....	1 (1)
Bacillary angiomatosis .....	1 (1)
Candidiasis .....	1 (1)
Unknown .....	62 (33)
Total .....	189 (100)

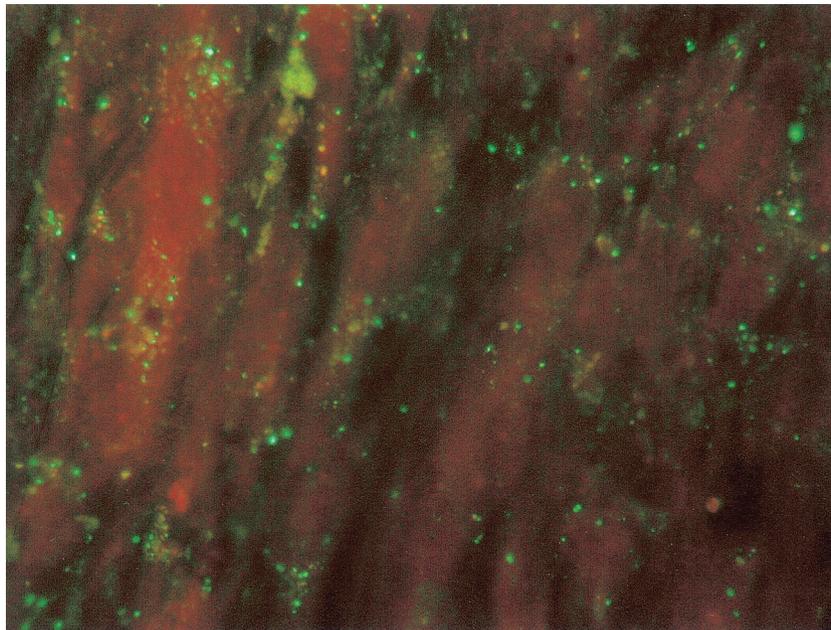


FIG. 1. Detection of *B. henselae* by IFD test in the lymph node of a patient with CSD by using a MAb directed against *B. henselae* as viewed by fluorescence microscopy (magnification,  $\times 400$ ).

in the CSD group and for 58 of 137 (42.3%) patients in the non-CSD group. The sensitivity of the IFA test using a cutoff titer of  $\geq 64$  for the detection of anti-*B. henselae* IgG antibodies was 86.8%, and the specificity was 74.1%. For a cutoff titer of  $\geq 128$ , the sensitivity was 60.5% and the specificity reached 86.2%. A final diagnosis for the 15 non-CSD patients who had positive CSD serology (titer of  $\geq 1:64$ ) was established in seven cases, including two cases of mycobacterial infection, one of lymphoma, one of sarcoidosis, one of Kaposi's sarcoma, and two of *Staphylococcus aureus* infection. In one case, *Propionibacterium acnes* was obtained from the lymph node and could be considered as a contaminant.

The 200 lymph nodes were tested on smears by IFD test. For the IFD assay, smears were viewed totally at a  $\times 400$  magnification and each smear was viewed for 10 min by two different operators. Among the 54 *B. henselae* PCR-positive lymph nodes, 43 were also positive by IFD (Fig. 1) and 11 were negative. For the 43 lymph nodes positive by IFD test, 13 were treated with antibiotics and 30 were not, whereas 3 of the 11 samples negative by IFD test were treated and 8 were not treated ( $P > 0.05$ , the Student *t* test). Among the 146 PCR-negative lymph nodes, 135 were IFD negative and 11 were IFD positive. Overall, the sensitivity was 79.6% and the specificity was 92.5%, with a positive predictive value of 79.6% and a negative predictive value of 92.5%.

A final diagnosis for the 11 patients in the non-CSD group with IFD-positive smears was identified in seven cases, including two cases of mycobacterial infection, one of lymphoma, one of cancer, and three of *Staphylococcus aureus* infection. These false-positive results were obtained with samples different from those that produced false-positive results with the IFA serological test. Moreover, for the 11 patients with a negative immunofluorescence smear but who were positive by PCR, serology was available for 10 and was positive for 9 patients

when the IFA cutoff titer was  $\geq 64$ . Sensitivity and specificity could be improved with the coupling of tests as follows: sensitivity was 97.4% with a positive IFA (cutoff titer,  $\geq 64$ ) or a positive IFD, whereas specificity was 100% with a negative IFA (cutoff titer,  $\geq 128$ ) and negative IFD (Fig. 2).

## DISCUSSION

CSD is usually suspected clinically, and it is confirmed by the detection of *Bartonella* DNA in lymph nodes. The infection is usually a self-limited disease, with frequent development of extensive regional lymph node enlargement lasting typically 2 to 3 months and occasionally longer. Surgical extirpation or lymph node aspiration is rarely needed. However, in cases of atypical presentation, lymph node biopsy is often performed to rule out more severe diseases such as malignant disease or mycobacterial infection. The isolation of *B. henselae* from lymph nodes of patients suffering from CSD has rarely been reported, since the bacteria are very fastidious (3, 6, 27). Thus, there is a need for suitable alternative tests or combinations of different tests to improve the diagnosis of *B. henselae* infections. Serological analysis by immunofluorescence or enzyme-linked immunosorbent assay is a useful noninvasive diagnostic method for the diagnosis of CSD, but specificities and sensitivities may vary according to the antigens used and disease case definition (5, 11, 32). To avoid this problem in the present study, all *B. henselae* cases were unambiguously diagnosed by PCR amplification of *B. henselae* from a lymph node biopsy sample by using two different target genes as previously reported (48). This study evaluated a new direct method for diagnosing CSD, i.e., an IFD assay using a MAb directed against *B. henselae*. This MAb (H2A10) was of the IgG2a subclass, reacted with a 43-kDa epitope present only in *B. henselae* strains, and did not cross-react with other *Bartonella*

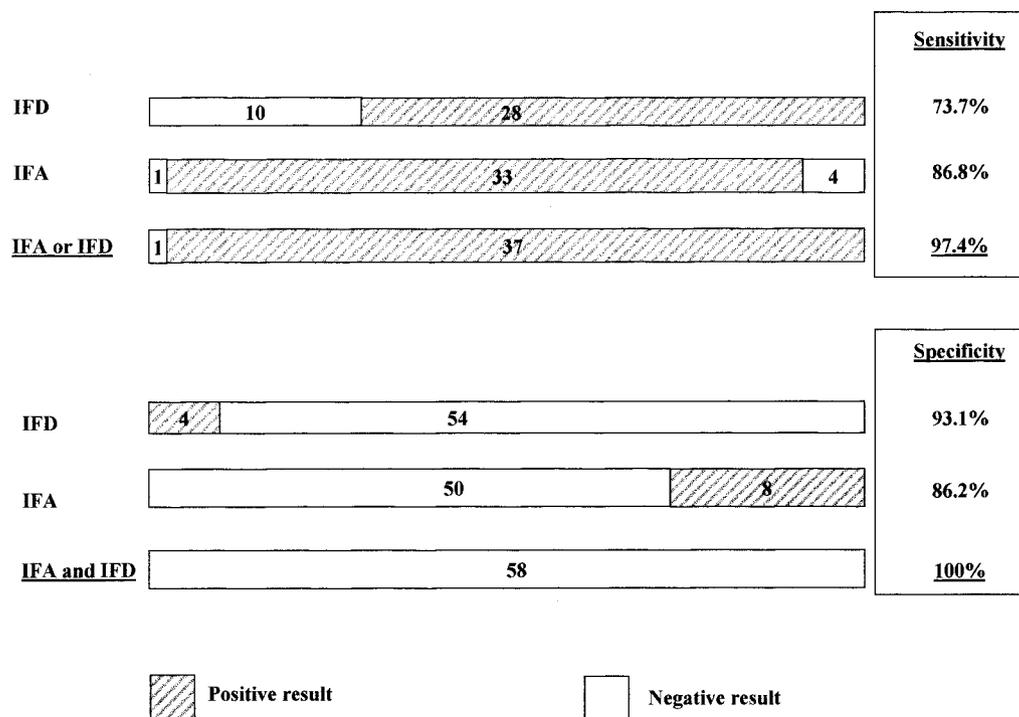


FIG. 2. Sensitivities and specificities obtained with IFA and/or IFD tests. For sensitivity, the cutoff titer for IFA was set at  $\geq 64$ , whereas for specificity, it was set at  $\geq 128$ .

species (41). This MAbs has been successfully used to specifically demonstrate the presence of *B. henselae* in erythrocytes from bacteremic cats (41). Moreover, we also evaluated a commercial IFA test to compare the two methods and to propose a strategy for the diagnosis of CSD.

We found in this study that about 28% of patients suspected of having CSD were found to have proven CSD (clinical and PCR-based detection). This proportion is very close to the result of a previous report which found that 39% of 274 patients with lymphadenopathy had CSD confirmed by PCR (48). *B. henselae* has been described as the main agent of CSD, but *B. quintana* and *B. clarridgeiae* have also been proposed as possible agents of the disease (13, 26, 29; D. Raoult, M. Drancourt, A. Carta, and J. A. Gastaut, Letter, Lancet 343:977, 1994). *B. clarridgeiae* and *B. quintana* were not amplified from the lymph nodes in this study or in a previous study by our group (48). The only differences found in the patients' characteristics between the two groups (CSD and non-CSD) were in the mean age and sex ratio, which were both lower in the CSD group. These results are in accordance with the fact that the disease usually occurs in young patients (48). In the non-CSD group, patients were older and some of the chronic cases were due to more serious diseases such as tuberculosis or lymphoma.

The sensitivity and specificity of the serological test used in this study were similar to those previously reported (32) and confirm the results of studies showing the high sensitivity of the IFA test for the diagnosis of CSD (44, 46). We report a sensitivity of 86.8% at a cutoff titer of  $\geq 64$ . However, specificity was low, since we found malignant processes and mycobacterial infections in the non-CSD group. Giladi et al. and Ridder

et al. have reported four cases of patients with malignant tumors and high antibody titers against *B. henselae* (18, 39). The study of Sander et al. excluded patients with malignancy (43). Moreover, the seroprevalence in a particular geographic area as well as the antigen preparation of the respective IFA tests used should be determined before interpretation of the results (43). Even when two different serological tests were coupled, the specificity and the sensitivity were not sufficient for the diagnosis of CSD (32). False-negative PCR results could be due to a previous infection, with disappearance of the bacteria in the lymph nodes while antibodies against *B. henselae* remained present, suggesting that the results of PCR strongly depend on the duration of illness (39). Since CSD lymphadenopathy may last for months, it is indeed possible that patients with positive serology and negative PCR results had a previous infection due to *B. henselae* which was not acute at the time of sampling. This may also explain why isolation of the bacteria from lymph nodes is so difficult (27), because bacteria have disappeared from the node and positive serology results reflect a previous infection. If so, then the sensitivity and specificity of PCR and IFD would be less if we consider a retrospective diagnosis. However, for an acute diagnosis at the time of sampling, PCR and IFD were more predictive of the disease. For these reasons, we believe that diagnosis of *B. henselae* infections should not be made only by serology. Exclusion of other diagnoses, especially of more severe diseases such as lymphoma and mycobacterial infections, should be performed in atypical cases by histological analysis of lymph nodes.

Our new IFD assay was very easy to perform since we only needed lymph node smears. Interpretation of clinical samples,

compared to negative and positive controls, was reproducible between operators. This method can be used in all laboratories with an epifluorescence microscope. Moreover, this method can be easily coupled with histological analysis and conventional culture to exclude more severe diseases, especially malignant processes and other bacterial infections. One of the problems with the IFD test was that fluorescence was not homogeneously distributed in the smears, as the bacteria were only found focally. Thus, all experiments were carried out twice to confirm the results. One may explain the 11 false-negative IFD tests by the focal presence of bacteria in the lymph node. Quantitative detection of *Bartonella* DNA would be of great interest to support this hypothesis but was not performed in this study. For the non-CSD group, this assay is 92.5% specific, but this is not enough since we have also found severe diseases such as mycobacterial infections and malignant processes in the non-CSD group. This type of result can lead to lack of treatment and serious consequences. Nevertheless, misdiagnosis for these cases was avoided by histological analysis of the lymph nodes. The three cases of *S. aureus* infection were also easily identified since culture of the bacteria was rapid and since this bacterium is known to cause false-positive results in immunofluorescence assays due to nonspecific reactions with protein A (14).

Interestingly, false-positive results with the IFA test and the IFD test were obtained with different samples and thus the two tests appear to be complementary. From our study, it is possible to propose a strategy for the diagnosis of CSD. To obtain a specificity and a positive predictive value of 100%, both IFA and IFD should be positive (using a cutoff titer for the serology of  $\geq 128$ ), whereas a sensitivity higher than 95% can be achieved when only one of the two tests is positive. These results for sensitivity and specificity should be interpreted cautiously since they are based on a small number of patients.

In conclusion, the present study shows the high specificity of the IFD using a MAbs directed against *B. henselae* and performed with lymph node smears from CSD patients, especially when associated with histological analysis and conventional bacterial culture. The test described in this study could play an important role in the diagnosis of CSD since it requires only an epifluorescence microscope and can reduce costs and delays for diagnosis. Since PCR-based detection with biopsy samples is available only in reference laboratories, we suggest using this test coupled with the commercial serology test for the diagnosis of CSD.

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