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Evaluation of IgG ELISA using *N*-lauroyl-sarcosine–soluble proteins of *Bartonella henselae* for highly specific serodiagnosis of cat scratch disease

Keiko Tsuruoka, Hidehiro Tsuneoka *, Mitsunobu Kawano, Masashi Yanagihara, Junzo Nojima, Tatehiko Tanaka, Misa Yamamoto, Kiyoshi Ichihara

Department of Clinical Laboratory Science, Division of Health Science, Yamaguchi University Graduate School of Medicine, Ube, Yamaguchi 755-8505, Japan

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ABSTRACT

Conventional IgG-ELISA methods for diagnosing cat scratch disease (CSD) caused by *Bartonella hensela* are still poor in sensitivity and specificity, which generally employ bacterial whole-cell proteins or *N*-lauroyl-sarcosine–insoluble proteins as the antigen. By Western blot analysis, we found that sarcosine-soluble fraction of proteins (SSP) showed highly specific reaction to immunofluorescence assay (IFA)–positive sera obtained from CSD patients compared with the above antigens. Clinical utility of the new ELISA employing SSP was evaluated using sera from 118 patients with clinically suspected CSD (sera positive by IFA: titers ≥1:256, $n = 46$; negative: titers <128, $n = 72$) and 88 sera from healthy individuals. Sensitivity and specificity of distinguishing IFA-positive patients from healthy individuals were 95.7% and 97.7%, respectively. Fifteen discordant results were observed (13 ELISA(+)/IFA(−); 2 ELISA(−)/IFA(+)). However, all 15 sera reacted with SSP by Western blot analysis, indicating superiority of the new ELISA over IFA. The ELISA employing SSP greatly improved the accuracy of diagnosing CSD.

1. Introduction

Cat scratch disease (CSD), an infection caused by Gram-negative bacillus *Bartonella henselae*, is a worldwide zoonosis associated with various clinical manifestations (Anderson and Neuman, 1997; Maurin et al., 1997; Murakami et al., 2002). The predominant clinical feature at onset is regional lymphadenopathy. Occasionally, the infection may manifest atypically with systematic symptoms such as hepatic–splenic granuloma, endocarditis, encephalopathy, and neuroretinitis (Anderson and Neuman, 1997; Maurin et al., 1997; Murakami et al., 2002). Because isolation of *B. henselae* from patients is difficult, laboratory diagnosis of CSD depends mainly on serologic analysis or PCR-based genetic identification.

In 1992, Regnery et al. (1992) developed a sensitive and specific immunofluorescence assay (IFA) to detect anti–*B. henselae* IgG for the serologic diagnosis of CSD. Since then, IFA has become the most commonly used diagnostic test for CSD, both as in-house and as commercial methods (Nadal and Zbinden, 1995; Sander et al., 1998; Tsuneoka et al., 1998), but this test has several limitations. Antigen preparation requires cultivation of *B. henselae* in Vero cells, results are often influenced by interobserver variability, and the test is time consuming. Additionally, IFA is not suitable for automation or screening large numbers of specimens.

The ELISA method has been proposed as an alternative to IFA. Several ELISA methods use sonically disrupted *B. henselae* (whole-cell proteins) or the putative outer membrane proteins (OMPs) that were fractionated by *N*-lauroyl-sarcosine of *B. henselae* (sarcosine-insoluble proteins) as the antigen (Giladi et al., 2001; Not et al., 1999; Vermeulen et al., 2007; Welch et al., 1993). However, several studies of ELISA-based CSD serodiagnosis showed that IgG ELISA was significantly inferior to IFA or even flawed because of its high false positivity and low sensitivity (Bergmans et al., 1997; Szelc-Kelly et al., 1995; Vermeulen et al., 2007). Therefore, an improvement of the antigen preparation for use in IgG ELISA will increase its clinical accuracy.

We found that sarcosine-soluble proteins of *B. henselae* are significantly more specific than whole-cell or sarcosine-insoluble proteins as an antigen for IgG ELISA. There have been no published studies on ELISA that use *N*-lauroyl-sarcosine–soluble proteins of *B. henselae* as a solid-phase antigen. In this study, we compared the results of 2 methods for diagnosing CSD, ELISA for IgG that uses the *N*-lauroyl sarcosine–soluble antigen, and IFA.

2. Materials and methods

2.1. Preparation of 3 antigens of B. henselae for ELISA

Three different antigens—whole-cell and sarcosine-insoluble and -soluble proteins of *B. henselae*—were prepared for ELISA. In the whole-cell antigen, *B. henselae* 49882 was grown on 10

* Corresponding author. Tel.: +81-836-22-2854; fax: +81-836-22-2854.
E-mail address: htsune@yamaguchi-u.ac.jp (H. Tsuneoka).

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chocolated agar plates for 4–5 days at 35 °C in CO_2. Bacteria were harvested in 10 mmol/L HEPES (*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid [pH 7.4]; Sigma) and sonicated for 20 min on ice. The lysate was centrifuged at 3000 rpm for 5 min at 4 °C, and the supernatant was prepared as whole-cell antigen. The supernatant was transferred to new tubes and centrifuged at 40,000 rpm for 45 min at 4 °C. The pellet was resuspended in 0.4% *N*-lauroyl-sarcosine with HEPES buffer and then placed on ice for 30 min. After another centrifugation at 60,000 rpm for 2 h at 4 °C, the pellet was prepared as the insoluble antigen. Additionally, the supernatant was dialyzed with a dialysis membrane in HEPES buffer overnight at 4 °C, and after another centrifugation at 60,000 rpm for 2 h at 4 °C, the supernatant was prepared as the soluble antigen for ELISA. These 3 antigens were stored at −80 °C until use.

2.2. Comparison of ELISA using 3 different antigens

As a test panel to estimate titers of IgG to *B. henselae* by ELISA using the 3 different antigens, 5 positive sera (positive control [PC]) were selected from clinical specimens with IgG titers to *B. henselae* of ≥1:256 by in-house IFA, and 5 negative sera (negative control [NC]) were obtained from normal healthy individuals with IgG titers of <1:64. Wells in Polysorb microtiter plates (Sumilon, Sumitomo Bakelite, Japan) were coated overnight at 4 °C with 100 μL of optimally diluted preparation of *B. henselae* antigen in 50 mmol/L of carbonate–bicarbonate coating buffer (pH 9.2). The optimal dilution of the antigen was defined as the concentration that gave comparable and reproducible results with the test panel. Plates were washed, blocked with 5% skim milk in phosphate buffered saline (PBS) with 0.1% Tween 20 (PBS-T), incubated for 4 h at room temperature, and washed again. Control panel sera (PC and NC sera) were diluted 1:100 in PBS-T, incubated for 1 h at 37 °C, and washed with PBS-T. After 100 μL of horseradish peroxidase–labeled goat anti-human IgG (Sigma) was added, the sera were incubated for 1 h and washed. Citrate buffer (pH 5.0) containing *ortho*-phenylenediamine was added and incubated at room temperature. Color development was stopped after 30 min by the addition of 4N H_2SO_4. Optical density (OD) of the plates was read at 490 nm. All controls were tested in triplicate. The average OD of each PC and NC by ELISA using the 3 different antigens, which were tested in triplicate, was estimated and compared between PC and NC for each of the 3 ELISAs.

2.3. Validation of ELISA using the most appropriate of the 3 candidate antigens

IgG antibody to *B. henselae* was determined by ELISA using the most appropriate of the 3 candidate antigens. Performance of the ELISA thus chosen was evaluated using sera from 118 patients (from 99 children and 19 adults) clinically suspected of having CSD because of lymphadenopathy or fever of unknown origin and with a previous history of contact with a cat or dog. These specimens were obtained when serologic diagnosis using IFA was ordered to our clinical laboratory in Yamaguchi University. Of the 118 serum samples, 46 were serologically confirmed positive for IgG antibodies to *B. henselae* with titers of ≥1:256 by IFA. The remaining 72 sera samples comprised 34 sera at the threshold level with titers of 1:64–1:128 and 38 negative sera with titers of <1:64 by IFA.

As a control, 88 adult serum specimens obtained from healthy individuals with no past history of overt lymphadenopathy or cat scratches/bites and which comprised serologically 5 sera at the threshold level with titers of 1:64–1:128 and 83 negative sera with titers of <1:64 by IFA were evaluated. All specimens were tested in triplicate. PC and NC were included in each assay. The ELISA index of each test was calculated as follows (Loa et al., 2005):

$$(\text{OD of sample} - \text{OD of negative control}) \,/\, (\text{OD of positive control} - \text{OD of negative control}).$$

2.4. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Western blot analysis

The 3 different antigens of *B. henselae* were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and Western immunoblotting. SDS-PAGE was carried out using a modification described by Laemmli (1970). Absorbance of the antigens at 280 nm was measured previously with NanoDrop2000 (Thermo Fisher Scientific, USA), and the amount of proteins was adjusted equally to 4 mg/mL. Antigens were suspended in equal volumes of sample buffer (0.05 mol/L Tris hydrochloride [pH 6.8], 2% SDS, 6% 2-mercaptoethanol, 10% glycerol, and 0.01% bromophenol blue), then heated for 5 min at 95 °C and centrifuged for 2 min at 10,000 rpm. The resulting suspension was separated by SDS-PAGE using a 5% to 20% gradient polyacrylamide gel (ATTO, Japan) in running buffer (25 mmol/L Tris, 192 mmol/L glycine, 0.1% SDS) for 85 min at 20 mA. Prestained protein ladder (Gene Direx, Taiwan) was used on each gel as a reference. The separated antigens were then transferred to a polyvinylidene difluoride membrane (Merck Millipore, Germany) at 120 V for 60 min using electrophoretic transfer cell (NIHON EIDO, Japan) with transfer buffer (25 mmol/L Tris, 192 mmol/L glycine, and 20% methanol). The membranes were then blocked overnight at 4 °C in Tris-buffered saline containing 1% Tween 20 (TBST) with 5% skim milk. Membranes were washed 3 times in TBST and incubated at room temperature for 60 min with a 1:100 dilution of the test sera in TBST containing 1% skim milk. After washing as described above, membranes were incubated at room temperature for 60 min with a 1:800 dilution of horseradish peroxidase–labeled anti-human IgG rabbit serum (heavy and light chains; polyclonal rabbit anti-human IgG HRP; Dako Cytomation, Denmark) in TBST containing 1% skim milk. After washing as before, antigens on the membrane were detected with a diaminobenzidine buffer (Merck Millipore, Germany). This reaction was carried out for 1 to 3 min and then stopped by the addition of distilled water.

2.5. Immunofluorescence assay

In-house antigen slides for detection of IgG antibody to *B. henselae* were determined by IFA using *B. henselae* ATCC 49882 cocultivated with Vero cells for 96 h at 35 °C in an atmosphere containing 5% CO_2, as previously described (Regnery et al., 1992; Tsuneoka et al., 1998). Briefly, these antigens were spotted onto Teflon-coated slides, which were then air-dried, fixed with acetone, and stored at −80 °C until use. Serum samples were diluted in PBS. Commercial fluorescein conjugated goat anti-human IgG (MBL, Japan) was used for detection of specific IgG antibody. Slides were observed with a fluorescence microscope (OPTIPHOT-2; Nikon, Japan). Antibody titer was expressed as the highest dilution of serum that resulted in positive staining. Sera with IFA titers of ≥1:256 were considered positive, whereas sera with IFA titers of 1:64 to 1:128 were regarded as threshold level and those with titers of <1:64 were considered negative (Tsuneoka et al., 1998; Tsuneoka and Tsukahara, 2006). One operator performed IFA and another performed ELISA; both were unaware of the clinical and laboratory findings.

2.6. Data analysis

The relative sensitivity of the ELISA was calculated as the percentage of positive sera by ELISA among 46 sera from CSD patients positive by