Chapter 3

Evaluation of an in-house Cat-scratch disease IgM ELISA to detect Bartonella henselae in a routine laboratory setting

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ABSTRACT

Cat-scratch disease (CSD) is caused by *Bartonella henselae* infection and is a common cause of regional lymphadenopathy. The diagnosis of CSD largely depends on serology, but detection of *B. henselae* in an affected lymph node by PCR is also an important diagnostic tool. We evaluated an IgM in-house ELISA protocol and analyzed its performance in routine CSD serology. Serum samples from PCR-positive patients (n = 126), PCR-negative patients (n = 123), and age-matched controls (n = 126) were used for evaluation. The sensitivity of the IgM ELISA was only 56%, showing that the performance of *B. henselae* serology under routine laboratory settings is low, probably caused by the wide variability in disease duration in patients suspected of CSD whose samples were submitted to our laboratory. Most patients (46%) with a positive IgM response were between 0 and 20 years of age. We conclude that the serodiagnosis of *B. henselae* is hampered by the low sensitivity and specificity of the assays when used in a routine laboratory setting. For this reason, a negative IgM or PCR result can never exclude CSD, especially with late sample collection.
INTRODUCTION

Although the clinical profile of cat-scratch disease (CSD) was described by 1950, the causal pathogen *Bartonella henselae* was only identified 43 years later in 1993 [1-5]. The domestic cat is the major reservoir of *B. henselae*, with a confirmed link to disease in humans [6,7]. There is evidence to suggest that the cat flea is a vector for *B. henselae* transmission from cat to cat [8]. Fleas may be involved in transmission to humans because approximately 30% of patients do not recall traumatic cat contact, but evidence to support this conclusion is lacking [9-12]. The majority (60%) of patients with CSD are children under the age of 20 [13], and a seasonal peak in the fall and winter months has been described [14].

Regional lymphadenopathy (axillary, head and neck, inguinal) is the predominant clinical feature of CSD. It develops 2-3 weeks after exposure (with a range of 7-50 days) [12, 15-17] and resolves spontaneously after several months (with an average of 6 weeks) [13]. The node is usually tender and markedly swollen, and suppurates in 15% of cases [13]. Thirty percent of the patients report low-grade fever (< 39 °C), and 25-60% of the patients report a small papule (2-3 mm) 3 to 6 days after exposure at what is believed to be the primary inoculation site [12,17]. The conjunctiva may be another port of entry in a small number of cases [15,17]. The lymphadenopathy is often accompanied by myalgia, malaise, and fatigue, and usually does not respond to antibiotics, perhaps because the bacteria are already dead by this phase of the disease. This may also explain the negative lymph node culture results usually reported in CSD patients [18]. Because of the nonspecific nature of the presentation of CSD (lymphadenopathy) a wide differential diagnosis is considered. The detection of *B. henselae* in lymph node biopsies or pus by PCR is useful, with a reported sensitivity between 58% and 96% [19,20]. However, pus can only be collected from approximately 15% of the patients. For these reasons, laboratory diagnosis mainly depends on serology.

Both IFA and ELISAs have been described. The evaluation of serological tests in earlier studies reported various specificities (95-100%) and sensitivities (40-100% for IgG and 60-74% for IgM), depending on the different study populations, cut-off levels and methods, antigens, and definitions of CSD [4,19,21-23]. *Bartonella*-specific IgM remains present for approximately 3 months [24-26].

Our laboratory has used an in-house ELISA for the serodiagnosis of *B. henselae* since 1995 [24]. We recently improved and re-evaluated the test performance of the IgM and IgG ELISAs using samples sent to our laboratory from patients suspected of having CSD.
Chapter 3

MATERIALS AND METHODS

Clinical samples
We examined several groups of sera during our re-evaluation of the improved protocol of the *B. henselae*-specific IgM ELISA for use as a routine diagnostic tool. The sensitivity of the assay was determined using a panel of sera collected from CSD patients with a positive PCR result (n = 126). Detection of *B. henselae* by PCR was the only inclusion criterion used and was considered to be proof of infection. Additional clinical symptoms reported by the physicians were reported in 76 patients, but these were not used as inclusion criteria, to allow a closer comparison to the patient groups that are sent to the laboratory for analysis. No clinical data were available for the 50 remaining patients in this group. The average age in the patient group was 26 years (with a range of 3 to 74 years).

A total of 126 age-matched controls were used to determine the cut-off value and to calculate the specificity of the IgM and IgG ELISAs. These sera were obtained from a cross-sectional epidemiological survey in the general population of The Netherlands [27]. For a further evaluation of test performance, serum samples from 123 patients suspected of having CSD, but with a negative PCR, were also examined. Clinical symptoms were reported in 69 PCR-negative patients. The average age in this patient group was 29 years (with a range of 1 to 77 years). All groups were also tested using the IgG ELISA to evaluate the additional value of IgG testing for diagnosing an active *B. henselae* infection.

To study the test performance of the IgM ELISA in the daily routine of CSD serology, all serum samples from patients with a known date of birth sent to our laboratory during 2004 (n = 1,785) were included in the analysis of seroprevalence of *B. henselae*-specific IgM in the different age groups. Other parameters, such as possible seasonal influences and reported clinical symptoms, were also analyzed. Follow-up samples from this group (80 patients) were also analyzed to study IgM and IgG kinetics.

*Bartonella henselae*-specific IgM and IgG ELISA
The IgM and IgG ELISAs were performed based on previously described methods, with several modifications to improve sensitivity [24,28]. The *B. henselae* strain ATCC 49882 (genotype I) was grown on Columbia agar plates containing 5% sheep blood for 7-10 days at 35 °C in an atmosphere containing 5% CO2. Colonies were scraped from the plates, suspended in phosphate buffered saline (PBS) and sonificated for 30 min, and stored at -20 °C. Wells of polysorb microtiter plates (Nunc, Copenhagen, Denmark) were coated overnight at +4 °C with 100 μl of an optimal dilution of the prepared *B. henselae* antigen in PBS. Parallel wells were coated with PBS alone to control for the non-specific binding of serum components. After incubation, the plates were washed twice with PBS with 0.05% Tween 20 and blocked with blocking reagent (Boehringer, Mannheim, Germany) according to the manufacturer's instructions. Serum dilutions (1:100) in PBS with 1% w/v bovine serum albumin and 8% v/v normal goat serum were added and were incubated for 1 h at 37 °C. Plates were washed four times between each incubation step. Prior to testing in the IgM assay, sera were depleted of
IgG with IgG-RF sorbent (Biotest, Soest, The Netherlands) according to the manufacturer’s instructions to prevent possible inter-isotype competition. Bound antibody was detected by horseradish-peroxidase-labeled goat anti-human IgM (Biorad, Veenendaal, The Netherlands) or rabbit anti-human IgG (Dako, Glostrup, Denmark; 1 h, 37 °C). Tetramethylbenzidine was used as a substrate, and color development was stopped after 10 min by the addition of 2 M H2SO4. The plates were read at 450 nm (second filter 690 nm). A high positive, low positive and negative control serum was included in each assay. The background from each sample was deducted from its reaction with the \textit{B. henselae} antigen to correct for non-specific binding.

The cut-off value was defined as the average optical density (OD) of three negative samples run in each assay + a factor based on the detected standard deviation (SD) in the age-matched control group (\(n = 126\)). A factor of 3x the SD for IgM and 2x the SD for IgG was used. A serum sample was considered positive if the calculated ratio (OD sample/cut-off value) was \(\geq 1\). An intermediate IgM result was defined as that of samples with OD values between the average +2xSD and the average +3xSD.

**PCR**

Clinical material for the PCR detection of \textit{B. henselae} DNA consisted of pus aspirates and biopsy specimens from lymph nodes. The extraction of DNA and subsequent PCR detection using the \textit{B. henselae} 16S rRNA gene as a target were performed as described earlier [19].

**Statistical methods**

A Chi-squared analysis was conducted to determine the significance of the difference in seroprevalence between two groups. P-values < 0.01 were considered significant.

**RESULTS**

**IgM and IgG ELISA results in the age-matched controls, PCR-positive CSD patients, and in a group of PCR-negative patients suspected of having CSD**

One hundred and twenty-six age-matched controls were tested in the IgM and IgG ELISA (Figure 1). Only 2 individuals tested positive for IgM (1.6%) and 3 had IgG levels above the cut-off (2.4%). The specificities of the IgM and IgG ELISAs were therefore 98.4% and 97.6% respectively. The sensitivities of the IgM and IgG ELISAs were determined with sera from 126 PCR-positive patients. Seventy-one patients (56.3%) were IgM-positive and 45 patients (35.7%) were IgG-positive. These responses were significantly higher than those detected in the age-matched control group.

In total, 123 patients suspected of having CSD with negative PCR results were also analyzed for their serologic response to \textit{B. henselae}. A considerable number in this group tested positive for IgM 14.1% (\(n = 18\)). The IgG distribution of the PCR-negative patient group resembled the distribution in the PCR-positive CSD patients, but the IgM distribution was, in general, lower (Figure 1). A percentage of 38.3 was IgG-positive above the cut-off, and this was significantly higher than the control group and very similar to the percentages observed in the PCR-positive group.
Seroprevalence of IgM antibodies to *Bartonella henselae* by age group

A large percentage of our incoming patients (33.2%) were children below the age of 19. The number of IgM-positive distribution over the age groups reflects the knowledge that most reported CSD patients are children (Figure 2). In total numbers, 46% of our patients with a positive IgM response were between 0 and 20 years of age. The number of CSD patients identified by a positive IgM response peaked between 11 and 20 years of age (19.3%). After the age of 20, the number of patients identified declined, with only 3.2% positive in the age group above 60. There were no indications that the IgM ELISA had a different sensitivity depending on the age group tested. The overall number of IgM-positive results was 14.1%. However, the number of IgM-positive patients was not equally distributed throughout the year, and a significantly lower seroprevalence of IgM (p-value < 0.01) was found during the months of April, September, and October.
Evaluation of routine *B. henselae* IgM ELISA

**Evaluation of the use of follow-up serology in serum samples from patients suspected of having CSD**

Consecutive serum samples from 55 patients with a negative or intermediate (inconclusive) IgM result and a negative IgG result in their first samples were examined for IgM and IgG antibody kinetics. About half of these samples were sent in within 4 weeks of the first sample date (50%), 68.8% within 8 weeks, and 76.3% within 12 weeks.

Only 10 (18.2%) of the 55 patients with an initial inconclusive IgM result showed results that were indicative of an active *B. henselae* infection during the investigation of a follow-up sample. In total, a seroconversion or clear increase from an intermediate to a positive IgM response was detected in 8 patients (15%), while a seroconversion or clear increase in the IgG titer was observed in only 2 patients (4%) from this group. However, in the majority of cases (82%), no proof of an active infection was detected based on the absence of antibody kinetics.

![Figure 2. Seroprevalence of IgM antibodies to *Bartonella henselae* by age group. The total numbers of sera tested per group were: 0-10 years, n=408; 11-20 years, n=244; 21-30 years, n=245; 31-40 years, n=330; 41-50 years, n=249; 51-60 years, n=151; > 60 years, n=158.](image-url)
Analysis of clinical data reported in PCR-positive CSD patients and PCR-negative and IgM-positive patients suspected of having CSD

In the majority of the 76 PCR-positive CSD patients with reported clinical symptoms, lymphadenopathy was reported (97.4%). One patient had endocarditis and in one patient only fever was reported (Table 1). The location of the affected lymph node was known in 41 PCR-positive CSD patients. In the PCR-negative patients clinical symptoms were reported in 67 cases (Table 1). Lymphadenopathy was reported in 53 cases (79.1%), and in 14 patients (20.9%) other CSD-related symptoms were reported, such as liver abnormalities, uveitis, and osteomyelitis (Table 1).

Clinical symptoms were reported in 212 IgM-positive patients whose findings were sent to our laboratory (Table 1). Lymphadenopathy was reported in 56.6% (n = 120) and a total of 33 patients (15.6%) had other CSD-related symptoms such as skin, eye, or heart infections, fever, or neurological abnormalities (Table 1). We identified 2 patients with endocarditis, 6 patients with eye infections, 3 patients with neurological problems, and 20 patients with fever caused by a *B. henselae* infection. An unexpectedly high incidence of 25.9% of the IgM-positive patients reported other symptoms such as gut-related symptoms (n = 9), stomach ache, and other clinical findings (Table 1).

Table 1.
Analysis of the clinical data reported in the PCR-positive and -negative patient groups used as control groups, and in IgM-positive patients suspected of having cat-scratch disease sent to our laboratory for *Bartonella henselae* serology.

<table>
<thead>
<tr>
<th>Findings</th>
<th>PCR+, n (%)</th>
<th>PCR-, n (%)</th>
<th>IgM+, n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total</strong></td>
<td>76 (100)</td>
<td>67 (100)</td>
<td>212 (100)</td>
</tr>
<tr>
<td><strong>Lymphadenopathy</strong></td>
<td>74 (97.4)</td>
<td>53 (79.1)</td>
<td>120 (56.6)</td>
</tr>
<tr>
<td>Head and neck</td>
<td>12</td>
<td>12</td>
<td>38</td>
</tr>
<tr>
<td>Axillary</td>
<td>20</td>
<td>10</td>
<td>16</td>
</tr>
<tr>
<td>Inguinal</td>
<td>9</td>
<td>3</td>
<td>11</td>
</tr>
<tr>
<td>No location reported</td>
<td>33</td>
<td>28</td>
<td>55</td>
</tr>
<tr>
<td><strong>Other symptoms</strong></td>
<td>2 (2.6)</td>
<td>14 (20.9)</td>
<td>33 (15.6)</td>
</tr>
<tr>
<td>Endocarditis</td>
<td>1</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Fever</td>
<td>1</td>
<td>2</td>
<td>20</td>
</tr>
<tr>
<td>Neurological</td>
<td>0</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Uveitis</td>
<td>0</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>Skin papules</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Liver abnormalities</td>
<td>0</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Osteomyelitis</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td><strong>Other clinical findings</strong></td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>55 (25.9)</td>
</tr>
</tbody>
</table>

Reported neurological symptoms were encephalitis and meningitis; reported liver abnormalities were hepatomegaly and peliosis. Other reported clinical findings consisted of gut-related symptoms, chronic fatigue, and animal and tick bites.
diarrhoea, vomiting or nausea) or chronic fatigue (n = 9). Animal and tick bites were found in 16 and 2 patients respectively. As expected, axillary, inguinal, and head and neck lymphadenopathies were most frequently reported in the three different groups (Table 1).

**DISCUSSION**

*Bartonella henselae* serology is notoriously difficult, and is hampered by both low sensitivity and specificity of the assays when used in a routine laboratory setting. The PCR testing of samples from patients suspected of being infected is helpful, but not always feasible, since biopsy material is not always available. For these reasons, CSD diagnostics largely depend on serology.

The high seroprevalence of IgG in the general population hampers enormously the use of IgG for serodiagnosing *B. henselae* [29]. This IgG background was also found to increase with age. To determine the cut-off level, we used age-matched controls, leading to the inclusion of older age groups as well. We observed a wider background than that reported by others [4,19,21,23]. This might be partly explained by the wider age range of our control group than is usually seen (many studies use younger blood donors, for example). Alternatively, a higher background seroprevalence may exist in The Netherlands compared with studies performed in other areas. To be able to reach an acceptable specificity level, a high cut-off level was required, leading to sensitivity of only 36% in PCR-positive CSD patients. This sensitivity level was considered to be too low to offer the IgG ELISA as a routine test for the serodiagnosis of CSD in our laboratory. Further research is needed to improve the IgG assay for clinical purposes; in particular, improvement of the antigen preparation might be of value.

We reached a sensitivity of 56% with the IgM ELISA in a group of PCR-positive CSD patients. This sensitivity is low, but the method is still a valuable tool for serodiagnosing an active *B. henselae* infection. The low sensitivity will be difficult to increase, because the time range in which CSD patients develop symptoms can be relatively wide, and it is not unusual for patients to develop clinical symptoms more than 8 weeks after traumatic cat contact [13]. The IgM levels in patients who present late are already declining, and may even be under the detection limit of the assays. IgM stays present in serum for approximately 3 months [23,25,26]. The IgM ELISA was recently compared with an IFA protocol applied for the serodiagnosing of CSD in another laboratory [26]. The results showed comparable levels of sensitivity and specificity for both assays [26].

Little is known about the time scale during which PCR is successful after the onset of CSD. The sensitivity of PCR is reported to be between 58 and 96% [19,20]. The IgG distribution of the PCR-negative patient group resembled the distribution in the PCR-positive CSD patients, but the IgM distribution was, in general, lower. The response in the PCR-negative group was, however, significantly different from that of the control group and very comparable to the
responses observed in the PCR-positive group. In addition, *Bartonella*-specific IgM was detected (14.4%) in the PCR-negative patients, indicating recent infection. Because the IgG response persists longer than IgM, the most likely explanation is that the PCR-negative patient group also contains a high number of true CSD patients, but sample collection was probably too late to detect *Bartonella*-specific IgM in most cases. The seroprevalence for IgM (14.1%) and IgG (38.3%), with 78% positive for both IgM and IgG in the group of patients with a negative PCR result, clearly indicates that CSD cannot be excluded in patients with a negative PCR result. Not surprisingly, we detected an age-dependent IgM positivity rate in the 11- to 20-year age group (19%), which is consistent with the fact that it is mostly children and young adults who are affected by CSD. On the basis of IgM seropositivity, 46% of our identified CSD patients were between 0 and 20 years of age. This is lower than the 60% reported in the literature, again reflecting the lower sensitivity of IgM ELISA when used in a routine laboratory setting [13]. It is reported in the literature that CSD is more prevalent in the fall and the winter [14]. We also observed a peak during November and December. This effect is most likely the result of colder weather conditions (bringing cats and humans together inside the house).

The value of the investigation of follow-up samples is limited. The examination of sera from patients with negative or inconclusive results shows that most of them remain negative or inconclusive on follow-up (82%). Therefore, the analysis of a second sample is only recommended when intermediate IgM responses are detected, to separate infection from false-positive results. It could also be considered if the sample collection time is not optimal, especially in patients with a strong clinical suspicion of CSD.

Analysis of the reported symptoms in the PCR-positive group shows that our CSD population is, as expected, not clinically different from those described in the literature (97.4% lymphadenopathy and 2.6% atypical CSD infections). Results in the IgM-positive patients suspected of having CSD sent to our laboratory, however, show a much larger number of other reported symptoms. Sometimes these symptoms were related to CSD symptoms other than lymphadenopathy (15.6%), but other clinical findings were also reported (25.9%). This difference can be partly explained by the fact that biopsies for PCR analysis are not always (directly) available for some of these reported symptoms (for example, fever, chronic fatigue, eye or neurological infections). However, possible cross-reactivity with other pathogens cannot be excluded and should be further investigated. It is interesting that other symptoms previously not recognized as being related to *B. henselae* infection have recently been described [30]. It is possible that other symptoms that might be caused by *B. henselae* infection will be recognized in the future.

In conclusion, the detection of specific IgM in a patient suspected of CSD is of diagnostic value, although the overall sensitivity of this method is low under routine laboratory settings, especially when serum samples are collected late after the onset of disease. Unfortunately, many CSD patients are seen relatively late during the infection, when IgM levels are already declining or below the detection limit. Since early sample collection is crucial for the successful serodiagnosis of CSD, clinicians who suspect a patient of having CSD should send in a serum sample as soon as possible.
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REFERENCES


