Chapter 5

Evaluation of sensitivity, specificity and cross-reactivity in *Bartonella henselae* serology

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Submitted
Laboratory diagnosis of cat-scratch disease (CSD) mainly relies on serological testing for *Bartonella henselae* antibodies by indirect immunofluorescence (IFA) or enzyme-linked immunosorbent assay (ELISA). In this study the current diagnostic value of seven *B. henselae* serological tests was evaluated, including both commercial and in-house IFA’s as well as in-house ELISA. Fifty patients with proven CSD, based on the presence of lymphadenopathy, positive PCR and no other diagnosis, were compared to 55 controls initially suspected for CSD but tested negative in PCR and who ultimately received a different diagnosis. Cross-reactivity was tested in serum samples positive for infection with Epstein-Barr virus (n = 141), cytomegalovirus (n = 39), *Toxoplasma gondii* (n = 20), *Streptococcus pyogenes* (n = 54), *Chlamyphila pneumoniae* (n = 14) and *Coxiella burnetii* (n = 21).

Sensitivity was higher in the two IgG tests (88-98%) than in the IgM tests (50-64%). Adding genotype II strains to the IgM IFA did not improve sensitivity and resulted in higher cross-reactivity. Specificity was higher in the IFA IgM tests (up to 95-96%) than in the Focus IgG tests (69%). The concurrent use of both IgM and IgG Euroimmun IFA improved sensitivity to 92% with a specificity of 84%. Cross-reactivity in lymph node diseases was 0-5% in the Houston-strain IgM IFA’s, 0-8% in IgG IFA and 5-24% in the IgM ELISA. Cross-reactivity was 0-36% in *C. pneumoniae* and 13-30% in *C. burnetii*, depending on the test. The results of this study indicate that the combined use of IgM and IgG IFA’s based on co-cultivation can be useful in serodiagnosis of CSD.
INTRODUCTION

The laboratory diagnosis of cat-scratch disease (CSD), caused by *Bartonella henselae* (*B. henselae*) infection, is mainly based on serological testing by indirect immunofluorescence assay (IFA) and enzyme-linked immunoassay (ELISA) and PCR [1-5]. *B. henselae* IgG detection for diagnosing CSD is often reported to have low specificity [2-6], with high seroprevalence (up to 66%) reported in the normal population. This could be due to cross-reactivity, but no large studies have evaluated this yet [5]. In fact, cross-reactivity has only been clearly confirmed with *B. quintana* and *Coxiella burnetii* [7,8]. *B. henselae* IgM detection for diagnosing CSD is often reported to have low sensitivity, that might be due to regional distribution of different *Bartonella* genotypes [2-6,9]. For example, in Dutch CSD patients, both *B. henselae* 16S genotype I (serotype Houston I) and genotype II (corresponding to serotype Marseille) have been found [10]. Currently applied serological tests only include genotype I (ATCC 49882) and might improve in sensitivity by addition of genotype II strains [3].

The aim of this study was to evaluate a new IFA, using both genotype I and II *B. henselae* strains and to compare the sensitivity, specificity and cross-reactivity of different serological assays that are used in The Netherlands.

MATERIALS AND METHODS

Patients and samples

Laboratory testing for sensitivity and specificity was completed using serum samples from a *B. henselae* patient and control panel as described earlier [3]. From that panel, one patient and one control were excluded, as no serum was available for retesting. In summary, *B. henselae* patients (n = 50) were selected based on the following criteria: lymphadenopathy, a positive PCR for *B. henselae* on lymph node material and exclusion of other causes of lymphadenopathy. The control group (n = 55) was selected from patients who initially presented with symptoms suspected for diagnosis of CSD, but ultimately were diagnosed having another disease with negative *B. henselae* PCR on lymph node material. The diagnoses in the control group were infection (36%), malignancies (38%), immunological disorders (13%), congenital cyst/fistula (9%) and other (4%) [3]. Of the 20 controls with infections, 9 were infected with atypical Mycobacteria, 4 with *Mycobacterium tuberculosis*, 3 with *Staphylococcus aureus*, and 4 with other microorganisms. No statistical differences were found between the CSD and control group regarding age (median of 27 years with a range 0-83) and gender (63% were male).

To assess the presence of cross-reactivity, serum samples were selected from patients with laboratory proven causes of lymphadenopathy, i.e. Epstein-Barr virus (EBV) (n = 141), cytomegalovirus (CMV) (n = 39), *Toxoplasma gondii* (n = 20) and *Streptococcus pyogenes* (n = 54). Additionally, sera were selected from patients who tested positive for two diseases.
that are previously suggested to cause cross-reactivity in Bartonella serology: *Chlamydophila pneumoniae* (n = 14) and *Coxiella burnetii* (n = 21). As no material was left to perform all tests, tests E and F were performed in 30 other serum samples of *Coxiella burnetii* positive patients. All sera were anonymised and stored at minus 20 °C.

**Laboratory techniques**

In total, seven serological tests (Test A to G) were performed to detect *B. henselae* antibodies, 5 tests for IgM and 2 for IgG detection (Table 1). Of these 7 tests, 5 were commercial tests and 2 were in-house prepared, 6 were an IFA and one an ELISA. All tests were prepared with *B. henselae* genotype I (ATCC 49882, Houston-I strain), with the exception of Test A which was prepared with *B. henselae* genotype II (‘Marseille strain’). All commercial tests were prepared with *B. henselae* strains co-cultivated with mammalian cells. Test A, B, C, and F were available from Euroimmun (Euroimmun AG., Lübeck, Germany) and Test G from Focus (Focus Technologies, Cypress, CA, USA).

The three commercial tests for IgM were combined in one test kit for this study, with slides containing mosaics of 4 ‘biochips’ with Test A, B, C and non-infected cells (negative control). The Euroimmun IgG test (Fl 219b-1005G) was used in this study for both IgM detection (Test C) and IgG detection (Test F). For that, Test C was performed and judged conform the other Euroimmun IgM tests (A and B), according to the manufacturer’s instructions (Fl 219b-1005M). This included application of immunoabsorption solution (Eurosorb, Euroimmun ZF 1270-0145) ultimately resulting in a 1:100 serum dilution. For IgG detection, two commercial IFA tests were used (Test F and G) according to the manufacturer’s instructions. Thus, Test F (Euroimmun, Fl 219b-1005G) was used with a 1:320 serum dilution and Test G (Focus, IF1300G) with a 1:64 serum dilution.

The two in-house tests used *B. henselae* strains with no co-cultivation. Test D is a validated in-house IFA for anti-*B. henselae* IgM detection, that was prepared as described earlier with a 1:8 serum dilution [2, 3]. All IFA tests were performed in the Regional Public Health Laboratory, Tilburg, The Netherlands. The validated in-house ELISA test (Test E) was prepared as described earlier for IgM detection [3] and was performed in the National Institute for Public Health and the Environment, Bilthoven, The Netherlands.

PCR detection of *B. henselae* DNA was used as described earlier on lymph node material of the CSD patients [3, 10]. This 16S rRNA genotype-specific PCR was performed to detect the *B. henselae* type I and type II genotypes in pus aspirates and lymph node biopsy specimens [3,10,11].

**Data analysis**

The data were analysed using SPSS 17.0 for Windows (SPSS Inc., Chicago, Illinois, USA). Sensitivity and specificity were calculated for each test. Categorical data were compared by use of the Pearson’s Chi-square Test or, when necessary, the Fisher’s Exact Test and continuous variables by use of the independent-samples t-Test. A two-sided p-value of < 0.05 was considered statistically significant.
RESULTS

The characteristics of the serological tests are shown in Table 1. Sensitivity was 50-64% in the IgM tests (A-E) which was significantly lower than in the IgG tests (88 and 98% for F and G respectively) (p < 0.01). Specificity was significantly higher in the IgM tests (87-96%) than in Test G (69%, p < 0.05), but not significantly higher than in Test F (89%, p = 0.3-0.8). Although sensitivity and specificity seemed to be lower in Test A ('Marseille strain'), differences were not statistically significant when compared to the other IgM tests.

Table 1.
Characteristics of B. henselae serological tests, as performed in 50 cat-scratch disease patients and 55 controls (with CSD-like symptoms, but no CSD).

<table>
<thead>
<tr>
<th>Test</th>
<th>Antibody</th>
<th>Assay</th>
<th>Manuf.</th>
<th>Strain</th>
<th>Co-cult.</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test A</td>
<td>IgM</td>
<td>IFA</td>
<td>Euroimmun</td>
<td>M.</td>
<td>yes</td>
<td>50%</td>
<td>87%</td>
</tr>
<tr>
<td>Test B</td>
<td>IgM</td>
<td>IFA</td>
<td>Euroimmun</td>
<td>H.</td>
<td>yes</td>
<td>54%</td>
<td>96%</td>
</tr>
<tr>
<td>Test C</td>
<td>IgM</td>
<td>IFA</td>
<td>Euroimmun</td>
<td>H.</td>
<td>yes</td>
<td>64%</td>
<td>95%</td>
</tr>
<tr>
<td>Test D</td>
<td>IgM</td>
<td>IFA</td>
<td>In-house</td>
<td>H.</td>
<td>no</td>
<td>54%</td>
<td>93%</td>
</tr>
<tr>
<td>Test E</td>
<td>IgM</td>
<td>ELISA</td>
<td>In-house</td>
<td>H.</td>
<td>no</td>
<td>62%</td>
<td>91%</td>
</tr>
<tr>
<td>Test F</td>
<td>IgG</td>
<td>IFA</td>
<td>Euroimmun</td>
<td>H.</td>
<td>yes</td>
<td>88%</td>
<td>89%</td>
</tr>
<tr>
<td>Test G</td>
<td>IgG</td>
<td>IFA</td>
<td>Focus</td>
<td>H.</td>
<td>yes</td>
<td>98%</td>
<td>69%</td>
</tr>
</tbody>
</table>

<sup>a</sup> Test C is a test for IgG detection but in this study used for IgM detection; <sup>b</sup> Data published earlier [3]. Manuf. indicates manufacturer, Co-cult. co-cultivation, IFA Immunofluorescence assay, M. B. henselae Marseille strain, H. B. henselae Houston strain.

A calculation was made combining the results of the Marseille and Houston-1 strain IgM tests, defining positivity as one test or both tests being positive. Combining the Marseille (Test A) and the Houston IgM IFAs (B and C) did not significantly increase sensitivity. The sensitivity was 64% for Test A and B and 72% for Test A and C, with a specificity of 87% for both combinations. Combining the two commercial IgM Houston strain IFAs (B and C) resulted in a sensitivity of 66% with a specificity of 95%, which was not a significant improvement compared to the use of one single test.

Combining IgM and IgG resulted in increased sensitivity, while decreasing specificity. Combining Test C and F increased sensitivity to 92% (p = 0.001), at a decrease in specificity to 84% (not significant compared to Test C alone; p = 0.07). Other combinations of IgM and IgG tests gave significant reductions in specificity. For example, sensitivity of Test B and G increased to 98% (p < 0.001) while specificity decreased to 66% (p < 0.001). Subanalyses were made for the CSD patients infected with B. henselae genotype I and genotype II. PCR in lymph node material determined that 31 CSD patients were infected with B. henselae genotype I (Houston-I strain), and 15 with genotype II (Marseille strain).
In 4 patients, material was no longer available or genotype analysis was not possible [3].

Sensitivity of Test A (Marseille strain, genotype II) was not higher in the genotype II infected patients than in genotype I, as shown in Table 2. In fact, sensitivity for Test A was higher in the patients infected with genotype I than in genotype II (p < 0.01), as it was for Test B (p = 0.03) and C (p < 0.01). No significant differences between the genotype I and II infected groups were found in the other tests.

**Cross-reactivity**
Table 3 shows the percentage of sera in the cross-reactivity study that were positive in the different *B. henselae* serological tests. In the sera of patients with lymph node diseases (EBV, CMV, *T. gondii*, *S. pyogenes*) cross-reactivity was lowest for Test B, C and D (0-5%). Test A and E showed the highest cross-reactivity, with values varying from 3-20% and 5-36% respectively for different patient groups. In the *C. burnetii* sera, cross-reactivity occurs in all tests more often than in the other samples, varying from 13-30% with no significant differences between the different tests.

None of the 289 serum samples tested positive in all tests. Seventy-eight percent of the positive samples was positive in only one single test. Agreement between the tests varied from 79-99% in the positive samples and was highest between the Houston-strain IgM IFA’s (93-99%).

**Table 2.**
Sensitivity of the Marseille strain test (A) and the Houston strain tests (B and C), for patients infected by *B. henselae* genotype I (Houston) and genotype II (Marseille).

<table>
<thead>
<tr>
<th></th>
<th>Genotype I (n = 31)</th>
<th>Genotype II (n = 15)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test A</td>
<td>68%</td>
<td>27%</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Test B</td>
<td>68%</td>
<td>33%</td>
<td>0.03</td>
</tr>
<tr>
<td>Test C</td>
<td>81%</td>
<td>40%</td>
<td>&lt; 0.01</td>
</tr>
</tbody>
</table>
In this study seven different serological assays were evaluated for diagnosis of CSD, in terms of sensitivity, specificity and cross-reactivity. Sensitivity is low in all tested IgM assays (50-64%), and does not improve by adding Marseille (genotype II) strain to the assay. Sensitivity of the Marseille strain test was even lower in patients infected with the Marseille strain than in those with Houston strain. This finding suggests that genetypic differences in the 16S RNA gene may not always reflect significant antigenetic differences. Alternatively, the Marseille strain may show less antigen expression than the worldwide used Houston strain. Sensitivity is significantly higher in the IgG tests (88-98%) than in the IgM tests. This can be explained by IgM being detectable earlier and for a shorter period than IgG, making the moment of sample taking of importance [2,3,6,8,12]. Furthermore, some patients appear to show no IgM response to *B. henselae* infection at all, as described by Bergmans et al. and others [2,12].

Specificity is higher in the IFA IgM tests (up to 95-96%) than in the Focus IgG test (Test G) (69%), but not significantly higher than the Euroimmun IgG test (Test F) (89%). Earlier reports differ widely, with high specificity reported in studies using healthy donors as controls [5]. In contrast, we used non-healthy controls who were initially suspected for CSD, but with different diagnosis and negative PCR. As this is the group of patients that need to be discriminated in CSD patients, this study clarifies that specificity in *B. henselae* serology is still not ideal in clinical practice. The additional value of IgG detection compared to IgM detection alone should be critically reviewed. The subsequent reduction of specificity from 95% to 84% (p = 0.07) may be significant in a larger study population and can be clinically relevant in patients with other serious pathologies. Therefore, *B. henselae* IgG detection in

<table>
<thead>
<tr>
<th>Cause of infection</th>
<th>Test A</th>
<th>Test B</th>
<th>Test C</th>
<th>Test D</th>
<th>Test E</th>
<th>Test F</th>
</tr>
</thead>
<tbody>
<tr>
<td>EBV</td>
<td>141</td>
<td>9%^BD</td>
<td>1%</td>
<td>1%</td>
<td>24%^**</td>
<td>8%^BD</td>
</tr>
<tr>
<td>CMV</td>
<td>39</td>
<td>3%</td>
<td>0%</td>
<td>0%</td>
<td>24%^**</td>
<td>5%</td>
</tr>
<tr>
<td><em>Toxoplasma gondii</em></td>
<td>20</td>
<td>20%</td>
<td>0%</td>
<td>0%</td>
<td>5%</td>
<td>0%</td>
</tr>
<tr>
<td><em>Streptococcus pyogenes</em></td>
<td>54</td>
<td>19%^BCDF</td>
<td>2%</td>
<td>4%</td>
<td>8%</td>
<td>2%</td>
</tr>
<tr>
<td><em>Chlamydia pneumoniae</em></td>
<td>14</td>
<td>14%</td>
<td>0%</td>
<td>7%</td>
<td>36%^ED</td>
<td>0%</td>
</tr>
<tr>
<td><em>Coxiella burnetii</em></td>
<td>21</td>
<td>20%</td>
<td>20%</td>
<td>30%</td>
<td>15%</td>
<td>20%&lt;^</td>
</tr>
</tbody>
</table>

Table 3. Serological cross-reactivity in *B. henselae* IgM serology in patients with different infections.

N, number of tested serum samples; *(A,B,..)* significantly higher than mentioned test; ** significantly higher than all other test; ^ due to limited amount of material, this was performed in 30 other Coxiella sera.
a single sample is not standard practice in our laboratories and is applied only in selected cases. In 2001 Sander et al. [5] reviewed 19 studies on IgG serology from 11 different countries and reported that sensitivity varied from 14-100% and specificity from 34-100% depending on the studied patient and control groups, the used tests and procedures. Other studies confirm that serological tests are difficult to compare [4,13].

Cross-reactivity in sera of patients with other lymph node diseases is low (0-5%) in the Houston-strain IgM IFA’s, but high in the IgM ELISA (5-24%) depending on the tested patient group. Cross-reactivity in IgG IFA’s was 0-8% in the lymph node diseases, which was not significantly higher than in the IgM IFA’s. Cross-reactivity was most frequent in the C. burnetii sera (13-30%) that might be explained by antigenetic similarity [14]. This finding is of limited clinical relevance as these patients, as well as those with C. pneumoniae infection, do not present with symptoms similarly to classic CSD.

The patient groups in the cross-reactivity study were selected based on serological diagnoses that were not confirmed by culture or PCR. We think it is unlikely that we coincidently selected CSD patients falsely diagnosed by cross-reacting B. henselae antibodies. Further analyses revealed that agreement was low in the positive samples, with no sample being positive in all B. henselae tests. The differences between the tests may be explained by different antigen preparation, e.g. in the ELISA’s sonification of bacteria may increase antigen expression compared to the IFA’s using intact bacteria. The two patients that tested positive by the in-house IgM IFA were further analysed. Both patients, one positive for EBV and one for S. pyogenes, had lymphadenopathy and cat contact and may have had CSD. In the EBV case, polyclonal B-cell stimulation may have played a role. We have no additional information about the positive patients in the other tests. This study shows that cross-reactivity is dependent on the test and should be taken into account when interpreting B. henselae serology. A limitation of this study is that we did not test sera of patients with tuberculosis, atypical mycobacteria or malignancies, although they were represented in small numbers in the control group. Insight in cross-reactivity in large groups of patients suffering these more serious diseases would certainly be of clinical importance.

The number of earlier reports on cross-reactivity in B. henselae serology is limited. Zbinden et al. reported on cross-reactivity in IFA in 20 EBV positive patients, reporting 95% and 45% B. henselae IgM positivity in two different IFA protocols [15]. Studies on cross-reactivity in IFA IgG are difficult to summarise as most report on small numbers. Sander et al. found low cross-reactivity in IgG IFA to EBV (2 out of 9), CMV (0/16), T. gondii (0/15) and C. pneumoniae (3/15), and concluded that cross-reactivity plays no important role in B. henselae serology [7]. Giladi et al. found no cross-reactivity by the use of IgM and IgG ELISA in 46 EBV, 9 CMV and 31 T. gondii patients [4]. In comparison to previous studies, our study offers the advantage of a larger study population and the comparison of different tests.

In conclusion, the diagnostic performance of serology for CSD is less than ideal. Adding genotype II does not improve the performance of the test. The combined use of IFA IgM and IgG improves sensitivity, although specificity is probably reduced. As falsely diagnosing
CSD can have major impact for the patient, we believe that IgG results must be interpreted with caution. Cross-reactivity is low in the tested IgM and IgG IFA’s, but should be taken into account when interpreting CSD serological results in clinical practice. In difficult cases we advise the exclusion of differential diagnoses, repeated serological testing after a few weeks or additional use of PCR.

ACKNOWLEDGEMENTS

We would like to thank L. Broers, I. Michelbrink-Hooijen, G. van den Hardenberg and M. Kuijer for performing the tests and J. Gaultney for critically reviewing the statistical methods and the manuscript. We thank Prof D. Raoult for making the Marseille strain available.

TRANSPARENCY DECLARATION

Data were generated as part of routine laboratory activities without any funding. All authors have no commercial relationship or potential conflict of interest to disclose.
REFERENCES