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

The laboratory diagnosis of cat-scratch disease (CSD), caused by *Bartonella henselae* infection, is mainly based on serological testing by indirect immunofluorescence assay (IFA) or ELISA, and by PCR (Bergmans et al., 1997; Sander et al., 2001). However, the sensitivity and specificity of the serological tests for diagnosing CSD are often reported to be low. The low specificity of *B. henselae* IgG detection is related to high seroprevalence (reported up to 66 %) in the normal population (Sander et al., 2001). This could be due to cross-reactivity, which has been reported in patients with *Coxiella burnetii* infection, *Chlamydia pneumoniae* infection and in patients with non-*henselae* Bartonella infections (Avidor et al., 2004; La Scola & Raoult, 1996; Maurin et al., 1997). Cross-reactivity in *B. henselae* serology has not yet been evaluated in large studies (Sander et al., 2001). The reported low sensitivity of *B. henselae* IgM tests might be due to regional distribution of different *Bartonella* genotypes. For example, in Dutch CSD patients, both *B. henselae* genotype I (serotype Houston I) and genotype II (corresponding to serotype Marseille) have been found (Bergmans et al., 1996). Currently employed serological tests only include genotype I (ATCC 49882) and sensitivity might improve by addition of genotype II strains (Vermeulen et al., 2007). The aim of this study was to evaluate a new commercial 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.

*B. henselae* patients (*n*=50) were selected based on the following criteria: lymphadenopathy, a positive PCR for *B. henselae* with lymph node material and exclusion of other causes of lymphadenopathy. The control group (*n*=55) was selected from patients who initially presented with symptoms consistent with a diagnosis of CSD, but who had negative *B. henselae* PCR with lymph node material and for whom another disease was diagnosed ultimately. The final diagnoses in the control group were infection (36 %), malignancies (38 %), immunological disorders (13 %), congenital cyst/fistula (9 %) and other (4 %) (Vermeulen et al., 2007). Of the 20 controls with infections, 9 were infected with an atypical *Mycobacterium*, 4 with *Mycobacterium tuberculosis*, 3 with *Staphylococcus aureus* and 4 with other micro-organisms. No statistically significant differences were found between the CSD group and control group regarding age (median of 22 and 34 years, respectively) and gender (70 and 58 % male, respectively).

To assess the presence of cross-reactivity, serum samples were selected based on serological diagnosis from patients with infections associated with lymphadenopathy: Epstein–Barr virus (EBV) (viral capsid antigen IgM positive, *n*=141), cytomegalovirus (CMV) (IgM positive, *n*=39), *Toxoplasma gondii* (IgM positive, *n*=20) and *Streptococcus pyogenes* (antistreptolysin O titre ≥ 1 : 800, *n*=54). Additionally, sera were selected from patients who tested positive for *Chlamydia pneumoniae* (IgM positive, *n*=14) and *Coxiella burnetii* (phase 2 antibodies detected by complement fixation, *n*=21). Since the amount of stored *Coxiella burnetii* serum samples was insufficient for all serological assays, two assays (D and E, described below) were performed on an additional 30 new *Coxiella burnetii*-positive serum samples.

The characteristics of the six *B. henselae* serological tests (test A to F) performed in this study are shown in Table 1. Test A is a commercial IgM IFA (Euroimmun; Fl 219b-1005G) containing co-cultivated Houston-1 *B. henselae* strains. Test D is a validated in-house prepared IgM IFA with agar-derived Houston-1 *B. henselae* strains, performed as described previously (Bergmans et al., 1997; Vermeulen et al., 2007). Test E is a commercial IgG IFA (Euroimmun; Fl 219b-1005G) containing co-cultivated Houston-1 *B. henselae* strains. All commercial tests were performed according to the manufacturer’s instructions.

PCR detection of *B. henselae* DNA was used as described elsewhere with lymph node material from the CSD patients (Bergmans et al., 1996). 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.

The sensitivity and specificity results of six serological assays for diagnosis of CSD are presented in Table 1. Sensitivity was only 50–62 % in the IgM tests (A–D), which was significantly lower than in the IgG tests (88 and 98 % for E and F, respectively, *P*<0.01). This can be explained by IgM being detectable earlier and for a shorter period than IgG, making the moment of sample taking of importance.

Furthermore, some patients may show no apparent IgM response to *B. henselae* infection at all (Bergmans et al., 1997; Metzker-Gotter et al., 2003).

Specificity was significantly higher in the IgM tests (87–96 %) than in the Focus IgG manufactured by Euroimmun with co-cultivated *B. henselae* culture ('Marseille strain'). Test A and B were combined in one test kit for this study with slides containing mosaics of ‘biochips’ with test A, B and non-infected cells (negative controls).
test (test F; 69 %, \( P<0.05 \)), but not significantly higher than in the Euroimmun IgG test (test E; 89 %). Earlier reports differ widely, with high specificity reported in studies using healthy donors as controls. Sander et al. (2001) reviewed 19 studies on IgG serology from 11 different countries and reported that sensitivity varied from 14 to 100 % and specificity from 34 to 100 % depending on the patient and control groups studied, tests and procedures.

When combining IgM-positive results and/or IgG-positive results for CSD diagnosis, sensitivity was increased while specificity was decreased, compared to the use of a single test. For example, the combination of test A (IgM) with test E (IgG) increased sensitivity to 90 % (\( P<0.001 \), compared to test A alone), while specificity decreased to 86 % (\( P<0.05 \)). Combining test A with test F resulted in sensitivity of 98 % (\( P<0.001 \)) and specificity of 66 % (\( P<0.001 \)). This reduction of specificity is clinically relevant in patients with other serious pathologies.

Sensitivity and specificity did not improve by use of the Marseille strain IgM IFA (test B) (Table 1). Also combining the commercial Houston-1 and Marseille strain IgM tests (test A and B), defining positivity as a positive result of test A and/or test B, did not significantly change sensitivity (64 %) or specificity (87 %) when compared to a single test.

We further analysed the results for the two different genotypes. A total of 31 CSD patients were infected with \( B. \) henselae genotype I (Houston-1 strain), 15 with genotype II (Marseille strain) and for 4 patients genotype analysis was not possible, based on lymph node PCR (Vermeulen et al., 2007). The commercial Houston strain (test A) and the Marseille strain (test B) IgM IFA showed higher sensitivity in the patients infected with the Houston strain (68 and 68 %, respectively) than in those with Marseille strain (33 and 27 %, \( P<0.05 \)). No significant differences between the two patient groups were found in the other tests (C–F). The lower sensitivity of the Marseille strain test, even found in patients infected with the Marseille strain, suggests that genotypic differences in the 16S rRNA gene do not reflect significant antigenic differences. Maybe this strain shows less antigen expression under cultivation conditions than the Houston strain used worldwide.

Table 2 shows the percentage of sera in the cross-reactivity study that were positive in the different \( B. \) henselae serological tests. Because of the low specificity (69 %) we considered test F not reliable for clinical use in our laboratory and did not evaluate it for cross-reactivity. Cross-reactivity was most common in the \( Coxiella \) burnetii sera, in accordance with earlier reports (Graham et al., 2000).

Early reports on cross-reactivity in \( B. \) henselae serology generally describe case reports or small studies and are difficult to compare. Zbinden et al. (1998) reported on cross-reactivity in IFA in 20 EBV positive patients, reporting 95 and 45 % \( B. \) henselae IgM positivity in two different IFA protocols. Sander et al. (2001) found low cross-reactivity in IgG IFA to EBV (2/9), CMV (0/16), \( T. \) gondii (0/15) and \( Chlamydia \) pneumoniae (3/15), and concluded that cross-reactivity does not play an important role in \( B. \) henselae serology. Giladi et al. (2001) found no cross-reactivity by the use of IgM and IgG ELISA in 46 EBV, 9 CMV and 31 \( T. \) gondii patients. In comparison to previous studies, our work offers the advantage of a larger study population and the comparison of different tests.

The patient groups in our cross-reactivity study were selected based on serological diagnoses that were not confirmed by culture or PCR. In the positive samples agreement between the tests varied from 79 to 99 %, with no sample being positive in all \( B. \) henselae tests. Therefore, we think it is unlikely that we coincidently selected CSD patients falsely diagnosed by cross-reacting \( B. \) henselae antibodies. However, in the two patients who tested positive by the in-house IgM IFA (test C) CSD cannot be excluded. Both patients, one positive for EBV and one for \( S. \) pyogenes, had lymphadenopathy and cat contact, and may have had CSD. However, polyclonal B-cell stimulation may have played a role in the EBV case. Still, the significant differences in cross-reactivity between the tests remain unexplained. Differences in antigen preparation may play a role, e.g. sonification of bacteria in the ELISA may increase antigen expression compared to the use of intact bacteria in the IFA.

In conclusion, this study underscores the difficulties of serodiagnosis of CSD by in-house and commercial tests. The combined use of (Houston strain) IFA IgM and IgG improves sensitivity with significant reduction of specificity. As falsely diagnosing CSD can have a major impact for the patient, we believe that IgG results must be interpreted with caution.

### Table 1. Results of serological tests performed for 50 CSD patients and 55 controls without CSD

<table>
<thead>
<tr>
<th>Test</th>
<th>Antibody</th>
<th>Assay</th>
<th>Manufacturer</th>
<th>Strain</th>
<th>Co-cultivation</th>
<th>Cut-off</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test A</td>
<td>IgM</td>
<td>FIA</td>
<td>Euroimmun</td>
<td>Houston</td>
<td>Yes</td>
<td>1:100*</td>
<td>54</td>
<td>96</td>
</tr>
<tr>
<td>Test B</td>
<td>IgM</td>
<td>FIA</td>
<td>Euroimmun</td>
<td>Marseille</td>
<td>Yes</td>
<td>1:100*</td>
<td>50</td>
<td>87</td>
</tr>
<tr>
<td>Test C</td>
<td>IgM</td>
<td>FIA</td>
<td>In-house</td>
<td>Houston</td>
<td>No</td>
<td>1:8†</td>
<td>54†</td>
<td>93†</td>
</tr>
<tr>
<td>Test D</td>
<td>IgM</td>
<td>ELISA</td>
<td>In-house</td>
<td>Houston</td>
<td>No</td>
<td>Ratio &gt;1†</td>
<td>62†</td>
<td>91†</td>
</tr>
<tr>
<td>Test E</td>
<td>IgG</td>
<td>FIA</td>
<td>Euroimmun</td>
<td>Houston</td>
<td>Yes</td>
<td>1:320*</td>
<td>88</td>
<td>89</td>
</tr>
<tr>
<td>Test F</td>
<td>IgG</td>
<td>FIA</td>
<td>Focus</td>
<td>Houston</td>
<td>Yes</td>
<td>1:64*</td>
<td>98</td>
<td>69</td>
</tr>
</tbody>
</table>

*According to the manufacturer’s instructions.
†As described previously (Vermeulen et al., 2007).
The use of an IFA based on Marseille strain *B. henselae* (genotype II) for IgM detection showed no additional value. Serological cross-reactivity is dependent on the test and can be high in certain patient groups.

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### Table 2. Serological cross-reactivity: results of five *B. henselae* tests performed with sera from patients with various infections

<table>
<thead>
<tr>
<th>Cause of infection</th>
<th>n</th>
<th>Test A (%)</th>
<th>Test B (%)†</th>
<th>Test C (%)</th>
<th>Test D (%)†</th>
<th>Test E (%)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>EBV</td>
<td>141</td>
<td>1</td>
<td>9*AC</td>
<td>1</td>
<td>24**</td>
<td>8*AC</td>
</tr>
<tr>
<td>CMV</td>
<td>39</td>
<td>0</td>
<td>3</td>
<td>0</td>
<td>24**</td>
<td>5</td>
</tr>
<tr>
<td>Toxoplasma gondii</td>
<td>20</td>
<td>0</td>
<td>20</td>
<td>0</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Streptococcus pyogenes</td>
<td>54</td>
<td>2</td>
<td>19*ACE</td>
<td>2</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>Chlamydia pneumoniae</td>
<td>14</td>
<td>0</td>
<td>14</td>
<td>0</td>
<td>36*ACE</td>
<td>0</td>
</tr>
<tr>
<td>Coxiella burnetii</td>
<td>21</td>
<td>20</td>
<td>20</td>
<td>15</td>
<td>20‡</td>
<td>13‡</td>
</tr>
</tbody>
</table>

n, Number of serum samples tested.

†Significance is indicated as follows: **AC significantly higher than test A and C; *ACE significantly higher than tests A, C and E; ** significantly higher than all other tests.

‡Due to a limited amount of material, this test was performed using 30 additional *Coxiella burnetii* sera.