Chapter 10

General discussion
GENERAL DISCUSSION

The aim of this thesis was to evaluate and improve the current laboratory techniques for the diagnosis of infection with *B. henselae* and to contribute to the knowledge of the clinical spectrum of cat-scratch disease (CSD).

The diagnostic value of *B. henselae* serology and its pitfalls

Studies in this thesis revealed that serological testing for *B. henselae* infection by immunofluorescence assay (IFA) and enzyme-linked immunosorbent assay (ELISA) is generally hampered by low sensitivity and specificity and by cross-reactivity. It is concluded that detection of anti-*B. henselae* IgM antibodies by the use of IFA and ELISA shows low sensitivity (50-64%) when cut-offs were set at high specificity. However, due to acceptable positive predictive values, we consider anti-*B. henselae* IgM detection to remain useful in clinical practice. However, negative IgM does not exclude CSD. In patients infected with genotype II, which constituted about one-third of our CSD population, sensitivity of serological testing tended to be lower. This could be due to the use of the genotype I reference strain in the tests. However, our attempt to improve sensitivity of IFA-based IgM serology by the use of *B. henselae* genotype II (Marseille strain) failed. Remarkably, sensitivity was lower in the Marseille IFA than in the Houston IFA, even in Marseille strain infected patients. This generates new questions regarding the differences between the genotypes in antigenic expression and requires further study.

The value of anti-*B. henselae* IgG detection in CSD diagnosis remains controversial. In our studies, we showed that detection of IgG had low specificity (69-91%) and may reflect either past exposure to *B. henselae* or cross-reactivity rather than recent infection. Anti-*B. henselae* IgG seroprevalence was high in the normal population and increased with age. We showed that combining IgM and IgG results, compared to IgM alone, improved sensitivity at the cost of specificity in CSD diagnosis. This reduction of specificity may be crucial in clinical practice, as it can potentially lead to false positive results and delay of the correct diagnosis. We showed that the value of IgG detection is age-dependent. Its ability to predict recent *B. henselae* infection was highest for patients below 20 years of age. A diagnostic model using both IgM and IgG results that also takes age into account might be clinically useful but still needs to be validated in a prospectively selected group of patients and controls. Worldwide, most laboratories use IgG detection alone for diagnosis of acute CSD. We criticize this policy and state that IgG results must be interpreted with great caution.

The moment of sample collection for serological testing relative to disease onset is important. We showed that IgM sensitivity was highest within 6 weeks after onset of symptoms, with
a sharp decrease after 8 weeks, while IgG sensitivity peaked at 6-8 weeks and then slowly decreased. Earlier studies have shown that IgG can remain positive over 1 year in about a quarter of patients [1,2]. In general practice, most CSD patients present with mild symptoms and therefore diagnostic tests are often initiated weeks to months after the initial symptoms. This late diagnostic approach, which is inherent to the mild course of the disease, is bound to have a negative effect on the value of serological testing.

Advice on repeated serological testing in cases of negative or inconclusive serology requires insight into the individual course of illness. We showed that in 82% of cases, consecutive samples revealed no subsequent seroconversion of anti-*B. henselae* antibodies. Repeated serology only seems useful in cases of strong suspicion of CSD and should be done preferably within 2 months after onset of disease.

Seroprevalence in the normal population differs per country and region, but is also likely to be influenced by the serological techniques used and the cut-off values. IgG seroprevalence is reported to be as high as 30-66% in some European countries, emphasizing the problem of discriminating CSD patients from patients with other diseases by the use of IgG [3,4]. These high seroprevalences are unlikely to reflect past infection in the majority of cases, as the earlier mentioned studies on antibody kinetics have shown that IgG disappeared after 1 year in about three quarters of proven CSD patients [1,2]. The high rate of seropositivity is more likely to be the result of cross-reactivity. In this thesis we were able to show that cross-reactivity is 0-5% and 0-8%, respectively, for *B. henselae* IgM and IgG IFA’s in Epstein-Barr virus, cytomegalovirus, *Toxoplasma gondii*, and *Streptococcus pyogenes*. We found that cross-reactivity in IgM serology can be as high as 30% and 36% in *Coxiella burnetii* and *Chlamydia pneumoniae* patients, respectively, in certain tests. These last two diseases usually do not manifest with lymphadenopathy, making cross-reactivity, therefore, less relevant in clinical practice. However, it is known that CSD can present as fever of unknown origin, which may clinically resemble Q-fever (*C. burnetii*). We did not study the role of aspecific reacting antibodies that may contribute to the high seroprevalence.

The low sensitivity and specificity combined with the presence of cross-reactivity found in our studies pose questions about the value of the numerous published seroprevalence studies. Studies on seroprevalence in different populations, patient groups and animals often lack critical remarks on the reliability of the used tests. Also, the choice of the control group strongly influences the results and should be critically taken into account when comparing data from different studies. High IgM or IgG seroprevalence is often interpreted as proof of (recent) infection, but cross-reactivity is also a known phenomenon. This is illustrated by our prospective study on Henoch Schönlein purpura (HSP) patients that used both serology and PCR and did not find any evidence for an etiological role for *B. henselae* in HSP. This was in contrast to earlier reports that found higher IgG seroprevalence in HSP patients than in controls [5,6]. In our opinion, these earlier results merely reflect the pitfalls of IgG serology and selection bias rather than a link between *B. henselae* and HSP.
In conclusion, *B. henselae* serology has many pitfalls and is still far from ideal at the moment. Interpreting serological results requires insight into the test characteristics as well as the clinical aspects of the patient's disease. Keeping the quality of serological tests high requires a continuous and labor-intensive process of further development and repeated evaluation of the used tests. Availability of commercial products may enhance quality assurance and may stimulate comparison of different populations and laboratories. However, commercial tests also need to be evaluated and validated in clinical settings.

The value of PCR in the diagnosis of CSD

Detection of *B. henselae* DNA in pus or tissue samples by PCR is highly specific and is increasingly used for CSD diagnostics, especially when serological results are inconclusive. Sensitivity of PCR is often presumed to be very high but is variably reported from 43-100% [1,7-11]. Results in this thesis may suggest that sensitivity can be low in clinical practice. In one of our studies, we found IgM and IgG serological profiles in PCR negative patients (IgM positivity rate of 14% for IgM and 38% for IgG) that resemble the profiles of CSD patients more closely than that of negative controls. This suggests that negative PCR can be found in CSD patients and that sensitivity is probably lower than previously assumed due to timing of sample collection or sampling error. In other words, because we know that PCR is able to detect even small amounts of *B. henselae* DNA, negative testing suggests that these materials are obtained either from a CSD patient in a phase when the bacteria are no longer present or from non-infected tissue. The optimal timing of PCR is probably within 6 weeks after disease onset but requires further investigation [12].

The main disadvantage of the PCR technique in CSD diagnosis is the need for an invasive sample by means of lymphadenectomy, needle aspiration or biopsy. It was earlier shown that *B. henselae* DNA was detectable in blood samples by the use of PCR in 10% of patients with human immunodeficiency virus (HIV) and in an immunocompetent CSD patient up until 4 months after a cat-scratch [13,14]. This raised the possibility that PCR for *B. henselae* in blood samples might be a useful alternative for more invasive procedures. Serum samples are easily available, and detection of bacterial DNA in serum has proven to be useful in other diseases like Legionnaires’ disease [15]. We, therefore, studied the use of PCR in serum samples in immunocompetent patients, which resulted in proven CSD. *B. henselae* DNA was detectable in three out of eighteen serum samples, suggesting that PCR in serum samples has low sensitivity. Maybe the duration of bacteraemia in immunocompetent patients is of short duration and therefore easily missed. Another explanation might be that *B. henselae* DNA is lacking in serum due to centrifugation of the samples or due to intracellular presence of the bacteria in blood cells. Therefore, we studied the use of PCR in plasma and full blood samples. No *B. henselae* DNA was detectable by the use of PCR in a small number of plasma and full blood samples of CSD patients, suggesting that it is merely the short duration of bacteraemia that limits the use of PCR in blood from immunocompetent patients. Further evaluation of PCR applications may
be rewarding, as the real-time PCR technique provides quick, quantitative and specific results. In conclusion, PCR in pus and tissue samples has a clear additional role in CSD diagnostics. As the ideal moment of sample taking is not yet clear, physicians should realise that a negative PCR result does not exclude CSD in all cases.

**Clinical aspects of CSD**

The largest overview on clinical aspects of CSD dates from 1985 when diagnostics still relied on clinical criteria and the skin test. Over the last few decades, diagnostic techniques have improved and much has been learned about the clinical spectrum of *B. henselae* infection with its various atypical manifestations. Meanwhile, an increasing number of questions has been raised concerning the patterns of occurrence, frequency and distribution.

Several authors, who focused on certain atypical presentations of CSD, have reported relatively high incidences of atypical presentations. For example, previous reports reveal 10% of CSD patients presenting with musculoskeletal symptoms [16,17], 5% with atypical skin lesions [18], 5% with Parinaud’s oculoglandular syndrome, 2-3% with neuroretinitis, and 2% with neurological manifestations [19]. Consideration of these results would suggest that atypical CSD is relatively common. However, unreliable serology as well as possible selection and publication bias might have overstated the incidence of atypical CSD. On the other hand, the incidence of some atypical presentations of CSD might be underestimated. Our review on vertebral osteomyelitis showed that patients usually present with atypical symptoms, such as fever, often without lymphadenopathy. The lack of clinical hallmarks in atypical CSD can easily lead to missing the diagnosis when *B. henselae* infection is not specifically included in the differential diagnosis. In immunocompromised patients, the clinical spectrum of *B. henselae* infection is likely even wider than in healthy persons. Early reports focused mainly on HIV-patients with angioproliferative disease, such as bacillary angiomatosis [20]. Recent reports show that also patients using immunosuppressive therapy can have severe *B. henselae* disease with a varied presentation. In conclusion, updated epidemiological information on the manifestations of *B. henselae* infection in both immunocompetent and immunocompromised patients is needed.

A review of the literature revealed that there is just one controlled study on antimicrobiotical therapy for CSD. No benefit was shown of azithromycin for treatment of uncomplicated classical CSD after 30 days of follow-up [21]. A gap in our knowledge regarding effective therapy for complicated infections and atypical presentations of CSD remains. Also, clear evidence for the different therapeutic options in immunocompromised patients is still lacking. With an increasing number of immunocompromised patients due to HIV infection and immunosuppressive therapy, this may become more important in the future. Due to the relatively low incidence, multi-center randomized controlled trials are required for the development of evidence-based therapeutic decisions.
Methodological remarks

The studies in this thesis have several methodological drawbacks. Inherent to the problems with culturing *B. henselae*, a generally accepted gold standard for detecting CSD infection is lacking. In the studies described in chapters 2 and 5, we considered the combination of clinical data, including the ultimate diagnosis, in combination with PCR to be the most reliable gold standard but realise that this is still not ideal. In these two studies we choose to use a control group that is clinically relevant, namely patients initially suspected of CSD but with other diagnosis and negative PCR. As clinical data were collected after receiving patient material in our laboratory, we cannot exclude the possibility that the clinicians based their diagnosis on the laboratory results that we were evaluating. This may have biased our selection, possibly leading to inclusion of CSD patients in the control group and negative patients in the CSD group. Nevertheless, we believe that our inclusion criteria are the best possible for the moment. Most studies on CSD diagnostics reported in the literature, as are the two described in chapters 3 and 4, include CSD patients based on PCR without clinical data. Even more commonly, healthy control groups are used that are clinically less relevant.

Future perspectives

The main conclusion of this thesis is that laboratory diagnosis of CSD remains problematic. Culture of the bacterium is difficult and histological findings are not specific. Serological testing for anti-*B. henselae* antibodies for the diagnosis of CSD has many pitfalls. Sensitivity is low, and specificity barely reaches the desirable values for safe diagnostic conclusions. Detection of *B. henselae* DNA by PCR also has drawbacks, as often invasive procedures are needed to obtain material and the sensitivity is likely dependent on the moment of sample collection during the course of disease. As serious diseases are included in the differential diagnosis, reliable diagnostic methods are required. Therefore, further improvement is needed in the diagnostics of CSD.

We suggest that a promising new technique to be developed falls back on the principle of the formerly used skin test, which relies on cellular rather than on humoral immunity. The CSD skin test was based on the same principle as the widely used tuberculin skin test (Mantoux test) for identifying tuberculosis infection. Specifically, in both skin tests an intradermal injection of antigen leads to a delayed type hypersensitivity response, resulting in a measurable skin reaction after 48-72 hours. Drawbacks of these tests include the need for a return visit to allow reading, cross-reactivity with other species, the booster effect, and immunosuppression giving false-negative results, as well as the variability in application and reading [22]. For the CSD test, heat-inactivated pus of CSD patients was used for injection, risking contamination with infectious material. When sensitivity, specificity and safety were seriously questioned and serology and PCR became reasonable alternatives, the CSD skin test was considered obsolete [7]. Meanwhile, using laboratory-derived antigens, the tuberculin
skin test remained in use. Serological testing for tuberculosis proved not useful and no reliable alternatives for diagnosis of tuberculosis evolved. However, in 1998, an alternative method for detection of immunological reactivity to mycobacterial antigens was developed. This in vitro test of cell-mediated immunity, the tuberculin interferon gamma release assay (IGRA), is based on detection and quantization of the cytokine interferon-gamma (IFN-γ). In patients with (latent) tuberculosis, IFN-γ is produced by T-lymphocytes after stimulation with tuberculin antigens [23]. This assay overcomes some of the shortcomings of the Mantoux test, as it has the potential to provide a result within 24 hours, does not require return visits and is less susceptible to inter-observer variability. Furthermore, recent studies show that it is likely to be more sensitive and specific in the diagnosis of tuberculosis than the skin test [24]. An IGRA based on stimulation of T-cells with *B. henselae* antigens has not yet been described. We hypothesize that the development of an IGRA, similar to that developed for the diagnosis of tuberculosis, could improve the laboratory diagnosis of *B. henselae* infections.
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SUMMARY

Part I
Introduction

In the introduction of this thesis (Chapter 1) an overview is given of the different aspects of cat-scratch disease (CSD) as described in the literature. History, microbiological aspects, epidemiology, immunology and pathogenesis are discussed. In addition to the mild classical presentation, CSD can present atypically with severe disease. An overview is given of the broad spectrum of clinical manifestations of infection with *B. henselae*. The importance of reliable laboratory diagnosis is noted, as both classical and atypical CSD can be difficult to differentiate from other infectious diseases and malignancies on clinical grounds. Laboratory diagnosis of CSD mostly relies on serology and polymerase chain reaction (PCR), as culturing of the bacterium is difficult and histological findings are not specific. A review is given of the literature on different diagnostic techniques and the current knowledge of treatment.

The aims of this thesis were to evaluate and improve current serological techniques and to evaluate the usefulness of testing serum samples by use of a new real-time PCR for CSD diagnosis. Moreover, we aimed to clarify the role of *B. henselae* infection in the etiology of Henoch Schönlein purpura and to review the literature on vertebral osteomyelitis, one of the atypical presentations of CSD.

Part II
*B. henselae* serology for diagnosis of cat-scratch disease

In Chapter 2, the diagnostic value of five serological tests was studied in a group of 51 proven CSD patients and 55 negative controls. The diagnosis of CSD was based on clinical data, a positive PCR, and the lack of another diagnosis that might explain the symptoms. The controls were patients initially suspected of CSD but with another diagnosis and negative PCR. We concluded that testing for anti-*B. henselae* IgM (IFA and ELISA) had low sensitivity (53-65%) and that specificity (91-93%) was not ideal. Detection of anti-*B. henselae* IgG has limited clinical value in diagnosing acute CSD due to a low sensitivity and high seroprevalence in controls. Approximately two-thirds of the CSD patients were infected with genotype I and one-third with genotype II. Although not significant for all tests, sensitivity seemed to be higher in patients infected with genotype I than in those infected with genotype II. This might be explained by the use of genotype I strains in the tests. IgM sensitivity was highest within 6 weeks after onset of symptoms, with a sharp decrease after 8 weeks, while IgG
Summary

sensitivity peaked at 6-8 weeks and then slowly decreased. A commercial IgM IFA proved of no clinical value in our population.

In Chapter 3, *B. henselae* ELISA was evaluated in a routine laboratory setting in PCR-positive CSD patients (n = 126) and age-matched controls (n = 126). Lymphadenopathy was reported in 97% of CSD patients, with 3% of patients showing atypical CSD. Low sensitivity was found for anti-*B. henselae* IgM (56%) and IgG (36%) detection when cutoffs were set at a high specificity level (98%). Sensitivity of IgG testing was considered too low for practical use. Of 55 CSD patients with negative or inconclusive *B. henselae* serology, consecutive samples showed no seroconversion of antibodies in 82% of cases. We concluded that, despite low sensitivity, IgM detection still could be useful in a clinical setting, although negative IgM does not exclude CSD. Anti-*B. henselae* IgM seroprevalence peaked in fall and winter and was highest in the 10-20 year age group. Relatively high IgM and IgG seroprevalences in a group of *B. henselae* PCR negative patients suggested that also a negative PCR does not exclude CSD. We concluded that the moment of sample collection influences the sensitivity level of both the serology and PCR test.

In Chapter 4, predictive models were designed to study the value of ELISA-based IgM and IgG detection in CSD and the influence of age on diagnostic performance. No age-dependency of IgM levels was found in CSD patients (n = 155) nor in controls (n = 244). However, with age, a highly significant increase of IgG response was found in controls. Analysis of several *B. henselae*-specific diagnostic models showed that IgM is a better predictor of CSD than IgG, with higher sensitivity when using both. Although the use of an age-factor further increased the sensitivity of IgM and IgG, it did not result in a clinically relevant increase of positive and negative predictive values (PPV and NPV) in the total population. However, in children, the use of IgM and IgG with an age-factor improved the PPV by 2% and the NPV by 8% compared to the use of IgM alone, which might be relevant in clinical practice.

In Chapter 5, seven different *B. henselae* serological techniques were evaluated in the CSD and control group that were described in Chapter 2. In this chapter, new commercial tests, including a new test containing the Marseille strain, were studied. Serological cross-reactivity was evaluated in 289 patients with other infectious diseases.

In both in-house and commercial tests, sensitivity of anti-*B. henselae* IgM detection was lower than that for IgG detection. We showed that combining the results of IgM and IgG detection, compared to IgM alone, improved sensitivity but lowered specificity in CSD diagnosis. The reduction of specificity, which was not statistically significant in this study, may be clinically significant. The Houston-strain (genotype I) tests were compared to a new IFA test containing Marseille strain (genotype II). The additional use of the Marseille strain IFA did not improve sensitivity or specificity and resulted in higher cross-reactivity. In fact, sensitivity of the Marseille strain test was lower in Marseille strain infected patients than in those infected with the Houston strain. In samples of patients with infectious lymph node diseases (i.e. Epstein-Barr virus, cytomegalovirus, *Toxoplasma gondii*, and *Streptococcus pyogenes*), cross-reactivity in *B. henselae* serology varied per test and disease: 0-5% in 5 IgM tests and 0-8% in an IgG test. Cross-reactivity also differed between tests and diseases and proved to be highest (13-30%) in sera of *Coxiella burnetii* patients. We concluded that the Marseille
strain test was of no additional value and that cross-reactivity should be taken into account when interpreting CSD serology.

Part III

*B. henselae* PCR for diagnosis of cat-scratch disease

In Chapter 6, an in-house real-time PCR targeting the *groEL* gene was validated in patients with suspected CSD. This new PCR showed 100% agreement with the conventional 16S rDNA-based PCR in seventy-three clinical samples. Testing was only positive for several *Bartonella* strains, while negative in many other bacteria. We concluded that this real-time PCR proves to be a sensitive, specific and quick method that is suitable for routine CSD diagnostics.

In Chapter 7, real-time *groEL* PCR was used for the detection of *B. henselae* DNA in serum samples of proven CSD patients. In a few cases (3/18), DNA was detectable in serum samples of CSD patients, but sensitivity seems too low for clinical diagnosis. In a small number of full blood and plasma samples of CSD patients, no *B. henselae* DNA was found.

Part IV

Clinical aspects of cat-scratch disease

In Chapter 8, we studied the role of *B. henselae* infection in Henoch Schönlein purpura (HSP). We compared *B. henselae* antibodies in forty-five HSP patients and ninety, age and gender matched, controls. Anti-*B. henselae* IgM was not detectable in HSP patients, and IgG seroprevalence was not higher in HSP patients than in controls. No *B. henselae* DNA was found in the acute phase serum samples. We found no evidence for acute or earlier *B. henselae* infection in HSP patients, which is in contrast to earlier reports in the literature. Also, no difference in seroprevalence of streptococcal antibodies was found between patients and controls. We concluded that *B. henselae* infection plays no significant etiological role in HSP.

In Chapter 9, we reported a case of vertebral osteomyelitis caused by *B. henselae* and reviewed the literature on this atypical presentation of CSD. We concluded that *B. henselae* vertebral osteomyelitis is a rare disease, mostly presenting with fever and sometimes with neurological symptoms. Most cases were treated with antibiotics and some required neurosurgical treatment. Generally, the prognosis of *B. henselae* vertebral osteomyelitis was good.
Part V
General discussion and summary

In chapter 10 the findings of this thesis are discussed and recommendations for future studies are made.