

EPIDEMIOLOGY

Seroprevalence of *Bartonella henselae* in cats in Germany

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Bartonella henselae and *B. quintana* infections in man are associated with various clinical manifestations including cat-scratch disease, bacillary angiomatosis and bacteraemia. While cats are the natural reservoir for *B. henselae*, the source of *B. quintana* is unclear. In this study, the sera of 713 cats from Germany were examined for the presence of antibodies against *B. henselae*, *B. quintana* or *Afipia felis* by an indirect immunofluorescence assay (IFA). *Bartonella*-specific antibody titres of ≥ 50 were found in 15.0% of the cats. There was substantial cross-reactivity among the various *Bartonella* antigens, although single sera showed high titres against *B. henselae* but not against *B. quintana* and *vice versa*. Antibodies against *A. felis* were not detected in any of these cats. Statistical analysis indicated that there is no correlation between *Bartonella* infections and the sex, age or breed of the cat or its hunting behavior. There was also no correlation between bartonella and toxoplasma infections in cats. However, whereas 16.8% of cats from northern Germany had *B. quintana*-specific antibodies, only 8.0% of cats from southern Germany were seropositive for *B. quintana*. No statistically significant difference was found for *B. henselae*. IFA-positive and IFA-negative sera were used for immunoblot analysis including *B. henselae* and *B. quintana*. Marked reactivity was observed with protein bands at 80, 76, 73, 65, 37, 33 and 15 kDa. The results of this study suggest that *B. henselae*, and possibly a *B. quintana*-related pathogen, but not *A. felis*, are common in cats in Germany, and that there are differences in the geographic distribution of bartonella infections in cats.

Introduction

Bartonella species are gram-negative, facultative intracellular bacteria of the $\alpha 2$ -Proteobacteria group. At present, 11 *Bartonella* species are known, five of which – *B. henselae*, *B. quintana*, *B. elizabethae*, *B. bacilliformis* and *B. clarridgeiae* – are pathogenic for man [1–3]. *B. bacilliformis* is endemic in the Andes region of South America and causes a diphasic illness designated as Carrion's disease, presenting with acute Oroya fever and chronic verruga peruana [4, 5]. *B. quintana* and *B. henselae* probably occur world-wide and are associated with a variety of clinical manifestations including cat-scratch disease, bacillary angiomatosis and pelliosis [1–3]. Trench fever, bacteraemia, endocarditis and chronic lymphadenopathy may also be

associated with *B. henselae* or *B. quintana* infections [6–12]. Less frequent clinical manifestations such as lesions in bone tissue, liver, lungs and eyes, as well as various neurological symptoms, have been attributed to bartonella infections [13–20]. More recently, *B. clarridgeiae* has been associated with cat-scratch disease and septicaemia [21, 22].

Afipia felis has also been associated with cat-scratch disease in man [23, 24]. However, although one study reported a seroreactivity of 5–7% in 430 human sera with *A. felis* antigen [25], others argued against an important role for this organism in man [26–28].

While cats are clearly recognised as the natural reservoir of *B. henselae*, the reservoirs of *B. quintana*, *B. bacilliformis* and *A. felis* have not yet been identified [1, 3, 29, 30, 31]. The prevalence of *B. henselae* antibodies in sera of domestic cats in various regions of North America varied between 3.7 and 54.6%, depending on the temperature and humidity of the

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different regions [29, 32]. In man, contact with cats or cat fleas, childhood and immunosuppression are epidemiological risk factors for *B. henselae* infections, while HIV infection, homelessness, chronic alcoholism, infestation with lice (*Pediculus humanus*) and close contact between individuals are risk factors for *B. quintana* infections [7, 33–36]. The seroprevalence of bartonella infections in man varies between 1 and 20% [26–28, 37–39]. A seroprevalence of 12% was found in healthy blood donors in Germany (Autenrieth *et al.*, unpublished observations).

Two *B. henselae* variants have been demonstrated recently in domestic cats in Germany by 16S rRNA sequence analysis, enterobacterial repetitive intergenic consensus (ERIC)-PCR and SDS-PAGE [40]. In 13% of cats, *B. henselae* could be isolated from the blood [40]. However, the epidemiology of bartonella infections in man or in cats in Germany has not yet been studied and the aim of this study was to determine the seroprevalence of *B. henselae* in comparison with *B. quintana* and *A. felis* in domestic cats in Germany.

Materials and methods

Sera and animals

Sera were collected from 713 cats during 1993 and 1994 (Table 1); 200 of these sera were collected at a veterinary clinic in southern Germany (district of Schweinfurt), the other 513 sera were collected at various veterinary practices in northern Germany (Niedersachsen, Hamburg, Bremen, Schleswig-Holstein and Nordrhein-Westfalen). The owners of the 513 cats

Table 1. Epidemiological characteristics of pet cats included in this study*

| Characteristic | Percentage |
|---------------------------------|------------|
| Age | |
| 1–6 months | 4.5 |
| 7–12 months | 17.2 |
| 1–2 years | 15.2 |
| 2–5 years | 19.1 |
| 6–10 years | 16.6 |
| >10 years | 10.5 |
| Breed | |
| European short hair | 73.5 |
| Pedigree cats | 16.6 |
| Sex | |
| Male | 52.0 |
| Female | 37.2 |
| Environment | |
| Indoor cats | 25.9 |
| Outdoor cats | 47.9 |
| Hunting behaviour | |
| Hunting | 37.8 |
| No hunting | 33.3 |
| <i>T. gondii</i> seroprevalence | |
| Positive | 27.7 |
| Negative | 72.3 |

*A total of 713 cats was studied: 200 (28.1%) were from southern Germany and 513 (71.9%) were from northern Germany. The characteristics detailed above are derived only from the cats from northern Germany.

from northern Germany were requested to provide the following information: age, breed and sex of the cat, the type of environment and the cat's hunting behaviour (Table 1). The sera had previously been examined for *Toxoplasma*-specific antibodies and were stored at -20°C .

Bacteria

The following strains of bacteria were used: *B. henselae* (Houston strain) ATCC 49793; *B. henselae* (Marseille strain; a kind gift from Professor D. Raoult, Marseille, France); a *B. quintana* isolate from a patient in Munich [41] and *A. felis* ATCC 53690. *B. henselae* and *B. quintana* were grown at 37°C for 8–10 days on chocolate agar plates containing defibrinated sheep blood 10% until the colonies reached confluence. Bacteria were harvested by rinsing the plates with phosphate-buffered saline (PBS, pH 7.4). Suspensions of bacteria were washed three times with PBS and then centrifuged for 10 min at 11 950 *g*. The pellets were suspended in 1 ml of NaCl 0.9% solution containing sodium azide 0.1% and further diluted to the appropriate concentration and stored at 4°C . Pili expression of *B. henselae* was confirmed by transmission electron microscopy (data not shown). *A. felis* was grown on charcoal-yeast extract (BCYE) agar plates at 30°C for 5 days, harvested and suspended as described for *Bartonella* spp.

Cell cultures

Vero cells (ATCC, Rockville, MD, USA) were grown in tissue-culture flasks (Nunc) in DMEM (Seromed, Germany) supplemented with fetal calf serum (Seromed) 10%, glutamine (Biochrom) 0.1%, sodium pyruvate (Biochrom) 0.1% and non-essential amino acids (Gibco) 0.1% at 37°C in a humidified atmosphere containing CO_2 5%. Cells were grown without antibiotics to a density of *c.* 10^5 cells/ml. They were infected with *B. henselae* at a bacteria:cell ratio of *c.* 100:1. Infected Vero cells were subcultured every 2–3 weeks and cells from the fourth subculture were harvested. The resulting suspension containing Vero cells and bacteria was washed in PBS, centrifuged and suspended as described above for bacteria harvested from agar plates.

Immunofluorescence assay (IFA)

The IFA was performed according to standard methods. Antigen preparations as described above (cell culture and conventional agar plate cultures) were applied to glass slides, air-dried and fixed by incubation in methanol for 15 min at -20°C . Serial dilutions of sera starting at 1 in 50 were prepared in PBS containing bovine serum albumin (Biomol) 0.5% and Tween 20 (Sigma) 0.1%. Antigen slides were overlaid with the serum dilutions and incubated for 30 min at 37°C in a humidified chamber. After washing with the PBS buffer

described above, FITC-conjugated (F(ab')₂-fragment goat anti-cat IgG (H + L) (Dianova, Hamburg, Germany) diluted 1 in 100 in PBS buffer containing Evans blue 0.1% was added to the slides and incubated for 30 min at 37°C. After final washing steps, the slides were air-dried, mounted in buffered glycerol (Fluoprep; Merck) and examined by fluorescent microscopy (Leitz Aristoplan, Heidelberg, Germany).

Immunoblot analysis

Bacterial suspensions of *B. henselae* (Houston, Marseille) were prepared as described above. Bacterial lysates were prepared by heat treatment (100°C for 5 min), separated on SDS polyacrylamide 12.5% gels and transferred to nitrocellulose by Western blotting as described previously [42]. After incubation with blocking buffer, nitrocellulose strips were incubated with cat sera diluted 1 in 100 in PBS containing Tween 20 0.5% overnight at ambient temperature. They were washed three times with PBS containing Tween 20 0.5% and incubated with a 1 in 100 dilution of F(ab')₂-fragment goat anti-cat IgG (H + L) conjugated to alkaline phosphatase (Dianova) for 90 min at ambient temperature. After final wash steps, the reactions were developed as described elsewhere [42].

Statistical analysis

Statistical analysis was performed with the computer programme SAS (SAS Institute, Cary, NC, USA). The relationships between the prevalence of antibodies to different bartonella antigens and various parameters that may be of importance for the epidemiology of bartonella infections in cats were examined by Fisher's exact test and the χ^2 test.

Results

A total of 713 sera samples was obtained from German pet cats and serological tests were used to determine the presence of anti-*Bartonella* IgG antibodies. The IFA results were considered positive when immunofluorescence was clearly noted at a titre of ≥ 50 . In immunofluorescence assays, two types of antigens were used: conventional bacterial antigen derived from bacteria grown on agar plates and *B. henselae* grown and harvested in co-culture with Vero cells.

A total of 107 (15.0%) sera gave positive results for *Bartonella* spp. Of these sera, 40 (5.6%) had antibodies against *B. henselae* (Marseille strain) and 44 (6.2%) against *B. henselae* (Houston strain); 102 (14.3%) exhibited antibodies against *B. quintana* ($p < 0.01$; Fig. 1). There was no significant difference in seroprevalence between the two *B. henselae* antigens (Marseille strain versus Houston strain). The maximum IFA titre detected was 400 (Fig. 2). None of the sera contained antibodies against *A. felis*.

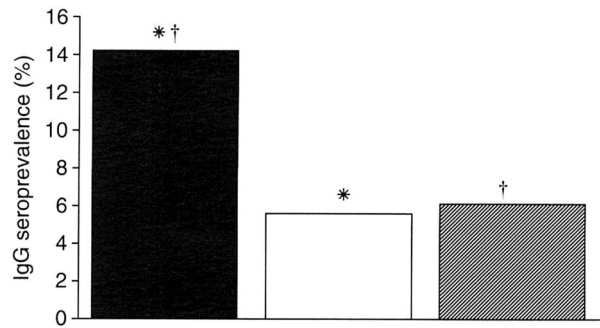


Fig. 1. Prevalence of anti-*B. quintana* (■), anti-*B. henselae* Houston strain (□) and anti-*B. henselae* Marseille strain (▨) antibodies in sera of 713 German cats as revealed by IFA assay. Sera with a titre ≥ 50 were considered positive. *† $p < 0.01$.

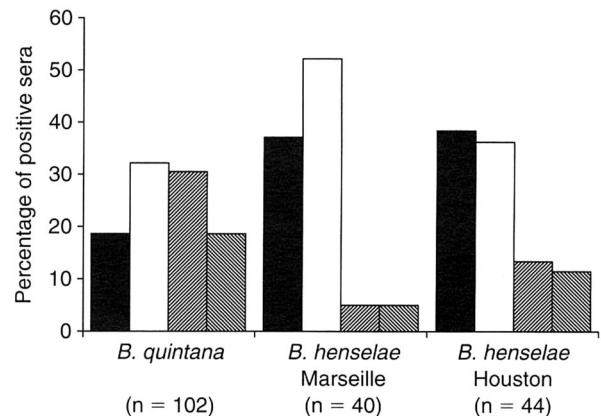


Fig. 2. *Bartonella*-specific antibody titres of 713 sera from German cats as revealed by IFA including conventionally grown bacteria as an antigen: ■, 50; □, 100; ▨, 200; ▤, 400.

Eighteen IFA-negative and 13 IFA-positive sera were analysed in parallel by an IFA including cell-culture antigen. Comparison of antibody titres of cat sera for these two antigens revealed 46% higher antibody titres with the cell culture antigen when compared with the conventional antigen (data not shown). However, the sera with negative antibody titres in the conventional IFA did not show positive antibody titres in the IFA with cell-culture antigen.

Details of age, breed, sex, environment, hunting behaviour and *T. gondii*-specific serum antibody prevalence were recorded for 513 cats. None of the cats aged 1–6 months had serum antibodies against *B. henselae* (Table 2). Seroprevalence in 7–12-month-old cats against *B. henselae* was 5.7% and remained at this level in older cats, suggesting that in most cases seroconversion occurred within the second part of the first year of life. The seroprevalence of anti-*B. quintana* antibodies was higher than that of *B. henselae* antibodies. Thus, 13.0% of 1–6-month-old cats and 19.3% of cats aged 7–12 months had antibodies against *B. quintana*, suggesting that seroconversion for *B. quintana* antigen occurred earlier in life than seroconversion for *B. henselae* antigen.

Table 2. Prevalence of *Bartonella*-specific IgG antibodies in sera from pet cats at different ages*

| Antigen | Number (%) of positive sera from cats aged | | | | | |
|------------------------------|--|-------------------------|-----------------------|-----------------------|------------------------|-----------------------|
| | 1–6 months (n = 23) | 7–12 months (n = 88) | 1–2 years (n = 78) | 2–5 years (n = 98) | 6–10 years (n = 85) | >10 years (n = 54) |
| <i>B. quintana</i> | 3 (13.0) | 17 (19.3) | 17 (21.8) | 14 (14.3) | 14 (16.5) | 8 (14.8) |
| <i>B. henselae</i> Marseille | 0 (0) | 5 (5.7) | 4 (5.1) | 4 (4.1) | 8 (9.4) | 4 (7.4) |
| <i>B. henselae</i> Houston-1 | 0 (0) | 5 (5.7) | 6 (7.7) | 5 (5.1) | 7 (8.2) | 5 (9.3) |

*Prevalence of *B. henselae*-specific IgG antibodies in sera of cats was determined by IFA.

Whereas 16.8% of cats from northern Germany had *B. quintana*-specific IgG serum antibodies at a titre of ≥ 50 , only 8.0% of cats from southern Germany had positive *B. quintana*-specific antibody titres ($p = 0.003$; Fig. 3). The higher seroprevalence in cats from northern Germany coincided with slightly higher rainfall in northern Germany compared with southern Germany (not shown). Moreover, the average temperature was slightly higher in southern Germany. The other epidemiological factors recorded including age, breed, sex, environment, hunting behaviour and *T. gondii*-specific serum antibody prevalence had no impact on *Bartonella*-specific IgG antibody prevalence in cat sera.

It is well known that the IFA is not specific for a particular *Bartonella* sp. In fact, considerable cross-reactivity to either of the antigens is found in sera from patients with *B. henselae* or *B. quintana* infection [37, 43]. Similarly, the results of the present study suggest considerable cross-reactivity between the two bartonella antigens in cat sera. Conversely, many of the sera revealed a differential reactivity with either *B. henselae* or *B. quintana*, respectively (data not shown). Thus, while five sera were shown to have anti-*B. henselae* IgG titres of 100, of these sera were negative when *B. quintana* antigen was used. Likewise, 14 sera showed significant anti-*B. quintana* antibody titres of 200 and 400, respectively, but were negative against *B.*

henselae. Moreover, single sera were preferentially reactive with *B. henselae* Houston strain when compared with *B. henselae* Marseille strain, and *vice versa*.

Immunoblot analysis was performed on 30 cat sera with *B. henselae* antigens, and revealed a marked reactivity with protein bands with a M_r in the range 13–80 kDa. A representative immunoblot analysis of sera with negative or positive anti-*B. henselae* IFA titres is depicted in Fig. 4a. Comparison of *B. henselae* IFA-positive and IFA-negative sera applied to immunoblot analysis with *B. henselae* revealed that IFA-positive sera more frequently ($p < 0.05$) recognised bands at 80, 76, 73, 65, 37, 33 and 15 kDa (Table 3). Moreover, the higher the IFA antibody titre of the sera, the more protein bands were usually recognised.

To examine the specificity of the differential reaction of sera with *B. quintana* and *B. henselae* antigen as revealed by IFA, these sera were applied to immunoblot analysis, including both *B. henselae* and *B. quintana* as antigen. As depicted in Fig. 4b, *B. quintana* IFA-positive-*B. henselae*-negative sera showed a marked reactivity with protein bands of both *B. quintana* and *B. henselae*. Although the pattern of recognised protein bands is different for both bacterial species, there was nevertheless considerable variation in the protein patterns recognised by sera with the same IFA titre.

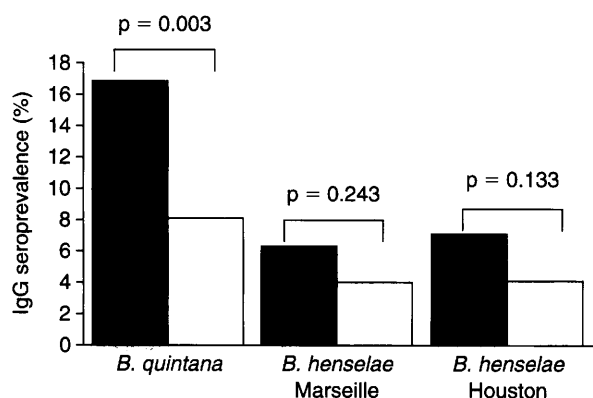


Fig. 3. Prevalence of anti-*B. quintana*, anti-*B. henselae* Houston strain and anti-*B. henselae* Marseille strain antibodies in sera of 200 cats from southern Germany (□) and 513 cats from northern Germany (■) as revealed by IFA assay. Sera with a titre ≥ 50 were considered positive.

Discussion

The purpose of this study was to determine the seroprevalence of anti-*B. henselae* antibodies in cats in Germany. Although the natural reservoir of *B. quintana* has not yet been established, *B. quintana* was included as an antigen in the study. Furthermore, as *A. felis* has been claimed as an aetiological agent in cat-scratch disease, this bacterium was also included in the present study, although the relevance of *A. felis* in cat-scratch disease remains obscure [23, 24].

It has been well established that cats are the natural reservoir of *B. henselae* [29–31]. In particular, young cats may have *B. henselae* bacteraemia for varying periods without developing clinical signs of disease [29, 30, 40]. In fact, experimental infection of cats with *B. henselae* causes bacteraemia persisting for up to 32

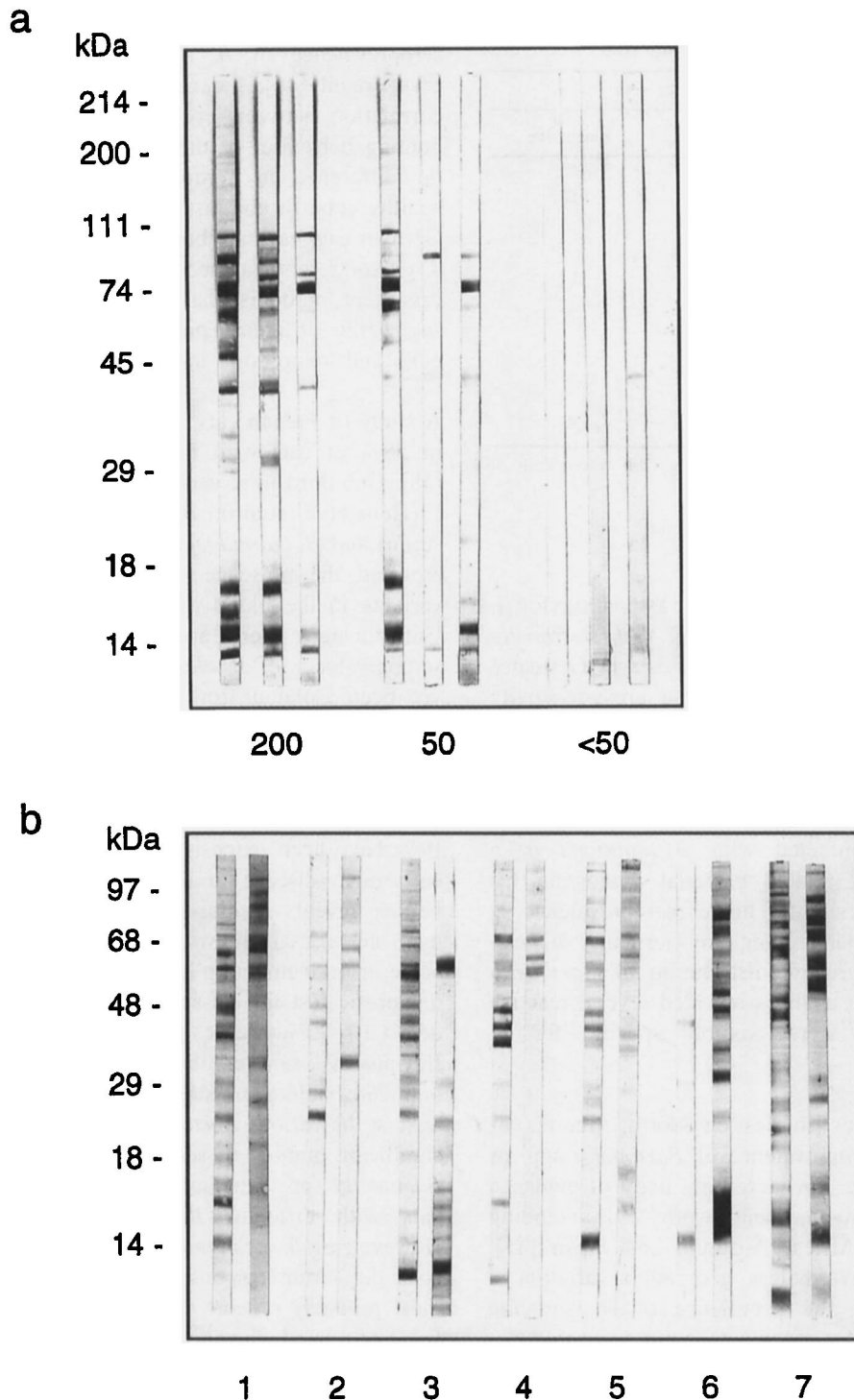


Fig. 4. (a) Immunoblot reactivities of whole cells of *B. henselae* with various IFA-negative or IFA-positive sera. Lanes 1–3, sera from cats with IFA titre of 200; 4–6, sera from cats with IFA titres of 50; 7–9, sera from cats with a negative IFA titre. (b) Immunoblot reactivities of whole cells of *B. quintana* (left lanes) and *B. henselae* (right lanes) with various IFA (*B. quintana*)-positive sera (titres 200–400).

weeks [44]. Transmission of *B. henselae* from cats to man probably occurs directly *via* bites and scratches as well as *via* cat fleas [30]. Therefore, contact with cats, the presence of cat fleas, as well as childhood are, in addition to immunosuppression, the most important epidemiological risk factors for *B. henselae* infection in man [29, 30, 36]. On the other hand, a recent study demonstrated that the seroprevalence of IgG antibodies

to *B. henselae* is similar in cat owners and controls. Thus, *c.* 20% of sera in both controls and cat owners contained antibody levels of ≥ 64 [38]. Several studies revealed an overall seroprevalence of *B. henselae* in man between 0 and 26% [26–28, 37, 39].

The most salient findings of the present study are as follows. (i) In all, 6–15% of sera contained anti-

Table 3. Anti-*B. henselae* IgG reactivity of sera from pet cats revealed by immunoblotting and IFA

| Reactive band (kDa) | Percent of sera | |
|---------------------|-----------------|--------------|
| | IFA-positive* | IFA-negative |
| 90 | 12.5 | 0 |
| 80 | 75.0† | 25.0† |
| 76 | 43.8† | 0† |
| 73 | 68.8† | 0† |
| 65 | 43.8† | 0† |
| 48 | 68.8 | 37.5 |
| 40 | 56.3 | 37.5 |
| 37 | 37.5† | 0† |
| 33 | 18.8 | 0 |
| 30 | 31.3 | 12.5 |
| 15 | 31.3 | 0 |
| 13 | 25.0 | 12.5 |

Sera were applied to IFA and immunoblotting with whole cells of *B. henselae* as antigen.

*IFA titres >50 were considered positive.

† $p < 0.05$.

Bartonella IgG antibodies, but none was positive for *A. felis* as revealed by IFA, suggesting that *Bartonella* spp. but not *A. felis* are common in cats in Germany. (ii) Although there was considerable cross-reactivity between the two bartonella antigens used, several sera had *B. henselae*- but not *B. quintana*-specific antibodies, and *vice versa*. Although it is yet quite speculative, these data suggest that at least single cats might have been infected with *B. quintana* or a serologically closely related bacterial pathogen. (iii) The study found a slightly higher seroprevalence in northern Germany than in southern Germany, suggesting a different geographic distribution of *Bartonella* spp. (iv) Immunoblot analysis revealed several reactive protein bands that were possibly specific for *B. henselae*.

In line with previous studies on North America or European cats, a seroprevalence of *Bartonella* spp. of c. 6–15% was found. An increasing body of evidence suggests that *B. henselae* occurs world-wide including the USA, Europe, Africa, Australia and Japan [45], although there is, nevertheless, geographic variation in seroprevalence, e.g., the prevalence of *B. henselae* antibodies in North American cats is more variable (5–55%; overall incidence 27.9%) depending mainly on the geographic distribution [32]. Thus, cats living in warm and humid areas showed a higher seroprevalence than cats in dry or cold regions. In California, 81% of cats were seropositive for *B. henselae* and 39.5% had bacteraemia [29]. The authors of these studies claimed that the varying seroprevalence might reflect the different frequency of potential arthropod vectors. Likewise, the results of the present study suggest a geographic impact on the prevalence of bartonella infections in cats, although it is not yet clear whether the slightly different humidity and temperature in northern and southern Germany may account for these results. Unfortunately there were no data available on the presence of possible vectors such as cat fleas in the present study.

In keeping with these data, a recent study revealed a seroprevalence of *B. henselae* of 8.3% in cats in Switzerland [46], but the authors did not observe a correlation between positive titre and sex, breed or hunting behaviour of the cats. Furthermore, there was no difference in seroprevalence between sick and healthy cats. In contrast to *Bartonella* spp. c. 50% of German cats have antibodies against *T. gondii*, and the *T. gondii* seroprevalence is higher in older cats and in cats kept outdoors that frequently go hunting [47], suggesting different epidemiological factors in bartonella and toxoplasma infections in cats.

A study of French cats revealed positive blood culture in 56% of cats with three different *Bartonella* spp. [48]. One-third were each positive for *B. henselae* type I (Houston-1 strain), *B. henselae* type II ('BA-TF' strain) and *B. clarridgeiae*. In Germany, a recent study reported the presence of two different *B. henselae* variants in the blood of 13% of domestic cats [40]. Unfortunately there are no data available on the seroprevalence of *B. clarridgeiae*. *B. quintana* has not yet been isolated from cats. There is accumulating evidence that man may be the only reservoir for this pathogen and that it is most probably transmitted by lice [1–3].

IFAs have been extensively evaluated for diagnosis of cat-scratch disease in man. However, the IFA assay neither reveals species-specific antibody responses in man, nor does it allow a clear differentiation between acute or prior infection [37, 43, 49]. Moreover, IFA as a diagnostic test for cat-scratch disease in man may not detect 10–30% of cases [3, 49]. In the present study 14 IFA-positive sera exhibited equal antibody titres for both *Bartonella* spp., suggesting cross-reactivity of sera against the various *Bartonella* spp. On the other hand a significant number of sera had different titres for both *Bartonella* spp. Whether this is due to co-infection of cats with different *Bartonella* spp. is not clear. However, as *B. quintana* has never been isolated from cats, the serum responses of cats against *B. quintana* most probably reflects cross-reactivity against *B. henselae* or related *Bartonella* spp. such as *B. clarridgeiae* rather than actual infection with *B. quintana*. However, a recent case report argues against this possibility as the authors observed no significant cross-reactive serum antibody response against *B. quintana* in a cat and a patient with *B. clarridgeiae* infection [22]. On the other hand, cross-reactions of bartonella-specific antibodies with other bacterial species, including *Chlamydia* and *Coxiella* spp., may occur [3] and this possibility cannot be excluded in the present study.

Nevertheless, as observed in the present study, there might be strain- or serotype-specific antibody responses [8]. A French study demonstrated significantly higher and more frequent positive *B. henselae*-specific antibodies in sera from human patients when they used a European isolate (Marseille strain) of *B. henselae* than

with an American strain (Houston-1) [50]. Furthermore, there is evidence that the presence or absence of pili on the bacterial surfaces of these strains might account for these differences, and that phase variation in antigen expression paralleled by transition from rough to smooth phenotypes of *Bartonella* may account for misleading results in serological tests [2].

Immunoblot analysis revealed a broad spectrum of protein bands recognised by cat sera when whole cells of *B. quintana* or *B. henselae* were used as antigen. Protein bands at 80, 73, 65, 37, 33 and 15 kDa were recognised more frequently by IFA-positive sera than by IFA-negative sera, suggesting that certain protein bands are dominant antigens and may be *B. henselae*-specific. However, to date, there are no recombinant antigens available which would make immunoblot analysis much more sophisticated.

This study suggests that *B. henselae* is common in cats in Germany, although there may be geographic variations, and that possibly *B. quintana*-related pathogens (but not *A. felis*) may occur in cats. The seroprevalence of *B. clarridgeiae* remains to be investigated.

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