Bartonella spp. in Small Mammals and Their Fleas in Differently Structured Habitats From Germany

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Most Bartonella spp. are transmitted by fleas and harbored by small mammals which serve as reservoirs. However, little is known about the composition of fleas and their Bartonella spp. from small mammals in Central Europe. Therefore, the aims of this study were to investigate flea communities on small mammals from three differently structured sites (urban, sylvatic, renatured) in Germany as well as the prevalence of Bartonella spp. in small mammals and their parasitizing fleas. In total, 623 small mammals belonging to 10 different species (the majority were Myodes glareolus and Apodemus flavicollis) were available. Fleas were removed from the small mammals’ fur, morphologically identified and DNA was extracted. To detect Bartonella spp., two conventional PCRs targeting the gltA gene and the 16S–23S rRNA intergenic spacer were carried out followed by sequencing. Obtained sequences were compared to those in GenBank. In total, 1,156 fleas were collected from 456 small mammals. Altogether, 12 different flea species (the majority were Ctenophthalmus agyrtes, Nosopsyllus fasciatus, and Megabothris turbidus) were detected. At the urban site mostly Leptopsylla segnis and N. fasciatus were collected which may be vectors of zoonotic pathogens to companion animals. The overall prevalence for Bartonella in small mammals was 43.3% and in fleas 49.1%. Five different Bartonella spp. were detected in small mammals namely B. grahamii, B. taylorii, B. doshiae, Bartonella sp. N40 and uncultured Bartonella sp. whereas in fleas four Bartonella spp. were found which were with the exception of B. doshiae identical to the Bartonella species detected in their small mammal hosts. While B. grahamii was the only zoonotic Bartonella sp. most Bartonella strains found in fleas and small mammals belonged to uncultured Bartonella spp. with unknown zoonotic potential. This study showed a high diversity of flea species on small mammals from Germany. Further, high prevalence rates of Bartonella species were detected both in fleas and in their mammalian hosts. Several different Bartonella species with a high genetic variability were discovered. Especially at the urban study sites, this may pose a risk for Bartonella transmission to companion animals and humans.

Keywords: Apodemus flavicollis, Clethrionomys glareolus, Ctenophthalmus agyrtes, Megabothris turbidus, Bartonella grahamii, Europe
INTRODUCTION

Bartonellosis, which can result in severe clinical symptoms in humans and their companion animals, is caused by the facultative intracellular alpha-proteobacteria Bartonella spp. (order Rhizobiales, family Bartonellaceae) (1). Bartonella spp. are arthropod-borne bacteria and mainly transmitted by fleas, lice, deer keds, and sandflies (2–5). Bartonellae are highly adapted to one specific or few closely related mammalian reservoir hosts in which they can cause long-lasting bacteremia. In contrast, infections in incidental hosts may evoke disease with a broad range of symptoms (6, 7). Amongst the most common reservoir hosts are cats, rodents and other small mammals. Phylogenetic analyses based on sequence data from rpoB, gltA, ribC, and groEL genes revealed four different deep-branching Eubartonella lineages and additionally Bartonella australis (8). Bartonella tamiiae and Bartonella apis could build two additional separate lineages, which is however not yet confirmed. Lineage 4 is the most diverse group regarding the variety of Bartonella spp. as well as reservoir host species. Thus far, the highest prevalence and highest diversity of Bartonella spp. were described in rodents. Five of these rodent-associated Bartonella spp. are known to be hazardous to human health (Bartonella grahamii, Bartonella elizabethae, Bartonella vinsonii subsp. arupensis, Bartonella washoensis, and B. tamiiae) (9). In studies from Poland (10, 11), Sweden (12), France (13), and the UK (14) the prevalence of Bartonella spp. in rodents ranged from 0 to 72.2%. Fleas are suggested to serve as main vectors for Bartonella spp. which are associated with rodents (15, 16). Previous studies showed that fleas may transmit Bartonella spp. experimentally to their mammalian hosts (17, 18). Moreover, there are several epidemiologic studies based on the molecular analysis showing that rodent-associated fleas are also infected with Bartonella spp. in nature (19–21). Studies from the USA, Afghanistan, and Israel reported prevalences between 15.5 and 95% for Bartonella spp. in fleas collected from rodents and small mammals (19–22). The knowledge on the species diversity of fleas on small mammals and the Bartonella prevalence are very scarce in Central Europe. Small mammal species build the vast majority of hosts for over 50 different flea species (23). In Germany, there are only four reports about small mammal fleas from the last century and only one report which is more recent (19). Recently, our group reported high prevalences of Bartonella spp. in rodents (68.8%) and their associated fleas (54.1%) in Germany (24). Further studies on the prevalence and species diversity of Bartonella in rodents and especially their parasitizing flea species are scarce in Germany. The previous study by our group showed results from one location and the sample size examined did not allow statistical associations. Thus, the objectives of the present study were: (1) detection of flea species parasitizing small mammals and (2) detection of Bartonella spp. in small mammals and their fleas and (3) detection of associations between small mammals, fleas and Bartonella species.

MATERIALS AND METHODS

Study Areas

To collect small mammals, traps were placed at three sites of urban, sylvatic or recultivated character. These locations were previously selected for field studies by our group (25, 26). The urban area (R1) “Dörrnbergpark” (7.4 ha, 49°00’55.72”N, 12°05’08.89”E) is situated in the city centre of Regensburg, Bavaria, Southern Germany. It is a small well-tended park which was described in detail before (26, 27). The sylvatic area (T) “Angelberger Forst” (661 ha, 48°06’36.42”N, 10°34’33.40”E) is a large forest located in Bavaria, Southern Germany (28). The recultivated site (S) consisted of three trapping localizations (51°15’32.2”N, 12°21’02.5”E; 51°17’01.3”N, 12°21’00.6”E; 51°26’97.2”N, 12°32’25.6”E) which were previously examined by our group and named as sites “E,” “F,” and “G” (25). This area is surrounding a lake which was a former open pit brown coal mining region near Leipzig, Saxony, Eastern Germany (436 ha).

Sampling of Small Mammals and Their Fleas

Altogether, 50 Sherman® live animal traps (H. B. Sherman Traps, Inc., Tallahassee, Fl., U.S.A.) were placed at each Bavarian site between July and October in 2012 and between April and September in 2013. In Saxony, 60 traps were placed between March and October in 2012 and between January and September in 2013 (official permit Site S: AZ 36.11–36.45.12/4/12-001, Site R1: 55.1-8646-4-140, Site T: 55.1-8646-2/30). Traps were placed for two consecutive nights per month and site and checked twice a day. Collected small mammals were anesthetized with CO2, then euthanized by cervical dislocation and stored at −80°C. Small mammals were morphologically identified using taxonomic keys (29). Additionally, randomly selected rodents (15 Apodemus sylvaticus, 14 Myodes glareolus, and 23 A. flavicollis, 5 Microtus arvalis, 1 Mi. agristis) as well as all shrews (Sorex spp.; n = 5) (by-catch found dead in traps) and least weasels (Mustela nivalis; n = 2) were identified by conventional PCR targeting the cytchrome b gene (354 bp) (30). A complete necropsy was performed with the collection of spleen samples. Cross contamination may be ruled out during dissection as each small mammal was handled with its own set of dissection instruments. Disinfection of working surfaces was performed after each individual and gloves were changed. Fleas were collected with tweezers from the fur during small mammal dissection. Fleas were stored individually in 100 μl RNALater (Qiagen, Hilden Germany) until morphological identification under a stereomicroscope (31, 32). Detailed information about trapping procedures and sampling sites have been given before (25–28).

Small Mammal Samples Made Available From Previous Studies

In a previous study by our group, 623 small mammals belonging to 10 different species (395 My. glareolus, 172 A. flavicollis, 6 A. agrarius, 35 A. sylvaticus, 6 Mi. arvalis, 1 Mi. agristis, 2 M. nivalis, 51
4 Sorex coronatus, one Sorex araneus, and one Talpa europaea) were captured (26, 33).

DNA Extraction
DNA was extracted from all collected rodents’ spleens and from a preselected number of fleas (n = 450) which were collected from the small mammals. The DNA extraction was carried out for each sample individually with the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) as previously described (20). The quality and quantity of the extracted DNA samples were measured spectrophotometrically (NanoDrop ND-1000, Erlangen, Germany). DNA samples exceeding a concentration of 40 ng/µl were additionally diluted with elution buffer in order to avoid false negative results.

Polymerase Chain Reaction and Sequence Analysis
DNA samples were tested for the presence of Bartonella spp. via conventional polymerase chain reaction (PCR) targeting the gltA gene with BhCS.1137n (5′-AATGCAAAAAGAACAGTAAACA-3′) as forward and BhCS.781p (5′-GGGGaCCaGCCTATGGTGG-3′) as reverse primer (34). All samples were further processed by an additional PCR targeting 453–780 base pairs (bp) of the 16S–23S rRNA intergenic spacer (ITS) region with the forward primer Ba325s (5′-CTTCAGATGATGATCCCAAGCCTTCTGGCG-3′) and the reverse primer Ba1100as (5′-GAACCGACGACCCCCTGCTTGCAAAGCA-3′) (35, 36). Visualization of PCR products followed under UV-light on 2% agarose gel dyed with GelRedTM (Biotium, Hayward CA, USA). As the gltA gene is considered to be more sensitive, only samples which were positive in both genes were considered positive and further processed by sequencing. Purification of PCR products of the samples positive in ITS was carried out with the QIAquick PCR purification Kit (Qiagen) according to the manufacturer’s recommendations. Purified amplicons were sequenced by Eurofins MWG Operon (Martinsried, Germany) with both primers and sequences were analyzed with Chromas Lite® (Technelysium Pty Ltd, South Brisbane, Australia) as formerly described (35). Obtained sequences were aligned with sequences from GenBank using BLASTn (National Center for Biotechnology Information, Bethesda MD, USA) and deposited in GenBank under following Acc. No.: MT551048-MT551101 and MT913158-MT913206. In total 33% of the positive rodent samples and 29% of the positive flea samples were sequenced. Sequences were considered as a matching result in GenBank with at least a similarity of 97.7%.

Statistical Analysis
Confidence intervals (95%CI) for the prevalence of Bartonella spp. in small mammals and fleas were determined by the Clopper and Pearson method using Graph Pad Software (Graph Pad Software Inc., San Diego, CA., USA). Independence of compared small sample sizes (n < 30) was tested with Fisher’s exact test, respectively with the chi-squared test for sample sizes n > 30. The t-test was used to test significant differences of flea infestation on My. glareolus and A. flavicollis. The significance threshold was set at p ≤ 0.05.

TABLE 1 | Flea burden per small mammal species.

| Small mammal species | Total No.1 of small mammals | Total No.1 of small mammals infested by fleas (%) |
|----------------------|------------------------------|--------------------------------------------|
| Apodemus flavicollis | 172                          | 146 (84.9)                                 |
| Myodes glareolus     | 395                          | 276 (69.9)                                 |
| Apodemus agrarius    | 6                            | 6 (100)                                    |
| Apodemus sylvaticus  | 35                           | 24 (68.6)                                  |
| Mustela nivalis      | 2                            | 1 (50)                                     |
| Microtus agrestis    | 1                            | 1 (100)                                    |
| Microtus arvalis     | 6                            | 2 (33.3)                                   |
| Total No.1           | 617                          | 456 (73.9)                                 |

No.1, Number.
RESULTS

Flea Collection

In total, 1,156 fleas were collected from 456 small mammals. Altogether, twelve different flea species were detected (873 Ctenocephalides agyrtes, 3 Ctenocephalides biocodontatus, 62 Ctenocephalides congener, 98 Megabothris turvidus, 8 Megabothris walkeri, 9 Hystrichopsylla talpe talpae, 25 Peromyscopsylla silvatica, 3 Paleopsylla soricis, 44 Nosopsyllus fasciatus, 11 Typhlocyclus popei, 17 Leptopsylla segnis) (Table 1).

Except for individuals belonging to the insectivore species T. europaea and Sorex spp., all other small mammal species were infested with fleas. The infestation prevalence ranged from 20 to 100% per small mammal species. The most prevalent species was C. agyrtes, which was found on all infested small mammal species. The flea burden was significantly higher on A. flavicollis compared to My. glareolus (t = −91.32; p < 0.0001). Megabothris turvidus was significantly more often collected from My. glareolus than from all other small mammal species (t = −5.65; p < 0.0001). Nosopsyllus fasciatus was significantly more frequently collected from specimens belonging to the family Muridae (Apodemus spp.) than from those belonging to the family Cricetidae (Microtus spp.; Myodes spp.) (t = −4.16; p = 0.00021). Leptopsylla segnis was exclusively found on A. sylvaticus, which were trapped only in the urban habitat. Peromyscopsylla silvatica were significantly more often collected from My. glareolus compared to all other small mammal species (t = −3.23; p = 0.0006).

Bartonella spp. in Rodents

Only samples yielding a positive result in both PCR approaches were considered positive in the following analysis. In total, 43.3% (95%CI: 39.5–47.3) of all small mammals were positive for Bartonella spp. Though not infested with fleas, positive T. europaea (100%; 95%CI: 16.75–100) and Sorex spp. (20%; 95% CI: 0–11.5) were detected. The prevalences were quite high in all small mammal species. The flea burden was significantly higher in A. flavicollis compared to My. glareolus (p < 0.0001; Table 2).

Sequence Analysis for Bartonella spp. in Rodents

A total of 88 out of 270 (33%) PCR products were selected by small mammal species and location to be further processed in order to determine the Bartonella species via sequence analysis. Altogether five different Bartonella species were detected in rodents and Sorex araneus (Table 2). The most prevalent species group (n = 56) which was detected in small mammals were uncultured Bartonella species. Altogether 56 sequences obtained in this study showed 100% identity to altogether 16 different uncultured Bartonella spp. sequences deposited in GenBank (Table 3), and these sequences showed 27–99% homology to one another. Bartonella grahamii was significantly more often detected in My. glareolus compared to A. flavicollis (p = 0.0370).
**TABLE 3** | Number of Bartonella sp. sequences based on the 16S–23S rRNA ITS of Bartonella found in small mammals and fleas in this study in comparison to sequences from GenBank.

| Host in this study (number of sequences per host species) | Number of Bartonella sequences detected | Bartonella sp. detected | Identity to following Accession number in Genbank | Host in GenBank | Country of origin | Citation of GenBank Accession number |
|----------------------------------------------------------|-----------------------------------------|-------------------------|-----------------------------------------------|----------------|------------------|-------------------------------------|
| Apodemus flavicollis                                    | 2                                      | Bartonella sp. uncultured | DQ155391                                       | Apodemus flavicollis | Slovenia         | (38)                                |
| Ctenophthalmus agyrtes                                  | 3                                      | Bartonella sp. uncultured | DQ155391                                       | Apodemus flavicollis | Slovenia         | (38)                                |
| Apodemus flavicollis (1); Myodes glareolus (1); Apodemus sylvaticus (2) | 4                                      | Bartonella sp. uncultured | DQ155384                                       | Apodemus sylvaticus | Slovenia         | (38)                                |
| Apodemus flavicollis (1); Myodes glareolus (2);         | 3                                      | Bartonella sp. uncultured | AJ269792                                        | Apodemus sylvaticus | UK               | (54)                                |
| Ctenophthalmus agyrtes                                  | 1                                      | Bartonella sp. uncultured | AJ269794                                        | Apodemus sylvaticus | UK               | (54)                                |
| Apodemus flavicollis (2); Myodes glareolus (3);         | 5                                      | Bartonella sp. uncultured | DQ155380                                        | Myodes glareolus  | Slovenia         | (38)                                |
| Ctenophthalmus agyrtes                                  | 2                                      | Bartonella sp. uncultured | DQ155380                                        | Myodes glareolus  | Slovenia         | (38)                                |
| Myodes glareolus                                        | 2                                      | Bartonella sp. uncultured | DQ155381                                        | Myodes glareolus  | Slovenia         | (38)                                |
| Apodemus flavicollis (2); Apodemus sylvaticus (3)       | 5                                      | Bartonella sp. uncultured | KU886433                                        | Ctenophthalmus nobilis | Germany        | (24)                                |
| Ctenophthalmus agyrtes                                  | 2                                      | Bartonella sp. uncultured | KU886488                                        | Apodemus flavicollis | Germany        | (24)                                |
| Nosopsyllus fasciatus;                                  | 6                                      | Bartonella sp. uncultured | KU886411                                        | Megabothris turbidus | Germany        | (24)                                |
| Myodes glareolus                                        | 1                                      | Bartonella sp. uncultured | KX267701                                        | Ixodes ricinus     | Slovakia        | (55)                                |
| Apodemus flavicollis                                    | 3                                      | Bartonella sp. uncultured | MF039571                                        | Rodent            | Slovakia        | (58)                                |
| Myodes glareolus (6); Apodemus flavicollis (5)          | 11                                     | Bartonella sp. uncultured | MN056366                                        | Myodes glareolus  | Germany         | (43)                                |
| Myodes glareolus                                        | 2                                      | Bartonella sp. uncultured | MN056367                                        | Myodes glareolus  | Germany         | (43)                                |
| Myodes glareolus                                        | 1                                      | Bartonella sp. Uncultured | MN056369                                        | Myodes glareolus  | Germany         | (43)                                |
| Myodes glareolus (4); Apodemus flavicollis (1)          | 5                                      | Bartonella sp. uncultured | MN056373                                        | Myodes glareolus  | Germany         | (43)                                |
| Myodes glareolus                                        | 2                                      | Bartonella sp. uncultured | MN056376                                        | Myodes glareolus  | Germany         | (43)                                |
| Apodemus flavicollis                                    | 4                                      | Bartonella sp. uncultured | MN056378                                        | Apodemus agrarius | Germany         | (43)                                |
| Myodes glareolus                                        | 1                                      | Bartonella sp. uncultured | MN056390                                        | Apodemus sylvaticus | Germany        | (43)                                |
| Apodemus sylvaticus                                     | 5                                      | Bartonella sp. uncultured | MN056393                                        | Microtus arvalis   | Czech Republic  | (43)                                |
| Myodes glareolus                                        | 2                                      | Bartonella taylor | MH547342                                        | Apodemus flavicollis | Lithuania    | (57)                                |
| Myodes glareolus (3); Apodemus flavicollis (5); Apodemus sylvaticus (1); Apodemus agrarius (1); Ctenophthalmus agyrtes (14) Peromyscopsylla sylvatica (1) | 10                                     | Bartonella taylor | MH547337                                        | Apodemus flavicollis | Lithuania    | (57)                                |
| Apodemus flavicollis (4); Apodemus sylvaticus (1);     | 15                                     | Bartonella taylor | MH547337                                        | Apodemus flavicollis | Lithuania    | (57)                                |
| Apodemus flavicollis                                    | 5                                      | Bartonella sp. N40      | AJ269787                                        | Genomic DNA       | UK              | (54)                                |

(Continued)
Bartonella doshiae was mainly detected in My. glareolus. There were four very short sequences (below 430 base pairs) which were not taken into consideration for Bartonella species identification.

**Bartonella spp. in Fleas**

Overall, 221 out of 450 fleas were tested positive for Bartonella spp. [49.1% (95%CI: 44.5–53.7)]. Every tested flea species was positive for Bartonella spp. with a prevalence ranging from 18.8 to 100% (Table 3). The prevalence levels of Bartonella spp. did not differ significantly comparing the most prevalent flea species (C. agyrtes, M. turbidus, N. fasciatus, C. congner; χ² = 1.8; p = 0.6121). Comparing small mammals and fleas, the prevalence with Bartonella spp. was almost identical and thus not significantly different (p = 0.9018). Positive fleas derived from 53 negative and 54 positive small mammals.

**Sequence Analysis for Bartonella spp. in Fleas**

In total, 74 positive samples (29%) were further determined to species level by sequencing which revealed four different Bartonella species in the examined fleas. All confirmed Bartonella species detected in fleas were the same Bartonella species as described for their small mammal hosts. However, most samples were positive for uncultured Bartonella spp. which showed 100% identity to 13 different sequences deposited in GenBank (Table 3). Almost all strains found in fleas were identical to those already found in their small mammal hosts (Table 4). Even though the distribution of the Bartonella species found in fleas was not completely identical compared to Bartonella spp. in small mammals, the prevalence of each Bartonella species did not differ significantly between small mammals and fleas (p = 0.2418–0.7631). Due to very small sample sizes of most flea species, statistical comparisons between the flea species were not carried out.

**DISCUSSION**

This study reports high prevalence rates of different Bartonella species in small mammals (43.3%) and their fleas (49.1%) from Germany. The Bartonella species detected in the current study were the same as earlier described by our group in small mammals from one of the investigated study sites (urban, renatured, and sylvatic) (24). The prevalences in small mammals from the current study as well as the detected Bartonella spp. are in line with those from Poland, France, the Netherlands, Slovenia, and Germany (11–72%) (10, 13, 24, 37, 38). Small mammals are known to be the main reservoirs for over 22 different Bartonella species (7, 8). The current study reports four Bartonella species belonging to lineage four in small mammals (B. grahamii, B. doshiae, B. taylorii, Bartonella sp. N40). Of the detected Bartonella spp., only B. grahamii is yet known to be zoonotic. Bartonella grahamii was isolated from My. glareolus from the UK for the first time (39). Since, it was found in rodents from almost all over the world and also caused disease in humans (40). Bartonellosis caused by B. grahamii displays
### TABLE 4 | Bartonella spp. detection based on the gltA gene and the 16S–23S rRNA ITS and species determination based on the 16S−23S rRNA ITS in fleas collected from small mammals.

| Flea species                      | Total No. | No. examined for Bartonella | Total No. of fleas infected with Bartonella based on ITS(%) | Total No. of fleas infected with Bartonella based on gltA (and ITS)(%) | No. of Bartonella-positive samples further investigated by sequencing | No. of detected Bartonella species based on the 16S−23S rRNA ITS |
|----------------------------------|-----------|-----------------------------|------------------------------------------------------------|------------------------------------------------------------------------|---------------------------------------------------------------------|---------------------------------------------------------------|
|                                  |           |                             |                                                            |                                                                        |                                                                 | Uncultured Bartonella sp. B. grahamii B. taylorii Bartonella sp. N40 |
| Ctenophthalmus agyrtes           | 873       | 322                         | 213 (59.5)                                                 | 165 (51.2)                                                             | 55                                                                  | 35 1 15 4                                                    |
| Megabothris turbidus             | 98        | 31                          | 15 (48.4)                                                  | 11 (35.5)                                                              | 3                                                                   | 2 1 0 0                                                    |
| Nosopsyllus fasciatus            | 44        | 31                          | 18 (58.1)                                                  | 16 (51.6)                                                              | 6                                                                   | 5 0 1 0                                                    |
| Ctenophthalmus congener          | 62        | 23                          | 12 (52.2)                                                  | 12 (52.2)                                                              | 3                                                                   | 2 0 1 0                                                    |
| Megabothris Walkeri              | 8         | 3                           | 1 (33.3)                                                   | 1 (33.3)                                                               | 1                                                                   | 0 0 1 0                                                    |
| Typhloceras poppei               | 11        | 2                           | 2 (28.6)                                                   | 2 (100)                                                                | 1                                                                   | 0 1 0 0                                                    |
| Paleopsylla soricis              | 3         | 1                           | 1 (100)                                                    | 0 (0)                                                                  | 0                                                                   | -- -- -- --                                                 |
| Leptopsylla segnis               | 17        | 16                          | 6 (37.5)                                                   | 3 (18.8)                                                               | 1                                                                   | 1 0 0 0                                                    |
| Ctenophthalmus bisoctodentatus   | 3         | 1                           | 1 (100)                                                    | 1 (100)                                                                | 0                                                                   | -- -- -- --                                                 |
| Hystrichopsylla talpae talpae    | 9         | 3                           | 3 (100)                                                    | 3 (100)                                                                | 1                                                                   | 0 0 0 1                                                    |
| Peromyscopsylla silvatica        | 25        | 18                          | 7 (38.9)                                                   | 7 (38.9)                                                               | 3                                                                   | 2 0 1 0                                                    |
| Megabothris rectangulatus        | 3         | 0                           | --                                                         | --                                                                     | --                                                                  | -- -- -- --                                                 |
| Total                            | 1156      | 450                         | 258 (57.3)                                                 | 221 (49.1)                                                             | 74                                                                  | 47 3 19 5                                                   |

**Note:** Number.
similar symptoms as cat scratch disease such as enlarged lymph nodes, fever and fatigue. Even though cats are not considered competent reservoirs for \textit{B. grahamii}, reports showed that they may still transmit the pathogen to humans via cat scratches when carrying infected rodent tissue on their claws (40). Most cases of bartonellosis caused by \textit{B. grahamii} are likely to remain undiagnosed due to the mild unspecific symptoms, insufficient diagnostic measures and the lack of awareness of practitioners (40). Further, 16 different uncultured \textit{Bartonella} spp. strains of yet unknown pathogenic potential were found in small mammals from the current study. This finding makes it obvious why clinical and public awareness of this zoonotic threat have to be increased.

Regarding the investigated small mammal species, \textit{Apodemus} spp. showed a significantly higher infection rate compared to \textit{My. glareolus} providing evidence that \textit{My. glareolus} is able to resolve \textit{Bartonella} infection after a certain time, while resolving a \textit{Bartonella} infection has not been observed in \textit{Apodemus} spp. yet (10). Moreover, it has been described that the re-infection rate in \textit{Apodemus} spp. is higher than in \textit{My. glareolus} which could also explain the higher prevalence in \textit{Apodemus} spp.

The infestation rate with fleas may also influence the \textit{Bartonella} prevalence in small mammals. In the current study, \textit{Apodemus} spp. were more often infested with fleas and the infestation rate of fleas was higher compared to \textit{My. glareolus}, which was also described in earlier studies from Germany and explained by the larger body size of \textit{Apodemus} spp. (41). This higher infestation rate may have resulted in a higher \textit{Bartonella} prevalence in \textit{Apodemus} spp. High \textit{Bartonella} prevalence rates (36–42%) were reported in \textit{Mi. arvalis} and \textit{Mi. agrestis} from Finland and Poland, respectively (9, 42). Further, another study by our group showed moderate to high prevalence rates in \textit{Microtus} spp. from the Czech Republic and Germany (43). In the current study, both \textit{Microtus} species (n = 8) were the only rodent species found negative for \textit{Bartonella} spp. However, the sample size tested was rather low. Although no fleas were found on the insectivores analyzed in this study, all insectivore species were positive for \textit{Bartonella} spp. In Sweden, it has been reported that insectivores may serve as reservoirs for certain \textit{Bartonella} species (37). Future studies need to be conducted in order to confirm this observation. The urban study site was the only site where \textit{A. sylvaticus} were trapped and \textit{L. segnis} were detected. Further \textit{L. segnis} is known to be a vector of \textit{Rickettsia felis} and \textit{Rickettsia typhi} and to occur mainly on small mammals which live synanthropic such as \textit{Mus musculus} and \textit{Rattus norvegicus} (44). The name “\textit{A. sylvaticus}” is misleading as this species is likewise synanthropic and a well-known host for \textit{L. segnis} (45). As the urban study site is a small park surrounded by walls and a high-traffic road, this study suggests it has basically a small ecological niche on its own. The proximity of small mammals to human settlements may pose a risk thus to the health of companion animals and humans.

The flea species may vary in their host specificity of being highly host-specific to being only host-opportunistic (46). The variety of flea species found in this study was high with twelve identified species. This high diversity is quite unexpected as previous studies from Poland, the UK and Germany found only 4–10 different flea species on the mentioned small mammal species (24, 41, 47, 48). However, one should consider that the current study covered three completely differently structured study sites which may have led to a higher variety of flea species. The flea burden was higher on \textit{Apodemus} spp. than on all other small mammal species. It is known that there is a higher immune resistance against flea burden in \textit{Microtus} spp. compared to \textit{Apodemus} spp. and \textit{My. glareolus} (49) which could also explain why the \textit{Microtus} spp. in the current study were all negative for \textit{Bartonella} spp. In our study, \textit{M. turbidus} and \textit{P. silvatica} were found significantly more often on \textit{My. glareolus} compared to \textit{A. flavicollis} which confirms that \textit{My. glareolus} is the main reservoir host for \textit{M. turbidus} (50). Moreover, the occurrence of \textit{P. silvatica} is quite rare and known to occur on \textit{My. glareolus} suggesting host specificity (51).

The \textit{Bartonella} species detected in small mammals were almost identical compared to those obtained from fleas. Half of the positive fleas were collected from negative small mammals. This observation indicates that the infection status of fleas can be independent from that of the current small mammal host. A previous study reported the vertical transmission in fleas which could explain how Bartonellae maintain in flea populations independently from a mammalian reservoir (52). Furthermore, frequent host changes by the fleas may have led to high infection levels. Only a few other studies report \textit{Bartonella} prevalence in the examined flea species (15, 17). However, it should be considered that some of these flea species may parasitize companion animals such as cats and dogs (53) and thus may pose a health threat as direct vectors of zoonotic pathogens such as \textit{Bartonella} spp. and \textit{Rickettsia} spp.

To conclude, this study shows a high diversity of flea species on small mammal hosts from Germany. Though none of the detected vector-host species combinations was unusual, the number of flea species found was unexpectedly high. In addition to small mammals, some of them especially the ones collected at the urban site also parasitize companion animals such as cats and dogs and may pose a risk for the transmission of zoonotic \textit{Bartonella} spp. Though \textit{B. grahamii} was the only confirmed zoonotic \textit{Bartonella} in this study, a very high variety of uncultured \textit{Bartonella} spp. of yet unknown zoonotic potential was also detected. Especially at the urban study site, a health risk in encountering \textit{Bartonella} infections is possible as infested rodents live there in close proximity to human settlements.

**DATA AVAILABILITY STATEMENT**

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ncbi.nlm.nih.gov/genbank/, MT551048-MT551101; MT913158-MT913206.

**AUTHOR CONTRIBUTIONS**

CS and MP organized and planned the study. AO and MP organized and participated in the fieldwork for the collection of wildlife samples. MK and DK carried out the morphologic determination of fleas. AO prepared the samples in the
laboratory. AO and NK tested the samples for *Bartonella* spp. AO, NK, and CS performed the sequence analysis. AO, NK, CS, and MP drafted the manuscript and wrote the final version. All authors read and approved the final manuscript.

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