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

Anna Obiegala[1,2]*, Martin Pfeffer[2], Daniel Kiefer[1], Matthias Kiefer[3], Nina Król[2] and Cornelia Silaghi[1,4]

[1] Comparative Tropical Medicine and Parasitology, Ludwig-Maximilians-Universität München, Munich, Germany, [2] Institute of Animal Hygiene and Veterinary Public Health, University of Leipzig, Leipzig, Germany, [3] Bavarian State Collection of Zoology, Munich, Germany, [4] Institute of Infectiology (IMED), Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Greifswald, Germany

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 Eubartonellae lineages and additionally *Bartonella australis* (8). *Bartonella tamiae* 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. tamiae*) (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 (65.8%) 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) "Dörnbergpark" (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" (641 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, Fla., 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 CO_2, 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. agrestis*) 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 cytochrome 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. agrestis*, 2 *M. nivalis*,

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′-AATGCAAAAAGAACAGT AAACA-3′) as forward and BhCS.781p (5′-GGGGaCCaGC TCATGGTGG-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′-CTTCAGATGATGATCC CAAGCCTTCTGGCG-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 $p \leq 0.05$.

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 (%)	Flea species No.[1] of small mammals infested/Total No.[1] of fleas												
			Ctenophthalmus agyrtes	*Megabothris turbidus*	*Paleopsylla soricis*	*Nosopsyllus fasciatus*	*Ctenophtalmus congener*	*Ctenophthalmus bisoctodentaus*	*Megabothris rectangulatus*	*Typhloceras poppei*	*Hystrichpsylla talpae talpae*	*Leptopsylla segnis*	*Megabothris walkeri*	*Peromyscopsylla silvatica*
Apodemus flavicollis	172	146 (84.9)	128/298	12/12	–	15/20	6/9	3/3	1/1	2/3	4/4	–	3/3	1/1
Myodes glareolus	395	276 (69.9)	232/507	61/80	2/3	4/4	36/51	-	1/2	1/1	4/4	–	5/5	21/24
Apodemus agrarius	6	6 (100)	5/8	1/1-	–	–	1/1	–	–	–	–	–	–	–
Apodemus sylvaticus	35	24 (68.6)	16/50	2/2	–	12/20	–	–	–	2/7	1/1	10/17	–	–
Mustela nivalis	2	1 (50)	1/4	1/1	–	–	–	–	–	–	–	–	–	–
Microtus agrestis	1	1 (100)	1/4	1/2	–	–	–	–	–	–	–	–	–	–
Microtus arvalis	6	2 (33.3)	1/2	–	–	–	1/1	–	–	–	–	–	–	–
Total No.[1]	617	456 (73.9)	384/873	78/98	2/3	31/44	43/62	3/3	2/3	5/11	9/9	10/17	8/8	22/25

No.[1], Number.

TABLE 2 | 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 spleen samples from different small mammal species.

Small mammal species	Total No.	Total No. of small mammals infected with Bartonella tested by ITS (%)	Total No. of small mammals 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.	Bartonella sp. N40	B. grahamii	B. taylorii	B. doshiae
Myodes glareolus	395	188 (47.5)	144 (36.5)***	44	25	0	7	5	7
Apodemus flavicollis	172	129 (73.3)	106 (61.6)***	31	21	4	0	5	1
Apodemus sylvaticus	35	14 (41.2)	13 (37.1)	12	10	1	0	1	0
Apodemus agrarius	6	3 (50)	3 (50)	1	0	0	0	1	0
Microtus arvalis	6	0 (0)	0 (0)	–	–	–	–	–	–
Microtus agrestis	1	0 (0)	0 (0)	–	–	–	–	–	–
Mustela nivalis	2	2 (100)	2 (100)	0	–	–	–	–	–
Sorex spp.	5	2 (40)	1 (20)	0	–	–	–	–	–
Talpa europaea	1	1 (100)	1 (100)	0	–	–	–	–	–
Total	623	339 (51.4)	270 (43.3)	88	56	5	7	12	8

***A. flavicollis significantly higher than in My. glareolus (p = 0.0001).
No., Number; B., Bartonella; sp., species; Cand., Candidatus.

RESULTS

Flea Collection

In total, 1,156 fleas were collected from 456 small mammals. Altogether, twelve different flea species were detected (873 *Ctenophthalmus agyrtes*, 3 *Ctenophthalmus bisoctodentatus*, 62 *Ctenophthalmus congener*, 98 *Megabothris turbidus*, 8 *Megabothris walkeri*, 9 *Hystrichopsylla talpae talpae*, 25 *Peromyscopsylla silvatica*, 3 *Paleopsylla soricis*, 44 *Nosopsyllus fasciatus*, 11 *Typhloceras poppei*, 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 turbidus* 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 captured small mammal species (20–100%) with the exception of *Microtus* spp. which were all negative and thus significantly less often infected than other species ($p = 0.007$). Considering the two most frequently captured species, the prevalence 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 (1); *Peromyscopsylla sylvatica* (1)	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	(56)
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 taylorii*	MH547342	*Apodemus flavicollis*	Lithuania	(57)
Myodes glareolus (3); *Apodemus flavicollis* (5); *Apodemus sylvaticus* (1); *Apodemus agrarius* (1);	10	*Bartonella taylorii*	MH547337	*Apodemus flavicollis*	Lithuania	(57)
Ctenophthalmus agyrtes (14) *Peromyscopsylla sylvatica* (1)	15	*Bartonella taylorii*	MH547337	*Apodemus flavicollis*	Lithuania	(57)
Apodemus flavicollis (4); *Apodemus sylvaticus* (1);	5	*Bartonella* sp. N40	AJ269787	Genomic DNA	UK	(54)

(Continued)

TABLE 3 | Continued

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
Hystrichpsylla talpae talpae (1) *Ctenophthalmus agyrtes* (4)	5	*Bartonella* sp. N40	AJ269787	Genomic DNA	UK	(54)
Myodes glareolus	7	*Bartonella grahamii*	CP001562	Genomic DNA	Sweden	(58)
Ctenophthalmus agyrtes (1) *Megabothris turbidus* (1) *Ctenophthalmus congener* (1)	3	*Bartonella grahamii*	CP001562	Genomic DNA	Sweden	(58)
Hystrichpsylla talpae talpae (1) *Ctenophthalmus agyrtes* (2)	3	*Bartonella* sp. uncultured	MN056412	*Apodemus flavicollis*	Germany	(43)
Ctenophthalmus agyrtes	4	*Bartonella* sp. uncultured	MK562487	*Apodemus* sp.	Italy	(59)
Leptopsylla segnis (1) *Ctenophthalmus congener* (2) *Ctenophthalmus agyrtes* (2)	5	*Bartonella* sp. uncultured	MN056379	*Apodemus sylvaticus*	Germany	(43)
Ctenophthalmus agyrtes	1	*Bartonella* sp. uncultured	MN056375	*Myodes glareolus*	Germany	(43)
Ctenophthalmus congener (1) *Ctenophthalmus agyrtes* (1) Megabothris walkeri (1) *Nosopsyllus fasciatus* (1)	4	*Bartonella taylorii*	MH547339	*Apodemus flavicollis*	Lithuania	(57)
Ctenophthalmus agyrtes	11	*Bartonella* sp. uncultured	MT551074	*Apodemus sylvaticus*	Germany	Current study
Myodes glareolus (7) *Apodemus flavicollis* (1)	8	*Bartonella doshiae*	AJ269786	genomic DNA	UK	(54)

Bartonella doshiae was mainly detected in *My. glareolus*. There were four very short sequences (below 430 base pairs) which were not considered as a positive sequencing result and therefore 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. congener*; $\chi^2 = 1.8$; $p = 0.6121$). 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

No., Number.

similar symptoms as cat scratch disease such as enlarged lymph nodes, fever and fatigue. Even though cats are not considered competent reservoirs for *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 *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 *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, *Apodemus* spp. showed a significantly higher infection rate compared to *My. glareolus* providing evidence that *My. glareolus* is able to resolve *Bartonella* infection after a certain time, while resolving a *Bartonella* infection has not been observed in *Apodemus* spp. yet (10). Moreover, it has been described that the re-infection rate in *Apodemus* spp. is higher than in *My. glareolus* which could also explain the higher prevalence in *Apodemus* spp.

The infestation rate with fleas may also influence the *Bartonella* prevalence in small mammals. In the current study, *Apodemus* spp. were more often infested with fleas and the infestation rate of fleas was higher compared to *My. glareolus*, which was also described in earlier studies from Germany and explained by the larger body size of *Apodemus* spp. (41). This higher infestation rate may have resulted in a higher *Bartonella* prevalence in *Apodemus* spp. High *Bartonella* prevalence rates (36–42%) were reported in *Mi. arvalis* and *Mi. agrestis* from Finland and Poland, respectively (9, 42). Further, another study by our group showed moderate to high prevalence rates in *Microtus* spp. from the Czech Republic and Germany (43). In the current study, both *Microtus* species ($n = 8$) were the only rodent species found negative for *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 *Bartonella* spp. In Sweden, it has been reported that insectivores may serve as reservoirs for certain *Bartonella* species (37). Future studies need to be conducted in order to confirm this observation. The urban study site was the only site where *A. sylvaticus* were trapped and *L. segnis* were detected. Further *L. segnis* is known to be a vector of *Rickettsia felis* and *Rickettsia typhi* and to occur mainly on small mammals which live synanthropic such as *Mus musculus* and *Rattus norvegicus* (44). The name "*A. sylvaticus*" is misleading as this species is likewise synanthropic and a well-known host for *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 *Apodemus* spp. than on all other small mammal species. It is known that there is a higher immune resistance against flea burden in *Microtus* spp. compared to *Apodemus* spp. and *My. glareolus* (49) which could also explain why the Microtus spp. in the current study were all negative for *Bartonella* spp. In our study, *M. turbidus* and *P. silvatica* were found significantly more often on *My. glareolus* compared to *A. flavicollis* which confirms that *My. glareolus* is the main reservoir host for *M. turbidus* (50). Moreover, the occurrence of *P. silvatica* is quite rare and known to occur on *My. glareolus* suggesting host specificity (51).

The *Bartonella* species detected in small mammals were almost identical compared to those obtained from fleas. However, 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 *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 *Bartonella* spp. and *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 dogs and cats and may pose a risk for the transmission of zoonotic *Bartonella* spp. Though *B. grahamii* was the only confirmed zoonotic *Bartonella* in this study, a very high variety of uncultured *Bartonella* spp. of yet unknown zoonotic potential was also detected. Especially at the urban study site, a health risk in encountering *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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