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Anaplasma phagocytophilum in Questing *Ixodes ricinus* Ticks: Comparison of Prevalences and Partial 16S rRNA Gene Variants in Urban, Pasture, and Natural Habitats

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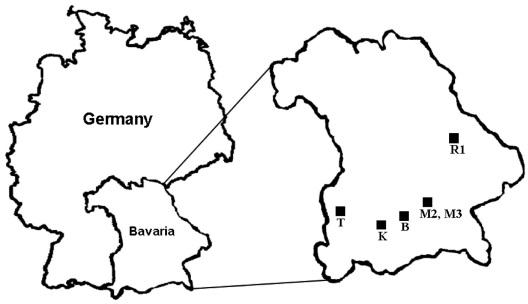
Urban, natural, and pasture areas were investigated for prevalences and 16S rRNA gene variants of *Anaplasma phagocytophilum* in questing *Ixodes ricinus* ticks. The prevalences differed significantly between habitat types, and year-to-year variations in prevalence and habitat-dependent occurrence of 16S rRNA gene variants were detected.

The obligate intracellular bacterium *Anaplasma phagocytophilum* is transmitted by *Ixodes ricinus* ticks in Europe (1) and causes granulocytic anaplasmosis in humans and several other mammalian species (2–5). Reservoir hosts are necessary to maintain the endemic cycle of *A. phagocytophilum* (transstadial but not transovarial transmission [6]). Different host species seem to be susceptible to different genetic variants of this pathogen, with potentially differing pathogenicity (1, 7). The aim of this study was to compare the prevalences and the variability in 16S rRNA gene variants of *A. phagocytophilum* in *I. ricinus* ticks, as well as the densities of questing *I. ricinus* ticks, collected in 2011 and 2012 from six study sites in Bavaria, Germany (structured in three different habitats—urban, pasture, and natural), with regard to the occurrence of various potential reservoir hosts (Fig. 1; further description of study sites will be published elsewhere [E. Overzier, K. Pfister, C. Thiel, I. Herb, M. Mahling, and C. Silaghi, submitted for publication]).

In April, May, and June of 2011 and 2012, questing ticks were collected with the flagging method on 300 m^2 transects on each study site. DNA was extracted automatically with the Maxwell 16 system (Promega, Mannheim, Germany). The DNA concentration was measured spectrophotometrically (NanoDrop ND-1000; PeqLab, Erlangen, Germany). The *msp2* gene of *A. phagocytophilum* was detected by SYBR Green real-time PCR in the AB 7500 real-time PCR system (Applied Biosystems, Darmstadt, Germany) as described previously (8). An assortment of positive samples was investigated with a nested PCR targeting the 16S rRNA gene to detect different gene variants (9, 10). Positive PCR products were purified, sequenced, and analyzed as described previously (8). All sequences were compared with sequences from previous studies by our group (4, 8, 10–13).

Exact 95% confidence intervals (95% CI) of prevalences in ticks were computed with the Clopper and Pearson method (14). Two logistic regression models were estimated to investigate the effects of gender, month, and year on the probability of ticks being positive for *A. phagocytophilum*. One model also included habitat, and another one the different sites. A simultaneous test for general linear hypotheses based on multiple comparisons of means with Tukey contrasts (15) was used to test for differences between gender, month, habitat, and site, respectively. Statistical analysis was performed with R, version 2.15.1 (16).

Overall 9,672 *I. ricinus* ticks were collected, with average tick densities in the urban area (ticks/100 m^2) ranging from 21 to 97 ticks per 100 m^2 in 2011 and 15 to 51 ticks per 100 m^2 in 2012. On the pasture, the tick densities were 50 and 38 ticks per 100 m^2 in 2011 and 2012, and in the natural area, the densities were 73 and 34 ticks per 100 m^2 (Table 1). The mean values of the ratio of adults to nymphs were 1:2.3 (2011) and 1:0.9 (2012) (Table 1).



Urban area	M2	Munich/'English Garden'	375 ha	48°09'01.73"N,	11°35'24.19"E
	M3	Munich/'Nymphenburger Schlosspark'	141 ha	48°09'38.93"N,	11°29'33.31"E
	R1	Regensburg/'Dörnbergpark'	7.4 ha	49°00'55.72"N,	12°05'08.89"E
	B	Lake Starnberg/'Schlosspark Berg'	30 ha	47°57'43.85"N,	11°20'53.35"E
Pasture area	K	'Kerschlach'	16 ha	47°54'57.18"N,	11°12'44.04"E
Natural area	T	Tussenhausen/'Angelberger Forst'	641 ha	48°06'36.42"N,	10°34'33.40"E

FIG 1 Locations of sampling sites.

A total of 214 of 4,064 questing-tick samples were positive for DNA of *A. phagocytophilum*, with mean prevalences in 2011 and 2012, respectively, as follows: urban, 4.9% and 7.4%; pasture, 1.1% and 2.8%; and natural area, 4.0% and 5.8% (Tables 2 and 3). No significant difference was detected between collection months, but the overall prevalence of *A. phagocytophilum* was significantly higher in 2012 than in 2011 ($P < 0.01$) (Tables 2 and 3). Adults showed a significantly higher prevalence than nymphs ($P < 0.001$). The prevalence was significantly lower on the pasture than

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TABLE 1 Absolute and average tick density (*Ixodes ricinus*: adults and nymphs/100 m^2) per site in April, May, and June and relationship of stages in 2011 and 2012

		Tick density/100 m^2													
		April		May		June		Mean		2011			2012		
Habitat	Site	2011	2012	2011	2012	2011	2012	2011	2012	Adults	Nymphs	Ratio	Adults	Nymphs	Ratio
Urban	English Garden (M2)	17	6	89	35	69	28	58	19	25	33	1:1.3	17	6	1:0.4
	Nymphenburger Schlosspark (M3)	22	14	31	21	10	10	21	15	7	14	1:2.0	8	7	1:0.9
	Dörnbergpark (R1)	93	15	91	114	108	23	97	51	56	41	1:0.7	33	18	1:0.5
	Berg (B)	31	13	105	21	134	20	90	18	10	80	1:8.0	14	4	1:0.3
	Mean	41	12	79	48	80	20	67	26	25	42	1:1.7	18	9	1:0.5
Pasture	Kerschlach (K)	68	26	70	53	12	33	50	38	14	36	1:2.6	16	22	1:1.4
Natural	Angelberger Forst (T)	65	28	74	38	80	36	73	34	5	68	1:13.6	9	25	1:2.8
All sites	Mean	49	17	77	47	69	25	65	29	20	45	1:2.3	16	14	1:0.9

in the urban ($P < 0.001$) and natural ($P < 0.01$) areas. Study site R1 showed a significantly higher prevalence than all other study sites ($P < 0.001$). Study site B showed a significantly lower prevalence than study sites M2 ($P < 0.05$) and T (natural area) ($P < 0.01$).

A total of 116 of 214 positive samples were sequenced. Alignment of 497 bp of the partial 16S rRNA gene sequences revealed 9 variants with 99 to 100% identity to each other and to sequences previously deposited in GenBank (Table 4). Nucleotide sequence accession numbers: The partial 16S rRNA gene sequences found in this study were submitted to GenBank under the accession numbers given in Table 4.

Our results support the hypothesis that the prevalence and genetic variants of *A. phagocytophilum* vary depending on habitat structures and the occurrence of different potential reservoir hosts. In a comparison of the results from the urban sites with the results from the same sites from a previous study during 2009 and 2010 (8), an overall continuous decrease followed by an increase in prevalence was detected. This might depend on more global factors affecting all sites, such as the weather conditions during those years, the overall appearance of the vector *I. ricinus*, and/or the appearance of common reservoir hosts, and not on habitat structure or other factors on single sites. The prevalence of *A. phagocytophilum* in ticks (which had their last blood meal 1 or 2 years prior to this investigation) depends on its prevalence in the reservoir hosts. Consequently, changes of the *A. phagocytophilum* prevalence in reservoir hosts are detected with a temporal delay by the prevalence in questing ticks.

We detected 9 different 16S rRNA gene variants of *A. phagocytophilum* with variations in study sites. In urban sites R1 and M2,

TABLE 2 Prevalence and 95% confidence interval of *Anaplasma phagocytophilum* in *Ixodes ricinus* ticks per site for 2011 and 2012[a]

			Adult ticks		Females			Males			Nymphs		
Habitat	Site	Yr	No. pos/total no.	%	No. pos/total no.	%	95% CI	No. pos/total no.	%	95% CI	No. pos/total no.	%	95% CI
Urban	M2	2011	9/240	3.8	2/120	1.7	0.2–5.9	7/120	5.8	2.4–11.6	1/120	0.8	0.0–4.6
		2012	20/240	8.3	8/120	6.7	2.9–12.7	12/120	10.0	5.3–16.8	5/118	4.2	1.4–9.6
	M3	2011	14/259	5.4	9/135	6.7	3.1–12.3	5/124	4.0	1.3–9.2	0/140	0.0	0.0–2.6
		2012	10/232	4.3	6/115	5.2	1.9–11.0	4/117	3.4	0.9–8.5	2/120	1.7	0.2–5.9
	R1	2011	39/240	16.3	16/120	13.3	7.8–20.7	23/120	19.2	12.6–27.4	2/120	1.7	0.2–5.9
		2012	54/235	23.0	26/115	22.6	15.3–31.3	28/120	23.3	16.1–31.9	7/120	5.8	2.4–11.6
	B	2011	2/150	1.3	2/79	2.5	0.3–8.8	0/71	0.0	0.0–5.1	1/120	0.8	0.0–4.6
		2012	6/240	2.5	3/120	2.5	0.5–7.1	3/120	2.5	0.5–7.1	0/106	0.0	0.0–3.4
	Total	2011	64/889	7.2	29/454	6.4	4.3–9.1	35/435	8.0	5.7–11.0	4/500	0.8	0.2–2.0
		2012	90/947	9.5	43/470	9.1	6.7–12.1	47/477	9.9	7.3–12.9	14/464	3.0	1.7–5.0
Pasture	K	2011	4/225	1.8	2/93	2.2	0.3–7.6	2/132	1.5	0.2–5.4	0/140	0.0	0.0–2.6
		2012	10/234	4.3	7/114	6.1	2.5–12.2	3/120	2.5	0.5–7.1	0/120	0.0	0.0–3.0
Natural	T	2011	7/79	8.9	2/33	6.1	0.7–20.2	5/46	10.9	3.6–23.6	1/120	0.8	0.0–4.6
		2012	17/226	7.5	11/109	10.1	5.1–17.3	6/117	5.1	1.9–10.8	3/120	2.5	0.5–7.1
All sites	Total	2011	75/1,193	6.3	33/580	5.7	3.9–7.9	42/613	6.9	5.0–9.1	5/760	0.7	0.2–1.5
		2012	117/1,407	8.3	61/693	8.8	6.8–11.2	56/714	7.8	6.0–10.1	17/704	2.4	1.4–3.8

[a] pos, positive for *A. phagocytophilum*; 95% CI, 95% confidence interval.

TABLE 3 Prevalence and 95% confidence interval of *Anaplasma phagocytophilum* in *Ixodes ricinus* ticks per month for 2011 and 2012[a]

Month	Yr	Site	Adults		Female			Male			Nymph		
			No. pos/total no.	%	No. pos/total no.	%	95% CI	No. pos/total no.	%	95% CI	No. pos/total no.	%	95% CI
April	2011	Urban	18/283	6.4	8/141	5.7	2.5–10.9	10/142	7.0	3.4–12.6	3/160	1.9	0.4–5.4
		Pasture	2/54	3.7	1/21	4.8	0.1–23.8	1/33	3.0	0.1–15.8	0/40	0.0	0.0–8.8
		Natural	3/29	10.3	2/13	15.4	1.9–45.4	1/16	6.3	0.2–30.2	0/40	0.0	0.0–8.8
		Total	23/366	6.3	11/175	6.3	3.2–11.0	12/191	6.3	3.3–12.2	3/240	1.3	0.3–3.6
	2012	Urban	42/312	13.5	22/155	14.2	9.1–20.7	20/157	12.7	8.0–19.0	2/146	1.4	0.2–4.9
		Pasture	0/79	0.0	0/39	0.0	0.0–9.0	0/40	0.0	0.0–8.8	0/40	0.0	0.0–8.8
		Natural	10/74	13.5	6/34	17.6	6.8–34.5	4/40	10.0	2.8–23.7	0/40	0.0	0.0–8.8
		Total	52/465	11.2	28/228	12.3	8.3–17.3	24/237	10.1	6.6–14.7	2/226	0.9	0.1–3.2
May	2011	Urban	28/317	8.8	13/160	8.1	4.4–13.5	15/157	9.6	5.5–15.3	0/160	0.0	0.0–2.3
		Pasture	2/79	2.5	1/39	2.6	0.1–13.5	1/40	2.5	0.1–13.2	0/40	0.0	0.0–8.8
		Natural	1/28	3.6	0/8	0.0	0.0–36.9	1/20	5.0	0.1–24.9	0/40	0.0	0.0–8.8
		Total	31/424	7.3	14/207	6.8	3.8–11.1	17/217	7.8	4.6–12.2	0/240	0.0	0.0–1.5
	2012	Urban	23/320	7.2	8/160	5.0	2.2–9.6	15/160	9.4	5.3–15.0	4/158	2.5	0.7–6.3
		Pasture	3/80	3.8	2/40	5.0	0.6–16.9	1/40	2.5	0.1–13.2	0/40	0.0	0.0–8.8
		Natural	3/78	3.8	2/38	5.3	0.6–17.7	1/40	2.5	0.1–13.2	3/40	7.5	1.6–20.4
		Total	29/478	6.1	12/238	5.0	2.6–8.6	17/240	7.1	4.2–11.1	7/238	2.9	1.2–6.0
June	2011	Urban	18/289	6.2	8/153	5.2	2.3–10.0	10/136	7.4	3.6–13.11	1/180	0.6	0.0–3.1
		Pasture	0/92	0.0	0/33	0.0	0.0–10.6	0/59	0.0	0.0–6.1	0/60	0.0	0.0–6.0
		Natural	3/22	13.6	0/12	3.0	0.0–26.5	3/10	30.0	6.7–65.2	1/40	2.5	0.0–13.2
		Total	21/403	5.2	8/198	4.0	1.8–7.8	13/205	6.3	3.4–10.6	2/280	0.7	0.1–2.6
	2012	Urban	25/315	7.9	13/155	8.4	4.5–13.9	12/160	7.5	3.9–12.7	8/160	5.0	2.2–9.6
		Pasture	7/75	9.3	5/35	14.3	4.8–30.3	2/40	5.0	0.6–16.9	0/40	0.0	0.0–8.8
		Natural	4/74	5.4	3/37	8.1	1.7–21.9	1/37	2.7	0.1–14.2	0/40	0.0	0.0–8.8
		Total	36/464	7.8	21/227	9.3	5.8–13.8	15/237	6.3	3.6–10.2	8/240	3.3	1.4–6.5

[a] pos, positive for *A. phagocytophilum*; 95% CI, 95% confidence interval.

variant A was found most frequently. This confirms the 16S rRNA gene variants found on these study sites in 2009 and 2010 (8). Variant A has not yet been detected in wild ungulates in our investigations (12, 24). Furthermore, as wild ungulates are rare to nonexistent on study sites R1 and M2, there must be other reservoir hosts present, such as foxes, small rodents, hedgehogs, squirrels, or birds (13, 17–21). Variant A has also been detected in a human patient (22) and in granulocytic anaplasmosis cases in horses and dogs (3, 4). It has been discussed previously whether this variant may be less pathogenic (3). This might explain the discrepancy between the high prevalence in questing ticks in urban areas and the lack of clinical human cases in Germany. More in-depth and experimental studies on the pathogenic potential of this variant are needed to elucidate this hypothesis. Further efforts to find the main reservoir host for *A. phagocytophilum* in urban areas are necessary. In the urban site M3, wild game exists, and besides variant A, variant Y, previously found in roe deer, was detected (12, 22). Variant Y was also detected in the other study sites where wild ungulates exist, whereas it was not detected in those city parks without large ungulate species. These findings confirm results from 2009 and 2010 (8). On the pasture, where mainly cattle are kept, variants B and W were additionally found. Variant B is identical to the prototype variant of *A. phagocytophilum* from human clinical cases in the amplified part of the 16S rRNA gene and is also frequently detected in horses and dogs with granulocytic anaplasmosis (3, 4). Variant W was evidenced mainly in sheep and cattle with tick-borne fever in previous studies (5, 23). In the natural and pasture areas, as well as in the forest-like park (site B), variant X was also found. Furthermore, it was detected in 44% of sequenced *A. phagocytophilum*-positive roe deer samples in the natural area in 2010 and 2011 (24).

In conclusion, the prevalence rates and the occurrence of partial 16S rRNA gene variants of *A. phagocytophilum* differed in all habitats investigated in our study, most likely depending on the habitat structure and, therefore, the appearance and availability of typical reservoir hosts. Furthermore, a year-to-year variation could be detected that was unaffected by the habitat structure, suggesting the involvement of more global factors in the occurrence of *A. phagocytophilum* in ticks.

Nucleotide sequence accession numbers. The partial 16S rRNA gene sequences found in this study (except for JX627370)

TABLE 4 16S rRNA gene variants of *Anaplasma phagocytophilum* in 116 samples of questing ticks with single-nucleotide substitutions in the 497-bp sequence compared with GenBank sequences

Sequence variant[a]	Total no. of samples with variant	Habitat[b]	Sampling site (no. of samples with variant)	Hosts found in other studies (GenBank accession no.)[c]	GenBank accession no. of sequence found in this study	Nucleotide at indicated position[d]						
						74	76	78	80	84	170	376
A	80	U	M2 (16), M3 (9), R (55)	*Ixodes ricinus* (JN181064), *Dermacentor reticulatus* (JN181063), dog (*Canis lupus familiaris*) (FJ829761), European hedgehog (*Erinaceus europaeus*) (JN571156), cat (*Felis domesticus*) (HM138366), human (GU236655)	JX909353	A	A	A	A	G	C	**A**
P	1	U	M2	NM[e]	JX909354	**G**	A	A	A	G	C	**A**
Z	1	U	M3	*Ixodes ricinus* (EU490523), goat (FJ538290)	JX909355	A	A	A	A	G	**T**	**A**
W	1	P	K	*Ixodes ricinus* (JN181071), *Ixodes persulcatus* (HM366582), northern red-backed vole (*Myodes rutilus*) (HQ630622), bank vole (*Myodes glareolus*) (AY094353), common shrew (*Sorex araneus*) (HQ630623), hedgehog (JN571163), coyote (*Canis latrans*) (AF170728), dog (AY741098), horse (*Equus caballus*) (AF172167), cattle (*Bos taurus*) (JQ026308), lama (*Lama glama*) (AF241532), mouflon (*Ovis musimon*) (EU839851), chamois (*Rupicapra rupicapra*) (FJ812399), red deer (*Cervus elaphus*) (GQ428331), sheep (*Ovis aries*) (GQ428333), roe deer (*Capreolus capreolus*) (JX627363), wild boar (*Sus scrofa*) (GU391313), human (*Homo sapiens*) (AF093789)	JX909356	A	A	A	A	**A**	C	G
O	1	N	T	Roe deer (GU236538)	JX627370	A	**G**	**G**	A	G	C	G
V	3	U, N	M3 (1), T (2)	*Ixodes ricinus* (FJ788512), dog (JN656381), hedgehog (JN571162), roe deer (JX627362)	JX909357	A	A	A	**G**	**A**	C	G
B	3	P, N	K (2), T (1)	*Ixodes ricinus* (HQ629917), *Ixodes ovatus* (AY969015), *Hemaphysalis longicornis* (GU064899), vulture (*Falconiformes*) (JN217095), cottontail rabbit (*Sylvilagus floridanus*) (AY144728), white-footed mouse (*Peromyscus leucopus*) (U72878), European hedgehog (JN571159), dog (FJ829787), horse (JF893938), red deer (EU839850), roe deer (EU839848), human (AY886761, U02521)	JX909358	A	A	A	A	G	C	G
X	8	U, P, N	B (3), K (2), T (3)	*Ixodes ricinus* (HQ629923), *Ixodes scapularis* (AF311343), goat (*Capra aegagrus hircus*) (FJ538288), roe deer (HM480381)	JX909359	A	**G**	A	A	**A**	C	G
Y	18	U, P, N	M3 (7), B (2), K (3), T (6)	*Ixodes ricinus* (JN181069), cotton rat (*Sigmodon hispidus*) (JQ063025), goat (FJ538289), mouflon (FJ812409), roe deer (HM480385)	JX909360	A	**G**	A	A	G	C	G
				Anaplasma phagocytophilum HZ complete genome (NC_007797)		A	A	A	A	G	C	G

[a] Not official nomenclature but has been used in previous studies (8, 12).
[b] U, urban area; P, pasture area; N, natural area.
[c] The list is not exhaustive.
[d] *Anaplasma phagocytophilum* HZ (complete genome, GenBank accession no. NC_007797) was used as the reference strain; nucleotide positions indicate the position relative to bp 1433 of the *rrsA* 16S rRNA gene (Gene ID 3930754). Bold indicates nucleotide differences compared to the complete genome.
[e] NM, no match with sequences in GenBank.

were submitted to GenBank under the accession numbers given in Table 4.

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