“*Candidatus* Neoehrlichia mikurensis,” *Anaplasma phagocytophilum*, and Lyme Disease Spirochetes in Questing European Vector Ticks and in Feeding Ticks Removed from People

Dania Richter and Franz-Rainer Matuschka

Abt. Parasitologie, Institut für Pathologie, Charité Universitätsmedizin Berlin, Berlin, Germany

To estimate the likelihood of people coming into contact with the recently described tick-borne agent “*Candidatus* Neoehrlichia mikurensis,” we compared its prevalence to those of Lyme disease spirochetes and *Anaplasma phagocytophilum* in questing adult *Ixodes ricinus* ticks collected in various Central European sites and examined ticks, which had been removed from people, for the presence of these pathogens. Whereas spirochetes infected questing adult ticks most frequently (22.3%), fewer than a third as many ticks were infected by “*Ca. Neoehrlichia mikurensis*” (6.2%), and about a sixth harbored *A. phagocytophilum* (3.9%). On average, every twelfth encounter of a person with an *I. ricinus* tick (8.1%) may bear the risk of acquiring “*Ca. Neoehrlichia mikurensis*.” Although a fifth of the people (20%) had removed at least one tick infected by “*Ca. Neoehrlichia mikurensis*,” none displayed symptoms described for this pathogen, suggesting that its transmission may not be immediate and/or that immunocompetent individuals may not be affected. Because immunosuppressed patients may be at a particular risk of developing symptoms, it should be considered that “*Ca. Neoehrlichia mikurensis*” appears to be the second most common pathogen in *I. ricinus* ticks. In our survey, only *Borrelia afzelii* appears to infect Central European vector ticks more frequently.

Ticks of the *Ixodes ricinus* complex serve as vectors for a variety of pathogens in the temperate zones of North America, Asia, and Europe. The most common of these is the agent of Lyme disease, *Borrelia burgdorferi* sensu lato. Like Pandora’s Box, these ticks seem to reveal consistently novel pathogens. “*Candidatus* Neoehrlichia mikurensis,” derived from Norway rats (*Rattus norvegicus*), and *Ixodes ovatus* ticks in Japan, served to delineate a novel genus in the family of Anaplasmataceae (8). A closely related organism had already been identified in Dutch *I. ricinus* ticks (17). Indeed, the DNA of “*Ca. Neoehrlichia mikurensis*” has been detected in various other rodents and in European *I. ricinus* and Asian *I. persulcatus* ticks during the last decade (1, 22).

Most recently, several human cases severely affected by an infection with “*Ca. Neoehrlichia mikurensis*” have been described in Europe (5, 12, 23, 24). As are all other members of the family Anaplasmataceae, this pathogen is an obligate intracellular bacterium. It appears to display an endothelial cell tropism (8), and symptoms observed in some of these patients, such as aneurysm, thromboembolic complications, subcutaneous hemorrhages, and erythematous rashes (5, 12, 23, 24), may result from this affinity. Of the six patients infected by “*Ca. Neoehrlichia mikurensis*” reported hitherto, five were undergoing immunosuppressive therapy (12, 23, 24). In addition, a dog affected by “*Ca. Neoehrlichia mikurensis*” postoperatively suffered from chronic neutropenia (4). The contact to a tick infected with “*Ca. Neoehrlichia mikurensis*” could have occurred during immunosuppressive treatment or latent bacteria acquired from a previous tick contact could have been activated by immunosuppressive treatment or postoperative trauma.

To estimate the likelihood of people coming into contact with the newly described tick-borne agent “*Ca. Neoehrlichia mikurensis*,” we compared its prevalence to those of Lyme disease spirochetes and of *A. phagocytophilum* in questing adult *I. ricinus* ticks collected in various European sites. In addition, we examined

ticks, which had been removed from people, for the presence of these pathogens.

MATERIALS AND METHODS

Questing adult ticks were collected by flagging ground vegetation at various sites in northern (Flensburg), central (Berlin and Potsdam-Mittelmark) and southern Germany (Hohenloher Plane, Stuttgart, Heilbronn, Karlsbad, Gruibingen, Rangendingen, and Weisenbach), in two sites in eastern France (Petite Camargue Alsacienne and Northern Vosges), in southwestern Poland (Złoty Stok), in the central Czech Republic (Konopiste), and on Madeira Island (Paul da Serra), Portugal. These ticks, as well as nymphal and adult ticks removed from German patients, were preserved in 80% ethanol until processed.

To detect DNA of *Borrelia* sp., “*Ca. Neoehrlichia mikurensis*” and *A. phagocytophilum* in a random subsample of these questing ticks, as well as in ticks removed from patients, we amplified a fragment of the 16S rRNA gene for each of the first two pathogens by nested PCR and a fragment of the p44 gene for *A. phagocytophilum* by conventional PCR (for primers and references see Table 1). DNA was obtained by using a QIAamp DNA minikit (Qiagen, Hilden, Germany) and stored at -30°C . For the amplification of DNA of “*Ca. Neoehrlichia mikurensis*” and *A. phagocytophilum*, aliquots of DNA suspensions (1 μl) were diluted to 25 μl by using 200 μM concentrations of each deoxynucleoside triphosphate, 1 U of *Taq* polymerase (Qiagen), and 5 pmol of the outer primer pair EC9 and EC12A for “*Ca. Neoehrlichia mikurensis*” or 10 pmol of the primer pair MSP3F and MSP3R for *A. phagocytophilum* and Coral-PCR-buffer supplied with the *Taq* polymerase. The mixture was placed in a thermocycler (PTC 200; MJ Research Biozym, Diagnostic, Hess. Oldendorf, Germany),

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Address correspondence to Dania Richter, drichter@charite.de.

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TABLE 1 Primers used in this study

Species	Target gene	Fragment size (bp) ^a	Outer primer ^b		Inner primer		Reference
			Name	Sequence (5'–3')	Name	Sequence (5'–3')	
<i>Ca. Neoehrlichia mikurensis</i> ^a	16S rRNA	488*	EC9	TAC CTT GTT AGG ACT T	IS58-62f	GGA ATA GCT GTT AGA AAT GACA	7
			EC12A	TGA TCC TGG CTC AGA ACG AAC	IS58-594r	CTA TCC TCT CTC GAT CTC TAG TTT	
	groESL	315*	gro607F1	GAA GAT GCT GTD GGA TGT ACT GC	gro667F2	ATT ACT CAG AGT GCT TCT CAG TG	20
			gro1294R1	AGT GCT TCA CCT TCT ACA TCT TC	gro1121R2	TGC ATR CCR TCA GTT TTT TCA AC	
<i>A. phagocytophilum</i>	p44	334	MSP3F	CCA GCG TTT AGC AAG ATA AGA G			26
			MSP3R	GCC CAG TAA CAA CAT CAT AAG C			
<i>Borrelia</i> sp.	16S rRNA	659*	16S1A	CTA ACG CTG GCA GTG CGT CTT AAG C	16S2A	AGT CAA ACG GGA TGT AGC AAT ACA	15
			16S1B	AGC GTC AGT CTT GAC CCA GAA GTT C	16S2B	GGT ATT CTT TCT GAT ATC AAC AG	

^a *, The product size was obtained using the inner primers.
^b Boldface letters indicate modifications of the published primer.

heated for 1 min at 94°C, and subjected to 40 cycles of 20 s denaturation at 94°C, 1 min for the annealing reaction at 55°C with a 20-s extension at 72°C, and a final extension for 2 min at 72°C. For “*Ca. Neoehrlichia mikurensis*,” 0.2 µl of the amplification product was transferred to a fresh tube containing 24.8 µl of the reaction mixture described above, except that 5 pmol of the inner primer pair IS58-62f and IS58-594r was used. This mixture was subjected to the same amplification conditions as described above. PCR products were detected by electrophoresis in a 1.5% agarose gel stained with ethidium bromide. Any PCR products were sequenced. To detect and identify spirochetal genospecies, we amplified spirochetal DNA as described previously (15). Each PCR amplification product was purified by using a QIAquick-Spin PCR column (Qiagen) according to the manufacturer’s instructions. Amplified DNA fragments were directly sequenced in both directions using the inner primers by the dideoxy-nucleotide chain termination method with a Licor DNA4200 sequencer (Licor Biosciences, Bad Homburg, Germany). Each resulting sequence was compared to sequences of the same gene fragment from various spirochete genospecies. The following sequences (identified by the accession numbers under which they were deposited) were used for comparison: X85196 and X85203 for *B. burgdorferi* sensu stricto; X85190, X85192, and X85194 for *B. afzelii*; X85193, X85199, and M64311 for *B. garinii*; X98228 and X98229 for *B. lusitaniae*; X98232 and X98233 for *B. valaisiana*; AY147008 for *B. spielmanii*; and AY253149 for *B. miyamotoi*. A complete match (no more than two nucleotide changes) was required for identification. A nested PCR amplifying a fragment of the ospA gene served to differentiate *B. bavariensis* (cand. sp. nov.) from *B. garinii* (16). To examine variability, a fragment of the groESL gene was amplified in any sample harboring “*Ca. Neoehrlichia mikurensis*” DNA and sequenced (20). The DNA was extracted, reaction vials were prepared for amplification, templates were added, and products were electrophoresed in separate rooms. As an additional precaution, the reaction mixtures were prepared in a designated PCR workstation (Labcaire Systems, North Somerset, United Kingdom), and templates were added to the mixtures in a second PCR workstation. Benches and equipment were wiped down with a DNA decontamination solution (DNAerase; MP Biomedicals, Eschwege, Germany) after each use. In each sixth reaction mix, water was added instead of extracted DNA to serve as negative control.

RESULTS

We determined the infection rates of “*Ca. Neoehrlichia mikurensis*,” *A. phagocytophilum*, and spirochetes in questing adult *I. ricinus* ticks collected in Europe. On average, ca. 6% of the ticks harbored “*Ca. Neoehrlichia mikurensis*” (Table 2). Although this pathogen was detected in 8 and 10% of adult ticks collected in Germany and the Czech Republic, respectively, none of the examined adult ticks deriving from Poland and Madeira Island harbored it. “*Ca. Neoehrlichia mikurensis*” was least prevalent at our French study site, infecting only one of 60 examined ticks (1.7%). None of the French and Czech tick samples harbored *A. phagocytophilum*, but it infected ca. 4% of adult ticks in the other study sites. In contrast, the overall infection rates of spirochetes were similar in all central European sites, spirochetes infected about a quarter of the adult ticks (22.3%); on Madeira Island, however, none of a hundred ticks harbored spirochetes. In all other sites, *B. afzelii* was the most prevalent Lyme disease genospecies, followed by *B. garinii*, and *B. valaisiana*. *B. burgdorferi* sensu stricto, *B. lusitaniae*, *B. spielmanii*, the recently described isolate SV1 (3), and the non-Lyme disease spirochete *B. miyamotoi* were rare and not ubiquitously distributed. *B. bavariensis* was not detected in any tick. The prevalences of coinfecting pathogens reflected their individual prevalence. The two most common pathogens, *B. afzelii* and “*Ca. Neoehrlichia mikurensis*,” coinfect almost every 50th tick (1.8%). Any other coinfection occurred at a rate of or less than

TABLE 2 Infection rates of "Candidatus Neoehrlichia mikurensis," *Anaplasma phagocytophilum*, and spirochetes in questing adult *Ixodes ricinus* ticks in various European countries

Country	No. examined	% Ticks infected with:			% Ticks infected with <i>Borrelia</i> sp.							
		"Ca. Neoehrlichia mikurensis"	<i>A. phagocytophilum</i>	<i>Borrelia</i> sp.	<i>B. afzelii</i>	<i>B. garinii</i>	<i>B. valaisiana</i>	<i>B. lusitaniae</i>	<i>B. burgdorferi</i> sensu stricto	<i>B. spielmanii</i>	SV1 ^a	<i>B. miyamotoi</i>
Germany	542	8.1	4.1	25.8	12.4	4.1	3.3	3.5	1.1	0.4	0.2	1.5
France	60	1.7	0.0	26.7	6.7	13.3	6.7	0.0	0.0	0.0	0.0	0.0
Czech Republic	20	10.0	0.0	25.0	20.0	0.0	0.0	0.0	0.0	5.0	0.0	0.0
Poland	40	0.0	2.5	22.5	5.0	5.0	7.5	2.5	0.0	0.0	0.0	2.5
Portugal	101	0.0	6.9	0.0								
Total ^b	763	6.2	3.9	22.3	10.1	4.2	3.3	2.6	0.8	0.4	0.1	1.2

^a SV1, isolate SV1, provisionally named *B. finlandensis* (3).

^b Note that 28 ticks were coinfecting.

once every 200th tick (i.e., "Ca. Neoehrlichia mikurensis" with *B. lusitaniae* [0.4%], *B. valaisiana* [0.4%], or *B. garinii* [0.1%] or with both *B. garinii* and *B. valaisiana* [0.1%], *B. afzelii* with *B. garinii* [0.1%], and *B. garinii* with *B. valaisiana* [0.1%]; *A. phagocytophilum* with *B. afzelii* [0.3%] or "Ca. Neoehrlichia mikurensis" [0.3%]). Whereas spirochetes infected questing adult ticks most frequently (22.3%), fewer than a third as many ticks were infected by "Ca. Neoehrlichia mikurensis" (6.2%), and about a sixth harbored *A. phagocytophilum* (3.9%).

We examined whether any of 111 nymphal or adult *I. ricinus* ticks removed from 35 persons harbored "Ca. Neoehrlichia mikurensis," *A. phagocytophilum*, or spirochetes. About a fifth of patient-associated nymphs (19.1%) and a third of adults (31.8%) were infected by spirochetes (Table 3). Patient-derived nymphs appeared to be twice as likely to be infected by "Ca. Neoehrlichia mikurensis" as adults (9% versus 4.5%). No such tick was infected by *A. phagocytophilum*. *B. afzelii* was the most frequently detected genospecies, infecting more than half (54.2%) of all spirochete-infected ticks. *B. burgdorferi* sensu stricto and *B. garinii* were detected in 4.5 and 3.6% of the ticks, respectively, whereas the non-pathogenic spirochetes *B. valaisiana*, *B. lusitaniae*, and *B. miyamotoi* infected fewer than 1% of ticks removed from people. Three nymphs harbored *B. afzelii* and "Ca. Neoehrlichia mikurensis" simultaneously. None of the people from whom these ticks were removed displayed symptoms typical for any of these pathogens.

We compared the sequences obtained from "Ca. Neoehrlichia mikurensis"-infected ticks with each other as well as with published sequences. The sequences of the 488-bp 16S rRNA gene fragment of all our "Ca. Neoehrlichia mikurensis" samples were identical to that of the "Schotti" variant (GenBank accession number AF104680) detected in a Dutch *I. ricinus* tick. The amplified 315-bp fragment of the groESL gene of our samples did not

differ from that of the "Canis" variant or the "W330" variant detected in a dog or an *I. ricinus* tick in Germany, respectively (GenBank accession numbers EU432375 and EU810407). For the amplified gene fragments of "Ca. Neoehrlichia mikurensis," no heterogeneity was observed in any of the questing or patient-derived ticks.

DISCUSSION

The proportions of *I. ricinus* ticks infected with "Ca. Neoehrlichia mikurensis" and spirochetes were similar regardless of whether they were obtained from people or vegetation. Our observation that ca. 6% of adult ticks harbor "Ca. Neoehrlichia mikurensis" exceeds the averaged frequency derived from 11 studies examining *I. ricinus*, *I. persulcatus*, and *I. ovatus* ticks collected mainly from vegetation (Table 4). In the Netherlands and Russia, "Ca. Neoehrlichia mikurensis"-infected *I. ricinus* ticks appeared to be somewhat more common (1, 17, 21). A related, if not identical pathogen, designated "Ca. Ehrlichia walkeri" infected questing *I. ricinus* in Italy at about the same rate as did "Ca. Neoehrlichia mikurensis" in our study (9). *I. persulcatus* and *I. ovatus* may be somewhat less likely to carry this pathogen (1, 8, 14, 18). The variation of infection rates between 0% and 11.7%, reported for "Ca. Neoehrlichia mikurensis," may derive from different levels of sensitivity of the various detection methods used or from local differences. In contrast, the frequencies of *A. phagocytophilum*, reported in these studies, appear to vary little. Only ticks removed from roe deer were significantly more often infected with this pathogen (17) than the average questing ticks analyzed in the remaining eight studies. This observation is not surprising, since deer, and possibly other ruminants, may serve as reservoir hosts for *A. phagocytophilum* (11). On average, 20% of the ticks are infected by spirochetes among the six studies that examined the ticks also for the presence of these pathogens, but as many as

TABLE 3 Infection rates of "Candidatus Neoehrlichia mikurensis," *Anaplasma phagocytophilum*, and spirochetes in nymphal and adult *Ixodes ricinus* ticks removed from 35 persons in Germany

		% Ticks infected with:			% Ticks infected with <i>Borrelia</i> sp.					
		" <i>Ca.</i> Neoehrlichia mikurensis"	<i>A. phagocytophilum</i>	<i>Borrelia</i> sp.	<i>B. afzelii</i>	<i>B. garinii</i>	<i>B. burgdorferi</i> sensu stricto	<i>B. valaisiana</i>	<i>B. lusitaniae</i>	<i>B. miyamotoi</i>
Stage	No. examined									
Nymph	89	9.0	0.0	19.1	10.1 ^a	3.4	4.5	1.1	0.0	1.1
Adult	22	4.5	0.0	31.8	18.2	4.5	4.5	0.0	4.5	0.0
Total ^a	111	8.1	0.0	21.6	11.7	3.6	4.5	0.9	0.9	0.9

^a Note that three ticks were coinfecting with "Ca. Neoehrlichia mikurensis" and one was coinfecting with *B. burgdorferi* sensu stricto.

TABLE 4 Infection rates of “*Candidatus* Neoehrlichia mikurensis” in various *Ixodes* ticks in Eurasia, as well as coanalyzed rates of *Anaplasma phagocytophilum* and spirochetes^a

Tick species	Stage	Source	No. examined	% Ticks infected with:			Detection		Country	Source or reference
				“ <i>Ca.</i> Neoehrlichia mikurensis”	<i>A. phagocytophilum</i>	<i>Borrelia</i> sp.	Method	Target		
<i>I. ricinus</i>	Mostly adult	Fed on deer	121	6.6	21.5	13.2	PCR+RLB	16S, 23S-5S spacer	Netherlands	17
<i>I. ricinus</i>	Various	Vegetation	1,580	3.5	3.3	7.6	PCR+RLB	16S, 23S-5S spacer	Netherlands	25
<i>I. ricinus</i>	Various	Vegetation	180	11.7	ND	22.8	PCR-DGGE, PCR+RLB	16S, 23S-5S spacer	Netherlands	21
<i>I. ricinus</i>	Various	Vegetation	292	6.5 ^d	ND	ND	PCR	GltA	Italy	9
<i>I. ricinus</i>	ND	Vegetation	295	7.1	1.4	38.0	PCR+RLB	16S, 23S-5S spacer	Russia	1
<i>I. ricinus</i>	Adult	Vegetation	68	2.9	4.4	ND	PCR	16S	Slovakia	19
<i>I. ricinus</i>	Various	Various ^b	1,022	0.1	4.1	ND	Nested PCR	16S	Germany	22
<i>I. ricinus</i>	Adult	Vegetation	763	6.2	3.9	22.3	Nested PCR	16S	Various	This study
<i>I. persulcatus</i>	ND	Vegetation	336	0.0	0.0	67.3	PCR+RLB	16S, 23S-5S spacer	Russia	1
<i>I. persulcatus</i>	ND	Vegetation	107	1.9	2.8	ND	PCR	Various	Russia	18
<i>I. persulcatus</i>	Adult	Vegetation	2,590	0.2	2.4	ND	Nested PCR	16S	Russia	14
<i>I. ovatus</i>	Adult	Vegetation	164 ^c	2.4	ND	ND	Nested PCR	16S	Japan	8
Total			7,518	2.4	3.2 ^e	20.9 ^f				

^a ND, not determined; RLB, reverse line blotting; DGGE, denaturing gradient gel electrophoresis; GltA, citrate synthase.

^b Dogs, cats, deer, and patients.

^c Individually and pooled.

^d Designated as “*Candidatus* Ehrlichia walkeri.”

^e Of 6,882 ticks.

^f Of 3,275 ticks.

two-thirds (67.3%) of *I. persulcatus* ticks may be infected (1). Although in ticks collected on Madeira Island, *A. phagocytophilum* was most common, neither “*Ca.* Neoehrlichia mikurensis” nor spirochetes were detected. As suggested earlier (10), the scarcity of Lyme disease spirochetes may be related to the depauperate host composition at the collection site and “*Ca.* Neoehrlichia mikurensis” may parallel this. If the infection rate of these pathogens is calculated without the Madeiran samples, 7.1 and 25.7% of the adult ticks would be infected by “*Ca.* Neoehrlichia mikurensis” and spirochetes, respectively. Our observations comparing the frequencies of various pathogenic Lyme disease spirochetes, *A. phagocytophilum*, and “*Ca.* Neoehrlichia mikurensis” suggests that only *B. afzelii* infects questing adult *I. ricinus* ticks more frequently than does “*Ca.* Neoehrlichia mikurensis.”

On average, every twelfth encounter with an *I. ricinus* tick may bear the risk of acquiring “*Ca.* Neoehrlichia mikurensis” (8.1% infection rate). In ticks removed from people, this pathogen appeared to be somewhat more frequent than in questing ticks. The difference may result from a bias, because three quarters of the patient-derived ticks were acquired in a German region where we determined that 18% of adult ticks harbored “*Ca.* Neoehrlichia mikurensis,” or it may result from a higher infection rate in nymphs, constituting most of the patient-removed ticks, than in adults. In comparison, <3% of ticks removed from asymptomatic patients in Northern Italy were infected with “*Ca.* Ehrlichia walkeri” (2). The 16S rRNA fragment amplified in our patient-derived ticks differed only in one base pair from that of “*Ca.* Ehrlichia walkeri” and may indicate closely related, if not identical, pathogens. None of the seven different persons to whom at least one tick infected with “*Ca.* Neoehrlichia mikurensis” had attached reported symptoms related to an infection with this pathogen. We assume that its transmission by the feeding tick is not immediate.

Similarly, Lyme disease spirochetes, as well as *A. phagocytophilum*, appear to not be transmitted before the second day of feeding (6, 13). Alternatively, symptoms related to “*Ca.* Neoehrlichia mikurensis” infection may only become apparent in immunocompromised patients (12, 23, 24).

Because “*Ca.* Neoehrlichia mikurensis” appears to be the second most prevalent pathogen in *I. ricinus* ticks after *B. afzelii*, numerous people may come into contact with ticks harboring this recently recognized pathogen. “*Ca.* Neoehrlichia mikurensis” may not affect healthy people. However, considering that a large proportion of the Central European population is temporarily or permanently immunosuppressed and thus at risk of acquiring “*Ca.* Neoehrlichia mikurensis,” such an infection should be taken into account, if tick exposure cannot be excluded. In addition, immunocompromised patients should be made aware of the risk and be instructed in methods for personal tick prevention.

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