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Infections and Coinfections of Questing *Ixodes ricinus* Ticks by Emerging Zoonotic Pathogens in Western Switzerland

Elena Lommano, Luce Bertaiola, Christèle Dupasquier, and Lise Gern

Institute of Biology, Laboratory of Eco-Epidemiology of Parasites, University of Neuchâtel, Neuchâtel, Switzerland

In Europe, *Ixodes ricinus* is the vector of many pathogens of medical and veterinary relevance, among them *Borrelia burgdorferi* sensu lato and tick-borne encephalitis virus, which have been the subject of numerous investigations. Less is known about the occurrence of emerging tick-borne pathogens like *Rickettsia* spp., *Babesia* spp., "*Candidatus* Neoehrlichia mikurensis," and *Anaplasma phagocytophilum* in questing ticks. In this study, questing nymph and adult *I. ricinus* ticks were collected at 11 sites located in Western Switzerland. A total of 1,476 ticks were analyzed individually for the simultaneous presence of *B. burgdorferi* sensu lato, *Rickettsia* spp., *Babesia* spp., "*Candidatus* Neoehrlichia mikurensis," and *A. phagocytophilum*. *B. burgdorferi* sensu lato, *Rickettsia* spp., and "*Candidatus* Neoehrlichia mikurensis" were detected in ticks at all sites with global prevalences of 22.5%, 10.2%, and 6.4%, respectively. *Babesia*- and *A. phagocytophilum*-infected ticks showed a more restricted geographic distribution, and their prevalences were lower (1.9% and 1.5%, respectively). Species rarely reported in Switzerland, like *Borrelia spielmanii*, *Borrelia lusitaniae*, and *Rickettsia monacensis*, were identified. Infections with more than one pathogenic species, involving mostly *Borrelia* spp. and *Rickettsia helvetica*, were detected in 19.6% of infected ticks. Globally, 34.2% of ticks were infected with at least one pathogen. The diversity of tick-borne pathogens detected in *I. ricinus* in this study and the frequency of coinfections underline the need to take them seriously into consideration when evaluating the risks of infection following a tick bite.

Ixodes ricinus is the most abundant and widespread tick species throughout Europe. It is the vector of many emerging pathogens of veterinary and human medical importance, including viruses like tick-borne encephalitis virus (TBEV); bacteria like *Borrelia burgdorferi* sensu lato, spotted fever group (SFG) rickettsiae, and *Ehrlichia-Anaplasma*; and protozoa such as *Babesia* spp.

B. burgdorferi sensu lato is the agent of Lyme borreliosis, the most prevalent tick-borne disease in Europe. Ten *Borrelia* species of the *B. burgdorferi* sensu lato complex (*B. burgdorferi* sensu stricto, *B. afzelii*, *B. garinii*, *B. valaisiana*, *B. spielmanii*, *B. bavariensis*, *B. bissettii*, *B. finlandensis*, *B. carolinensis*, and *B. lusitaniae*) and *Borrelia miyamotoi*, related to the relapsing fever spirochetes, have been detected in *I. ricinus* (11, 13, 22, 37), and most of them are known as pathogens of humans (22). In Switzerland, all these *Borrelia* genospecies, except *B. bissettii*, *B. finlandensis*, and *B. carolinensis*, were detected in *I. ricinus* ticks (22).

Ehrlichia and *Anaplasma* species are intracellular bacteria. *Anaplasma phagocytophilum* was first established as a veterinary pathogen until the discovery of the first case of human granulocytic anaplasmosis in the United States (12, 63). In Europe, fewer than 100 clinical cases have been reported (22). In Switzerland, *A. phagocytophilum* has been detected in fewer than 2% of *I. ricinus* ticks, but no human case has been documented (36, 45). Recently, a newly described pathogen, "*Candidatus* Neoehrlichia mikurensis," in blood samples of a patient with signs of septicemia was reported in Switzerland (18). The bacterium, a member of the family *Anaplasmataceae*, has been detected primarily in *I. ricinus* ticks in the Netherlands and was designated an *Ehrlichia*-like "Schotti variant" (50). Afterwards, a similar organism, "*Candidatus* Neoehrlichia mikurensis," was isolated from wild rats in Japan, representing a novel genetic cluster together with the *Ehrlichia*-like Schotti variant (34). Ticks infected by members of this cluster were reported in Japan (34) and in Europe (1, 50, 59, 62) but never in Switzerland.

Tick-borne rickettsioses are caused by bacteria of the genus *Rickettsia*, belonging to the SFG. Rickettsiae are currently considered emerging pathogens, like, for example, *Rickettsia helvetica* and *R. monacensis* in Europe and Eurasia. The pathogenicity of *R. helvetica* remains unclear, although it was reported to be involved in some cardiac and neurological symptoms (19, 39, 40). *R. monacensis* was isolated from the blood of patients (31) and was found in *I. ricinus* ticks from different countries in Europe. In Switzerland, *R. helvetica* appears frequently in ticks (7), whereas *R. monacensis* is rare (9).

Babesiosis, caused by a protozoon of the genus *Babesia*, is a disease of veterinary importance but is getting increasing consideration as an emerging disease of humans. In Europe, *Babesia divergens*, *B. microti*, and *B. venatorum* (also known as *Babesia* sp. EU1) are of medical importance (27). In Switzerland, a recent study reported these three *Babesia* species in fewer than 2% of ticks (23).

Information on the occurrence of the above-mentioned pathogens, except *B. burgdorferi* sensu lato, is scarce, especially in Western Switzerland. Moreover, little is known about their coexistence in *I. ricinus* ticks. In Europe, the prevalence of ticks carrying multiple pathogens has been reported to vary between 3.2% (47) and 28.8% (57). Because mixed infections (involving species of same genus) and coinfections (involving species of different genera) are of medical relevance, by increasing the severity of symptoms in humans and animals (6), it is crucial to determine the prevalence

Received 22 December 2011 **Accepted** 11 April 2012
Published ahead of print 20 April 2012
Address correspondence to Lise Gern, lise.gern@unine.ch.

doi:10.1128/AEM.07961-11

of ticks infected by more than one pathogen. Hence, our aim was to gain an insight into the prevalence and geographic distribution of *Borrelia* spp., *A. phagocytophilum*, "*Candidatus* Neoehrlichia mikurensis," *Rickettsia* spp., and *Babesia* spp. as well as their coinfections in free-living *I. ricinus* ticks at various sites in the Western Swiss Plateau.(These results are part of the Ph.D. thesis of E. Lommano.)

MATERIALS AND METHODS

Study area and tick sampling. This study was carried out at 11 sites located between 400 and 900 m above sea level: Romanel (46°34.37′N, 6°35.59′E), Chalet-à-Gobet (46°34.04′N, 6°40.62′E), Agiez (46°43.23′N, 6°29.90′E), Montcherand (46°44.04′N, 6°29.26′E), Delémont (47°22.41′N, 7°19.40′E), Mormont (47°26.20′N, 7°02.61′E), Rosé (46°47.66′N, 7°04.68′E), Belfaux (46°50.07′N, 7°05.43′E), Matran (46°46.73′N, 7°05.24′E), Seedorf (46°48.10′N, 7°02.30′E), and Neuchâtel (47°01.00′N, 6°56.00′E). All sites were mixed deciduous forests. Questing ticks were collected by flagging vegetation in 2009 and 2010.

Amplification and detection of tick-borne pathogen DNA. Total DNA was extracted from nymphs and adults by using ammonium hydroxide (NH_4OH) (38). Each tick was tested individually for the presence of *A. phagocytophilum*, "*Candidatus* Neoehrlichia mikurensis," *Rickettsia* sp., *Babesia* sp., and *B. burgdorferi* sensu lato DNAs using specific primers and probes (Table 1). All PCR amplifications were performed with final volumes of 25 μl with 5 μl of template DNA, except for *A. phagocytophilum* (2 μl) and *Babesia* spp. (10 μl). Negative (sterile water) and positive controls were included for each PCR amplification.

"*Candidatus* Neoehrlichia mikurensis." A ~500-bp fragment of the 16S rRNA gene spanning the V1 region of *Anaplasma* spp. and *Ehrlichia* spp. was amplified as described previously (50), using modifications described previously by Bekker et al. (5). The positive control consisted of 3 μl of *A. phagocytophilum* DNA (Webster strain). A touchdown PCR program, modified from a method described previously by Bekker et al. (5), was performed (58). The reverse line blot (RLB) technique was used to identify "*Candidatus* Neoehrlichia mikurensis" (50).

***A. phagocytophilum*.** A 77-bp fragment of the *msp2* gene of *A. phagocytophilum* was amplified and detected by using a real-time PCR method reported previously (9), with modifications (14). The positive control consisted of 2 μl of *A. phagocytophilum* (Webster strain). For sequencing, the 16S rRNA gene was amplified with primers 16S8FE and B-GA1B (5) (Table 1).

***Rickettsia* spp.** A PCR targeting the 23S-5S internal spacer of *Rickettsia* spp. (30) and amplifying a 345-bp fragment was coupled with an RLB hybridization procedure (9), modified as described previously by Jado et al. (30). DNA of *Rickettsia conorii* was used as the positive control.

***Babesia* spp.** For the detection of *Babesia* spp., a 450-bp fragment of the 18S rRNA gene was amplified (20). The positive control consisted of 1 μl of *B. microti* or *B. divergens* DNA. A touchdown PCR program was performed as described previously (58), followed by RLB for the identification of *Babesia* species (23).

***Borrelia* spp.** A real-time PCR was used to amplify a 132-bp fragment of the flagellin gene of *Borrelia* spp. (26, 51). Isolates of *B. burgdorferi* sensu stricto (B31), *B. garinii* (NE11), *B. afzelii* (NE632), and *B. valaisiana* (VS116) were used as positive controls. Each sample that was positive by real-time PCR was further analyzed by PCR and RLB to identify *B. burgdorferi* sensu lato genospecies (26). The amplification of the intergenic spacer region between the 5S and 23S rRNA genes was performed as described previously (1), using a touchdown program (8) followed by RLB for the identification of *Borrelia* species (Table 1) (21).

DNA sequencing. Positive PCR products were purified with a purification kit (Promega, Madison, WI) and sent to Microsynth AG (Balgach, Switzerland) for sequencing. Each obtained sequence was compared with sequences from an international database (NCBI BLAST) by the use of ClustalW2.0.12 (56).

Statistical analysis. The influence of *I. ricinus* life stages on *Borrelia* infection and coinfection was assessed by a χ^2 test on a contingency table.

Nucleotide sequence accession numbers. Three "*Candidatus* Neoehrlichia mikurensis" sequences were submitted to the NCBI GenBank database under accession numbers JQ014620, JQ014621, and JQ014622. GenBank accession numbers for one *R. monacensis* sequence and one *A. phagocytophilum* sequence were JQ670878 and JQ277467, respectively.

RESULTS

A total of 1,476 *I. ricinus* ticks, including 1,194 nymphs and 282 adults (144 females and 138 males), were collected and individually screened for the presence of *A. phagocytophilum*, *Rickettsia* sp., *Borrelia* sp., and *Babesia* sp. DNAs (Table 2). The occurrence of "*Candidatus* Neoehrlichia mikurensis" was screened for in 818/1,476 ticks collected at 5/11 sites (648 nymphs, 91 females, and 79 males).

Borrelia spp. were the most frequently isolated pathogens in ticks, with a global prevalence of 22.5% (328/1,458) (Table 2). Some ticks ($n = 18/1,476$) could not be screened for *Borrelia* due to a lack of material. *Borrelia*-infected ticks were detected at all sites. Adults (30.8%; 87/282) were significantly more infected than nymphs (20.5%; 241/1,176) ($P < 0.001$ by χ^2 test). Due to a lack of material, the *Borrelia* species in 6 samples that were positive by real-time PCR could not be identified by RLB. Among the 328 *Borrelia*-infected ticks, 357 events of infection (including single and mixed infections) were detected by RLB, and 6 different genospecies were identified. *B. afzelii* was predominant (141/357; 39.5%), followed by *B. garinii* (92/357; 25.8%), *B. valaisiana* (59/357; 16.5%), *B. burgdorferi* sensu stricto (20/357; 5.6%), *B. bavariensis* (17/357; 4.8%), and *B. miyamotoi* (15/357; 4.2%). For 10 out of 357 (2.8%) infections, *Borrelia* isolates could not be identified to the species level. Additionally, *B. lusitaniae* was detected in two nymphs collected at Agiez and Delémont, and *B. spielmanii* was detected in one male (in a mixed infection with *B. afzelii*) at Montcherand.

Rickettsia-infected ticks were observed at all sites, with a global prevalence of 10.2% (150/1,476 ticks; 123/1,194 nymphs and 27/282 adults) (Table 2). *R. helvetica* was predominantly detected (136/150; 90.7%), followed by unidentified *Rickettsia* spp. (12/150; 8%). *R. monacensis* was identified in two nymphs (2/150; 1.3%) collected at two different sites (Agiez and Delémont) after sequencing the 23S-5S rRNA gene. Both sequences showed 100% homology with *Rickettsia* sp. strain 362 (accession number DQ139797).

A. phagocytophilum-infected ticks were recorded at 4/11 sites (Table 2). The global prevalence reached 1.5% (22/1,476; 18/1,194 nymphs and 4/282 adults), and the mean local prevalence (only sites where *A. phagocytophilum* was present are taken into consideration) was 2.7% (22/812). Sequencing (16S rRNA gene) was successful for one sample, which showed 100% homology with an *A. phagocytophilum* strain from a cat (accession number HM138366) and 99% homology (divergence of 1 base only) with strains isolated from human blood in Italy (accession number DQ029028), *I. ricinus* (accession number AF084907), and one horse (accession number AF057707) in Switzerland.

"*Candidatus* Neoehrlichia mikurensis" DNA was found at all sites, with a global prevalence of 6.4% (52/818; 41/648 nymphs and 11/170 adults) (Table 2). Sequencing of 20/52 samples (16S rRNA gene) revealed 100% homology to each other and to other strains isolated from human blood in Switzerland (GenBank ac-

TABLE 1 Primers and probes used in this study for PCR, RLB, and real-time PCR[a]

Primer or probe	Sequence (5′–3′)	Concn (pmol) for RLB	Type of sequence	Target gene	Target organism	Reference
RCK/23-5-F	Biotin-GATAGGTCRGRTGTGGAAGCAC		Primer	23S-5S rRNA gene	*Rickettsia* genus	30
RCK/23-5-R	TCGGGAYGGGATCGTGTGTTTC		Primer	23S-5S rRNA gene	*Rickettsia* genus	30
GP-RICK	TAGCTCGATTGRTTTACTTTG	100	Probe	23S-5S rRNA gene	*Rickettsia* genus	30
RCK-SFG	ACTCACAARGTTATCAGGT	500	Probe	23S-5S rRNA gene	SFG *Rickettsia*	30
P-HELV	CATGGCTTGATCCACGGTA	100	Probe	23S-5S rRNA gene	*R. helvetica*	30
RLB-F2	GACACAGGGAGGTAGTGACAAG		Primer	18S rRNA gene	*Babesia-Theileria* genus	20
RLB-R2	Biotin-CTAAGAATTTCACCTCTGACAGT		Primer	18S rRNA gene	*Babesia-Theileria* genus	20
Catch-all B/T	TAATGGTTAATAGGARCRGTTG	50	Probe	18S rRNA gene	*Babesia-Theileria* genus	20
B. venatorum	GAGTTATTGACTCTTGTCTTTAA	500	Probe	18S rRNA gene	*B. venatorum*	23
B. divergens	GTTAATATTGACTAATGTCGAG	500	Probe	18S rRNA gene	*B. divergens*	23a
B. microti	GCTTCCGAGCGTTTTTTTAT	500	Probe	18S rRNA gene	*B. microti*	23
ApM SP2f	ATGGAAGGTAGTGTTGGTTATGGTATT		Primer	*msp2* gene	*A. phagocytophilum*	14
ApM SP2r	TTGGTCTTGAAGCGCTCGTA		Primer	*msp2* gene	*A. phagocytophilum*	14
ApM SP2p	FAM-TGGTGCCAGGGTTGAGCTTGAGATTG-TAMRA		Probe	*msp2* gene	*A. phagocytophilum*	14
16S8FE	GGAATTCAGAGTTGGATCMTGGYTCAG		Primer	16S rRNA gene	*Anaplasma-Ehrlichia* genus	50
B-GA1B	biotin-CGGGATCCCGAGTTTGCCGGGACTTYTTCT		Primer	16S rRNA gene	*Anaplasma-Ehrlichia* genus	5
Catch-all A/E	GGGGGAAAGATTTATCGCTA	100	Probe	16S rRNA gene	*Anaplasma-Ehrlichia* genus	5
A-Eschot	GCTGTAGTTTACTATGGGTA	100	Probe	16S rRNA gene	"*Candidatus* Neoehrlichia mikurensis"	50
FlaF1A	AGCAAATTTAGGTGCTTTCCAA		Primer	Flagellin gene	*Borrelia burgdorferi* sensu lato	51
FlaR1	GCAATCATTGCCATTGCAGA		Primer	Flagellin gene	*Borrelia burgdorferi* sensu lato	51
Flaprobe1	FAM-TGCTACAACCTCATCTGTCATTGTAGCATCTTTTA		Probe	Flagellin gene	*Borrelia burgdorferi* sensu lato	51
B-5SBor	Biotin-GAGTTCGCGGGAGAGTAGGTTATT		Primer	5S-23S spacer	*Borrelia burgdorferi* sensu lato	1
23SBor	TCAGGGTACTTAGATGGTTCACTT		Primer	5S-23S spacer	*Borrelia burgdorferi* sensu lato	1
SL1	CTTTGACCATATTTTTATCTTCCA	75	Probe	5S-23S spacer	*Borrelia burgdorferi* sensu lato	48a
SS	AACACCAATATTTAAAAAACATAA	75	Probe	5S-23S spacer	*Borrelia burgdorferi* sensu stricto	48a
GA	AACATGAACATCTAAAAACATAAA	75	Probe	5S-23S spacer	*B. garinii*	48a
GANE	CAAAAACATAAATATCTAAAAACATAA	75	Probe	5S-23S spacer	*B. garinii*	43
AF	AACATTTAAAAAATAAATTCAAGG	75	Probe	5S-23S spacer	*B. afzelii*	48a
VSNE	TATATCTTTTGTTCAATCCATGT	75	Probe	5S-23S spacer	*B. valaisiana*	43
LusiNE	TCAAGATTTGAAGTATAAAATAAAA	75	Probe	5S-23S spacer	*B. lusitaniae*	43
LusiNE1	CATTCAAAAAAATAAACATTTAAAAACAT	100	Probe	5S-23S spacer	*B. lusitaniae*	21
LusiNE2	AAATCAAACATTCAAAAAAATAAAC	100	Probe	5S-23S spacer	*B. lusitaniae*	21
RFLNE	CTATCCATTGATCAATGC	100	Probe	5S-23S spacer	*B. miyamotoi*	21
SpiNE2	GAATGGTTTATTCAAATAACATA	100	Probe	5S-23S spacer	*B. spielmanii*	21
SpiNE3	GAATAAGCCATTTAAATAACATA	100	Probe	5S-23S spacer	*B. spielmanii*	21
GANE1	AAAATCAATGTTTAAAGTATAAAAT	100	Probe	5S-23S spacer	*B. garinii*	21
BisNE1	AAACACTAACATTTAAAAAACAT	100	Probe	5S-23S spacer	*B. bissettii*	21
BisNE2	AACTAACAAACATTTAAAAAACAT	100	Probe	5S-23S spacer	*B. bissettii*	21

[a] All probes for RLB were 5′-amino labeled. FAM, 6-carboxyfluorescein; TAMRA, 6-carboxytetramethylrhodamine.

TABLE 2 Prevalence of tick-borne pathogens detected in ticks according to sampling site[a]

Sampling site	No. of ticks analyzed	No. of ticks positive for pathogen/total no. of ticks tested (%)					No. of ticks infected with at least 1 pathogen/total no. of ticks tested
		A. phagocytophilum	"*Candidatus* Neoehrlichia mikurensis"	*Rickettsia* spp.	*Babesia* spp.	*Borrelia* spp.	
Romanel	86	0/86 (0)	6/86 (7)	12/86 (13.9)	1/86 (1.2)	8/86 (9.3)	26/86 (30.2)
Chalet-à-Gobet	129	3/129 (2.3)	3/129 (2.3)	15/129 (11.6)	3/129 (2.3)	22/129 (17)	39/129 (29.4)
Agiez	125	0/125 (0)	NA	11/125 (8.8)	3/125 (2.4)	26/122 (21.3)	39/125 (31.2)
Montcherand	156	5/156 (3.2)	NA	10/156 (6.4)	7/156 (4.5)	40/156 (25.6)	58/156 (37.2)
Delémont	145	0/145 (0)	6/145 (4.1)	16/145 (11)	2/145 (1.4)	38/145 (26.2)	51/145 (35.2)
Mormont	71	0/71 (0)	3/71 (4.2)	10/71 (14.1)	0/71 (0)	13/71 (18.3)	21/71 (29.6)
Rosé	131	0/131 (0)	NA	20/131 (15.3)	4/131 (3.1)	30/125 (24)	49/131 (37.4)
Belfaux	140	3/140 (2.1)	NA	22/140 (15.7)	4/140 (2.8)	34/140 (24.3)	52/140 (37.1)
Matran	24	0/24 (0)	NA	1/24 (4.2)	0/24 (0)	8/24 (33.3)	9/24 (37.5)
Seedorf	82	0/82 (0)	NA	9/82 (11)	2/82 (2.4)	21/82 (25.6)	29/82 (35.4)
Neuchâtel	387	11/387 (2.8)	34/387 (8.8)	24/387 (6.2)	2/387 (0.5)	88/378 (23.3)	132/387 (34.1)
Total	1,476	22/1,476 (1.5)	52/818 (6.4)	150/1,476 (10.2)	28/1,476 (1.9)	328/1,458 (22.5)	505/1476 (34.2)

[a] NA, not available. Minimum and maximum prevalences are underlined.

cession number GQ501090) and Germany (accession number EU810404) and from *I. ricinus* in Germany (accession number EU810405).

Babesia-infected ticks were detected at 9/11 sites, with a global prevalence of 1.9% (28/1,476; 25/1,194 nymphs and 3/282 adults). Local prevalence varied between 0.5% (2/387 ticks for Neuchâtel) and 4.5% (7/156 for Montcherand) (Table 2). *B. venatorum* was the most prevalent species (18/28; 64.3%), followed by *B. divergens* (5/28; 17.9%). Five out 28 (17.9%) *Babesia* sp. isolates could not be identified to the species level with RLB, and sequencing (18S rRNA gene) of these samples was not successful.

Mixed infections, involving two or three *Borrelia* genospecies, occurred in 2.1% (30/1,458) of ticks and in 9.1% (30/328) of *Borrelia*-infected ticks (Table 3). The *B. garinii-B. valaisiana* association was the most frequently found association (Table 3). Coinfections involving pathogens of different genera were detected in 4.7% (69/1,476) of ticks and in 13.7% (69/505) of infected ticks (Table 3). These coinfections involved mostly *B. afzelii* and *R. helvetica*. *B. afzelii* was found mostly in association with *R. helvetica* (13/36; 36.1%) and "*Candidatus* Neoehrlichia mikurensis" (12/36; 33.3%) (Table 3). Inversely, 60% of "*Candidatus* Neoehrlichia mikurensis" isolates were in coinfections with *B. afzelii* (12/20) (Table 3).

If we considered ticks to be infected by more than one pathogen species, we observed a global prevalence of 6.7% (99/1,476). Among infected ticks, 19.6% (99/505 ticks) were carrying more than one pathogen species. Globally, adult ticks (31/282; 11%) were more frequently infected with multiple pathogens than nymphs (68/1,194; 4.6%) ($P < 0.005$ by χ^2 test). Infections with three pathogen species were observed for 6 ticks (6/1,476). The *B. afzelii-B. garinii-B. valaisiana* association was found in two nymphs, and the *B. garinii-B. valaisiana-B. miyamotoi* association was observed in one male. The other three triple infections consisted of *B. afzelii-B. garinii-R. helvetica* (in one male), *B. garinii-B. valaisiana-R. helvetica* (in one nymph), and *B. valaisiana-R. helvetica*-"*Candidatus* Neoehrlichia mikurensis" (in one nymph).

B. valaisiana occurred frequently in multiple infections (27/59; 45%), mostly with *B. garinii* (11/59; 18.6%) and *R. helvetica* (8/59; 13.6%) (Table 3). Additionally, *B. valaisiana* was implicated in 5/6 triple infections (see above). Similarly, *A. phagocytophilum* was frequently involved in coinfections, since 9/22 (41%) *A. phagocytophilum* infections were coinfections (Table 3).

Globally, the prevalence of ticks infected with at least one pathogen reached 34.2% (505/1,476) (Table 2).

DISCUSSION

Here, we assessed the prevalences and geographic distribution of five human-pathogenic microorganisms (*Borrelia* spp., *Rickettsia* spp., *Babesia* spp., *A. phagocytophilum*, and "*Candidatus* Neoehrlichia mikurensis"), as well as their coinfections, in questing ticks at 11 sites situated in Western Switzerland.

Borrelia spp., *Rickettsia* spp., and "*Candidatus* Neoehrlichia mikurensis" were present at all sites, while *Babesia* spp. and *A. phagocytophilum* showed a more restricted geographic distribution. Ticks infected with *Borrelia* genospecies were largely predominant, and the mean prevalence reached 22.5%, followed by *Rickettsia* spp., which were identified in 10.2% of ticks. This is in line with prevalences previously reported for Switzerland (7, 33). In contrast, the prevalence of *Babesia* spp. and *A. phagocytophilum* in ticks was lower than 2%, and they were not present at all sites. The absence of *Babesia* spp. at two sites can be explained by the relatively few ticks examined at these sites ($n = 24$ and $n = 71$) in relation with the low mean prevalence observed (1.9%). However, *A. phagocytophilum* was absent from sites where it could be reasonably expected to occur considering its mean local prevalence (2.7%) and the number of collected ticks, indicating a patchy distribution. A similar patchy distribution was reported previously for Spain and Sweden (3, 52). On the other hand, despite the low prevalence of infected ticks and their patchy distribution within a region, the "pockets of infection" were quite stable over years. In fact, when *A. phagocytophilum* was present at a location, it was detected again the following year (data not shown). The low prevalence of *Babesia* spp. and *A. phagocytophilum* is in the range (0 to 1.7% and 0.8 to 2%, respectively) of what has been observed in Switzerland (9, 10, 23, 45). The *A. phagocytophilum* sequence obtained here was identical or closely related (99 to 100% identity) to those of other pathogenic strains isolated from domestic animals in Switzerland and the Czech Republic (44; D. Hulinska, unpub-

TABLE 3 Coinfections (involving two pathogens) observed in ticks[b]

Pathogen genus	Pathogen species	No. of ticks with coinfection with:														
		B. afzelii	*B. garinii*	*B. burgdorferi* sensu stricto	*B. valaisiana*	*B. bavariensis*	*B. miyamotoi*	*B. spielmanii*	SL	SP	EU1	*B. divergens*	*A. phagocytophilum*	"*Candidatus* Neoehrlichia mikurensis"	*Rickettsia helvetica*	*Rickettsia* sp.[c]
Borrelia	*B. afzelii*		5	3	2			1						12	13	
	B. garinii	5			11		2				1		2	1	6	1
	B. burgdorferi sensu stricto	3													2	
	B. valaisiana	2	11			3							1	1	8	1
	B. bavariensis				3											1
	B. miyamotoi		2											3		
	B. spielmanii	1														
	SL												2			
	SP															1
	EU1		1										1		4	
Babesia	*B. divergens*															1
Anaplasma-Ehrlichia	*A. phagocytophilum*		2		1				2		1			2	1	
	"*Candidatus* Neoehrlichia mikurensis"	12	1		1		3						2		1	
Rickettsia	*Rickettsia helvetica*	13	6	2	8	1					4		1	1		
	Rickettsia spp.[c]		1		1					1		1				
Total coinfections ($n = 93$)		36	29	5	27	4	5	1	2		1	6	1	9	20	36	4	
Total infections ($n = 505$)[a]		141	92	20	59	17	15	1	10		6	18	5	22	52	136	12	

[a] "Total infections" refers to the total number of infections involving the concerned pathogen.
[b] SL, *Borrelia* spp. matching only with the SL1 probe (RLB); SP, *Borrelia* spp. that could not be identified by RLB due to a lack of DNA; EU1, *Babesia venatorum*.
[c] Matching only with the generic probe.

lished data), from *I. ricinus* ticks in Switzerland (45), and from humans in Italy (15). Therefore, the presence of this pathogenic strain in ticks from Switzerland points out a real risk of infection for animals and humans.

This study reports for the first time the presence of "*Candidatus* Neoehrlichia mikurensis" in questing *I. ricinus* ticks from Switzerland. More than 6% of ticks were infected by this microorganism, which occurred at all sites. In Europe, the prevalence of "*Candidatus* Neoehrlichia mikurensis" is known to range from 3.5% to 7% (1, 50, 62). "*Candidatus* Neoehrlichia mikurensis" can currently be considered an emerging pathogen of veterinary and medical importance. In 2007, the bacterium was identified in a dog with hematological troubles in Germany (17). In 2010, four human cases were reported in Switzerland, Germany, and Sweden (18, 60, 61), one of which was fatal. All the sequences here were identical to sequences of strains detected in the blood samples of patients suffering from febrile bacteremia in Switzerland and Germany (18, 60) and of a strain from *I. ricinus* in Germany (59). Our findings indicate that "*Candidatus* Neoehrlichia mikurensis" is present in questing ticks in Western Switzerland, representing a risk of contracting "*Candidatus* Neoehrlichia mikurensis" for humans and pets.

Among the five pathogens identified here, *Borrelia* spp. and *Rickettsia* spp. demonstrated a high variability in their occurrences in ticks according to site. Their local prevalences ranged from 9.3% to 33.3% for *Borrelia* spp. and from 4.2% to 15.7% for *Rickettsia* spp. Similar variations were observed previously in Europe, where local prevalences of *Borrelia* spp. (33, 47) and *Rickettsia* spp. (24) differed greatly among areas. Ecological conditions present at each site, including reservoir host availability or a high density of reservoir-incompetent hosts, most certainly play a role in these local variations.

The prevalences of the different *Borrelia* genospecies in ticks were in agreement with previously reported findings in Switzerland (26), except that two uncommon genospecies (*B. lusitaniae* and *B. spielmanii*) were detected in this study. *B. lusitaniae*-infected ticks are very common around the southern limit of the distribution of *I. ricinus* ticks (16, 64) but are quite uncommon in other regions. In Switzerland, *B. lusitaniae* has been detected in free-living ticks in some localities of Canton Ticino (32), in ticks feeding on birds (43), and in Western Switzerland (33); our findings report its occurrence in two additional localities. In contrast, *B. spielmanii* has been reported only once in Switzerland (21). *B. spielmanii*, which is pathogenic to humans (4), has already been identified in ticks in France and Germany (46, 48).

Two different *Rickettsia* species were identified here. *R. helvetica* was the most common and the most abundant species. The other species, *R. monacensis*, was found in only two nymphs. Sequencing revealed 100% identity with *Rickettsia* sp. strain 362, isolated from the blood samples of patients with Mediterranean spotted fever in Spain (30) and recognized afterwards as *R. monacensis*, a novel *Rickettsia* species (54). The arrangement of *Rickettsia* species observed here (>90% *R. helvetica* and <2% *R. monacensis*) was similar to that reported previously for Switzerland, where few *R. monacensis*-infected ticks have been identified (7, 9), whereas *R. helvetica* is commonly identified in ticks (7, 41). *R. monacensis* is only sporadically present in Central Europe, while it tends to be more common in Southern Europe (49).

B. venatorum was by far the most prevalent *Babesia* sp. identi-

fied here, followed by unidentified *Babesia* spp. and *B. divergens*. The predominance of *B. venatorum* has also been recognized in other European countries (47, 49), including Switzerland (9, 23). Although most of the human clinical cases in Europe are due to *B. divergens*, *B. venatorum* was recently involved in clinical cases in Italy, Germany, and Austria (25, 27).

Mixed infections with two or three *Borrelia* genospecies occurred in 2.1% of ticks and in 9.1% of *Borrelia*-infected ticks. Moreover, coinfections with pathogens of different genera were detected in 4.7% of ticks and in 13.7% of infected ticks. Globally, 6.7% of ticks and 19.6% of infected ticks were carrying more than one pathogen (mixed infections and coinfections); among them, coinfections with *B. afzelii* and *R. helvetica* were the most frequent. This was not surprising, as they represented the most prevalent species. *B. afzelii* was strongly associated with *R. helvetica* and "*Candidatus* Neoehrlichia mikurensis," probably due to their common reservoir hosts. In fact, *B. afzelii* is associated with rodents (22), and some previous studies designated rodents as potential reservoirs for "*Candidatus* Neoehrlichia mikurensis" (2, 34). *I. ricinus* acts as both a vector and a reservoir host of *R. helvetica*, and no vertebrate host is really required for its maintenance. Nevertheless, it was demonstrated previously that mice and roe deer might also be reservoirs for *R. helvetica* (55).

B. valaisiana was frequently implicated in mixed infections with *B. garinii* and in coinfections with two or three pathogens, mostly with *R. helvetica*. *B. valaisiana* and *B. garinii* share a common reservoir host, songbirds (28). To our knowledge, only one previous study described coinfections involving *B. valaisiana* and *R. helvetica* (47). Interestingly, the level of involvement of *A. phagocytophilum* in coinfections was relatively high (0.6%), compared to its low prevalence (1.5%). Part of explanation for this finding is the immunosuppressive action of *A. phagocytophilum*, making infected animals and humans more susceptible to other infections (63). Triple infections were detected in two males and four nymphs, underlining the important role of hosts as sources of multiple infections for ticks by carrying various microorganisms. As expected, *I. ricinus* adults were more frequently infected with multiple pathogens than nymphs, probably due to consecutive feedings.

In our study area, five different pathogens were found to circulate, and surprisingly, a high frequency of ticks was infected with at least one pathogen (34.2%). Moreover, 19.6% of infected ticks were carrying more than one pathogen, and this prevalence could easily be expected to be higher if all collected ticks had been screened for "*Candidatus* Neoehrlichia mikurensis." Compared to a previous study of the same pathogens (except "*Candidatus* Neoehrlichia mikurensis") in Luxembourg (47), *Borrelia* spp. and *Rickettsia* spp. were found more frequently in ticks in this study (22.5% and 10.2%, respectively, compared to frequencies of 11.3% and 5.1%, respectively, reported previously [47]), while the prevalences of *Babesia* spp. and *A. phagocytophilum* were slightly lower (1.9% and 1.5%, respectively, compared to prevalences of 2.7% and 1.9%, respectively, reported previously [47]). Moreover, the global prevalence of ticks infected by at least one pathogen in this study (34.2%) and the prevalence of multiple infections in ticks (6.7%) were higher than those reported previously for Luxemburg (19.5% and 3.2%, respectively [47]). In conclusion, this study contributes to a better understanding of the occurrence and co-occurrence of human-pathogenic agents in questing *I. ricinus* ticks.

ACKNOWLEDGMENTS

This work was financially supported by the Swiss National Foundation (grant no 310030-127064/1).

We are grateful to Mégane Pluess for her technical assistance and to Olivier Rais and Paul Monnerat and his students for having collected ticks in Neuchâtel and Jura, respectively. We thank Ana Sofia Santos (CEVDI, Portugal), Simona Casati (Instituto di Microbiologia, Ticino, Switzerland), and Olivier Péter (Institut Central des Hôpitaux du Valais, Sion, Switzerland) for providing positive controls.

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