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Article

Swiss Army Survey in Switzerland to Determine the Prevalence of *Francisella tularensis*, Members of the *Ehrlichia phagocytophila* Genogroup, *Borrelia burgdorferi* Sensu Lato, and Tick-Borne Encephalitis Virus in Ticks

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Abstract A total of 6071 *Ixodes ricinus* ticks were collected on Swiss Army training grounds in five regions of Switzerland. The aim of the survey was to assess the prevalence of ticks infected with the human pathogens *Francisella tularensis*, members of the *Ehrlichia phagocytophila* genogroup, *Borrelia burgdorferi* sensu lato, and the European tick-borne encephalitis virus. TaqMan PCR (PE Biosystems, USA) and TaqMan RT-PCR (PE Biosystems) analyses were performed on DNA and RNA extracted from pools of ten ticks grouped by gender. Here, for the first time, it is shown that ticks may harbor *Francisella tularensis* in Switzerland, at a rate of 0.12%. Furthermore, 26.54% of the ticks investigated harbored *Borrelia burgdorferi* sensu lato, 1.18% harbored members of the *Ehrlichia phagocytophila* genogroup, and 0.32% harbored the European tick-borne encephalitis virus. A new instrumentation was applied in this study to carry out and analyze more than 2300 PCR reactions in only 5 days. Furthermore, the results reveal that people working in outdoor areas, including army personnel on certain training grounds contaminated with ticks containing tick-borne pathogens, are at risk for different tick-borne diseases.

Introduction

Worldwide, many infections caused by viruses, bacteria, or parasites are known to be tick-transmitted zoonoses. Tick-borne diseases are a major threat to mammals and humans during outdoor activities in spring and fall. In recent years, the impact of tick-borne diseases has gained in importance as humans have moved into rural areas and have increased their recreational outdoor activity. In Europe, *Francisella tularensis* occurs in localized geographic areas, and human granulocytic ehrlichiosis (HGE) is considered a new emerging infectious disease. The most important tick-borne diseases are Lyme borreliosis and tick-borne encephalitis (TBE).

Francisella tularensis is the causative agent of tularemia, a zoonotic bacterial disease endemic in the Northern Hemisphere [1]. Lagomorphs and rodents are thought to be natural reservoirs, and tick species serve as vectors [2]. The agent is extremely virulent, with as few as ten organisms capable of causing disease in humans [3]. Tularemia is not endemic in Switzerland; nevertheless, *Francisella tularensis* has been isolated from a small number of animals in Switzerland [4, 5]. Blood-feeding arthropods and flies are the most impor-

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tant vectors of tularemia [1, 6]. Ticks predominate as vectors in North America, whereas mosquitoes are the most frequent insect vector in Sweden [7]. Among the ticks, *Dermacentor* was found to harbor *Francisella tularensis* more frequently than *Ixodes* species isolated in the Czech Republic [6].

Ehrlichiae are obligate intracellular organisms that have been divided into three genogroups based on the nucleotide sequence of the 16S rRNA gene: *Ehrlichia phagocytophila*, *Ehrlichia canis*, and *Ehrlichia sennetsu* [8]. Members of the *Ehrlichia phagocytophila* genogroup are transmitted by *Ixodes* ticks. In Switzerland, *Ehrlichia phagocytophila* has been identified in cattle [9], and an agent with 100% homology in the 16S rRNA gene to the agent of HGE has been detected in dogs [10], horses [11], and ticks [12]. The causative agent of HGE is a recently discovered species that infects humans [13] and has been found in both the USA and Europe [14]. Seroepidemiological and polymerase chain reaction (PCR) studies indicate that the HGE agent also occurs in Switzerland and poses a potential threat to humans [12, 15–17].

Lyme borreliosis, caused by pathogenic spirochetes belonging to the species *Borrelia burgdorferi* sensu lato, is a multisystemic illness that occurs worldwide [18]. *Borrelia burgdorferi* sensu stricto has been found in ticks and patients infected in Europe as well as in North America, whereas *Borrelia garinii* and *Borrelia afzelii* have been found only in ticks and patients infected in Eurasia [19].

Tick-borne encephalitis virus (TBEV), a virus pathogenic for humans, is a member of the family *Flaviviridae*, which contains about 70 different viruses that can be divided into several serologic subgroups. In many countries in Europe and Asia, TBE represents an important arthropod-borne disease. Most flaviviruses pathogenic for humans are mosquito-borne, whereas TBEV is transmitted by ticks. In Switzerland, several natural foci of TBEV exist, and new endemic foci have been recognized in recent years [20]. In southern Germany, an increase in the number of cases as well as an expansion of TBE-endemic regions has been noted over the past several years [21].

The purpose of this study was to investigate the prevalence of *Francisella tularensis*, *Borrelia burgdorferi* sensu lato, and members of the *Ehrlichia phagocytophila* genogroup and to find new natural foci of TBEV in ticks collected from five regions in Switzerland.

Materials and Methods

Collection and Processing of Ticks. A total of 6071 free-living adult ticks of the species *Ixodes ricinus* were collected in the following five regions of Switzerland during spring of 1999: Kloten/Bülach (east Switzerland), Neuchatel (west Switzerland), Brugg and Thun/Spiez (central Switzerland), and Ticino (south Switzerland). The ticks were collected by the cloth-dragging method. After identification, ticks were pooled in groups of at least ten males, females, or nymphs and stored at –20 °C until DNA extraction was performed. Ticks were mechanically crushed, and nucleic acid extractions were performed with a QIAamp Tissue Kit (Qiagen, Switzerland) according to the manufacturer's instructions.

Commercial Oligonucleotide Probes and Primers and Real-Time Polymerase Chain Reaction. TaqMan (PE Biosystems, USA) systems for members of the *Ehrlichia phagocytophila* genogroup [22] and *Borrelia burgdorferi* sensu lato were used as described previously [17]. The sequences for *Francisella tularensis* were as follows: forward primer Ft.820f 5′-TTGGGCAAATCTAG CAGGTCA-3′, TaqMan probe Ft.851p: 5′-FAM-AAGACCAC CACCAACATCCCAAGCA-TAMRA-3′, and reverse primer Ft.921r 5′-ATCTGTAGTCAACACTTGCTTGAACA-3′. The 25 μl PCR mixtures contained 10 mM Tris (pH 8.3), 50 mM KCl, 5 mM $MgCl_2$, (200 μM) 2.5 mM deoxynucleotide triphosphates, 400 nM of each primer, 80 nM of fluorogenic TaqMan probe, 0.625 U of AmpliTaq Gold per reaction, 0.25 AmpErase UNG per reaction, and 10 μl of bacterial lysates, tick DNA, or plasmid standard. After AmpliTaq Gold activation for 10 min at 95 °C, amplification conditions were as follows: 45 cycles of 15 s at 95 °C and 60 s at 60 °C. The analytical specificity was tested by sequencing two cloned PCR products and running DNA from the following bacterial strains with the *Francisella* TaqMan assay: *Francisella tularensis* (ATCC 29684), *Francisella tularensis* (vaccine strain 410062), two *Francisella* field isolates from Switzerland [4], *Francisella philomiragia* (ATCC 25016), *Borrelia burgdorferi* sensu lato (VS215), *Bartonella henselae (*CIP 103737), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa (*ATCC 27853), and *Rickettsia rickettsii* (field isolate, USA). The cloned PCR products were used to run a standard curve.

The sequences for the TBEV TaqMan system were as follows: forward primer FS. 16f (5′TCCAGTGGAGCTGGAGTTGA G3′), TaqMan probe FS. 39p (5′AATAAGCTTGAACTACCT GGGCTGGCA3′), and reverse primer FS. 96r (5′TCCTCCAT TCAGGGTTGACAA3′). The 25 μl TBEV reverse transcriptase (RT)-PCR mixture for one reaction contained 5 μl of optimized single-buffer 5× (Access RT-PCR system; Promega, USA), 5 mM MgSO4, 500 μM deoxynucleotide triphosphates, 10 nM of the TaqMan probe, 1.2 μM of each primer, 2.5 U of Avian myoblastosis virus reverse transcriptase, 2.5 U of *Thermus flavus* polymerase per reaction, and 5 μl of nucleic acid preparation. After an initial reverse transcription step of 45 min at 48 °C and a denaturation step of 2 min at 94 °C, 40 cycles, each 30 s at 94 °C, 1 min at 64 °C, and 2 min at 68 °C, followed. All reactions were carried out in an ABI Prism 7700 Sequence Detector (PE Biosystems). To test for sensitivity, in vitro transcripts of a cloned TBEV fragment were serially diluted and tested with the TBEV TaqMan RT-PCR system.

Preparation of Bacterial Polymerase Chain Reaction Templates. Colonies from bacterial cultures were suspended in 10 mM Tris HCl (pH 8.0) containing SDS (5% w/v) and 1 μg/μl Proteinase K (Sigma, Switzerland). Lysates were incubated for 30 min at 70 °C and diluted 100-fold. Prior to performing the PCR, samples were boiled at 95 °C for 5 min and chilled on ice.

Data Evaluation. The estimation of the rate of infection of the four pathogens was calculated using the following formula [23]:

$$f(x) = 1 - \left(\frac{n - X^{1/m}}{n}\right) \times 100$$

where $f(x)$ = estimated rate of infection (in percent); n = number of pools tested; m = average number of ticks per pool; and X = number of pools positive.

Results

Optimization of the Francisella Polymerase Chain Reaction System. The *Francisella* TaqMan PCR assay detected ten molecules of the standard plasmid. Amplification of a standard plasmid dilution over eight orders of magnitude showed linearity over the whole range. The assay proved to be specific for *Francisella tularensis*. The ATCC strain, the two field isolates, and the vaccine strain generated fluorescence signals, but the other bacterial DNA tested, including *Francisella philomiragia*, did not. Specificity was also verified by sequencing two cloned PCR products obtained by amplification of the ATCC strain. The TaqMan systems that detected *Francisella tularensis*, *Borrelia burgdorferi*, and *Ehrlichia phagocytophila* had the same sensitivity of ten molecules per assay [17, 22]. In addition, the sensitivity of the TBEV RT-PCR was ten copies of an in vitro transcribed RNA.

Prevalence According to the Sample Structure. The sample structure of the 6071 ticks collected was as follows: 5336 nymphs, 390 adult males, and 338 adult females (Table 1). Of the 6071 ticks examined, seven pools (0.12%) were positive in the *Francisella* TaqMan PCR, all of which contained nymphs. Sixty-eight (1.18%) of the 607 pools were positive in the *Ehrlichia phagocytophila* genogroup TaqMan PCR and consisted of 47 (0.94%) nymph pools, 10 (2.61%) adult male pools, and 12 (3.72%) adult female pools. In the *Borrelia burgdorferi* TaqMan PCR, 563 (26.54%) pools gave a positive reaction: 495 (25.9%) nymph pools, 39 (21.1%) male pools, and 34 (20.16%) female pools. In the TBEV-specific TaqMan RT-PCR, 19 (0.32%) of the 607 tick pools tested positive. They consisted of 14 (0.27%) nymph pools, four (0.97%) adult male pools, and one (0.27%) adult female pool (Tables 2 and 3).

Prevalence According to Tick Stage Development. The prevalence of infected ticks varied with the stage of development and the geographical origin (Table 3). *Francisella* was found in nymphs (0.14%) but not in adult ticks. Members of the *Ehrlichia phagocytophila* genogroup were found more frequently in adult ticks (2.95%) than in nymphs (0.94%). In the chi-square test, this difference was significant ($P < 0.001$). *Borrelia* was found almost evenly distributed among the tick developmental stages, and TBEV was more frequent in adult males than in adult females and nymphs, but this was not statistically significant (Table 3). *Ehrlichia* and *Borrelia* were found in all five regions, whereas *Francisella tularensis* could be found in four of five regions and TBEV in two of four regions. The highest prevalence of members of the *Ehrlichia phagocytophila* genogroup, *Francisella tularensis*, and *Borrelia* was found in Neuchatel, with an absence of TBEV in this region. TBEV was found in Thun/Spiez and in Kloten/Bülach.

Table 1 Geographic origin and developmental stages of 6071 *Ixodes ricinus* ticks

Region (no. of ticks)	Nymphs ($n=5336$)	Adult males ($n=396$)	Adult females ($n=339$)
Brugg (710)	596	60	54
Kloten/Bülach (3717)	3201	320	196
Neuenburg (89)	80	3	6
Thun/Spiez (1099)	1009	10	80
Ticino (456)	450	3	3
Total	5336	396	339

Table 2 Total rates of tick infections

Pathogen	Prevalence	
	Percent	Positive pools/ total pools
Francisella tularensis	0.12	7/607
Ehrlichia phagocytophila[a]	1.18	68/607
Borrelia burgdorferi sensu lato	26.54	563/590
TBEV	0.32	19/607

[a] TaqMan PCR specific for members of the *Ehrlichia phagocytophila* genogroup
TBEV, tick-borne encephalitis virus

Discussion

The goals of the present study were as follows: (i) to apply a new instrumentation to detect pathogens in

Table 3 Results of TaqMan PCR for 6071 *Ixodes ricinus* ticks by geographic origin and developmental stage

Developmental stage	Prevalence							
	Francisella tularensis		*Ehrlichia phagocytophila*[b]		*Borrelia burgdorferi*		TBEV	
Nymphs	0.14	(7/521)[a]	0.94	(47/521)	25.9	(495/521)	0.27	(14/521)
Adult males	0	(0/43)	2.61	(10/43)	21.1	(39/43)	0.97	(4/43)
Adult females	0	(0/38)	3.72	(12/38)	20.16	(34/38)	0.27	(1/38)
Adult males and females	0	(0(81)	2.95	(22/81)	20.66	(73/81)	0.6	(5/81)

[a] Data expression: x (y/N), where x is the percentage of positive ticks, y is the actual number of positive pooled samples, and N is the total number of pools analyzed

[b] TaqMan PCR specific for members of the *Ehrlichia phagocytophila* genogroup

Table 4 Geographic distribution of tick infection

Region	Prevalence[a] (no. of infected ticks/total no. of ticks)							
	Francisella tularensis		*Ehrlichia phagocytophila*[b]		*Borrelia burgdorferi*		TBEV	
Brugg	0	(0/72)[a]	1.02	(7/72)	20.57	(63/70)	0	(0/72)
Kloten/Bülach	0.08	(3/370)	1.47	(51/370)	28.42	(335/370)	0.36	(13/370)
Neuenburg	0.95	(3/10)	3.13	(3/10)	100	(10/10)	0	(0/10)
Thun/Spiez	0.21	(3/96)	0.32	(3/96)	29.28	(93/96)	0.64	(6/96)
Ticino	0.21	(4/47)	0.89	(4/47)	31.65	(44/47)	nd	–

[a] Data expression: x (y/N), where x is the percentage of positive ticks, y is the actual number of positive pooled samples, and N is the total number of pools analyzed

[b] TaqMan PCR specific for members of the *Ehrlichia phagocytophila* genogroup

nd, not done

large numbers of samples in a short period of time, and (ii) to investigate the distribution of *Francisella tularensis*, members of the *Ehrlichia phagocytophila* genogroup, *Borrelia burgdorferi* sensu lato, and TBEV in five regions of Switzerland.

We have developed the TaqMan systems to detect tick-borne pathogens by PCR. The systems are based on a novel real-time PCR that measures PCR product accumulation through a dual-labeled fluorogenic probe (i.e. TaqMan probe) [24]. This system was successfully used to screen large numbers of samples for different pathogens in our laboratory and in others.

Two new TaqMan systems were developed for this study: the first system, which detects *Francisella tularensis*, was based on fopA sequences obtained earlier from a Swiss isolate (GenBank accession no. AF097542). The second system, which detects TBEV, is based on the NS5 gene for the Central European tick-borne encephalitis virus (GenBank accession no. S68159). The sensitivities obtained with all four systems were comparable. As pools of ten ticks were used for DNA extractions, the estimated prevalences may be an underestimation of the actual prevalences, especially for pathogens with a low actual prevalence. Comparisons of prevalences obtained with DNA from single purified ticks with our results showed that they are in agreement with the prevalences obtained from pooled ticks. This comparison argues against a dilution effect of DNA obtained from pooled ticks and suggests that this method leads to reliable estimations of the prevalence.

Francisella is efficiently transmitted by tick bites. Even though *Francisella* cases in animals were described earlier in Switzerland [4, 5], the pathogen itself had not yet been detected in ticks from Switzerland. In the present study, *Francisella* was found in nymph stages of *Ixodes ricinus* but not in adult stages. The presence of *Francisella tularensis* in ticks of Switzerland was expected, as this pathogen is endemic in several neighboring countries. Our results are in good agreement with results from the Czech Republic, where a minimal infection rate of 0.01% of *Francisella tularensis* was found in *Ixodes ricinus*, 1.4–2.6% in *Dermacentor reticulatus*, and 0.05% in *Dermacentor marginatus* [25]. This is in contrast to findings in Austria, where *Francisella tularensis* could only be found in *Dermacentor* species at an infection rate of 3.5% [2]. In the present study, only *Ixodes ricinus* ticks were included. *Dermacentor* ticks are more frequently found in northwest Switzerland. In view of the prevalences found in different tick species in other countries, it may be speculated that *Dermacentor* species harbor *Francisella* more frequently in Switzerland, too. Based on the similar prevalence of *Francisella* in *Ixodes ricinus* in Switzerland and the Czech Republic, speculation is open as to whether *Francisella* leads to infections in humans in Switzerland. If so, *Francisella* infection would be a nondiagnosed infectious disease in our country and should therefore be included in the differential diagnosis of the infectious processes observed in Switzerland.

In Europe, at least two granulocytic species of *Ehrlichia* are present: *Ehrlichia phagocytophila* and the agent causing HGE. In Switzerland, the prevalence of *Ehrlichia*-infected ticks varied from 0.4 to 2.1%, depending on the region [12]. In regions with sporadic occurrence of granulocytic ehrlichiosis in dogs and horses, 1.3% of ticks were infected with members of the *Ehrlichia phagocytophila* genogroup [26]. This is consistent with results from the regions of Brugg, Kloten/Bülach, Thun/Spiez, and Ticino, where we found prevalences of 1.02%, 1.47%, 0.32%, and 0.89%, respectively. In the region of Neuchatel, the prevalence was higher (3.13%), indicating a hot spot of members of the *Ehrlichia phagocytophila* genogroup. The distribution among the tick stages was similar, as reported previously, showing that predominantly adult ticks were infected [26]. HGE is an emerging tick-transmitted infection caused by a granulocytic species of *Ehrlichia*, which has 99.9% 16S rRNA gene sequence homology with *Ehrlichia phagocytophila*. This agent causes granulocytic ehrlichiosis in humans, and seroepidemiological studies indicate that HGE also occurs in Switzerland and poses a potential threat to humans [16,

27]. HGE is transmitted by ticks and may be associated with *Borrelia burgdorferi* within the same tick [12, 17].

The lowest prevalence of *Borrelia* in this study (26.54%) is in agreement with prevalences found earlier (5–34%) [28]. Lyme borreliosis is the most commonly reported tick-borne disease worldwide. In Switzerland, people at high risk for tick bites, such as orienteers, have a *Borrelia* antibody prevalence of 26.1% [29]. Among them, 1.9% had a history of definite clinical Lyme borrelosis and 3.1% a history of probable disease, indicating that most infections remain unapparent. This is in contrast to findings in other countries, where the ratio of apparent to unapparent infection is estimated to be 1:1. After 7 years, the percentage of cases with Lyme borreliosis increased to 4.9%, showing that chronic manifestations had occurred.

The incidence of TBE in the human population in Switzerland ranges between 0.46 and 0.9/100,000 per year [30]. Acquisition occurs only in well-known endemic areas through bites of infected ticks. Since death occurs in 0.5–2% [23] and nearly half of patients with meningitis/meningoencephalitis show residual symptoms, knowledge of the endemic areas is very important for immunoprophylaxis of TBE. The largest areas of endemicity are located in Canton Schaffhausen, the northern part of Canton Zurich, and northwest Canton Thurgau. Another known area of endemicity is the region of Thun in Canton Berne [20]. TBEV, in addition to being found in Thun, was also found in Bülach/Kloten, which had only been suspected as a region of endemicity in Switzerland [21]. Whether this newly described natural focus in ticks is also associated with clinical illness in humans should be carefully examined.

Coinfection of ticks with different tick-borne pathogens is well known and may pose a serious problem for diagnosis and treatment. Coexistence of antibodies to tick-borne pathogens, such as *Babesia microti*, the HGE agent, and *Borrelia burgdorferi*, has been reported [31, 32], indicating that humans and animals may be infected simultaneously by these pathogens through tick bites. Coinfection with *Borrelia* and TBEV may also occur [33]. Coinfection of ticks with *Borrelia burgdorferi* and HGE has been reported in the USA as well as in Switzerland [17, 34]. Infection with *Ehrlichia* can cause immunosuppression of mammals, which may increase the severity of *Borrelia burgdorferi* infection [36]. Coinfections may explain the variable clinical signs seen in humans and animals with Lyme borreliosis and ehrlichiosis [27, 36, 37]. Due to the high prevalence of *Borrelia* in ticks, the lowest risk – per area – of ticks being coinfected with *Borrelia* and members of the *Ehrlichia phagocytophila* genogroup is 1/330 ticks. As prevalences for TBEV and *Francisella* are low, the lowest risk of ticks being coinfected drops to 1/1250 and 1/3330 ticks, respectively. This study reveals that people, including army personnel, working in natural habitats of ticks possibly infected with pathogenic microorganisms are at risk of infection by different tick-borne pathogens. Greater awareness among local physicians of the existence of tick-borne transmitted infections in Switzerland, coupled with better availability of improved diagnostic techniques, is necessary to diagnose and treat potentially fatal tick-borne diseases.

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