



First metagenomic report of *Borrelia americana* and *Borrelia carolinensis* in Poland – a preliminary study

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Abstract

Introduction and objective. *Ixodes ricinus* (*I. ricinus*) and *Dermacentor reticulatus* (*D. reticulatus*) are the most common ticks in Poland. These ticks contain many bacteria, which compose a microbiome with potential impact on humans. The aim of the study was to discover the microbiome of ticks in Poland.

Materials and method. Ticks were collected in The Protected Landscape Area of the Bug and Nurzec Valley, Poland, in 2016–2018 by flagging. They were cleaned in 70% ethanol and damaged in mortar with PBS (without Ca²⁺ and Mg²⁺ ions). DNA was extracted from the homogenates with spin columns kits, and used as a matrix in end-point PCR for bacterial 16S rRNA fragments amplifications, and further for next generation sequencing (NGS) by ILLUMINA.

Results. In 22 ticks (3 *I. ricinus* and 19 *D. reticulatus*) 38 microorganisms were detected. The most common were *Francisella hispaniensis* and *Francisella novicida*. In 17 ticks, *Sphingomonas oligophenolica*, and in 12 *Rickettsia aeschlimanii* were found. In 2, *I. ricinus* specific DNA of *Borrelia americana* and *Borrelia carolinensis* were found. In one female, *D. reticulatus* *Anaplasma phagocytophilum* and *Anaplasma centrale* were found. *Pseudomonas lutea* and *Ps. moraviensis* were detected in 9 and 8 ticks, respectively.

Conclusions. Polish ticks microbiome contains not only well-known tick-borne pathogens, but also other pathogenic microorganisms. For the first time in Poland, *Borrelia americana* and *Borrelia carolinensis* in *I. ricinus* collected from the environment were detected. The dominant pathogenic microorganisms for humans were *Francisella* spp. and *Rickettsia* spp., and non-pathogenic – *Sphingomonas oligophenolica*. Knowledge of a tick microbiome might be useful in tick-borne biocontrol and tick-borne diseases prevention.

Key words

ticks microbiome; *Borrelia americana*; *Borrelia carolinensis*; Poland

INTRODUCTION

Ticks are ectoparasites that feed on the blood of vertebrates. During a meal, ticks can transmit several pathogenic microorganisms which makes them vectors of tick-borne diseases. About 90% of tick species are of veterinary or medical significance [1, 2].

Ixodes ricinus (*I. ricinus*) is a hard tick that dominates in Poland and in Europe. It is also present in north-west Africa, Middle East [3]. *I. ricinus* prefers wet environments such as broadleaf and mixed forests with a diverse spectrum of feeders, as well as bushes or grasslands with a diverse flora. *I. ricinus* appears more often in urban areas: parks, gardens, squares or wastelands [3]. It feeds on other mammals and birds, which makes them relevant to veterinary medicine [4]. Seasonal activity usually begins in April and ends in November; however, microclimate and recent climate change allow for tick activity throughout most of the year [5].

Dermacentor reticulatus (*D. reticulatus*) is the one of the most frequently detected ticks in Poland [6]. It is less well

known than *I. ricinus* but its role in pathogen transmission is comparable [4]. Its behavior and adaptive capabilities facilitate its spread and subsequent transmission of tick-borne diseases [7]. In Poland, similar to the rest of Europe, expansion of *D. reticulatus*' territory is evident in 2018 [8]. This process was linked to changes in its preferred habitats; previously, it favoured wet areas with a relatively high level of ground waters, swampy mixed forests, riverside valleys and meadows, but now it can be found with the same frequency in urban and suburban areas [5, 7]. *D. reticulatus* feeds on many different mammals, which facilitates its survival and expansion to new habitats. Humans might be incidental hosts of *D. reticulatus*, especially when other mammals are unavailable [5].

Microbiome (microbiota) is a group of diverse microorganisms present inside and outside another organism. They can be symbiotic, commensal or even pathogenic for the host [3, 9]. Tick microbiome includes different species of bacteria, viruses and protozoa; some of which are called tick-borne pathogens.

Francisella spp., *Rickettsia* spp., *Borrelia* spp., *Anaplasma* spp., *Ehrlichia* spp., *Babesia* spp., *Theileria* spp., *Bartonella* spp., *Coxiella* spp. and tick-borne encephalitis virus (TBEV) might be transmitted during tick feeding and cause single infection or co-infections [7, 9–12].

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Next generation sequencing (NGS) replaces older molecular techniques, such as culture or cloning-based methods, and allows for identification of the whole microbiome present in ticks [12, 13]. Previously, there was only a limited number of microorganism that could be identified in ticks [3]. New laboratory methods allow for simultaneous detection of multiple microorganisms in a single vector [9]. Microbiome of the tick's ecosystem is also crucial and it encompasses all microorganism in soil and water and in the vertebrates – potential feeders of ticks [3].

Endosymbionts such as *Francisella* spp., *Rickettsia* spp. and *Coxiella* spp. are part of tick microbiome [10, 14, 15]. Feeding increases tick microbial diversity [16] and it seems to be a self-propelling mechanism.

Tick microbiota exists in different parts of the arthropod, mainly in the entrails, midgut, salivary glands and ovaries. The presence of the microorganism in salivary glands facilitates their transmission to the vertebrate organism [1, 12, 13]. Microbiota diversity also depends on the gender and stage of the tick [17–19]: the microbiome of *I. scapularis* appears more diverse in adult males than in the female nymph or larva.

There has been limited research into interactions between ticks and their microbiota, and connections between pathogenic and non-pathogenic microorganism coexisting in different tick species [1, 2, 13, 14]. Modern molecular techniques, e.g. NGS and other genomic resources, can therefore be the first step towards identification of the determinants affecting tick's vector competence [1, 20, 21].

MATERIALS AND METHOD

Localization. Ticks were collected in a diverse environmental – the Area of Protected Landscape of the Bug and Nurzec Valley. This encompasses forests with a mosaic structure, Nurzec river, ponds, waterholes, grasslands, arable land and houses. It is an ideal habitat for many potential feeders for ticks, including humans. This area is rich in ecotones, which give rise to a wide range of microhabitats.

Ticks collection. Altogether 811 *I. ricinus* and *D. reticulatus* ticks were collected between May and October 2016, March and October 2017, and April and November 2018 (3 seasons of tick activity) using a flagging method (with a white flannel flag of 1m²) in the afternoon on a dry day. Each tick was placed in a separate Eppendorfs tube and transported to the laboratory in cool conditions. Humidity, air temperature at 5–10 cm from ground level, height of vegetation, and day duration were measured at the site of collection. Air temperature and humidity were measured to 0.1 °C and 0.1%.

Material initial preparation and DNA extraction. Each tick was checked under a stereomicroscope (Olympus, Germany) using dichotomous keys and character matrices and identified to *D. reticulatus* or *I. ricinus* species (including its life stage and gender). To avoid environmental contaminations and cross-contaminations, pre-PCR and PCR actions were prepared with sterile equipment and rooms. Ticks were cleaned for a minute in 70% ethanol (99.8% ethanol and ultra-pure water), in ultra-pure water per 1 min and again in 70% ethanol for 30 sec to remove all environmental microorganism from the tick surface. Homogenization was

achieved by grinding each tick with phosphate buffered saline PBS (without Ca²⁺ and Mg²⁺, pH=7.4). Homogenates were centrifuged and 300 µl of supernatant was used for DNA extraction with spin columns kits (EURx, Poland), according to the manufacturer's instructions. The quality and quantity of obtained DNA was checked with a WPA UV1101 spectrophotometer (WPA The Old Station, Linton, Cambridge, UK), to confirm the presence of a minimum of 10ng/µl of DNA. 100 µl of DNA extracts were stored at -20 °C until molecular analysis.

Molecular diagnostic – PCR for metagenomic (16S rRNA).

DNA isolates were a matrix for molecular biology techniques, such as end-point PCR for 16S rRNA fragments of bacterium nucleic acid (size ca. 550 bp). Amplification was performed with Start-Warm HS-PCR Mix (A&A Biotechnology, Gdynia, Poland), ddWater (aseptic, free from nucleases water, treated with DEPC), and appropriate primer sequences (F: sens 5' – TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CCT ACG GGN GGC WGC AG – 3', R: antisens 5' – GTC TCG TGG GCT CGG AGA TGT GTA TAA GAC ACA GGA CTA CHV GGG TAT CTA ATC C – 3') selected from Kindworth et al. [22], as the most promising bacterial primer pair for NGS. PCR protocol (95 °C for 3 min, 25 x (95 °C for 30s, 55 °C for 30s, 72 °C for 30s), 72 °C for 5 min, 4 °C holding) was performed on Sensoquest LabCycler (SensoQuest, Gemrany). Separation of PCR products on 2% agarose gel (Sigma-Aldrich, Germany) stained with Midori Green (Nippon Genetics Europe GmbH, Germany) by electrophoresis at 90V for 45 minutes. The results of the PCR amplicons were visualized under UV light in Gel Logic System 100 (Kodak Imaging System, Inc., USA). Amplification products (550 bp size) with a strong signal, and the high quality was chosen for further metagenomic investigation by Illumina MySeq (22 samples).

NGS (Illumina MySeq) – libraries preparation and sequencing.

Following the Illumina 16S Metagenomic Sequencing Library Preparation Guide (Illumina MySeq, Inc., San Diego, California, USA), DNA paired-end libraries were constructed with an insert size of ~550 bp using the primer pair sequences for the V3 and V4 variable regions of the 16S rRNA gene. The quantity and quality of all libraries were evaluated by capillary electrophoresis on a 2200 Agilent TapeStation Instrument with Genomic DNA ScreenTape Assay (Agilent Technologies Inc., St Clara, CA, USA). Samples were pooled in equal proportions and sequenced for 600 cycles (approx. 65-hour run) using a MiSeq Platform (Macrogen, Seoul, Korea) with v3 reagents with 2 x 300 bp paired-end reads. 10% of PhiX viral DNA was added to the sample pool as an internal control for a low diversity library. End-paired reads recorded in FASTQ formats were automatically demultiplexed, and Illumina adapters were removed by an Macrogen's in-house pipeline.

Bioinformatic analysis. Obtained on Illumina MySeq 16S rRNA sequences were quality-filtered, clustered at 97% similarity of sequences, and analyzed by the Quantitative Insights into Microbial Ecology 2 (QIIME2) (Swei and Kwan, 2017) software package, version 3.5.3. Filtration of sequences was based on length (200 – 500 bp) and multiple quality metrics. No sequences with primer mismatches were accepted. Also removed were reads with poor quality of

assembly, and misaligned, non-specific (such as eukaryotic) and chimeric signals.

RESULTS

For all 22 ticks (16 females and 6 males), high quality sequences for bacterial 16S *rRNA* gene amplicons using an Illumina MiSeq were obtained in very high numbers from each sample (range 15,870 – 139,436; minimum read length 200 bp) and uploaded into QIIME 2 (Quantitative Insights Into Microbial Ecology 2) software. The output reads number has a crucial role for appropriate evaluation of microbiome diversity – large number of this reads is linear with metagenome (taxonome) coverage. In this study, the number of output reads were mostly higher than 100,000 – mean 113,389.36, with standard deviation 47,741.3, what indicated very high alpha diversity refraction plots.

In 22 ticks (3 *I. ricinus*, 19 *D. reticulatus*), NGS confirmed the presence of 38 identified microorganisms (including 20 genera) (Tab. 1; Fig. 1), pathogenic as well as potentially non-pathogenic to humans. The most common pathogens, present in almost every tick were *Francisella hispaniensis* and *Francisella novicida* (21 and 20 ticks, respectively). *Sphingomonas oligophenolica* (non-pathogenic for immunocompetent individuals) was found in 17 ticks; and *Sphingomonas dokdonensis* in 2 and *Sphingomonas echinoides* in one. Within *Rickettsia* spp., *Rickettsia aeschlimanii* dominated and was detected in 12 ticks; *Rickettsia tamurae* was detected in 4, *Rickettsia marmionii* in 2, *Rickettsia typhi* in 2 and *Rickettsia monacensis* in one tick. In 2, *I. ricinus* ticks *Borrelia americana* and *Borrelia carolinensis* DNA was identified. In one, *D. reticulatus* female *Anaplasma phagocytophilum* and *Anaplasma centrale* were found. *Pseudomonas lutea* was present in 9, and *Pseudomonas moraviensis* in 8 ticks, although other *Pseudomonas* genospecies were also detected. Several genospecies from *Methylobacterium*, *Rhodococcus* and *Pedobacter* species were also observed in the analyzed ticks.

There exist some differences between bacterial community composition despite gender. *Rickettsia* spp., *Methylobacterium* spp., *Thermomonas* spp., *Calotrix* spp., *Rhodococcus* spp. and *Luteibacter* spp. dominated in females, while *Pseudomonas* spp., *Chitinophaga* spp. and *Pedobacter* spp. dominated in tick males.

Besides previously known species, NGS analysis revealed also new unclassified bacteria species: in one tick they amounted to 83.05% of species and in another – 6.64%; on average, 19% +/-22.3% of microorganism in a single tick were unclassified (median 10.45%).

DISCUSSION

Molecular analysis of Polish *D. reticulatus*' and *I. ricinus*' microbiome revealed the presence of various pathogenic microorganisms of proven medical significance, especially for the young, elderly and non-immunocompetent [23, 24]. Over the years, 40 pathogenic microorganisms have been isolated from *D. reticulatus*. Czech researchers cultured an additional 38 bacterial strains of unknown medical and veterinary significance [25]. Sui et al. [26] detected 70 different bacteria species in *I. scapularis* using NGS.

Metagenomic analysis by the authors of the current study identified 38 microorganisms (from 20 species) *D. reticulatus* and *I. ricinus*. In contrast, metagenomic identification of 50 male and 50 female *I. scapularis* conducted by Zhang et al. revealed the presence of 373 microorganisms in unfed and 289 in fed ticks [12].

The mechanism of *Dermacentor* and *Ixodes* feeding has a significant impact on pathogen transfer. These ticks' feeding is long-lasting and results in a large intake of vertebrate's blood [18, 26, 27]. Endosymbionts are also transmitted during tick meal [14]. At times, these endosymbionts can emerge as vertebrate pathogens [16].

Borrelia burgdorferi sensu lato is the most common tick-borne pathogen. Its several genospecies cause Lyme borreliosis (Lyme Disease – LD). In Poland, as in the whole of Europe, *B. garinii*, *B. afzelii* and *B. burgdorferi* sensu stricto dominate in ticks. It is surprising that *B. afzelii*, *B. garinii* and *B. burgdorferi* s.s., species commonly present in Polish ticks, were not detected in the current study. However, it should be emphasized that in Poland the prevalence of *B. burgdorferi* sensu lato in ticks fluctuates from a few to several dozen percent, depending on the region of the country. It is supposed that the reason why no *B. afzelii*, *B. garinii* and *B. burgdorferi* s.s. were detected was the sample size. Moreover, the aim of the current study was to analyze microbiome of ticks in Poland, which was achieved. In 2 of 3 *I. ricinus* analysed, *Borrelia americana* and *Borrelia carolinensis* were identified. This is a novel finding that confirms the presence of these species in Polish ticks. Recently, Michalik et al., found *B. carolinensis* in *Ixodes* ticks (Acari: Ixodidae) associated with cave-dwelling bats from Poland and Romania [28], which adds new information on the presence of *Borrelia* spp. in Poland as a new species, previously not seen, but which are being detected more often.

Borrelia americana, which is very closely related to *B. carolinensis*, was detected for the first time by Rudenko et al. in southern USA [29]. It is well known that *D. reticulatus* is a vector of *B. burgdorferi* sl. However, it is not a dominant pathogen, what can be explained by the observation that this tick inhibits *Borrelia* spirochetes; growth *in vitro* inactivates this bacterium via activation of its own immune system [25, 30]. This may explain the absence of *Borrelia* in *D. reticulatus* ticks analyzed in the current study. According to Mierzejewska et al. [30], *B. afzelii* was detectable in only 0.09% of ticks. In the current study, *Borrelia* DNA was observed in 9.1% of ticks. Gofton et al. [20] performed a metagenomic analysis of *I. ricinus* and detected *B. burgdorferi* ss, *B. afzelii* with 100% homology, and *B. miyamotoi* and *B. lonestari* with 99.3% and 97.7% homology, respectively. In Australia, Panetta et. al [21] found new *Borrelia* genospecies *Candidatus Borrelia tachyglossi* in 12% of studied ticks and other types of *Borrelia* from *sensu lato* complex.

There might be a close relationship between *A. phagocytophilum* and other microbiota present in ticks [21]. *Enterococcus* spp. and *Rickettsia* spp. decreases *A. phagocytophilum* infection rate, while *Pseudomonas* spp. seemed to increase it. In the presented study, one of the *D. reticulatus* in which *A. phagocytophilum* and *A. centrale* were detected, *Pseudomonas* and *Rickettsia* were also detected.

In the current study, comparable *Rickettsia* spp. prevalence to other Polish studies was observed. In the Area of Protected Landscape of the Bug and Nurzec Valley, 5 different *Rickettsia* genospecies (*Rickettsia aeschlimanii*, *Rickettsia*

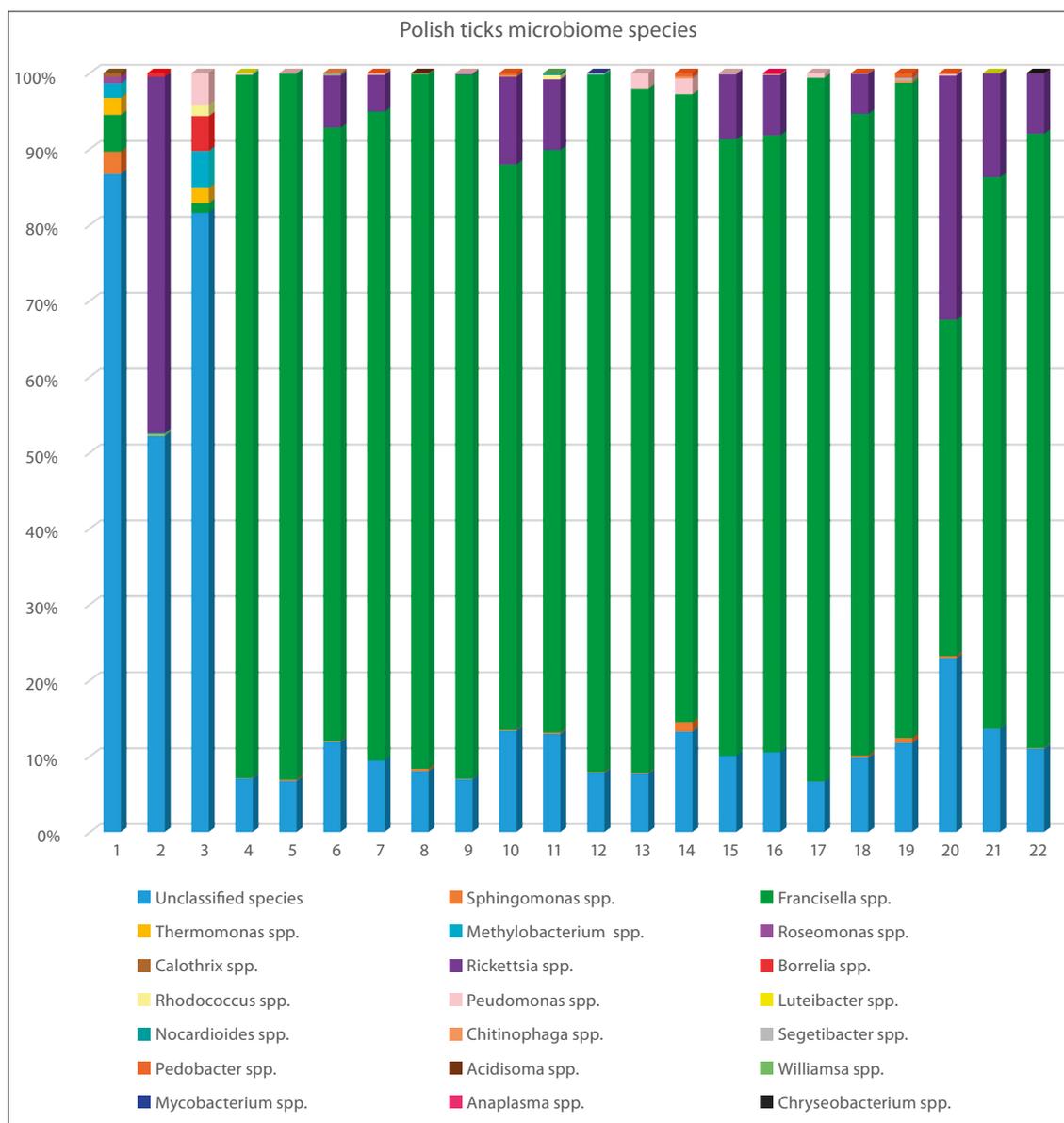


Figure 1. Species composition (in %) in 22 Polish ticks

tamurae, *Rickettsia typhi*, *Rickettsia marmionii* and *Rickettsia monacensis*) were found in 54.55% of examined ticks. Similar results were obtained in the Lublin Province in eastern Poland by Wójcik-Fatla et al. [31], who detected *Rickettsia* spp. DNA in 53% of ticks and in north-eastern Poland by Stańczak [32] where 41% of *D. reticulatus* were carriers of *Rickettsia* species. This data was confirmed by Chmielewski et al. [32] in the Białowieża Primeval Forest National Park, where 57% of ticks were infected with *Rickettsia raoulti*, and by Mierzejewska et al. [30] where 44.1% of *D. reticulatus* were infected with *Rickettsia raoulti*.

Rickettsia spp. is present in other European countries. In Germany, it varies depending on the region from 30% – 67%; in Slovakia – 26% and in Belarus – 22% ticks were infected with this pathogen. The lowest prevalence was in an isolated area of the UK where only 5% of ticks carried *Rickettsia* spp. DNA [30, 34]. TIBOLA (tick-borne lymphadenopathy) syndrome in people bitten by *Dermacentor reticulatus* (1 male, 5 females) infected with *R. raoulti* is well known. Thapa et al. [27] confirmed *Rickettsia* spp. domination in the

metagenomic analysis of *I. scapularis*: the majority were the endosymbiotic *Rickettsia buchneri* which was absent in the current research.

Ponnusamy et al. [35] confirmed the domination (35% of the bacterial population) and diversity of *Rickettsiales* in *Amblyomma americanum*. The presented study shows a great diversity of *Rickettsia* species (5 different genospecies). In the USA, new genospecies have been discovered in the *Rickettsiaceae* family: *Candidatus Rickettsia amblyommii* and *Candidatus Midichloria mitochondrii*.

The very high alpha diversity plots obtained by the authors of the current study in their research (mean ca. 113,000 reads) indicates a very high quality of microbiome diversity coverage. In comparison to other studies on ticks microbiome, this indicates the good recognition of genospecies presented in ticks in north-eastern Poland [36].

Almost all ticks in this study contained a high number of *F. hispaniensis* sequences reads, which is of medical relevance. Other authors have emphasized that *Dermacentor* spp. is associated mainly with *Francisella*-like endosymbionts,

Table 2. Characteristic of environmental conditions of ticks collection

Tick Lab No.	Date of collection	Tick species	Tick Study/Gender	Area	Tick activity	T [°C]	H [%]	Day length [min]	Flora type
T654	31.05.2018	IR	F	III/IV/V/VI	alive	22.9	70	989	3
T649	31.05.2018	IR	F	III/IV/V/VI	alive	22.5	62.1	989	2
T610	01.05.2018	IR	F	III/IV/V/VI	alive	20	56.1	898	2
T595	29.04.2018	DR	F	VII/VIII/IX	alive	20.7	71.1	891	2
T594	29.04.2018	DR	M	VII/VIII/IX	alive	20.7	71.1	891	2
T544	22.08.2018	DR	F	III/VII	alive	17.6	49	864	2
T543	22.08.2018	DR	M	III/VII	alive	17.6	49	864	2
T542	22.04.2018	DR	M	III/VII	alive	18.5	35.3	864	1
T468	01.10.2017	DR	F	III/VII	alive	14.3	96	696	2
T400	25.08.2017	DR	F	II/III	alive	26	51.6	846	2
T404	25.08.2017	DR	F	II/III	alive	25.7	52.2	846	2
T400	14.08.2017	DR	F	II/III	alive	19.8	68	888	2
T390	17.07.2017	DR	M	II/III	alive	21.3	95	977	1
T389	21.06.2017	DR	F	II/III	alive	20.1	61.1	1012	1
T297	04.04.2017	DR	M	III/VII	alive	33.6	41.5	792	2
T292	04.04.2017	DR	F	III/VII	alive	33.6	41.5	792	2
T244	31.03.2017	DR	F	II/III	alive	15.2	73.1	776	1
T117	02.09.2016	DR	F	II/III	alive	19.4	78.6	813	2
T116	02.09.2016	DR	F	II/III	alive	20.6	71.3	813	2
T115	02.09.2016	DR	M	III/IV/V/VI	alive	21.2	73.1	813	2
T114	02.09.2016	DR	F	III/VII	alive	20.1	70	813	2
T19	09.06.2016	DR	F	III/VII	alive	15.7	70.6	1005	2

DR – *Dermacentor reticulatus*; IR – *Ixodes ricinus*;

F- female; M- male; N- nymph;

II, VII – meadow; III, V – forest; IV – pasture; VI – waterhole; VIII – line of trees;

1 – short flora, up to 20 cm; 2 – medium high flora, 20 – 50 cm; 3 – high flora, 50 cm – 1 m

but their pathogenic potential needs to be taken into consideration. Vayssier-Taussat et al. coined the term ‘pathobiome’ for all pathogenic and possible pathogenic microorganism in ticks [2].

Gall et al. [18] emphasized the importance of tick microbiome fingerprints (similar to human microbiome) and confirmed comparable microbiome profiles in ticks collected from the environment (IN THE WILD), and those from the laboratory generation in the same population and from the same area. Information on tick microbiome from specific localities might have an important epidemiological meaning, and could be used for the veterinary and medical prevention of tick-borne diseases.

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