University of South Bohemia in České Budějovice

Faculty of Science

Detection and quantification of tick-transmitted pathogens from the selected localities of the Czech Republic

Bachelor thesis

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České Budějovice 2015

Bachelor thesis

Zemanová Z., 2015: Detection and quantification of tick-transmitted pathogens from the selected localities of the Czech Republic. Bc. Thesis, in English. – 44 p., Faculty of Science, University of South Bohemia, České Budějovice, Czech Republic.

Annotation

Prevalence of tick-borne pathogens (*Borrelia* and *Babesia*) in *Ixodes ricinus* ticks was determined in three localities of the Czech Republic. The pathogens were further classified into genospecies. Quantities of *Borrelia* spirochetes in nymphs was determined and subsequently compared with results of *Borrelia* quantification performed on nymphs artificially infected in the laboratory *Borrelia* transmission model.

Financial sources: GACR 585530/3181 and GACR 585510/3179

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Zuzana Zemanová

Acknowledgment

I would like to thank to my supervisor RNDr. Ondřej Hajdušek, Ph.D for the opportunity to work in his laboratory and for funding of my research. Big thanks also go to RNDr. Radek Šíma PhD. for readiness to answer all my questions and to consult any problem. I would also like to thank all the other people I worked with in our lab. I am also very thankful to my family, and my friends for their encouragement.

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Goals

- Detection of pathogens (*Borrelia*, *Babesia*) in *Ixodes ricinus* ticks from three selected localities of the Czech Republic (South Bohemia, South Moravia, Central Bohemia) – collection of ticks, isolation of DNA, PCR, nested PCR, sequencing.

- Quantification of *Borrelia* spirochetes from the collected nymphs by qPCR, and comparison of these numbers with number of *Borrelia* in laboratory-infected nymphs.

- Incorporation of the DNA samples into the laboratory sample collection for further analyses.

1. Introduction

1.2. Ticks

Ticks are blood-feeding ectoparasites and vectors trasmitting a variety of pathogens (viruses, bacteria, fungi, and protozoa) to humans and animals (de la Fuente *et al*., 2008). Ticks are arachnids belonging to the subclass Acari, superorder Parasitiformes, and order Ixodida (Krantz, 2009).

The order Ixodida is subdivided into 3 families (Nava *et al*., 2009):

- Ixodidae (692 species)
- Argasidae (186 species)
- Nuttallielidae (1 species)

Ixodidae

The family Ixodidae contains 13 genera. Genus *Ixodes* is the most important and is represented by the relevant vectors of Lyme disease and tick borne encephalitis in Europe (*I. ricinus*, *I. persulcatus*), and Lyme disease, human babesiosis, and anaplasmosis in the United States (*I. scapularis*, *I. pacificus*). *Dermacentor* is another important genus of the family Ixodidae. *D. variabilis* and *D. andersoni* are vectors of Rocky Mountain spotted fever and tularemia in the United States. *D. reticulatus* is mainly found in wooded areas across Europe and transmits *Babesia canis* to dogs and *Babesia divergens* to cattle (Sonenshine, 1991).

Ixodid ticks are characterized by a 3-host life cycle (Sonenshine, 1991) (Fig. 1). The life cycle of these ticks consists of the egg and 3 active stages – larva, nymph, and an adult (male and female). Each active stage must take once blood before it molts into the subsequent stage (Balashov, 1998). In contrary to the adult females, adult males do not take blood. Females are mated by males directly on the hosts or before feeding. Females ingest large amount of blood and grow their body mass up to 100 times. Then, fully fed females drop off their host and lay thousands of eggs. Most ixodid ticks are non-nidicolous, they are living in forests and meadows hidden in leaf litter and vegetation (Sonenshine, 2014).

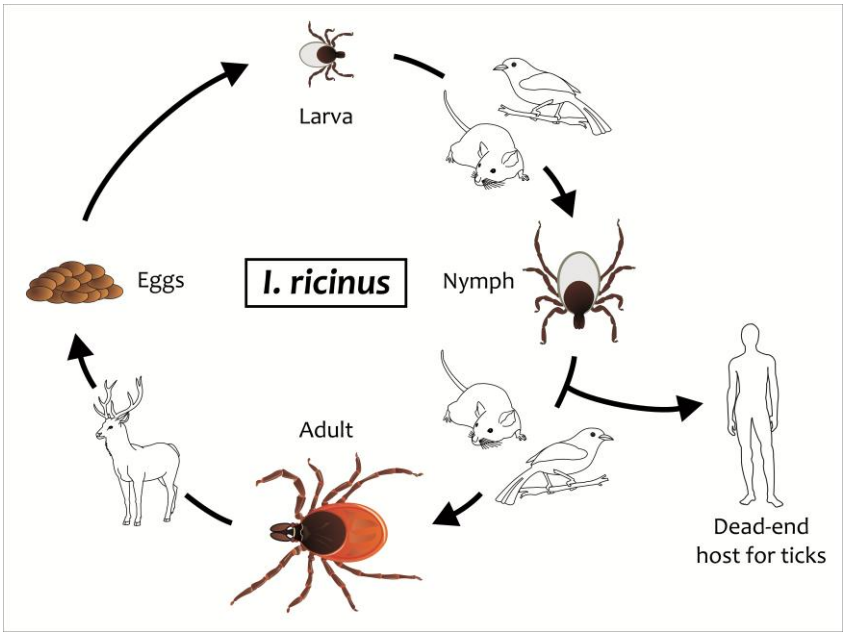

Figure 1. Schematic diagram representing the 3-host life cycle of *I. ricinus* ticks. Larvae feed on the first host (e.g., small rodents, birds), drop off, and molt to the nymphal stage. Subsequently, the unfed nymphs quest for a host (e.g., small rodents, birds), feed, drop off, and molt to the adult stage. The adults seek their hosts (large mammals), mated females feed, drop off the host, lay eggs, and die. Humans, considered as dead-end hosts for ticks, are mostly infested by the nymphal stages (Sonenshine, 1991). Scheme designed by M. Hajduskova (Biographix).

Argasidae

The family contains five genera: *Argas*, *Ornithodoros*, *Otobius*, *Antricola*, and *Nothoaspis*. Genus *Ornithodoros* is worldwide in distribution. *O. moubata* is an important species transmitting tick-borne relapsing fever in eastern and southern Africa (Sonenshine, 1991).

Argasid ticks are multi-host parasites with several nymphal stages. Females lay only a few hundred of eggs after each blood meal, but are capable of several cycles of feeding, mating, and oviposition. Most argasid ticks are nidicolous, living in nests, burrows, caves, or other shelters used by their hosts (Sonenshine, 2014).

Morfology

Tick body consists of three major segments: capitulum, idiosoma (body), and legs. Capitulum contains chelicerae, palps, and hypostome. Ventral side of the hypostome is covered by rows of recurved denticles that help to attach the tick in the host skin during blood feeding. Idiosoma contains walking legs, genital pore, and internal organs. Adults and nymphs bear four pairs of walking legs, larvae bear only three pairs of walking legs. First pair of legs carries Haller's organ, which is responsible for determining the host location and odors, recognizing pheromones, and other sensory functions.

In hard ticks of the family Ixodidae, dorsum in adults is covered by hard, sclerotized plate, called scutum. In males, the scutum covers entire dorsum, whereas in females, nymphs, and larvae covers only the anterior half of dorsum. Argasid ticks lack the scutum, instead of that they possess a leathery, folded cuticle (Sonenshine, 2014).

1.3. Tick-borne diseases

Ticks are vectors transmitting a wide spectrum of pathogens causing serious diseases to humans and animals, such as Lyme disease, ticks-borne encephalitis, rickettsiosis (also known as spotted fever), ehrlichiosis, anaplasmosis, theileriosis, or babesiosis (Hajdusek *et al*., 2013).

1.3.1. Lyme disease

Lyme disease is the most common and the most important human tick-borne disease in the United States and Europe. It is a multisystemic disease affecting many organs and causing various symptoms (Burgdorfer *et al*., 1982). Lyme disease is caused by *Borrelia burgdorferi* sensu lato (Fikrig and Narasimhan, 2006). *Borrelia* belongs to the order Spirochaetales, which also includes the agents causing syphilis or leptospirosis (Olsen *et al*., 2000).

Borrelia spirochetes are transmitted by ixodid ticks. *I. scapularis* is a vector of *B. burgdorferi* sensu stricto in the eastern North America, *I. pacificus* is the principal vector of Lyme Disease in the western North America. In Europe, *I. ricinus* is the main vector of the disease, but it can be

also transmitted by *I. hexagonus* and *I. persulcatus* in eastern Europe and Asia, respectively (Sonenshine, 2014).

Human Lyme disease is caused, in most cases, by one of these three principal genospecies – *B. burgdorferi* sensu stricto, *B. garinii*, and *B. afzelii* (Sonenshine, 2014). In Europe, *B. garinii* and *B. afzelii* were found to be more prevalent in patients, ticks, and hosts than *B. burgdorferi* sensu stricto (Moran Cadenas *et al*., 2007; Hanincova *et al*., 2003; Ruzić-Sabljić *et al*., 2002; Tarageľová *et al*., 2008).

Different genospecies utilize different reservoir hosts. Major reservoir hosts for *B. garinii* are birds (Tarageľová *et al*., 2008), while rodents are the most important reservoir hosts for *B. afzelii* (Hanincova *et al*., 2003). This dichotomy in the host distribution of *B. garinii* and *B. afzelii* is due to difference in the sensitivity of these genospecies to the host serum complement system (Kurtenbach *et al*., 1998). *B. burgdorferi* sensu stricto utilizes birds as reservoir hosts (Ginsberg *et al*., 2005) and also rodents can serve as reservoir hosts for this genospecies (Anderson *et al*., 1987; Donahue *et al*., 1987).

Except for these three above mentioned principal genospecies, human Lyme disease can be also caused by other, less common, genospecies, for example *B. spielmanii* (Richter *et al*., 2006). This genospecies utilizes garden dormice (*Eliomys quercinus*) as a reservoir host (Richter *et al*., 2004). Another genospecies is *B. lusitaniae* first described in Portugal (Le Fleche *et al*., 1997), and widespreaded mainly in southern Europe (De Michelis *et al*., 2000; Grego *et al*., 2007; Younsi *et al*., 2005). Lizards serve as reservoir hosts for this genospecies (Amore *et al*., 2007; Dsouli *et al*., 2006; Majlathova *et al*., 2006; Richter, 2006).

Borrelia spirochetes survive in the nature usually in a tick-mammal infection cycle (Anguita *et al*., 2003; Pal and Fikrig, 2003). *Borrelia* could be acquired during one of the life stages of its vector (larva, nymph) at the time of engorgement on infected hosts (Anderson, 1989; Munderloh and Kurtti, 1995; Pal and Fikrig, 2003).

Transovarial transmission of *Borrelia* in ticks is not likely (Rollend *et al*., 2013), so Lyme disease can be transmitted to humans mostly by feeding of infected nymphs or adults (Hajdusek *et al*., 2013). The nymphal stage of *I.*

ricinus tick is the most significant for the transmission of Lyme borreliosis to humans (Jaenson, 1991).

In the Czech Republic, 3000 – 4000 new cases of human Lyme disease are reported every year (Zvolský and Žofka, 2014).

1.3.2. Babesiosis

Babesiosis is a malaria-like opportunistic infection causing a serious health disorders in elderly, immunocompromised, and cirrhotic patients. It causes only subclinical symptoms in intact persons (Spielman *et al*., 1981). *Babesia* infect also a wide range of domestic and wild mammals (Vannier and Krause, 2012). Over half of the world's cattle population residing in tropical and subtropical regions is at the risk of contracting babesiosis (Sonenshine, 2014). Bovine babesiosis also known as redwater, tick fever, Texas fever, or cattle fever, is arguably the most economically important arthropod-borne disease of cattle globally (Bock *et al*., 2004). It is caused by intraerythrocytic parasites of genus *Babesia*, and transmitted by ixodid ticks (Homer *et al*., 2000). Genus *Babesia* belongs into the order Piroplasmida, class Haematozoea, and phylum Apicomplexa (Volf *et al*., 2007).

Genus *Babesia* involves at least three genospecies pathogenic for humans: *B. bovis*, *B. divergens*, and *B. microti* (Homer *et al*., 2000). *B. divergens* is responsible for the most cases of human babesiosis in Europe (Gorenflot *et al*., 1998). *B. microti* is now the most common, transfusion-transmitted pathogen in the United States (Gubernot *et al*., 2009; Young and Krause, 2009; Leiby, 2011). *B. venatorum* (also known as *Babesia* EU1) has been described in some human cases in Europe (Herwaldt *et al*., 2003; Haselbarth *et al*., 2007), whereas *B. bovis*, *B. bigemina*, *B. divergens*, and *B. major* are generally recognized as the most commonly found in cattle (Bock *et al*., 2004). The major reservoir hosts for *B. microti* are rodents (Healy *et al*., 1976; Spielman *et al*., 1981; Telford *et al*., 1990). Unlike *B. microti* that is transmitted by the vector trans-stadially, *B. bovis*, *B. divergens*, and *B. bigemina* are capable of transovarial transmission and newly hatched larvae are infectious to the hosts (Chauvin *et al*., 2009).

2. Materials and methods

2.1. Collection of ticks

Ixodes ricinus ticks were collected in three selected localities of the Czech Republic – locality 1 - South Bohemia (48°58'57.8"N 14°25'30.5"E), locality 2 - South Moravia (48°50'32.5"N 15°56'46.5"E), and locality 3 - Central Bohemia (49°58'30.0"N 13°54'55.9"E). Ticks were collected using method of flagging the vegetation in the period from May to September 2013. A total number of 387 *I. ricinus* ticks were collected. 110 nymphs and 25 females were collected in the localities 1 and 2, 110 nymphs, and 7 females were collected in locality 3. All ticks were frozen separately at -20 °C until further processing.

2.2. DNA isolation

Each tick was individually homogenised using sterile blade and the genomic DNA was isolated using NucleoSpin Tissue Kit (Macherey-Nagel) according to manufacturer's protocol.

Quality of DNA was verified by amplification of a 600bp fragment of *I. ricinus* DNA using primers IR-600F, and IR-600R. Primers are described in Table 11 in the supplement. The DNAs were labeled and stored at -20 °C for further analysis.

2.3. PCR for detection of *Borrelia* spirochetes

PCR was performed in a 25 μl reaction volume containing 12.5 μl of FastStart PCR Master (Roche), 4 μl of purified DNA, 1 μl of each primer (FlaF1, FlaR1, 10 μM). The remaining volume was adjusted with sterile water. Primers used for PCR detection are shown in Table 11 in the supplement. PCR was performed with conditions shown in Table 1.

Table 1. Amplification program for detection of *Borrelia* spirochetes by PCR using Fla primers.

	Temperature	**Time**	
initialisation	94°C	10 min	
denaturation	94°C	30 sec	40x
annealing	60°C	30 sec	
elongation	72°C	40 sec	
final elongation	72°C	7 min	
final hold	14°C	infinity	

2.4. Nested PCR

Nested PCR was used for detection of *Babesia* and for genotypization of *Borrelia* species. Nested PCR was performed in a 25 μl reaction volume containing 12.5 μl of FastStart PCR Master (Roche), 4 μl of purified DNA, 1 μl of each primer, the remaining volume was adjusted with sterile water. For detection of *Borrelia* species, following primers were used (first round: IGS F, IGS R, second round: IGS Fn, IGS Rn, 10 μM) specific for the *Borrelia* gene encoding small rRNA subunit. For detection of *Babesia*, following primers were used (first round: IR 270, IR 275, second round: IR 272, IR 273, 10 μM) specific for the *Babesia* gene encoding 18S rRNA. Primers used for nested PCR are described in Table 11 in the supplement. Nested PCR for genotypization of *Borrelia* species was performed with the temperature profile described in Table 2. Nested PCR for detection of *Babesia* was performed with the temperature profile described in Table 3.

Table 2. Amplification program for genotypization of *Borrelia* spirochetes by nested PCR using IGS primers.

	First round		**Second round**		
	Temperature	**Time**	**Temperature**	**Time**	
initialisation	94°C	10 min	94°C	10 min	
denaturation	94°C	30 sec	94°C	30 sec	
annealing	56°C	30 sec	60°C	30 sec	40x
elongation	72°C	60 sec	72°C	60 sec	
final elongation	72°C	7 min	72°C	7 min	
final hold	14°C	infinity	14°C	infinity	

Table 3. Amplification program for detection of *Babesia* by nested PCR using 18S rRNA primers.

	First round		**Second round**		
	Temperature	**Time**	**Temperature**	**Time**	
initialisation	94°C	10 min	94°C	10 min	
denaturation	94°C	30 sec	94°C	30 sec	
annealing	60°C	30 sec	60°C	30 sec	40x
elongation	72°C	60 sec	72°C	60 sec	
final elongation	72°C	7 min	72°C	7 min	
final hold	14°C	infinity	14°C	infinity	

2.5. Quantitative PCR

Quantitative PCR (qPCR) was used for the absolute quantification of *Borrelia* spirochetes. qPCR was performed in a 25 µl reaction volume containing 12.5 µl of FastStart Universal Probe Master (Rox) (Roche), 5 µl of purified DNA, 1 µl of each primer (FlaF1, FlaR1, 10 µM), 1 µl of Taqman probe (Fla probe1, 5 µM) and the remaining volume was adjusted with sterile water. Primers used for qPCR are described in Table 11 in the supplement. qPCR was performed with the temperature profile described in Table 4.

Table 4. Amplification program for quantification of *Borrelia* spirochetes.

	Temperature	**Time**	
initialisation	95°C	10 min	50x
denaturation	95°C	15 sec	
annealing + elongation	60°C	1 min	

2.6. Gel electrophoresis

The results of PCR reaction were analysed by a gel electrophoresis. Ethidium bromide (Sigma) stained, 1.5 % agarose gel in TAE buffer was used for separation and size determination of PCR products. 10 µl of each sample was mixed with DNA loading dye (Top-Bio) and subsequently loaded on the gel. PCR product size was determined according to the 100 bp DNA ladder (Thermo Scientific).

2.7. Sequencing

Amplified PCR products were extracted from the agarose gel using NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel). 50 ng of purified PCR product was mixed with 25 pmol of appropriate primer (Tab. 11) in a final volume of 10 µl and the mixture was sent for sequencing to SEQme company. Sequences were analysed by Chromas Lite 2.1.1 (Technelysium) and compared to online sequence database (BLAST).

3. Results

3.1. Tick collection

I. ricinus ticks were collected in three selected localities of the Czech Republic:

- locality 1 - South Bohemia
- locality 2 - South Moravia
- locality 3 - Central Bohemia

Disposition of the localities 1, 2 and 3 is shown in Figure 2.

110 nymphs and 25 females were collected in the localities 1 and 2, 110 nymphs and 7 females in locality 3. GPS device was used to specify exact position of the collection of each five ticks. Subsequently, tick position maps were created (Google maps) for respective location as shown in Figures 7, 8 and 9, whereas each point means approximately five ticks. Exact GPS locations are shown in Tables 12 – 14 in the supplement.

Figure 2. Map of selected areas for the tick sampling.

3.2. Detection of *Borrelia* spirochetes

330 collected *I. ricinus* nymphs were tested for the presence of *Borrelia* spirochetes by PCR. Total prevalence reached 22%. The highest prevalence 25.5% was in locality 2 – South Moravia. The lowest prevalence 15.5% was in locality 3 – Central Bohemia. In locality 1 – South Bohemia the

prevalence reached 24.5%. Comparison of prevalence of *Borrelia* spirochetes in localities 1, 2, and 3 are shown in Table 5 and Figure 3. Distributions of uninfected and *Borrelia*-infected ticks are shown in Figures 7, 8, and 9.

Table 5. Prevalence of *Borrelia* spirochetes in selected areas.

Locality	Number of nymphs	Infected nymphs	Prevalence (%)
1	110	27	24.5
2	110	28	25.5
3	110	17	15.5
Total	**330**	**72**	**22**

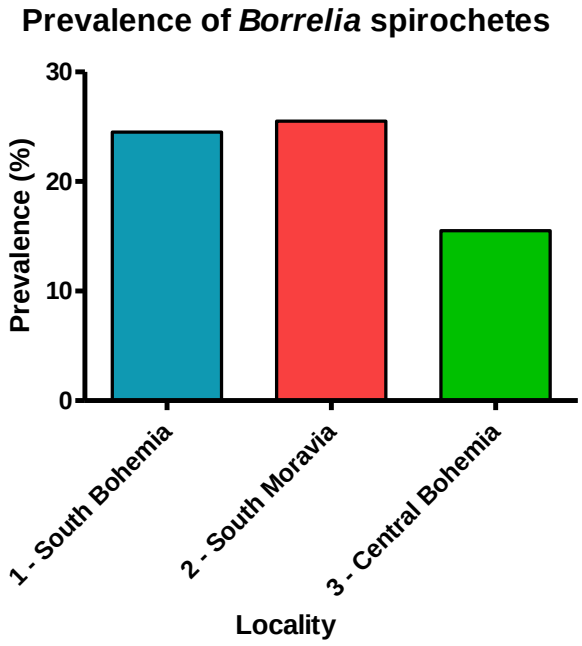

Figure 3. Prevalence of *Borrelia* spirochetes in localities 1, 2 and 3.

3.2.1. Determination of *Borrelia* into genospecies

The genospecies of *Borrelia* spirochetes were determined using method of nested PCR. Three positive controls were used – *B.afzelii*, *B. garinii* and *B.burgdorferi* sensu stricto. Genotyping was determined according to different lenght of PCR products as shown in Figure 4. Classification of samples of unclear lenght was confirmed by sequencing. The *I. ricinus* ticks examined were found to contain these three *Borrelia* genospecies:

- *B. afzelii*
- *B. garinii*
- *B. burgdorferi* sensu stricto

The number of *Borrelia* genospecies in tested nymphs including percentage status is shown in Table 6, and percentage comparison of respective *Borrelia* genospecies is shown in Figure 5.

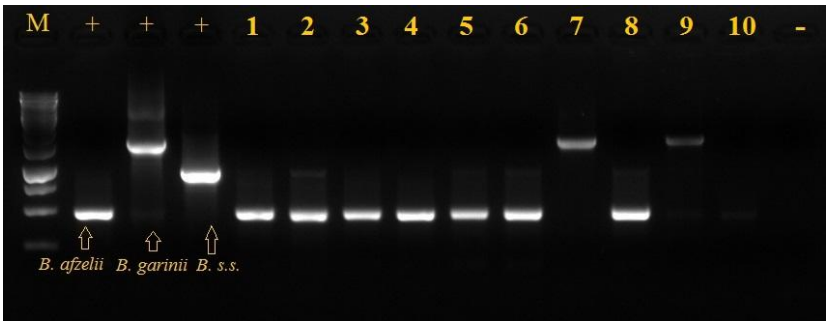

Figure 4. Genotyping of *Borrelia* genospecies (M = marker, + = positive controls, 1 – 10 samples, - = negative control).

Table 6. Representation of *Borrelia* genospecies in all analysed nymphs.

Genospecies	**Number**	**Prevalence (%)**
B. afzelii	48	66.7
B. garinii	18	25
B. burgdorferi s. s.	6	8.3

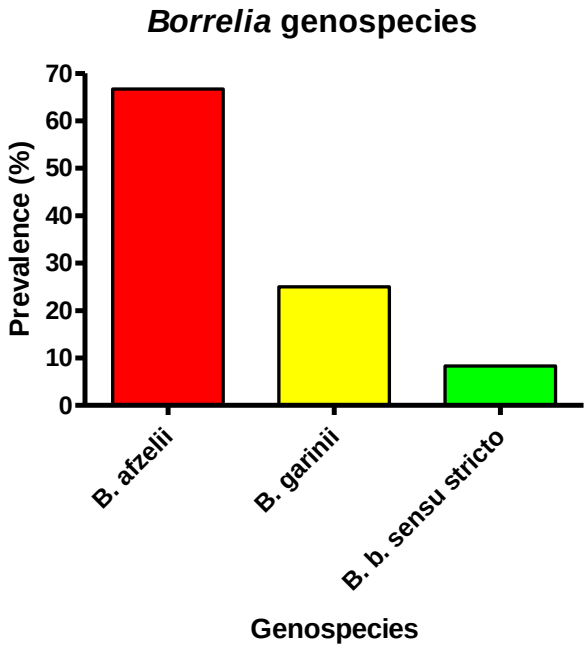

Figure 5. *Borrelia* genospecies found in *I. ricinus* nymphs from all three localities.

These three genospecies were present in all three selected areas of samples collection. Nevertheless, representation of genospecies in these areas were different. In locality 1, 81.5% of positive nymphs were infected with *B. afzelii*, 3.7% with *B. garinii*, and 14.8% with *B. burgdorferi* sensu stricto. In locality 2, 78.6% of positive nymphs were infected with *B. afzelii*, 17.9% with *B. garinii*, and 3.6% with *B. burgdorferi* sensu stricto, and in locality 3, 23.5% of positive nymphs were infected with *B. afzelii*, 70.6% with *B. garinii*, and 5.9% with *B. burgdorferi* sensu stricto. Percentage comparison of these genospecies in respective localities is shown in Table 7 and Figure 6. Distributions of *Borrelia* genospecies in respective localities are shown in Figures 7, 8, and 9.

Table 7. Percentage comparison of *Borrelia* genospecies in respective localities.

Locality	**Prevalence of *Borrelia* spp. (%)**	**Genospecies (%)**		
		B. afzelii	***B. garinii***	***B. burgdorferi* s.s.**
1	24.5	81.5	3.7	14.8
2	25.5	78.6	17.9	3.6
3	15.5	23.5	70.6	5.9

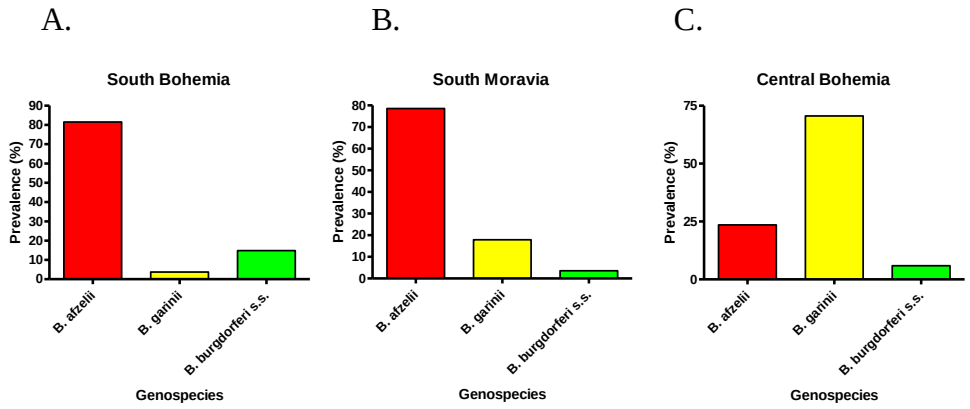

Figure 6. *Borrelia* genospecies in three selected localities – A = *Borrelia* genospecies found in locality 1 – South Bohemia. B = *Borrelia* genospecies found in locality 2 – South Moravia. C = *Borrelia* genospecies found in locality 3 – Central Bohemia.

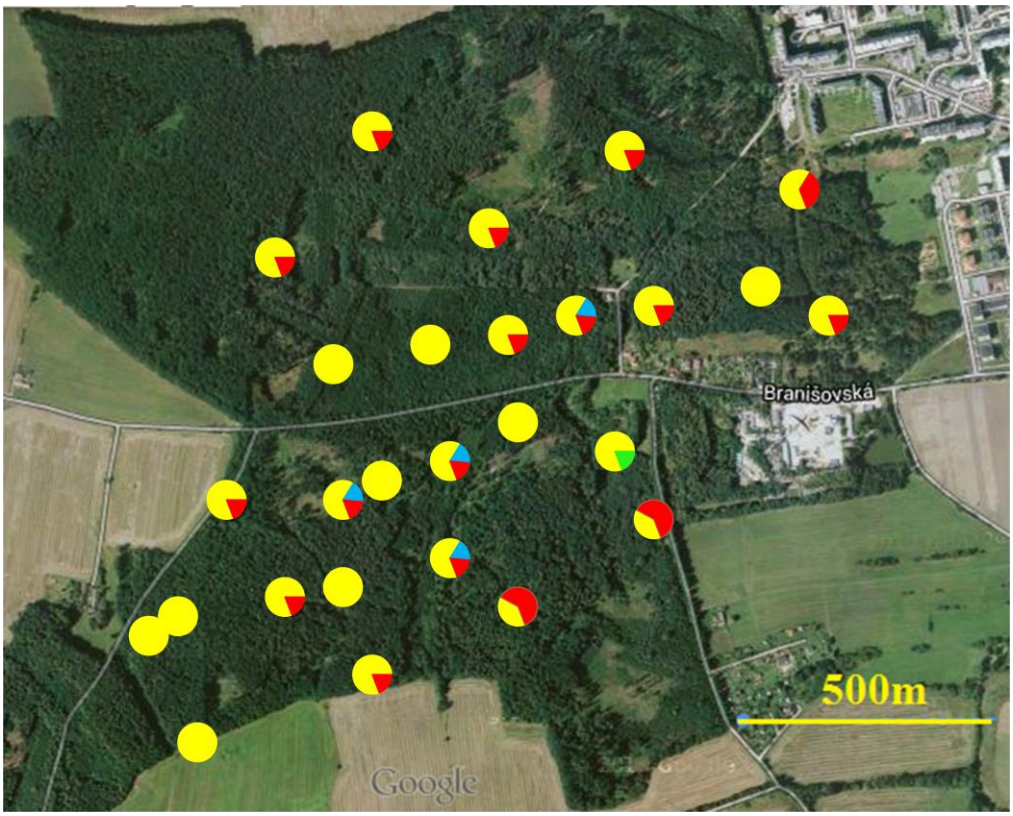

Figure 7. Distribution of uninfected and *Borrelia*-infected nymphs and genospecies in locality 1 – South Bohemia (yellow = negative samples, red = *B. afzelii*, blue = *B. burgdorferi* s. s., green = *B. garinii*). Each point shows rate between infected and uninfected nymphs.

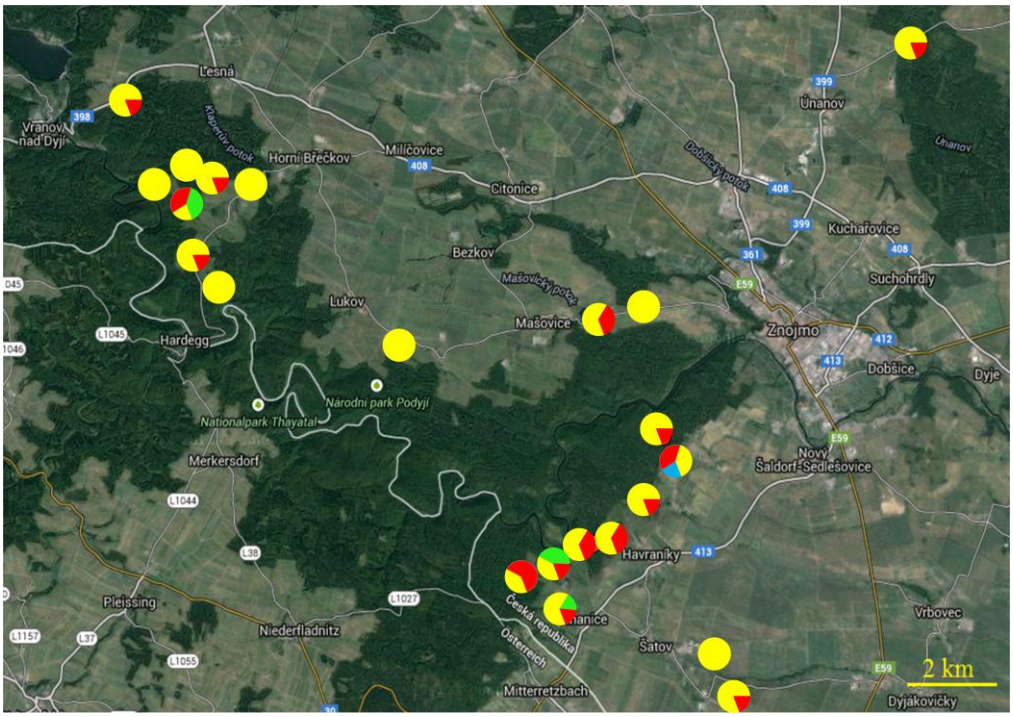

Figure 8. Distribution of uninfected and *Borrelia*-infected nymphs and genospecies in locality 2 – South Moravia (yellow = negative samples, red = *B. afzelii*, blue = *B. burgdorferi* s. s., green = *B. garinii*). Each point shows rate between infected and uninfected nymphs.

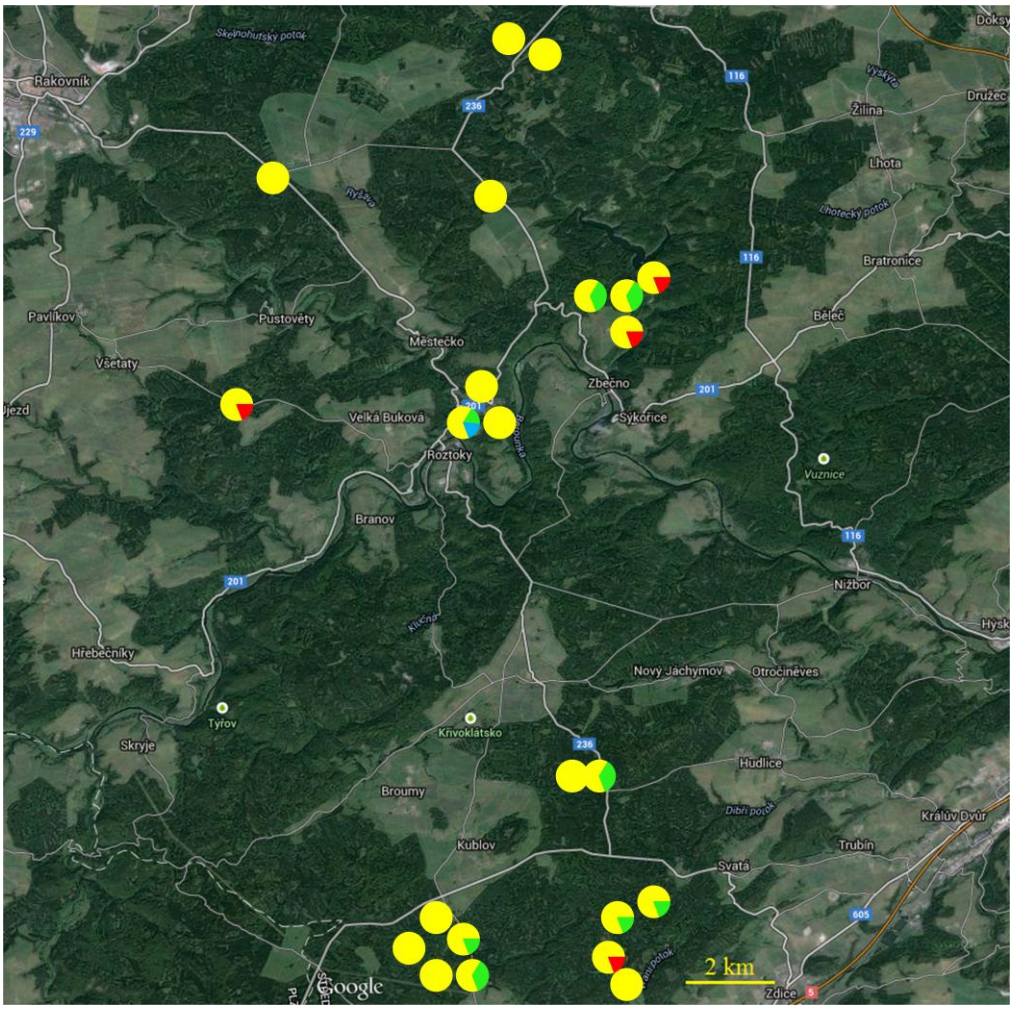

Figure 9. Distribution of uninfected and *Borrelia*-infected nymphs and genospecies in locality 3 – Central Bohemia (yellow = negative samples, red = *B. afzelii*, blue = *B.burgdorferi* s. s., green = *B. garinii*). Each point shows rate between infected and uninfected nymphs.

3.3. Quantification of *Borrelia* spirochetes in nymphs

Borrelia spirochetes in positive nymphs from localities 1, 2, and 3 were quantified by quantitative PCR. The results of *Borrelia* quantification in nymphs from all locations are presented in Figure 10 (red dots). The range of *Borrelia* numbers is from 12360 to 115080 spirochetes/nymph. The average number of spirochetes in one nymph is 46390 ± 3181.

Subsequently, these results from the locations were compared with results of *Borrelia* quantification performed on nymphs artificially infected with *B. afzelii* CB43. These data were obtained from laboratory archive and

are represented by blue dots in Figure 10. The range of *Borrelia* spirochete numbers in colony nymphs is from 4360 to 100600 and the average number of spirochetes in one colony nymph is 53550 ± 4513. Numbers of *Borrelia* spirochetes in laboratory infected nymphs correspond with spirochete counts in field collected *I. ricinus* nymphs as shown in Figure 10, differences are not statisticaly significant (t-test). Number of *Borrelia* spirochetes in single nymphs were also compared between three different localities, and different genospecies. No significant differences between these three localities and these three genospecies were found. (Fig. 11A and Fig. 11B).

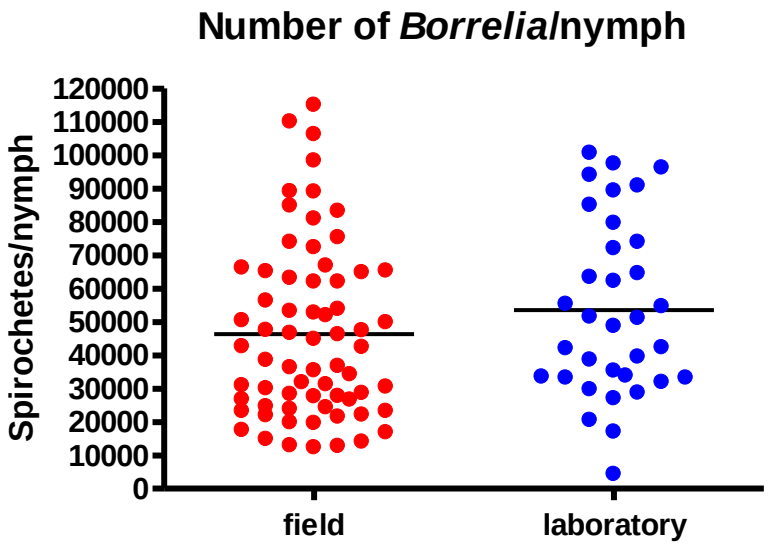

Figure 10. Number of *Borrelia* spirochetes per a nymph. Field collected nymphs (red dots), and artificially infected nymphs (blue dots).

A. B.

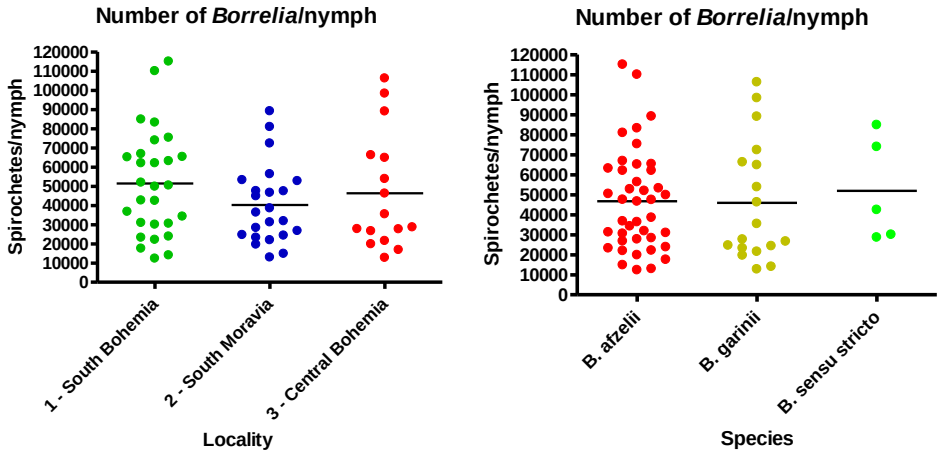

Figure 11. Number of *Borrelia* spirochetes in single nymphs from three different localities (A). Number of *Borrelia* spirochetes in single nymphs of different genospecies (B).

3.4. Detection of *Babesia*

Collected *I. ricinus* ticks were further tested for the presence of *Babesia* by nested PCR. The total prevalence of *Babesia* in nymphs reached 1.5%. The highest infection rate was in locality 1 – South Bohemia (2.7%). In locality 2 – South Moravia and locality 3 – Central Bohemia, the prevalence reached 0.9%. Comparison of prevalence of *Babesia* in localities 1, 2, and 3 is shown in Table 8 and Figure 12. Distribution of uninfected and *Babesia*-infected nymph is shown in Figures 15, 16, and 17.

Table 8. Prevalence of *Babesia* in the selected areas.

Locality	Number of nymphs	Infected nymphs	Prevalence (%)
1	110	3	2.7
2	110	1	0.9
3	110	1	0.9
total	**330**	**5**	**1.5**

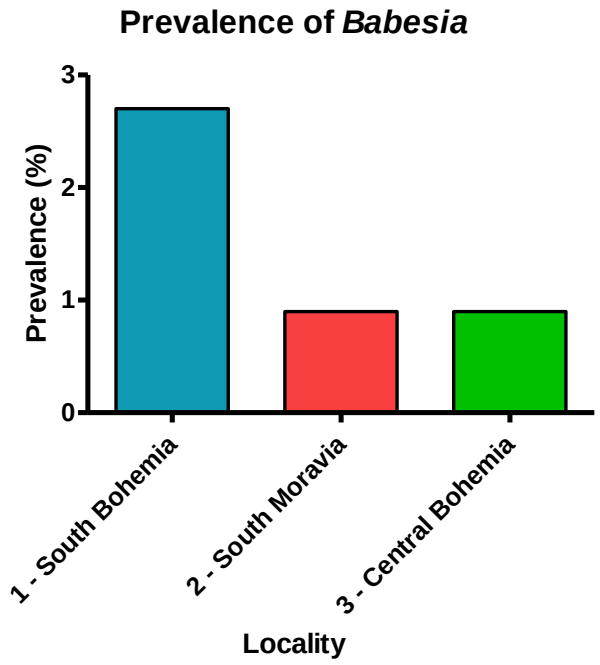

Figure 12. Prevalence of *Babesia* in localities 1, 2 and 3.

3.4.1. Determination of *Babesia* into genospecies

Specific PCR products (positive samples from nested PCR mentioned above) were further characterized by sequence analysis. The *I. ricinus* nymphs examined were found to contain these 2 *Babesia* genospecies:

- *Babesia microti*
- *Babesia venatorum* (EU1)

Representation of these two genospecies were different in three selected localities. While *B. venatorum* was presented in locality 1 – South Bohemia and 3 – Central Bohemia, *B. microti* was presented only in locality 2 – South Moravia. The representation of individual genospecies is shown in Table 9 and Figure 13. Percentage comparison of these two genospecies from positive nymphs in respective localities is shown in Table 10 and Figure 14. Distributions of *Babesia* genospecies in respective localities are shown in Figures 15, 16, and 17.

Table 9. Representation of *Babesia* genospecies in all analysed nymphs.

Species	Number	Prevalence (%)
B. microti	2	40
B. venatorum (EU 1)	3	60

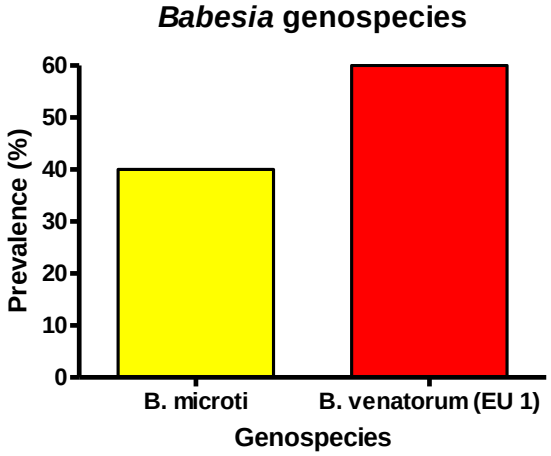

Figure 13. *Babesia* genospecies found in *I. ricinus* nymphs from all three localities.

Table 10. Percentage comparison of *Babesia* genospecies in respective localities.

Locality	Prevalence of *Babesia* spp. (%)	Genospecies (%)	
		B. microti	*B. venatorum* (EU 1)
1	2.7	37	74
2	0.9	100	0
3	0.9	0	100

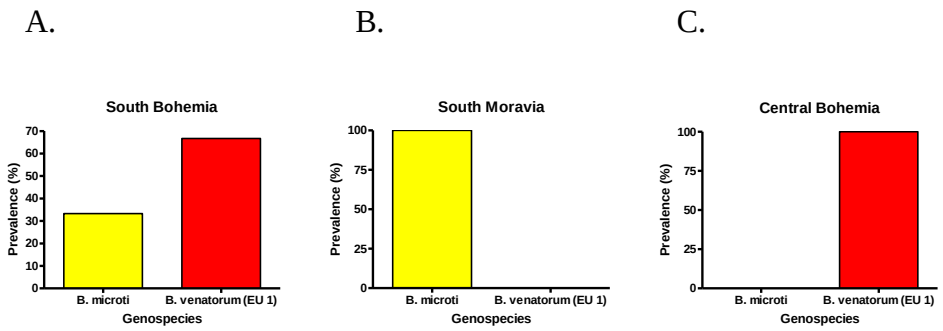

Figure 14. *Babesia* genospecies in three selected localities – A = *Babesia* genospecies found in locality 1 – South Bohemia. B = *Babesia* genospecies found in locality 2 – South Moravia. C = *Babesia* genospecies found in locality 3 – Central Bohemia.

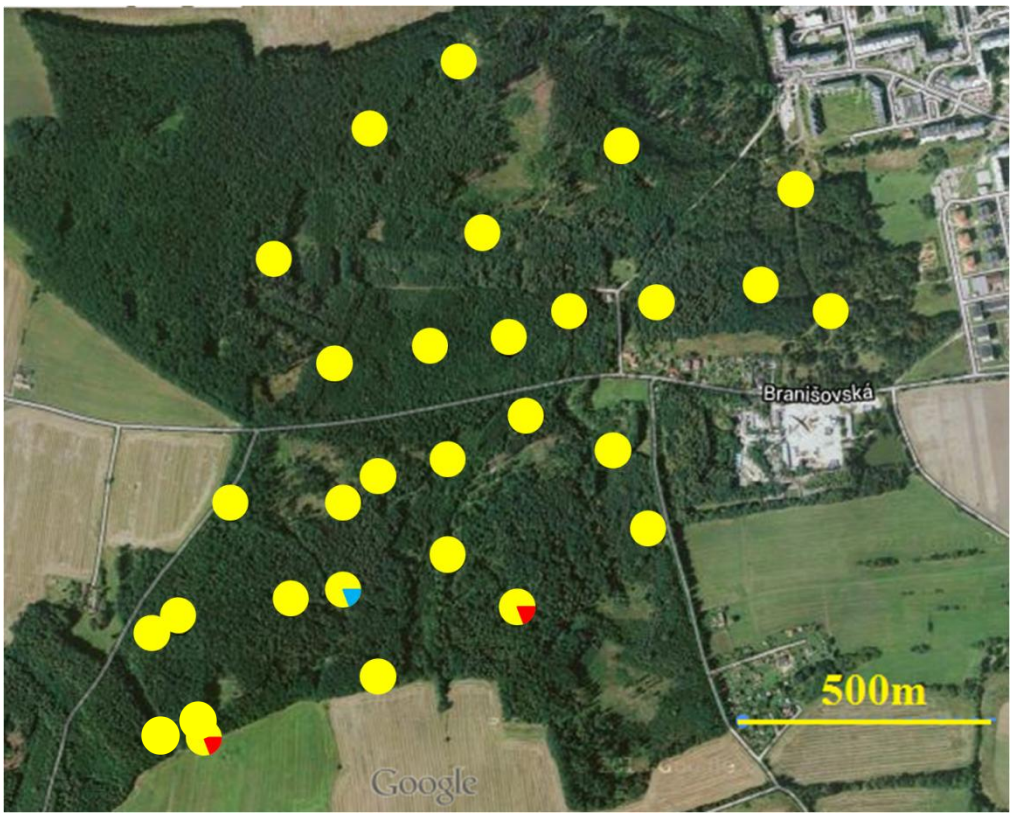

Figure 15. Distribution of uninfected and *Babesia*-infected nymphs and genospecies in locality 1 – South Bohemia (yellow = negative samples, red = *B. venatorum*, blue = *B. microti*). Each point shows rate between infected and uninfected nymphs.

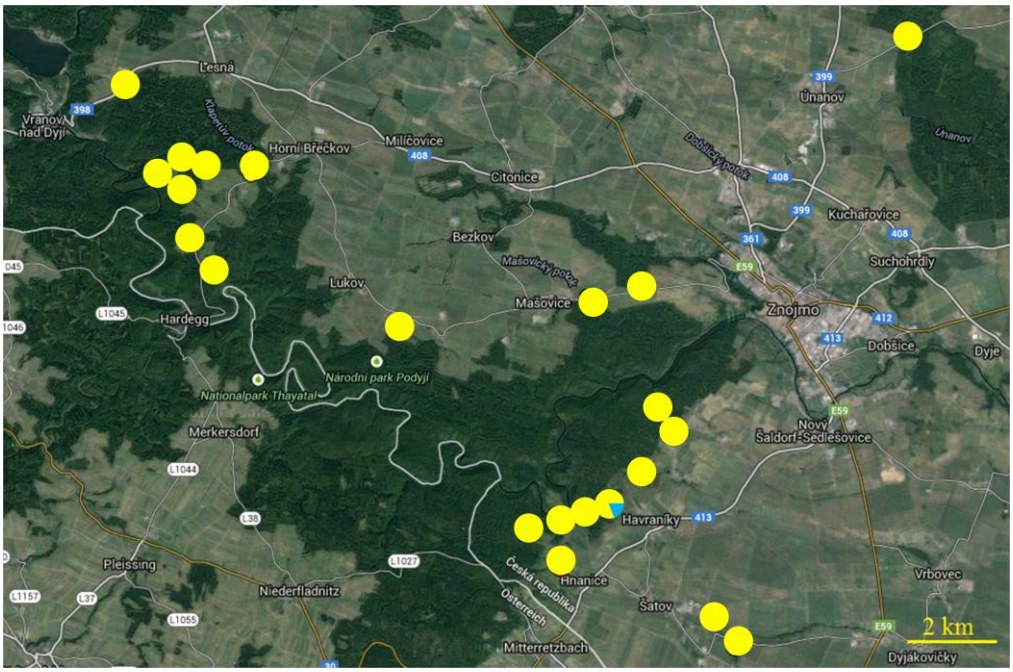

Figure 16. Distribution of uninfected and *Babesia*-infected nymphs and genospecies in locality 2 – South Moravia (yellow = negative samples, blue = *B. microti*). Each point shows rate between infected and uninfected nymphs.

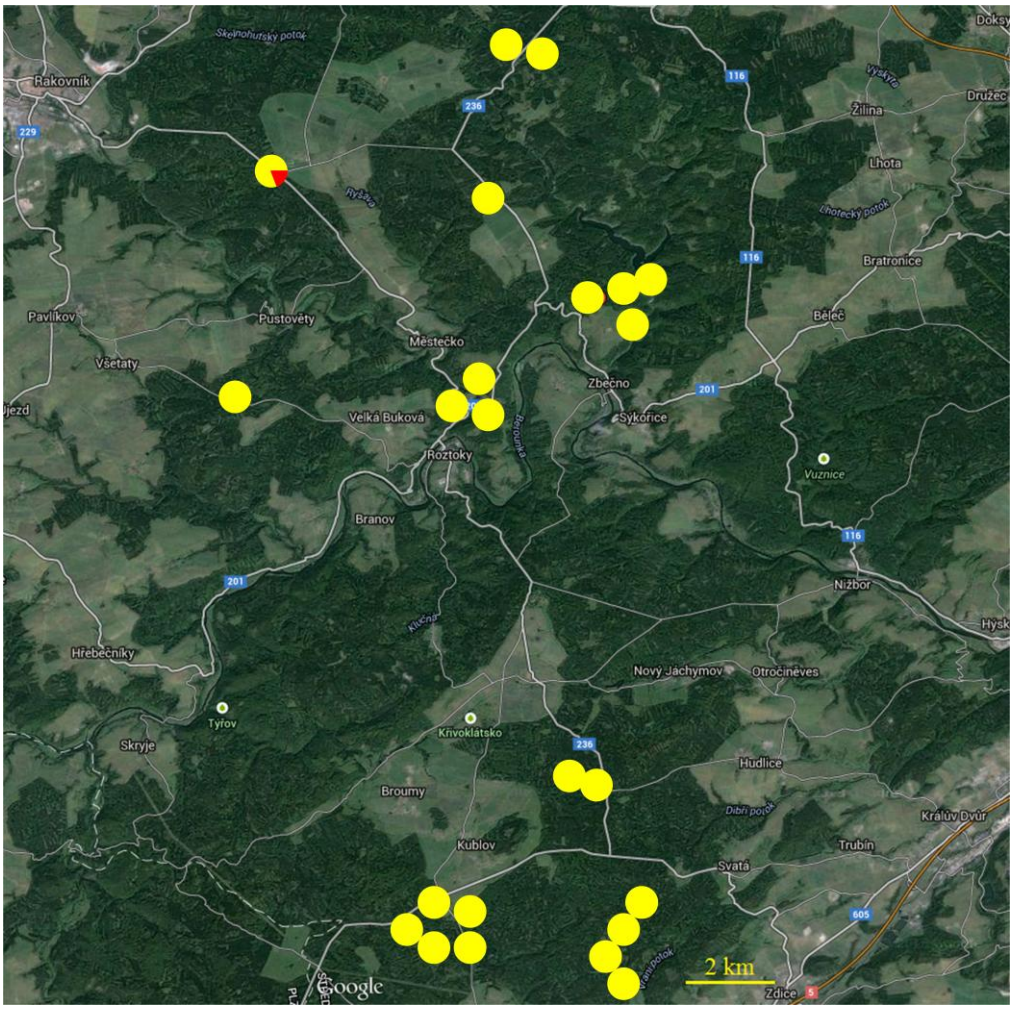

Figure 17. Distribution of uninfected and *Babesia*-infected nymphs and genospecies in locality 3 – Central Bohemia (yellow = negative samples, red = *B. venatorum*). Each point shows rate between infected and uninfected nymphs.

4. Discussion

Lyme borreliosis as well as babesiosis has become recognized as emerging worldwide diseases. No effective vaccine is currently available for the prevention of these diseases (Schuijt *et al*., 2011).

Purpose of this thesis was to monitor prevalence of pathogens (*Borrelia* and *Babesia*) in *I. ricinus* ticks in the selected localities of the Czech Republic and classify them into genospecies. Localities (South Bohemia, South Moravia, and Central Bohemia) for detection of pathogens in nymphs were selected to cover mainly turistic areas of the Czech Republic, and therefore dangerous areas for the people. GPS device was used to specify exact location of each collected tick to enable further location of areas with infected ticks. These data about ticks collections will be used by following researches in our laboratory. DNA from ticks was isolated using commercial DNA purification kit, which is suitable for long-term storage of isolated DNA. Purified DNA was placed into the laboratory sample collection for further detail studies over these samples.

In the first part of the thesis, presence of *Borrelia*-infected nymphs was determined. Total prevalence of *Borrelia* in nymphs reached 22%. This fact signals a real danger for human infections in the Czech Republic. This is similar to what was found by Eshoo *et al*., 2014 in Germany, where the prevalence of *Borrelia* was 21%. Prevalence of *Borrelia* spirochetes in Austria reached 25.7% (Glatz, 2014). In the present study, *B. afzelii*, *B. garinii*, and *B. burgdorferi* sensu stricto were found in 63%, 26%, and 10% of infected nymphs, respectively. Presence of these three genospecies corresponds to the previous studies conducted in Europe (Michelet., 2014). Results show that *B. afzelii* is the most common genospecies in the sampled areas of the Czech Republic. *B. afzelii* was the most common in locality 1 – South Bohemia, and locality 2 – South Moravia. Interestingly, *B. garinii* was the most abundant genospecies in locality 3 – Central Bohemia. This fact could be caused by the higher occurrence of birds that serve as hosts for this genospecies, but such research was not aim of this thesis. No co-infections with multiple *Borrelia* species were observed. Previous study performed in South Bohemia pointed on the highest prevalence of *B. burgdorferi* sensu

stricto (Danielova *et al*., 2004). On the contrary, results of this thesis showed low infection rate of this genospecies in this region. Nevertheless, the study by Danielova *et al.* was not performed exactly in the same area of South Bohemia. In Western Europe, *B. garinii* is the predominant species (44% infection rate), *B. afzelii* and *B. burgdorferi* sensu stricto represent 27% and 19% infection rate, respectively (Saint Girons, 1998). In Slovakia, the infection rate of *Borrelia* spirochetes is only 10.15% and is represented by *B. afzelii, B. garinii, B.valaisiana, and B. burgdorferi* sensu stricto *(*Pangracova, 2013). Cisak *et al*. observed 11.6% prevalence of *Borrelia* spirochetes in *I. ricinus* ticks collected in south-eastern Poland with different ratio of *Borrelia* genospecies. *B. burgdorferi* sensu stricto was present in 55.3%, *B. afzelii* was present in 38.3%, *B. garinii* was not found. Mixed infection (*B. burgdorferi* sensu stricto and *B. afzelii*) was found in 2.1% (Cisak *et al*., 2005). These data indicate, that the Czech Republic belongs to the countries in the Central Europe with high prevalence of *Borrelia* spirochetes. In Europe, tick infection of various *Borrelia* genospecies is different. This is probably due to the various occurrence of different reservoirs hosts for this genospecies.

The occurrence of *Babesia* genospecies in *I. ricinus* ticks has not been yet investigated in the Czech Republic. The purpose of this part of the thesis was to detect and determine prevalence of *Babesia* genospecies in *I. ricinus* ticks in selected localities of the Czech Republic. In the present study, the total prevalence of *Babesia* in nymphs reached 1.52%. *B. microti*, and *B. venatorum* (EU 1) were found in 40%, and 60% of *Babesia* infected *I. ricinus* nymphs, respectively. Severe human *B. divergens* infection was found in Norway (Mørch, 2015). In Germany prevalence of *B. microti* in *I. ricinus* ticks was 3.5% (Eshoo *et al*., 2014). In Poland, higher prevalence of *B. microti* was found (6.2 – 13.3%), (Karbowiak, 2004). Human babesiosis is rarely reported outside the United States and Europe. In addition to *I. ricinus* ticks, 7 *D. reticulatus* ticks were found in locality 2 – South Moravia (data not shown in the results). *D. reticulatus* tick is the main vector of canine babesiosis in Central Europe (Zygner, 2006). The most frequent causative agent of canine babesiosis in the Central Europe is *Babesia canis* (Kubelova, 2011). No pathogens were found in *D. reticulatus* ticks in this thesis. In Poland, *B. microti* was recently detected in 4.5% of *D. reticulatus* (Wojcik-

Fatla *et al*., 2012). In tropical regions, babesiosis is one of the most devastating arthropod borne infections of livestock causing enormous losses in cattle industry. In South Africa, the overall prevalence of babesiosis in cattle (*B. bigemina* and *B. bovis*) is 64.7% and 35.1% respectively (Mtshali, 2013). In Brazil, high prevalence of babesiosis in cattle is observed, 20.4% of the animals were found to be positive for *B. bovis* (Amorim, 2014). These data show, that the Czech Republic belongs to countries with low prevalence of babesiosis.

The sharing of reservoir and vector hosts by *Babesia* and *Borrelia* leads to co-infection in nature. The *Borrelia* DNA is much more persistent in the blood of the co-infection affected patients than in those infected by a single pathogen only (Krause *et al*., 1996). In the present study, two co-infections with *Borrelia afzelii* + *Babesia venatorum*, and *Borrelia afzelii* + *Babesia microti* were found in our set of *I. ricinus* nymphs.

In addition to the nymphs of *I. ricinus* ticks, adult females were also collected and tested for the presence of *Borrelia* and *Babesia* (data not shown in the results). In locality 1 – South Bohemia, *Borrelia* spirochetes were detected in four samples from the total number of 25. In locality 2 – South Moravia, *Borrelia* spirochetes were detected in ten samples from the total number of 25. In locality 3 – Central Bohemia, only 7 females were collected due to their lack in this locality. *Borrelia* spirochetes were detected in one sample from the total number of 7. *Borrelia* genospecies in females were present in similar proportions as in nymphs. One infected female with *Babesia* was detected in locality 3 – Central Bohemia and was represented by *B. venatorum* (EU1).

The second goal of this thesis was to determine the numbers of *Borrelia* spirochetes in single nymphs from nature and compare it with spirochete counts of the laboratory infected *I. ricinus* nymphs. Results showed that *Borrelia* spirochete counts in single nymphs from nature correspond with numbers in nymphs artificially infected in the laboratory (i.e. 46 386 and 53550, respectively). These results indicate, that our recently developed laboratory transmission model employing *I. ricinus* nymphs, European strains of *Borrelia*, and mice is suitable for testing of vaccine candidates and accurately simulates situation in nature.

Further detail studies over these samples have been already planed by other students. DNA samples will be used for identification of the zoonotic reservoirs of Lyme borreliosis and for detailed screening of other tick-borne pathogens.

5. Conclusion

In this thesis, prevalence of tick-transmitted pathogens (*Borrelia*, *Babesia*) was detected in the three selected localities of the Czech Republic – South Bohemia, South Moravia, and Central Bohemia. Furthermore, these pathogens were determined to genospecies. Results showed high prevalence of *Borrelia* in South Moravia. The most abundant genospecies was *B. afzelii*. Prevalence of *Babesia* was overall low, and was represented of two genospecies. The highest prevalence was in the locality South Bohemia. The most abundant genospecies was *B. venatorum*.

Moreover, quantification of *Borrelia* spirochetes in nymphs was conducted and subsequently compared with results of *Borrelia* quantification performed on nymphs artificially infected. Results showed that *Borrelia* spirochete counts in single nymphs from nature correspond with numbers in nymphs artificially infected in the laboratory.

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7. Supplement

Table 11. Oligonucleotides used for PCR.

Target	Name	Sequence (5'→3')	Product size	Annealing	Source
Borrelia flagellin	FlaF1	AGCAAATTTAGGTGCTTTCCAA	154 bp	60°C	Schwaiger M, 2001
	FlaR1	GCAATCATTGCCATTGCAGA			
	Fla probe1	TGCTACAACCTCATCTGTCAT TGTAGCATCTTTTATTTG			
Borrelia 5S, 16S rRNA	IGS F	GTATGTTTAGTGAGGGGGGTG	*B. afzelii* – 450 bp *B. garinii* – 1500bp *B. b. s. s.* – 760 bp	56°C	Bunikis J, 2004
	IGS R	GGATCATAGCTCAGGTGGTTAG			
	IGS Fn	AGGGGGGTGAAGTCGTAACAAG		60°C	
	IGS Rn	GTCTGATAAACCTGAGGTCGGA			
Babesia 18S rDNA	IR270	AACCTGGTTGATCCTGCCAGTAGTCAT	540 bp	60°C	Malandrin L, 2010
	IR275	GAATGATCCTTCCGCAGGTTCACCTAC			
	IR272	GYYTTGTAATTGGAATGATGG	559 bp		
	IR273	CCAAAGACTTTGATTTCTCTC			
I. ricinus	Ir-600F	GAGGCATGAGGGTGTGTTTT	600 bp	60°C	Šíma R. unpublished
	Ir-600R	GACCTGCACGAAAATGATTG			

Table 12. GPS localization and names of samples from locality 1 – South Bohemia.

GPS localization	Name of sample	Mark on the map
N 48° 58.803' E 14° 25.668'	1 – 2 NJ, 1 – 3 SJ	1
N 48° 58.822' E 14° 25.836'	3 – 5 NJ, 4 – 5 SJ	2
N 48° 58.794' E 14° 25.949'	6 – 7 NJ, 6 – 8 SJ	3
N 48° 58.919' E 14° 25.887'	8 – 12 NJ	4
N 48° 58.961' E 14° 25.625'	13 – 15 NJ, 9 – 10 SJ	5
N 48° 58.795' E 14° 25.543'	16 – 17 NJ, 11 – 13 SJ	6
N 48° 58.764' E 14° 25.440'	18 – 22 NJ	7
N 48° 58.761' E 14° 25.324'	23 – 26 NJ, 14 SJ	8
N 48° 58.738' E 14° 25.168'	27 – 30 NJ, 15 SJ	9
N 48° 58.850' E 14° 25.079'	31 – 33 NJ, 16 – 17 SJ	10
N 48° 58.980' E 14° 25.230'	34 – 38 NJ	11
N 48° 58.877' E 14° 25.404'	39 – 42 NJ, 18 SJ	12
N 48° 58.687' E 14° 25.463'	43 – 47 NJ	13
N 48° 58.544' E 14° 25.352'	48 – 52 NJ	14
N 48° 58.421' E 14° 25.235'	53 – 57 NJ	15
N 48° 58.359' E 14° 24.962'	58 – 59 NJ, 19 – 21 SJ	16
N 48° 58.480' E 14° 24.928'	60 – 63 NJ, 22 SJ	17
N 48° 58.652' E 14° 25.602'	64 – 68 NJ	18
N 48° 58.576' E 14° 25.663'	69 – 73 NJ	19
N 48° 58.496' E 14° 25.461'	74 – 78 NJ	20
N 48° 58.644' E 14° 25.350'	79 – 83 NJ	21
N 48° 58.625' E 14° 25.241'	84 – 87 NJ	22
N 48° 58.604' E 14° 25.192'	88 – 92 NJ	23
N 48° 58.512' E 14° 25.183'	93 – 97 NJ	24
N 48° 58.498' E 14° 25.102'	98 – 101 NJ, 24 SJ	25
N 48° 58.467' E 14° 24.888'	102 – 106 NJ	26
N 48° 58.596' E 14° 25.010'	107 – 110 NJ	27
N 48° 58.723' E 14° 25.555'	25 SJ	28

Table 13. GPS localization and names of samples from locality 2 – South Moravia.

GPS localization	Name of sample	Mark on the map
N 48° 54,133'E 15° 50,366'	1 – 4 N, 1S	1
N 48° 53,194'E 15° 51,887'	5 – 9 N	2
N 48° 53,222'E 15° 51,527'	10 – 14 N	3
N 48° 52,957'E 15° 51,518'	15 – 19 N	4
N 48° 53,055'E 15° 51,036'	20 – 23 N, 2 S	5
N 48° 52,768'E 15° 52,177'	3 S	6
N 48° 52,229'E 15° 51,674'	24 – 25 N, 4 – 5 S	7
N 48° 51,892'E 15° 52,044'	26 – 30 N	8
N 48° 53.093' E 15° 52.785'	31 – 34 N	9
N 48° 51,141'E 15° 55,684'	35 – 43 N, 7 S	10
N 48° 51,442'E 15° 59,457'	44 – 49 N	11
N 48° 51,639'E 16° 00,347'	50 – 54 N	12
N 48° 54,817'E 16° 05,454'	55 – 59 N	13
N 48° 49,869'E 16° 00,859'	60 – 64 N, 8 – 10 S	14
N 48° 49,327'E 16° 00,372'	65 – 69 N, 11 – 13 S	15
N 48° 48,789'E 15° 59,579'	70 – 74 N, 14 S, 1 D	16
N 48° 48,077'E 15° 58,765'	75 – 79 N, 15 S, 2 D	17
N 48° 47,067'E 16° 02,123'	80 – 84 N, 16 S	18
N 48° 47,464'E 16° 01,695'	85 – 88 N, 17 S, 4 – 5 D	19
N 48° 48,822'E 15° 59,404'	89 – 93 N	20
N 48° 48,738'E 15° 59,088'	94 – 98 N, 18 – 19 S	21
N 48° 48,625'E 15° 58,758'	99 – 103 N, 20 – 23 S, 6 D	22
N 48° 50,100'E 16° 00,724'	104 – 110 N, 24 – 25 S	23

Table 14. GPS localization and names of samples from locality 3 - Central Bohemia.

GPS localization	Name of sample	Mark on the map
N 49° 54.688' E 13° 55.479'	1 – 5 NS	1
N 49° 54.849' E 13° 55.435'	6 – 10 NS	2
N 49° 55.190' E 13° 55.320'	11 – 14 NS, 1SS	3
N 49° 55.345' E 13° 55.333'	15 – 18 NS, 2SS	4
N 49° 55.316' E 13° 52.417'	19 – 23 NS	5
N 49° 55.310' E 13° 52.208'	24 – 28 NS	6
N 49° 55.262' E 13° 52.051'	29 – 33 NS	7
N 49° 55.145' E 13° 52.008'	34 – 38 NS	8
N 49° 55.216' E 13° 52.364'	39 – 43 NS	9
N 49° 57.447' E 13° 54.921'	44 – 48 NS	10
N 49° 57.487' E 13° 54.566'	49 – 53 NS	11
N 50° 02.684' E 13° 52.786'	54 – 58 NS	12
N 50° 02.600' E 13° 52.622'	59 – 63 NS	13
N 50° 02.481' E 13° 52.759'	64 – 68 NS	14
N 50° 03.595' E 13° 55.012'	69 – 73 NS	15
N 50° 03.587' E 13° 55.309'	74 – 78 NS	16
N 50° 03.709' E 13° 55.581'	79 – 82 NS, 3SS	17
N 50° 03.807' E 13° 55.912'	83 – 87 NS	18
N 50° 04.916' E 13° 52.845'	88 – 92 NS	19
N 50° 06.754' E 13° 53.466'	93 – 96 NS, 4 – 6 SS	20
N 50° 06.835' E 13° 53.308'	97 – 101 NS	21
N 50° 05.145' E 13° 48.609'	102 – 105 NS, 7 SS	22
N 50° 02.285' E 13° 47.851'	106 – 110 NS	23

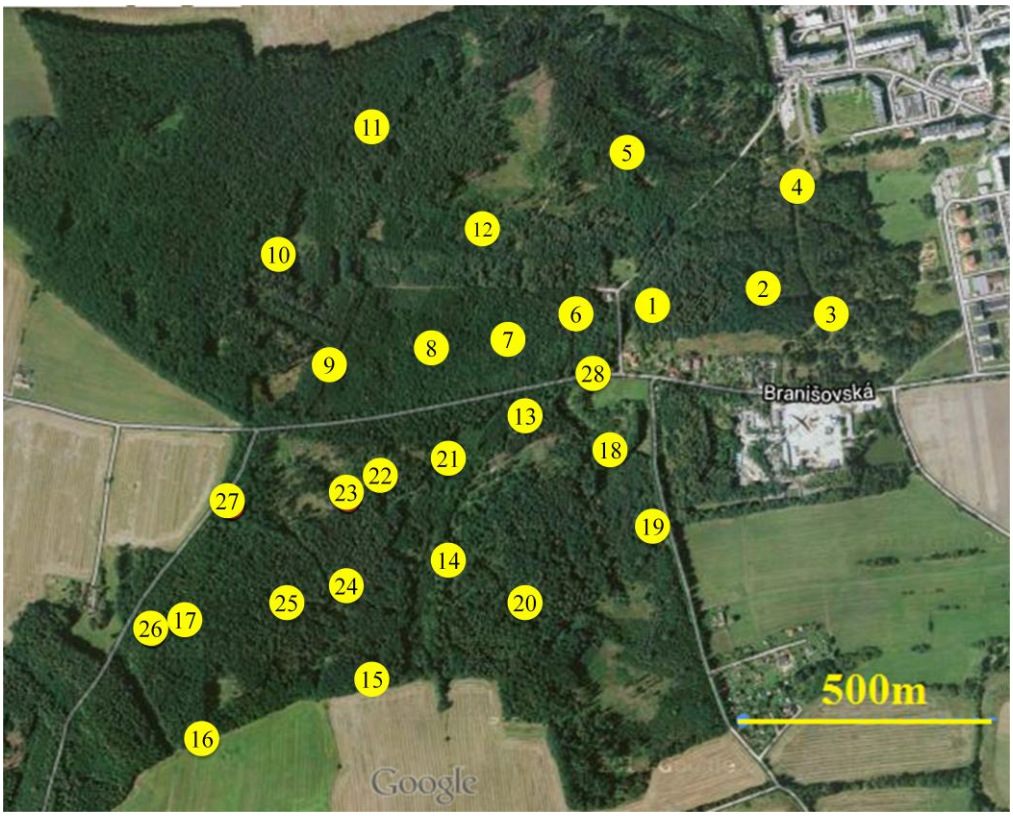

Figure 18. Distribution of tick samples in locality 1 –South Bohemia.

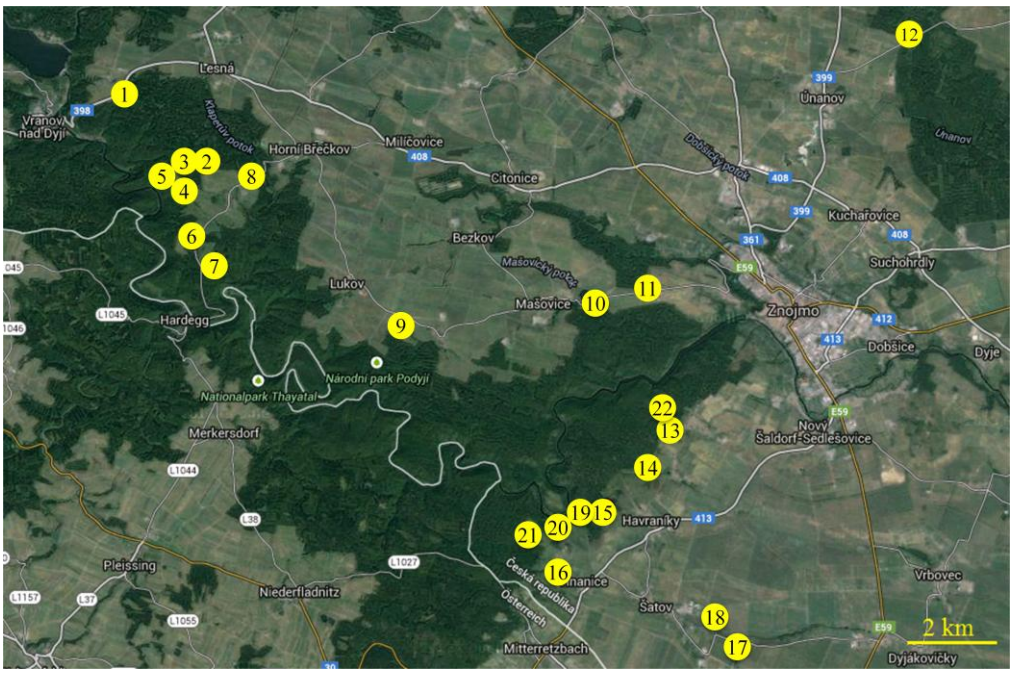

Figure 19. Distribution of tick samples in locality 2 –South Moravia.

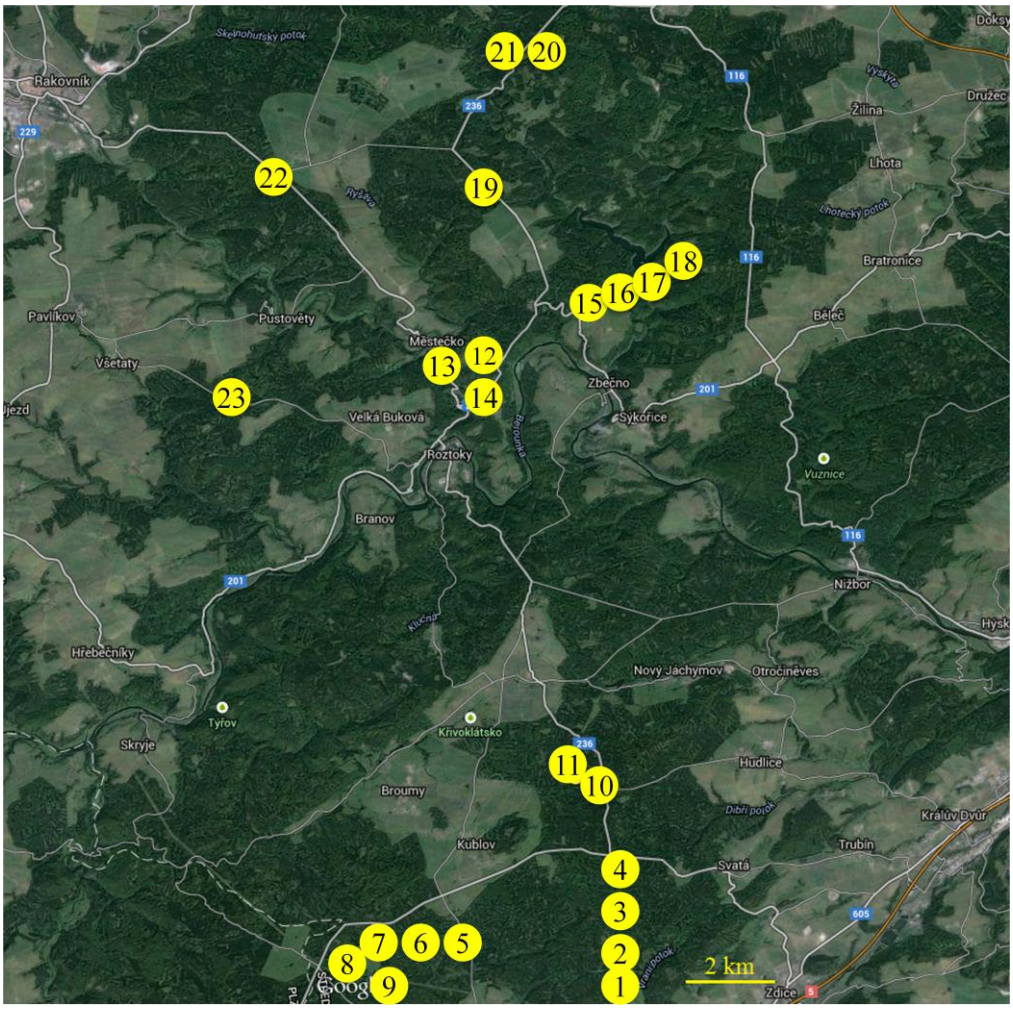

Figure 20. Distribution of tick samples in locality 3 –Central Bohemia.