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# Long-Term Study of *Borrelia* and *Babesia* Species Distribution in *Ixodes Ricinus* and *Dermacentor Recticulatus* Ticks Removed From Humans in Poland, 2016-2019

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## Abstract

Monitoring changes in the prevalence of different *Borrelia* genospecies/ species in ticks might be an important indicator of risk assessment and of differences in pathogenicity in humans. Furthermore, the evaluation of pathogens in feeding ticks represents the risk of human exposure better than studies on questing ticks. The objective of our study was to assess the prevalence and distribution of *Borrelia* and *Babesia* species in ticks removed from humans, in a larger sample collected for several months during four years of studies. We confirmed high *Borrelia* prevalence, including *B. miyamotoi*, in ticks removed from humans as well as the shift in *Borrelia* genospecies/ species frequency of occurrence during the four-year study. Despite the fact that *Babesia* prevalence was relatively low, the majority of tested isolates are considered to be pathogenic for humans. The results of our study have also shown that *Borrelia* and *Babesia* coinfections in ticks are more common in *Borrelia*-infected ticks. Even if the overall risk of developing Lyme borreliosis after a tick bite in Europe is rather low, the knowledge of prevalence and distribution of *Borrelia* and *Babesia* species in ticks might be an important indicator of both tick-borne disease risk and pathogenicity assessment.


## 1. Introduction

With 85,000 cases reported annually in Europe, Lyme borreliosis (LB) is the most common vector-borne disease in temperate zones of the northern hemisphere [1]. The estimated incidence of LB in Poland increased dramatically from 20.3 per 100,000 inhabitants in 2007 to 53.6 per 100,000 inhabitants in 2019 (an estimated average increased from 7,735 cases per year in 2007 to 20,614 cases per year in 2019) (National Institute of Public Health – National Institute of Hygiene, Epidemiological reports, www.pzh.gov.pl). However, the reliability of LB incidence data is uncertain due to diagnostic problems and limited reporting [2]. At least five species of *Borrelia* – *Borrelia burgdorferi* sensu stricto, *Borrelia garinii*, *Borrelia afzelii*, *Borrelia spielmani* and *Borrelia bavariensis* – are known to be pathogenic to humans and each genospecies is believed to be associated with different clinical manifestations. The heterogeneity among *B. burgdorferi* s.l. genospecies seems to be the main factor causing the regional differences in the clinical expression of human Lyme borreliosis [3]. *Borrelia burgdorferi* sensu stricto is particularly arthritogenic, *B. afzelii* primarily causes skin infections, and *B. garinii* is especially neurotropic. Infection usually begins with an expanding skin lesion, known as *erythema migrans* which, if left untreated, can be followed by early disseminated infection, particularly neurological abnormalities, and by late infection, especially arthritis or *acrodermatitis chronica atrophicans* (ACA). Recently, *Borrelia miyamotoi* has been identified as a human pathogen causing relapsing fever in Europe, and little is known about its local impact on human health. *Borrelia miyamotoi* disease (BMD) has also been confirmed in an immunocompetent patient, and BMD concurrent with Lyme disease has also been described [4].

In Europe, including Poland, other tick-borne diseases such as babesiosis are reported sporadically. About 60 confirmed cases of human babesiosis caused mainly by *Babesia divergens* have been described so far [5]. Non-specific clinical symptoms of babesiosis, such as fever, flu-like disease, headache, chills, sweats and myalgia, as well as diagnostic difficulties have a key impact on their correct diagnosis and, consequently, effective treatment [6]. Babesiosis in immunocompetent individuals often has an asymptomatic but chronic course [7]. In terms of safe blood donation, this is of fundamental importance especially if blood recipients are immunosuppressed. Transfusion-transmitted babesiosis is being increasingly described globally, mainly in the United States [8].

The *Ixodes ricinus* species is associated with deciduous and mixed forests, but the expansion of *I. ricinus* observed over the past decades allowed to extend the range of its occurrence to northern areas of the continent and areas located at a higher altitude [9]. Across Europe, *I. ricinus* typically make up 90–100% of all ticks removed from humans and nymphs are the most commonly detected life stage [10, 11]. The increase in the density of ticks, also in urban areas, and the prolonged period of activity of these arachnids are probably the result of changes occurring in the environment, e.g. in land use in agriculture, forest management, changes in abundance and distribution of free living animals, and climate change [12–15]. The observed phenomena translate directly into an increase in the risk of transmission of pathogens vectored by ticks,

which can be a significant problem for people with impaired immune system whose percentage in society is constantly increasing [16].

The *I. ricinus* ticks are competent vectors for many species of pathogenic viruses, bacteria and protozoa. An important problem in the epidemiology of tick-borne diseases is co-infection, i.e. simultaneous, multi-species infections, especially difficult to diagnose in humans [17]. Co-infection in humans and animals might enhance disease severity and may have significant consequences in terms of tick-borne disease treatment and diagnosis. For instance, co-infected Lyme disease patients harboured more influenza-like symptoms than those with Lyme disease alone [7]. In the case of concurrent babesiosis and Lyme disease, co-infected patients experienced a greater number of symptoms for a longer duration than those with Lyme disease alone.

The knowledge of *Borrelia* prevalence and genospecies distribution is crucial to understand epidemiology as well as the prevention and diagnosis of LB. There is a limited number of studies on particular species prevalence in ticks removed from humans, mainly providing information only on *B. burgdorferi* (s.l.) complex. In Poland, most of the previously conducted research concerned questing ticks or ticks collected from animals [18–25]. However, the evaluation of pathogens in feeding ticks represents the risk of human exposure better than studies on questing ticks. The aim of our study was to assess the prevalence and distribution of *Borrelia* and *Babesia* species in ticks removed from humans in Poland, in a larger sample collected for several months during four years of studies.

## 2. Results

## 2.1. *Ixodes ricnus* Ticks

During four years of study, 1890 *I. ricinus* ticks were collected from humans: 54 (2.9%) larvae, 1,298 (68.7%) nymphs, 524 (27.7%) females and 14 (0.7%) males. Most of them were collected in 2018–2019 (n = 762 and n = 775, respectively), whereas in 2016–2017 only 335 ticks were tested (n = 126 and n = 227, respectively). The main peak of tick activity was observed in June and the second one in October; however, the mean number of ticks collected in October was almost four times lower (Fig. 1). The number of ticks in each stadium (larvae, nymphs and adults) removed from humans has varied significantly between months of study (*month x number of I. ricinus tick in each stadium*: $\chi^2_{16} = 85.5$, $p < 0.000$). Overall, the median number of larvae collected by month was 6, with a minimum of 2 larva (in May), a maximum of 18 (in July), and no larvae were collected in March–April and November. The proportion of nymphs over the total number of ticks during a particular month of study increased from 67.4% (62/92) in April to 73.3% (173/236) in August, followed by a decrease to approximately 60.0% in September–November (89/153, 94/152 and 13/21, respectively). The proportion of females and males over the total number of ticks during a particular month of study decreased from approximately 33% in April–May (30/92 and 116/352, respectively) to 19% (45/236) in August, followed by an increase to the mean of 37.5% in October–November (60/162 and 8/21, respectively).

## 2.2. *Dermacentor reticulatus* Ticks

During the four-year study, 63 *D. reticulatus* ticks were collected: 41 (65%) females and 22 (35%) males. Most of *D. reticulatus* ticks were collected in 2018–2019 (n = 54; 85.7%). Overall, the median number of ticks collected monthly was 7; however, the highest number of ticks was noted from March to May (21%, 21% and 30%, respectively), and no ticks were observed in July and August ($\chi^2_8 = 14.8$; $p = 0.054$).

## 2.3. *Borrelia* Prevalence in *I. ricinus* Ticks

Overall, the *Borrelia* infection prevalence in the human-derived *I. ricinus* ticks determined by nested-PCR was 25.3% (479/1890, 95% CI: 23.4–27.3%). Annual prevalence ranged from 30.2% in 2016 to 23.4% in 2019 (Table 1). Statistical analysis of the long-term period revealed a significant decrease of *Borrelia* prevalence between 2016 (30.2% [38/126], 95 %

CI: 22.7–38.6 %) and 2019 (23.4% [181/775], 95% CI: 20.5–26.4%) ($\chi^2_3$ = 7.58; p = 0.051; Table 1). Furthermore, a significant effect of tick stage was also observed ($\chi^2_2$ = 11.9; p = 0.003). *Borrelia* DNA was detected in 9.3% (5/54, 95% CI: 3.6–19.1%) of larvae, 24.7% (321/1297, 95% CI: 22.5–27.2%) of nymphs, and 28.4% (153/539, 95% CI: 24.7–32.3%) of adult *Ixodes* ticks (Table 1). When analysing the effect of month on *Borrelia* prevalence in *I. ricinus*, no significant differences were detected (p = 0.085). The highest *Borrelia* prevalence was noted in May (27.6% [97/352], 95% CI: 23.1–32.4%), October (30.2% [49/162], 95% CI: 23.6–37.6%) and November (47.6%, 95% CI: 27.7–68.1%), where 10 out of 21 tested ticks were positive.

Table 1
Stage and year distribution of *Borrelia*-infected ticks removed from humans in 2016 and 2019

| | **No. of attested ticks** | ***Borrelia*-positive *I. ricinus* ticks** **No of positive ticks (%; 95% confidence interval)** | | | | | |
|---|---|---|---|---|---|---|---|
| | | **2016** | **2017** | **2018** | **2019** | **Total** | **P value** |
| Larvae | 54 | 2 (28.6; 6.5–64.8) | 0 (0.0) | 2 (8.7; 1.9–25.1) | 1 (7.7; 0.8–30.7) | 5 (9.3; 3.6–19.1) | p = 0.231 |
| Nymphs | 1298 | 22 (27.5; 18.6–38.0) | 38 (29.2; 21.9–37.4) | 129 (24.5; 21.0-28.3) | 132 (23.6; 20.2–27.2) | 321 (24.7; 22.5–27.2) | p = 0.550 |
| Adults | 538 | 14 (35.9; 22.3–51.5) | 28 (32.6; 23.4–42.9) | 63 (29.7; 23.9–36.1) | 48 (23.8; 18.3–30.0) | 153 (28.4; 24.7–32.3) | p = 0.247 |
| Total | 1890 | 38 (30.2; 22.7–38.6) | 66 (29.1; 23.5–35.2) | 194 (25.5; 22.5–28.6) | 181 (23.4; 20.5–26.4) | 479 (25.3; 23.4–27.3) | **p = 0.051** |
| | | *Borrelia*-positive *D. reticulatus* ticks No of positive ticks (%; 95% confidence interval) | | | | | |
| | | 2016 | 2017 | 2018 | 2019 | Total | P value |
| Adults | 63 | 1 (20; 2.3–62.9) | 1 (25; 2.8–71.6) | 2 (7.7; 1.6–22.5) | 4 (14.3; 5.0-30.5) | 8 (12.7; 6.1–22.2) | p = 0.740 |

## 2.4. *Borrelia* Prevalence in *D. reticulatus* Ticks

In total, 12.7% (8/63, 95% CI: 6.1–22.2%) of the *D. reticultaus* ticks delivered within 2016–2019 were tested positive for *Borrelia* infections (Table 1). Prevalence of infection decreased from 20–25% in 2016–2017 to 7.7–14.3% in 2018–2019; however, only 9 *D. reticulatus* ticks were tested within the first two years of study (Table 1). Females (9.8% [4/41], 95% CI: 3.6–21.5%) were less often infected than males (18.2% [4/22], 95% CI: 6.5–37.6%). No statistical differences between sex and month of study were observed.

## 2.5. *Borrelia* Genospecies/ Species in *I. ricinus* Ticks

Species typing was performed on the basis of sequencing of flagellin gene fragments (~ 600 bp product) or RFLP-PCR analysis. Species/genospecies differentiation of *Borrelia* infected ticks was successful in 251 out of 479 positive tick samples (52.4%), i.e. 38 out of 38 (100%) in 2016, 64 out of 66 (97%) in 2017, 77 out of 194 (40%) in 2018, and 72 out of 181 (39.8%) in 2019.

The most frequently detected *Borrelia* genospecies was *B. afzelii* (65.3%, 95% CI: 59.3–71.0%), followed by *B. burgdorferi* (10.8%, 95% CI: 7.4–15.0%), *B. garinii* (8.8%, 95% CI: 5.7–12.7%), *B. valaisiana* (5.2%, 95% CI: 2.9–8.4%), *B. spielmanii*

(1.2%, 95% CI: 0.3–3.2%), and *B. lusitaniae* (0.4%, 95% CI: 0.0–1.8%) (Table 2). The relapsing fever spirochete *B. miyamotoi* was identified in 8.4% (95% CI: 5.4–12.3%) of analyzed ticks.

Table 2
*Borrelia* genospecies/species distribution in infected *I. ricinus* ticks (n = 251) removed from humans between 2016 and 2019

| | | No of positive ticks (%; 95% confidence interval) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | **No of tested ticks** | ***B. afzelii*** | ***B. garinii*** | ***B. burgdorferi*** | ***B. miyamotoi*** | ***B. valaisiana*** | ***B. lusitaniae*** | ***B. spielmanii*** |
| Total | | 251 | 164 (65.3; 59.3–71.0) | 22 (8.8; 5.7–12.7) | 27 (10.8; 7.4–15.0) | 21 (8.4; 5.4–12.3) | 13 (5.2; 2.9–8.4) | 1 (0.4; 0.0-1.8) | 3 (1.2; 0.3–3.2) |
| Tick stage | larvae | 3 | 2 (66.7; 17.7–96.1) | 0 | 0 | 0 | 0 | 1 (33.3; 3.9–82.3) | 0 |
| | nymphs | 157 | 105 (66.9; 59.3–73.9) | 11 (7.0; 3.8–11.8) | 18 (11.5; 7.2–17.1) | 12 (7.6; 4.2–12.6) | 8 (5.1; 2.4–9.4) | 0 | 3 (1.9; 0.5-5.0) |
| | adults | 91 | 57 (62.6; 52.4–72.1) | 11 (12.1; 6.6–19.9) | 9 (9.9; 5.0-17.3) | 9 (9.9; 5.0-17.3) | 5 (5.5; 2.1–11.6) | 0 | 0 |
| Month of study | March | 1 | 0 | 1 (100) | 0 | 0 | 0 | 0 | 0 |
| | April | 6 | 4 (66.7; 28.6–92.3) | 0 | 2 (33.3; 7.7–71.4) | 0 | 0 | 0 | 0 |
| | May | 46 | 31 (67.4; 53.1–79.6) | 2 (4.3; 0.9–13.2) | 4 (8.7; 3.0-19.4) | 4 (8.7; 3.0-19.4) | 2 (4.3; 0.9–13.2) | 0 | 3 (6.5; 1.9–16.4) |
| | June | 72 | 51 (70.8; 59.7–80.4) | 7 (9.7; 4.5–18.1) | 5 (6.9; 2.7–14.6) | 6 (8.3; 3.6–16.4) | 3 (4.2; 1.2–10.7) | 0 | 0 |
| | July | 48 | 32 (66.7; 52.7–78.7) | 2 (4.2; 0.9–12.7) | 8 (16.7; 8.2–29.0) | 4 (8.3; 2.9–18.6) | 2 (4.2; 0.9–12.7) | 0 | 0 |
| | August | 31 | 22 (71,0; 53.7–84.6) | 3 (9.7; 2.8–23.6) | 3 (9.7; 2.8–23.6) | 2 (6.5; 1.4–19.1) | 1 (3.2; 0.4–14.1) | 0 | 0 |
| | September | 23 | 11 (47.8; 28.7–67.5) | 2 (8.7; 1.9–25.1) | 3 (13.0; 3.8–30.9) | 2 (8.7; 1.9–25.1) | 4 (174; 6.2–36.2) | 1 (4.3; 0.5–18.6) | 0 |
| | October | 20 | 11 (55.0; 33.8–74.9) | 5 (25.0; 10.2–46.4) | 2 (10.0; 2.1–28.4) | 1 (5.0; 0.5–21.1) | 1 (5.0; 0.5–21.1) | 0 | 0 |

| | | No of positive ticks (%; 95% confidence interval) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | No of tested ticks | *B. afzelii* | *B. garinii* | *B. burgdorferi* | *B. miyamotoi* | *B. valaisiana* | *B. lusitaniae* | *B. spielmanii* |
| | November | 4 | 2 (50; 12.3–87.7) | 0 | 0 | 2 (50; 12.3–87.7) | 0 | 0 | 0 |

Analysis of coinfection in multiple infected ticks was performed only using RFLP-PCR in 2018–2019. Overall, 2.0% (3/149) of analyzed ticks carried two *Borrelia* species (*B. afzelii* with *B. burgdorferi/ B. miyamotoi/ B. spielmanii*), while triple infections were observed only in 1 (0.7%) tick (*B. afzelii/ B. burgdorferii/ B. lusitaniae*).

*Borrelia* genospecies distribution showed no significant differences between tick stages (p = 0.231) and the month of study (p = 0.524) (Table 2). Adult ticks were more frequently infected with *B. afzelii* (57/91, 62.6%, 95% CI: 52.4–72.1%) and *B. garinii* (11/91, 12.1%; 95% CI: 6.6–19.6%). In nymphs, the most commonly detected genospecies were *B. afzelii* (105/157, 66.9%, 95% CI: 59.3–73.9%) and *B. burgdorferi* (18/157, 11.5%, 95% CI: 7.2–17.1%). Larvae were infected only *B. afzelii* (2/3, 66.7%, 95% CI: 17.7–96.1%) and *B. lusitaniae* (1/3, 33.3%, 95% CI: 3.9–82.3%).

The species distribution in different sampling years is shown in Fig. 2. ($\chi^2_{18} = 49.9$; $p < 0.000$). Throughout our 4-year study, the ticks were predominantly infected with *B. afzelii* (60.5% [95% CI: 44.7–74.8%], 60.9% [95% CI: 48.7–72.2%], 77.9% [95% CI: 67.2–86.1%], and 58.3% [95% CI: 46.3–69.2%] in 2016–2019, respectively. Nevertheless, the shift of the second most common genospecies/ species was observed during our study. In 2016, *B. myiamotoi* was detected in 15.8% [95% CI: 6.9–29.7%] of ticks, followed by a decrease of infected ticks in 2017 and 2018 (3.1% [95% CI: 0.7–9.6%] and 6.5% [95% CI: 2.5–13.6%]) and another increase to 11.1% [95% CI: 5.4–19.9%] in 2019. *Borrelia garinii* was the second most frequently noted species in 2017 (23.4% [95% CI: 14.4–34.8%]); however, only 1.3% [95% CI: 0.1–5.9%] and 4.2% [95% CI: 44.7–74.8%] ticks were infected in 2018 and 2019. *Borrelia burgdorferii* was the most frequently identified species after *B. afzelii* in 2018 and 2019 (9.1% [95% CI: 4.2–17.0%] and 20.8% [95% CI: 12.7–31.2%]) – despite the fact that in 2017 only 3.1% [95% CI: 0.7–9.6%] of ticks were infected.

Comparison of genospecies/ species distribution in diagnostic ticks removed from humans with those from questing ticks in our previous study [22] revealed that ticks removed from humans were by far more frequently infected with *B. myiamotoi* (p = 0.003), whereas questing ticks were more commonly infected with *B. garinii* (p = 0.0001). Detailed results are shown in Fig. 3.

## 2.6. *Borrelia* Genospecies/ Species Identification in *D. reticulatus* Ticks

Genospecies differentiation of *Borrelia* infected ticks was successful in 6 out of 8 positive tick samples (75%). All *Borrelia* isolates were identified on the basis of RFLP-PCR analysis as *B. afzelii*.

## 2.7. *Babesia* Prevalence in *I. ricinus and D. reticulatus* Ticks

In total, 1.3% (15/1100, 95% CI: 0.8–2.2%) of the *I. ricinus* ticks delivered in 2016–2018 were tested positive for *Babesia* infections. No significant statistical differences between sex and stage of ticks, as well as month and year of study, were detected. The prevalence of *Babesia* infection ranged from 0.9% (2/227, 95% CI: 0.2–2.8%) in 2017 to 2.4% (3/126, 95% CI: 0.7–6.2%) in 2016. Higher *Babesia* prevalence of 2.4 % (8/337, 95% CI: 1.1–4.4%) was found in adult *I. ricinus* than in nymphs (7/737, 0.9%, 95% CI: 0.4–1.9%); no infected larvae were noted. The percentage of infected ticks varied from 0.6% (1/172, 95% CI: 0.1–2.7%) to 1.9% (3/160, 95% CI: 0.5–4.9%) between May and October.

Species typing was performed on the basis of sequencing of 18S rRNA gene fragment (~ 540 bp product); all positive PCR samples were sequenced. Alignment and BLAST-NCBI analyses revealed the presence of three *Babesia* species. Nine out of 15 isolates (60%) have shown high similarity level (> 99.5%) to *B. microti* strain Jena isolated originally from human patients in Germany (EF413181). The nucleotide sequences of five isolates (33.3%) were identical to *B. venatorum* isolate from *I. ricinus* in France (FJ215873). One isolate was identified as *B. canis* with a similarity level of > 99% to another Polish isolate (JN107810).

During three years of study (2016–2018), one *D. reticulatus* tick (1/36, 2.8%) was infected with *B. canis* with a similarity level of > 99% to another Polish isolate (JN107810).

## 2.8. *Borrelia* and *Babesia* Coinfection in *I. ricinus* Ticks

Statistical analysis of coinfection in *I. ricinus* revealed significant differences among infected ticks ($\chi^2_1 = 4.81$; $p = 0.028$). *Babesia*-positive *I. ricinus* ticks were more frequently observed among *Borrelia*-positive ticks (2.7%; 8/290) than among ticks uninfected with *Borrelia* (0.8%; 7/810).

# 3. Discussion

Analysis of available data revealed the high socio-economic impact of Lyme borreliosis on public health systems as well as on quality of life for infected patients [29, 30]. In this study, we confirmed high *Borrelia* prevalence in ticks removed from humans as well as the shift in *Borrelia* genospecies/ species frequency of occurrence during the four-year study. The results of our study have also shown that *Borrelia* and *Babesia* coinfections in ticks are more common in *Borrelia*-infected ticks.

The ticks removed from humans in Poland were almost exclusively *I. ricinus* (97%), the most widespread and abundant ticks species in humans in Europe (European Centre for Disease Control & Prevention, 2019). Only a few specimens of *D. reticulatus* were collected (3%). While almost the whole of Europe is an endemic region for *I. ricinus*, the geographical range of *D. reticulatus* in Europe is discontinuous with two main macroregions, and the spreading of *D. reticulatus* is believed to be associated with the loss of forest area [31]. This tick species appeared to show bimodal activity pattern with the highest density in March–May and September–November, whereas no ticks were collected in summer, which is typical for this tick species [32]. *Dermacentor reticulatus* ticks were also removed from patients in Germany, Belgium and Poland [33–35]; however, the frequency of occurrence of this species does not exceed a few percent.

For *I. ricinus*, we observed the peak of activity in June which is congruent with the results of our previous study on questing ticks [22] and other studies on seasonality *of I. ricinus* bites on humans [11, 12]. The predominance of nymphs of up to 73% in dependence of month of study was similar to other European studies on ticks collected from humans [33–38]. The activity of larvae was the highest in August and September; however, only 54 specimens in total were removed from humans. It is worth noting that the highest number of tick bites occurred during the summer period when people are more likely to be exposed to ticks by spending time outdoors, not only in natural areas. Our previous analysis of the frequency of occurrence of *Borrelia* spirochetes in ticks collected from areas with varying degrees of anthropopression has shown that although the population density of ticks in natural areas was significantly higher, the prevalence of *Borrelia* infection in *I. ricinus* ticks collected from natural and urban areas was similar (12% vs. 11%) [22].

To observe a long-term trend, *Borrelia* spirochetes prevalence as well as species/ genospecies distribution in ticks removed from humans were compared in the course of four years. Surprisingly, between 2016 and 2019, annual *Borrelia* prevalence in ticks decreased significantly from 38–25%. At the same time, the number or Lyme borreliosis cases in Poland decreased slightly from 21,220 in 2016 to 20,614 in 2019 (National Institute of Public Health – National Institute of Hygiene, Epidemiological reports, www.pzh.gov.pl). Similar fluctuations in *Borrelia* prevalence in *I. ricinus* collected from humans were observed in Germany and Romania [33, 37, 38]; however, the differences were not so significant. Our previous studies

have shown that annual *Borrelia* occurrence in questing *I. ricinus* ticks in Poland varied from 8–15% between 2013 and 2014 [22]. These inter-annual fluctuations in *Borrelia* prevalence may be due to climatic or other ecological factors affecting tick density or the abundance and, as a result, the availability of reservoir hosts, such as rodents or birds. It has been proven that the relative abundance of the white-footed mouse is positively associated with nymphal infection prevalence value which is regarded as the most important indicator of Lyme borreliosis risk [39].

Overall in Europe, including Poland, the *Borrelia* prevalence in ticks removed from humans range from 5–29% [33, 34, 35, 37, 38, 40, 41, 42, 43, 44]. In our study, the *Borrelia* prevalence has differed significantly between *I. ricinus* ticks removed from humans (25%) and questing ticks (11%, [22]). Some results suggest that the abundance of spirochaetes in questing *Ixodes* ticks may be low (below 300 copies of bacteria) and, therefore, often undetectable, while blood repletion or simply the increased ambient temperature triggers bacteria growth and rises detectability, but possibly only within a short period (around 72 h after changing the conditions) [45, 46]. The knowledge of this phenomenon is still limited, and, in consequence, the number of infected *Borrelia* ticks removed from the host (human) may be higher than it has been evaluated in questing ticks, which could translate into higher risk of tick-borne infections.

The observed significant lower *Borrelia* infection rates in *I. riciunus* larvae (9%) compared to nymphs (25%) and in nymphs compared to adults (28%) is in accordance with previous studies on questing and engorged ticks [22, 23, 33, 34, 35, 37]. Since each tick stadium has only one blood meal from different hosts and the probability of acquiring pathogens increase with every blood meal, the highest prevalence of infection is noted in adults ticks. It is believed that transovarial transmission of *Borrelia* is rare or non-existent and larval ticks are not important vectors of Lyme borreliosis [47]. Richter et al. [48] have suggested that questing larvae in nature may have acquired *Borrelia* spirochetes from an interrupted host contact. In our study, we confirmed *Borrelia* infection in 9% of removed larvae; however, only 54 of them were collected. Detection of the spirochetes in larvae was previously noticed at low prevalence in questing ticks [49] as well as in ticks removed from humans [34, 37, 38], which strengthens the evidence for transovarial transmission of *Borrelia* under field conditions. Nonetheless, Faulde et al. [50] did not confirm the case of acquired Lyme borreliosis following the bite of an infected *I. ricinus* larva. Hence, the hypothesis of *Borrelia* transmission from larvae to human need further experimental studies.

*Borrelia* infection rate in *D. reticulatus* ticks does not exceed 13%; however, only 63 ticks were tested. The previous studies have shown that *Borrelia* prevalence in questing *D. reticulatus* ticks is significantly lower [51–53]. Nevertheless, the infection rates in engorged *D. reticulatus* ticks collected from dogs is similar to the results noted in this study [25].

Since different *Borrelia* species/genospecies are involved in distinct clinical manifestations, it is important to know accurate numbers for the prevalence of a particular species with regard to risk assessment. In our study, the species identification by sequencing or RFLP analysis was successful in 52.4% of *Borrelia*-positive *I. ricinus* ticks. The *Borrelia* species / genospecies differentiation revealed that *B. afzelii* was the most frequent species within four years of study with the prevalence ranging between 58% and 78%. The obtained results are comparable to data on questing and engorged ticks from other European countries (reviewed in 54, 23, 33, 35–37]. *Borrelia garinii* is believed to be the second dominant genospecies in *I. ricinus* ticks, followed by *B. afzelii* [55]. However, in our study, the second most frequent species were *B. burgdorferi* (10.8%), *B. garinii* (8.8%) and *B. miyamotoi* (8.4%). *Borrelia valaisiana* constituted only 5% of analyzed samples, while *B. spielmanii* and *B. lusitaniae* were even less common (1.2% vs. 0.4%, respectively). Similar *Borrelia* genospecies/ species distribution was noted in questing *I. ricinus* ticks in our previous studies (Fig. 3, [22]). The low frequency of *B. spielmanii* and *B. lusitaniae* could be explained by relatively low abundance of the competent reservoir host for those species, mainly dormice and lizards [56, 57]. Coipan et al. [58] have also shown that the infection peak in seasonal dynamics in questing ticks is different for different pathogens, including *B. afzelii* and non-*B. afzelii* spirochetes, suggesting that they were acquired from the distinct vertebrate hosts. However, we have not confirmed significant differences in *Borrelia* genospecies/ species distribution between the month of study what might be the result of limited number of non-*B. afzelii* isolates.

Interestingly, in our study *I. ricinus* ticks removed from humans were more frequently infected with *B. miyamotoi* than questing ticks (8.4% vs. 2.2%, p = 0.003) [22], whereas the latter were significantly more often infected with *B. garinii* (8.8% vs. 21.3%; p < 0.0001). Nevertheless, the questing ticks were collected between 2012 and 2015 from selected natural areas of North-Eastern Poland and urban areas of Central Poland, whereas ticks were removed from habitants of multiple regions of the country and were delivered to laboratory between 2016 and 2019. Therefore, the differences in *Borrelia* prevalence in questing and engorged ticks might be the result of specific eco-epidemiological conditions within the habitats affecting the availability and abundance of reservoir hosts for ticks as well as for *Borrelia* spirochetes. We have also observed that *B. afzelii* prevalence was noted more often in ticks removed from humans than in questing ticks (63% vs. 57%, p = 0.060). Similar results were obtained by Springer et al. [37] and Waindok et al. [33]. Coipan et al. [58] have shown that *B. afzelii* and *B. bavariensis* were significantly more frequent in human cases than in questing ticks, which is related with the fact that both are mammal-associated *Borrelia* species. Rodents are mainly reservoir hosts for *B. afzelii* as well as for *I. ricinus* larvae and nymphs; therefore, this phenomena might be also the result of spatial overlap between habitats of rodents with human activity areas and where the risk of tick bites is significant [37]. Nevertheless, no *B. bavariensis* isolates were observed in this study. It is likely due to using the single restriction enzyme DdeI which is not able to distinguish the recently described *B. bavariensis* from *B. garinii* [19]. However, the sequence analysis *Borrelia* isolates from 2016–2017 did not confirm the presence of *B. bavariensis* species.

Monitoring changes in the prevalence of different *Borrelia* genospecies/ species in ticks might be an important indicator of risk assessment and of differences in pathogenicity in humans [59]. The statistical analysis in our study has shown considerable annual variation in the frequency of non-*B. afzelii* genospecies/ species occurrence. Similar year-to-year variations were shown in *I. ricinus* ticks removed from humans in Germany [37, 38] and in questing ticks collected in Europe [9, 22, 60]. It is well-known that the distribution and prevalence of *Borrelia* spp. in ticks show significant temporal and spatial variations. Surprisingly, in our study, the annual prevalence of *B. miyamotoi* was relatively high (up to 15.8% in 2016) compared to other European studies in questing as well as feeding ticks where the prevalence usually did not exceeded 5% [19, 22, 23, 35, 61–65]. In contrast, Springer et al. [37] have confirmed *B. miyamotoi* infection in 7.4% of *I.ricinus* ticks removed from humans. Breuner et al. [66] have shown that single *I. scapularis* nymphs effectively transmit *B. miyamotoi* while feeding and transmission can occur within the first 24 h of nymphal attachment. Additionally, probably due to the overlap of endemic areas for *B. miyamotoi* with *B. burgdorferi* s.l. complex, co-infections of *B. miyamotoi* with other spirochete species in *I. ricinus* ticks and humans have been observed [22, 23, 37, 38]. Taken together, this data indicates that the risk of *B. myiamotoi* infection in Poland should not be underestimated. So far, only one case of human *B. miyamotoi* infection has been diagnosed [67]. However, Fiecek et al. [67] suggested that in case of the patients who do not meet the criteria for neuroboreliosis (presence of *B. burgdorferi* antibodies only in serum, no antibodies in PMR), *B. miyamotoi* disease should be considered. According to the National Institute of Public Health -National Institute of Hygiene in Poland (epidemiological reports), in 2013 only 14% of all reported cases with neurological symptoms (n = 1267) met the clinical and laboratory criteria of neuroborreliosis (detection of antibodies in PMR) [67].

Co-infections in ticks are frequently reported. This is likely due to a large variety of animals from which they can ingest blood, exposing the ticks to any pathogens currently infecting the hosts, including bacteria, parasites and viruses. In the present study, we have also investigated the occurrence of *Borrelia* coinfection. We have confirmed that 2% of tested *I. ricinus* ticks carried two *Borrelia* species and triple infections were detected only in 0.7% of ticks. The observed rate of coinfection prevalence was significantly lower than in feeding *I. ricinus* ticks in other European studies [33, 37]. The mechanism by which *Borrelia* co-exists with other microbial pathogens within the tick, including different *Borrelia* species, remains unexplored. Furthermore, the extent to which different *Borrelia* species or strain engage in interactions or how multi-species/strain infections might influence spirochete loads in ticks and, consequently, on transmission to humans and pathogenicity is yet to be discovered. Competition between strains of *B. burgdorferi* s.l. in the vertebrate host has been shown in field studies [68] and experimental infections [69]. Field studies on *I. ricinus* population have found in coinfected questing nymphs that the spirochete load per strain decreased with increasing strain richness, and this result provides

indirect evidence for competition [70]. Nonetheless, the low prevalence of coinfection with different *Borrelia* species has suggested that the risk of this type coinfection in humans in Poland is rather negligible.

In Europe, the majority of human babesiosis cases are caused by *Babesia divergens* [5]. However, in Poland so far only *B. microti* infections in humans have been noted [71–74]. Additionally, the molecular studies of questing *I. ricinus* ticks in Poland have shown that the *B. microti* species occurred significantly more often than *B. divergens* [75–77]. In the current study, we have confirmed the occurrence of three *Babesia* species, out two of them (*B. microti* and *B. venatorum*), are considered to be pathogenic for humans. Nonetheless, the *Babesia* prevalence in *I. ricinus* removed from humans is rather low (1.3%) and similar to other European studies on engorged as well as questing *I. ricinus* ticks [9, 35, 65, 78].

The recent studies concentrating on *Babesia microti* and *B. burgdorferi* infections in rodents and ticks have indicated that coinfection with these pathogens is common in vectors and enzootic hosts with a greater probability of coinfection than predicted by chance, and they have suggested that co-infection provides a survival advantage for both pathogens [17]. Alekseev et al. [79] went one step further and put forward that *B. microti* infection can only survive in *I. persulcatus* in combination with *Borrelia* spp. Serological studies indicate that coinfection with *B. microti* and *B. burgdorferi* is also common in humans [80]. In endemic regions in the United States, almost 40% of Lyme disease patients reported concurrent babesiosis, while up to 25% of babesiosis patients also had Lyme disease (reviewed in [17]). Co-infection in humans and animals might enhance disease severity and may have significant consequences in terms of tick-borne disease treatment and diagnosis. Moreover, babesiosis and borreliosis can present with similar clinical manifestations [17]. In our study, *Babesia*-positive *I. ricinus* ticks were significantly more often observed among *Borrelia*-positive ticks (2.7%) than among ticks non-infected with *Borrelia* (0.8%). Therefore, our results seem to confirm the presence of positive interaction among these two pathogens; however, the molecular mechanism of these facilitation remain still unclear.

In conclusion, our study confirmed relatively high *Borrelia* prevalence in ticks removed from humans with significant annual variation of spirochete genospecies/ species. In spite of low *D. reticulatus* abundance, the prevalence of *B. afzelii* in this tick species is significant. Although *B. afzelii* constitutes the majority of detected isolates, the risk of *B. miyamotoi* disease in humans should not be underestimated. Analysis of *Babesia* prevalence suggests that risk of human babesiosis is rather negligible, which is consistent with babesiosis cases reported in Poland. Even if the overall risk of developing Lyme borreliosis after a tick bite in Europe is 4% [81], the knowledge of prevalence and distribution of *Borrelia* and *Babesia* species in ticks might be an important indicator of both tick-borne disease risk assessment and varying pathogenicity in humans.

## 4. Materials And Methods

# 4.1. Ethics approval and consent to participate

Written informed consent was obtained from all individual participants included in the study. We confirmed that all experimental protocols were approved by Diagnostic Laboratory of Parasitic Diseases and Zoonotic Infections AmerLab Ltd, registered as medical entity in the National Chamber of Laboratory Diagnosticians (Poland), the University of Warsaw and the Medical University of Warsaw. We confirmed that the study was carried out under relevant guidelines and regulations (in accordance with the Resolution on the protection of animals used for scientific or educational purposes of January 15, 2015 [Journal of Laws of the Republic of Poland of 2015, item 266], and 2013 Declaration of Helsinki). We confirmed that the study were approved by the University of Warsaw and the Medical University of Warsaw.

# 4.2. Tick Collection and Identification

The ticks were delivered directly or by post to Diagnostic Laboratory of Parasitic Diseases and Zoonotic Infections AmerLab Ltd up to 5 days after removal from skin. Only ticks attached to skin were collected. The ticks were removed from habitants of multiple regions of the country and were collected from March to November in 2016–2019. Ticks were

morphologically identified in terms of species and developmental stage. Specimens that could not be identified due to extensive damage induced by the removal from the skin were not included in the study.

## 4.3. DNA Extraction and PCR Analysis

Individual larvae, nymph and adult ticks were sterilized to avoid contamination and then homogenised. Genomic DNA from ticks was isolated with Genomic Tissue Spin-Up kit (AA Biotechnology) or DNeasy Blood & Tissue Kits (Qiagen) according to the manufacturer's protocol. Genomic DNA was used for molecular screening for spirochetes by amplification flagellin gene (*flaB*) marker, with published primers [26]. Initial PCR conditions were modified as follows: initial denaturation in 95°C for 5 min, 35 cycles of denaturation in 95°C for 30 s, 30 s of primers annealing in 52°C, and elongation in 72°C for 80 s with the final elongation in 72°C for 7 min. Nested PCR was performed with minor modification: denaturation in 95°C for 20 s and annealing in 55°C for 20 s, elongation in 72°C for 60 s. For *B. miyamotoi* detection among positive samples, specific primers for flaB marker were used [22]. *Babesia* spp. were detected and identified using GR2 and GF2 primers targeting the fragment of 18S rDNA. The primers and thermal profiles used in this study were previously described [27]. Negative controls were performed in the absence of template DNA. PCR products were visualized on 1.5% agarose gels stained with Midori Green Stain (Nippon Genetics Europe, Düren, Germany).

## 4.4. *Borrelia* and *Babesia* species identification

A *Borrelia*-positive samples from ticks collected in 2016–2017 and *Babesia*-positive samples from ticks collected in 2016–2018 were sequenced by a private company (Genomed S.A., Poland) in both directions. Obtained nucleotide sequences were analyzed using BLAST NCBI and MEGA v. 7.0 software [28] for sequence alignment and species typing.

Restriction fragment length polymorphism (RFLP) was used to differentiate *Borrelia*-positive isolates at the genospecies level obtained in 2018–2019. Positive amplicons after nested-PCR were digested with the restriction enzyme HpyF3I (Thermo Fisher Scientific, USA), which recognizes the 5'C↓TNAG3' sequence [26]. The digestion was performed according to the producer's protocol in 37°C for 2 h. The enzyme was heat-inactivated at 65°C for 15 min. The digestion products were separated on 2% agarose gel, visualized and archived in the GelDoc-It imaging system (USA). The obtained restriction patterns enabled the recognition of the species of *B. burgdorferi* complex and *B. miyamotoi*.

## 4.5. Statistical Analysis

Statistical analysis was performed using IBM SPSS Statistics v. 25.0 software. Prevalence of *Borrelia* and *Babesia* infection (percentage of ticks infected) was analyzed by Maximum Likelihood techniques based on log-linear analysis of contingency tables (HILOGLINEAR). For analysis of the prevalence of *Borrelia* and *Babesia* in ticks, we fitted the prevalence of pathogens as a binary factor (infected = 1, uninfected = 0) and then year (4 levels: 2016–2019 for *Borrelia* and 3 levels: 2016–2018 for *Babesia*), month (March–November), and tick stadium (larvae, nymphs, adults). P-values < 0.05 were considered statistically significant.

## Declarations

**Conflict of interest:**

The authors declare that they have no conflict of interest.

**Author Contributions:**

RWF: conceptualization, analysis and interpretation of data, statistical analysis, supervision, writing - original draft, review & editing; AP, MB, AH, MP, ER and EM: methodology (tick collection and molecular analysis), visualization, analysis and interpretation of data, ; AP: analysis and interpretation of data, writing - original draft, review & editing. All authors read and approved the final manuscript.

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**Data Availability:**

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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# Figures

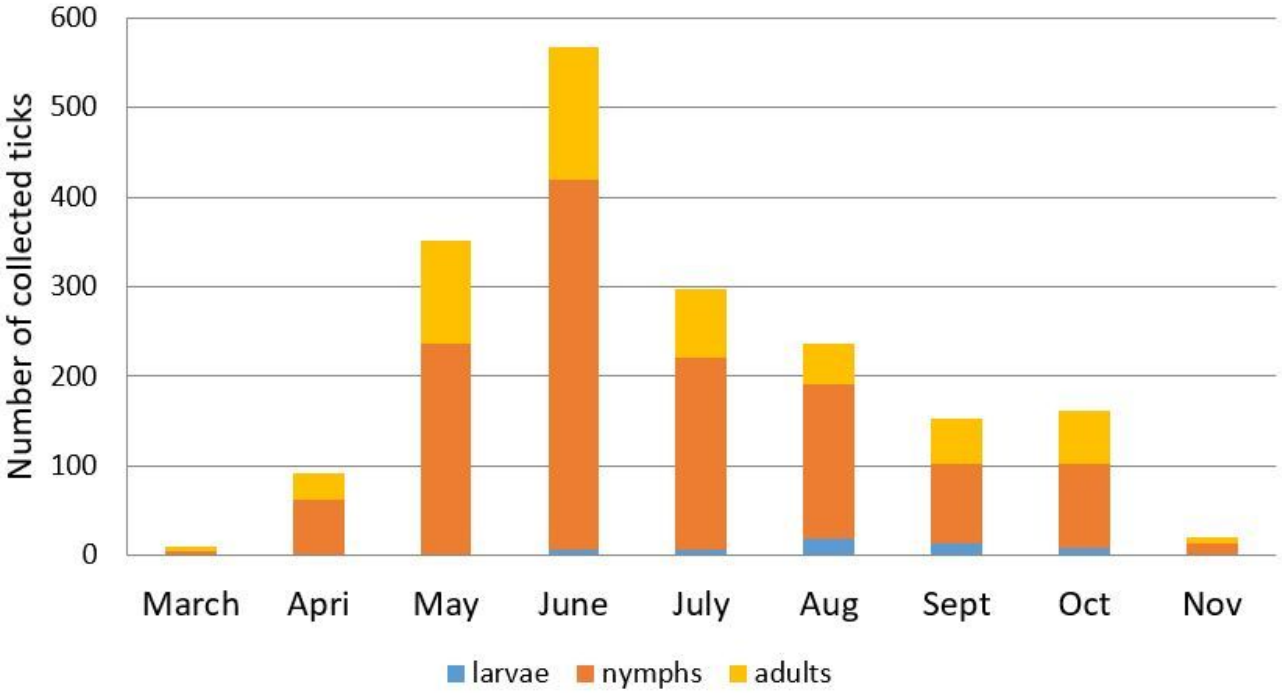
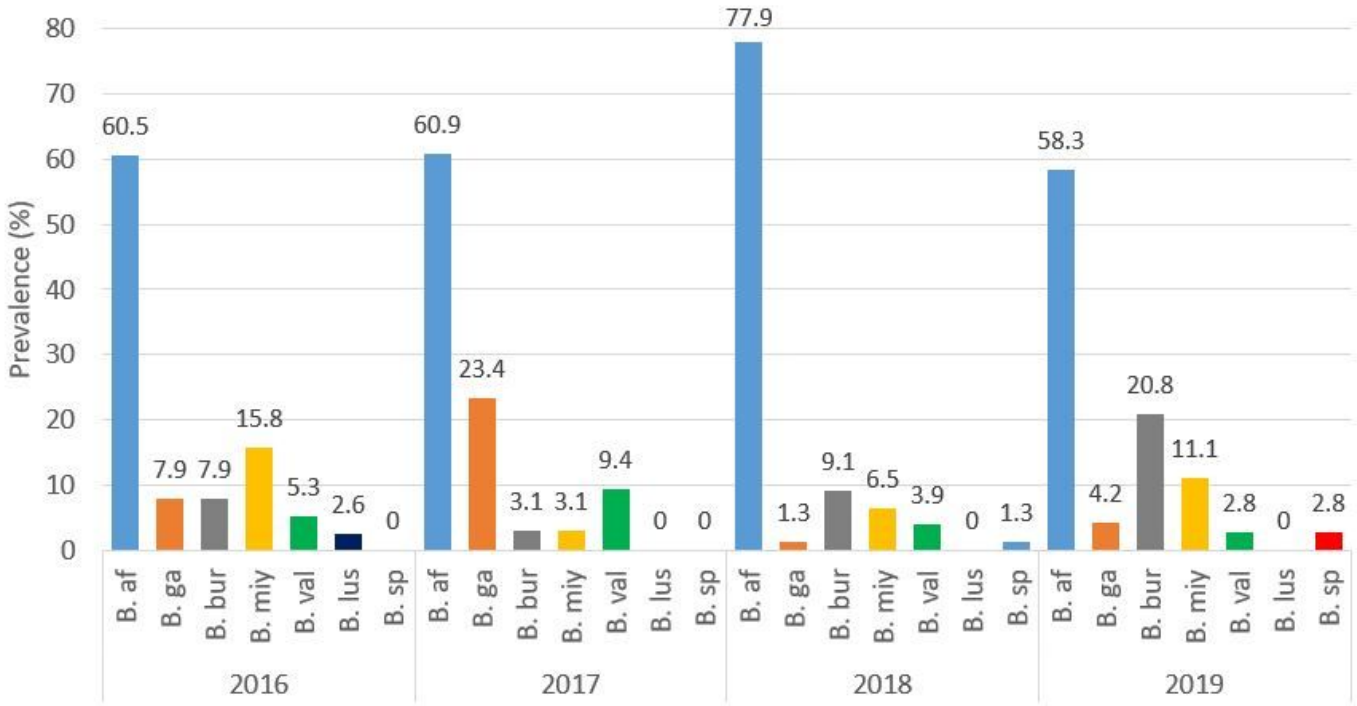



**Figure 1**

Number of I. ricinus ticks included in the study, by stage and month




## Figure 2

Borrelia burgdorferi genospecies/species distribution in different year of study in I. ricinus ticks removed from humans between 2016 and 2019

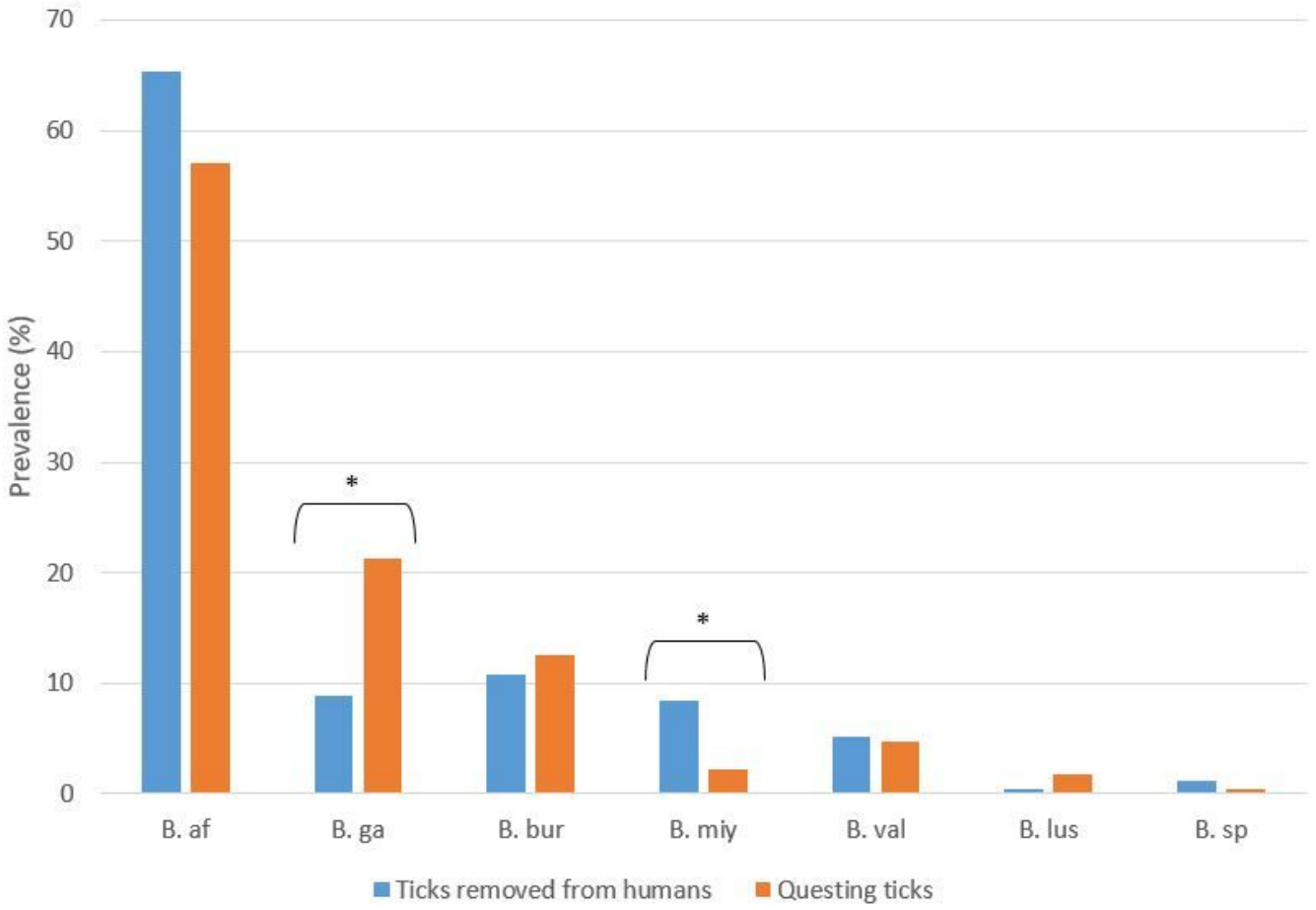



**Figure 3**

Comparison of the Borrelia genospecies/species distribution in I. ricinus ticks removed from humans between 2016 and 2019 (this study) and questing ticks collected in our previous study (Kowalec et al. 2017). Asterisks (*) indicate statistically significant differences ($p \leq 0.05$)