Article

Molecular Detection of *Borrelia burgdorferi* Sensu Lato and *Anaplasma phagocytophilum* in Ticks Collected from Dogs in Urban Areas of North-Eastern Poland

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Abstract: From 2016 to 2018, ticks were collected from 272 dogs admitted to veterinary clinics in the city of Olsztyn (north-eastern Poland). Among 522 collected ticks, 423 were identified as *Ixodes ricinus* (413 females and 10 males) and 99 as *Dermacentor reticulatus* (62 females and 37 males). Non-engorged (86 individuals) and engorged (436 individuals) ticks were screened for the presence of *Borrelia burgdorferi* sensu lato and *Anaplasma phagocytophilum* DNA. *Borrelia* and *A. phagocytophilum* species detection was determined based on the sequence of the *fla B* and 16S RNA genes, respectively. DNA of *B. burgdorferi* s.l. was identified in 31.6% (165/522, 95% CI: 27.6–35.8%) of ticks (*I. ricinus* 151/423, 35.7%, 95% CI: 31.1–40.4%; *D. reticulates* 14/99, 14.1%, 95% CI: 7.9–22.6%). *A. phagocytophilum* was identified in 0.96% (5/522, 95% CI: 0.3–2.2%) of specimens. All positive samples were engorged *I. ricinus* females (5/402, 1.2%, 95% CI: 0.4–2.9%). In 85.4% (141/165, 95% CI: 79.1–90.4%) of Borrelia infected ticks, the DNA of one genospecies was revealed. The DNA of at least two different genospecies was detected in 14.5% of specimens (24/165, 95% CI: 9.5–20.8). The coexistence of *B. burgdorferi* s.l. and *A. phagocytophilum* was not detected.

Keywords: *Borrelia burgdorferi* sensu lato; *Anaplasma phagocytophilum*; *Ixodes ricinus*; *Dermacentor reticulatus*; ticks; dogs; urban areas

1. Introduction

Along with mosquitoes, ticks are the most widespread vectors of pathogenic microorganisms (viruses, bacteria, and protozoa) for humans and domestic animals worldwide [1–3]. *Ixodes ricinus* and *Dermacentor reticulatus* are the most important tick species in Poland, but the role of *I. ricinus* in pathogen transmission is dominant [4]. One of the most frequently diagnosed zoonotic tick-borne diseases is Lyme borreliosis (LB). Worldwide, the main vectors of the *B. burgdorferi* s.l. are *I. ricinus*, *I. scapularis*, and *I. persulcatus*. In Central Europe, *I. ricinus* represents the main health risk to humans and many other vertebrate species as a vector of multiple pathogens, including *Borrelia* spirochetes [5]. The pathogens causing LB are spirochetes included in the *Borrelia burgdorferi* complex, which now comprises ca 20 *Borrelia* species. Nine of them have been detected in European *I. ricinus* ticks. The most
common genospecies of *Borrelia* in Europe are *B. afzelii*, *B. garinii*, *B. burgdorferi* sensu stricto (*B. burgdorferi* s.s.), *B. valaisiana*, and *B. lusitaniae* [6]. Three of these genospecies (*B. garinii*, *B. afzelii*, and *B. burgdorferi* s.s.) are clearly pathogenic to humans [7–9]. These species differ in organotropism and they cause different LB clinical symptoms in humans: *B. afzelii* is mainly associated with skin manifestations of LB-migratory erythema (EM) and chronic atrophic dermatitis (ACA), *B. burgdorferi* s.s. with changes in the osteoarticular system, and *B. garinii* with neurological symptoms [9].

The reservoirs of the *B. burgdorferi* spirochetes are rodents, medium-sized mammals (mainly from the Cervidae and Canidae families), birds, and lizards [10,11]. Domestic and farm animals often undergo a mild, usually undiagnosed, form of LB. Clinical LB caused by *B. burgdorferi* s.s. has nonetheless been reported in dogs, horses, and cats [12,13]. Domestic and wild animals usually play a passive role in the epizootic chain by transmitting ticks, the main vector of infection. Most often, wild animals are a reservoir of *B. burgdorferi* and they themselves show a tolerance to this bacterium. They do not get sick, but they are the source of infection for feeding ticks [11].

Anaplasmosis is a zoonotic multi-organ disease of humans and animals. This disease is caused by *Anaplasma phagocytophilum*. It is an obligatory intracellular, Gram-negative bacterium that mainly inhabits the granulocytes of peripheral blood [14,15]. *I. ricinus* is the only known vector for *A. phagocytophilum* in Central Europe [16,17]. Reservoir animals for *A. phagocytophilum* are predominantly roe deer, livestock (cattle, sheep, horses), small rodents (mice, shrews, voles), and pet animals, mainly dogs [14]. Human granulocytic anaplasmosis (HGA) may occur in the absence of associated clinical signs, and cases may not always be detected. In symptomatic patients, most present with fever, headache, fatigue or malaise, myalgia, arthralgia, and nausea. Other clinical observations in humans have included renal, pulmonary, and neurological complications, which may be accompanied by thrombocytopenia, leukopenia, and normocytic anemia [18,19]. *A. phagocytophilum* may cause canine granulocytic anaplasmosis (CGA) [20]. Most dogs naturally infected with this pathogen show no symptoms of the disease, despite serological evidence of infection [14]. After an incubation period of 1–2 weeks, the most common clinical signs are lethargy and fever. Less commonly reported symptoms also include coughing, diarrhea, anorexia, reluctance to move, lameness, enlargement of lymph nodes, pale mucous membranes, and hemorrhage [14,15,20–23].

Recreational green areas within city agglomerations could be a favorable habitat for ticks and their hosts [24–26]. In these areas, tick hosts, i.e., reservoirs of pathogens and primary sources of infection, are mainly small mammals (rodents, hedgehogs, squirrels) and birds [26,27]. A similar role of pets (dogs and cats) is highly probable [26,28,29]. In Poland, 30% of city residents declare owning a dog (in Olsztyn there are currently about 9000 dogs) [30], which can be parasitized by five tick species: *I. ricinus*, *I. hexagonus*, *D. reticulatus*, *I. crenulatus*, and *I. rugicollis* [28,31]. This justifies the regular prophylactic screening of dogs for tick infestation and for tick-borne diseases. In addition, the collection of ticks from companion animals combined with a screening for tick-borne pathogens can provide information about the potential infection risk for people [27].

*I. ricinus* has a three-host life cycle. Before it molts, it ingests the blood of another host in each life stage. In the case of *Borrelia* spp. and *A. phagocytophilum*, transovarial transmission of a pathogen is very rare and DNA detection of these agents in feeding larvae is proof of pathogen transmission from an infected reservoir host to the tick [15,32,33]. Therefore, pathogens have the ability to persist throughout the molting process to the next developmental stage of tick vectors by transstadial transmission [13]. The most numerous tick species isolated from dogs in Europe is *I. ricinus* [25,34]. Many studies have shown that adult ticks are significantly more infected with spirochetes and *A. phagocytophilum* than nymphs [5,35–38]. Additionally, dogs can be useful for collecting ticks in a way similar to flagging, and the prevalence of infection in ticks removed from dogs provides an estimate of the risk of dogs becoming infected by tick-borne disease agents [38].

*D. reticulatus*, the second most abundant tick species in many parts of Europe after *I. ricinus*, can transmit to a host the protozoa *Babesia canis*, bacteria from the *Rickettsia* and *Anaplasma* genera, or the tick-borne encephalitis virus [39]. The participation of *D. reticulatus* in the transmission of *B. burgdorferi*
s.l. is still pending, although the specific DNA of this pathogen has been detected in these ticks [40,41]. However, there is still no evidence of its role as a vector of spirochetes [4].

Based on the “One Health” theory, tick-borne diseases are associated with close relationships among ticks, humans, and companion animals. Additionally, the monitoring of tick-borne pathogens in ticks attached to animals is important to determine disease distribution and possible transmission to humans [42]. Considering the information above, the aim of this study was the detection of _B. burgdorferi_ s.l. and _A. phagocytophilum_ in non-engorged and engorged _I. ricinus_ and _D. reticulatus_ ticks collected from dogs in the urban areas of north-eastern Poland, a region endemic for tick-borne diseases.

2. Results

2.1. Tick Collection

Vets from three veterinary clinics in Olsztyn collected non-engorged and engorged ticks from dogs between spring 2016 and autumn 2018. A total of 522 adult ticks were identified: 81% _I. ricinus_ (423/522—413 females and 10 males) and 19% _D. reticulatus_ (99/522—62 females and 37 males). All of the ticks were removed from a total of 272 dogs (on average there were 1.92 ticks per dog). These ticks were stored at −4°C for further analysis.

2.2. Molecular Identification of Pathogens

Overall, 522 adult ticks were analyzed (Table 1). _B. burgdorferi_ DNA was detected in 165 (31.6%, 95% CI: 27.6–35.8%) ticks. Statistically significant differences ($\chi^2 = 350.2, p < 0.05$) were noted between the mean tick infections in _I. ricinus_ (151/423, 35.7%, 95% CI: 31.1–40.4%) and _D. reticulatus_ (14/99, 14.1%, 95% CI: 7.9–22.6%, Table 1). The percentage of _Borrelia_-positive ticks was significantly higher statistically in engorged ticks in comparison to non-engorged ticks (153/436, 35.1%, 95% CI: 30.6–39.8%; 12/86, 13.9%, 95% CI: 7.4–23.1%, respectively; $\chi^2 = 14.85, p < 0.05$, Table 1). In 85.4% (141/165, 95% CI: 79.1–90.4%) of _Borrelia_-positive ticks, the DNA of one genospecies was revealed. The DNA of at least two different genospecies was detected in 14.5% of specimens (24/165, 95% CI: 9.5–20.8%). _B. garinii_ was the predominant mono-infective species (114/141, 80.9%, 95% CI: 73.4–86.9%), while the less numerous species were _B. afzelii_ (14/141, 9.9%, 95% CI: 5.5–16.1%) and _B. burgdorferi_ s.s. (13/141, 9.2%, 95% CI: 5.0–15.2%). The results were significant at $p < 0.05$ for the occurrence of _B. garinii_ and the other two genospecies ($\chi^2 = 214.91$). _B. garinii_ and _B. afzelii_ co-occurred most often (12/24, 50%, 95% CI: 29.1–70.9%). _B. afzelii/B. burgdorferi_ s.s. (6/24, 25%, 95% CI: 9.7–46.7%) and _B. garinii/B. burgdorferi_ s.s. (5/24, 20.8%, 95% CI: 7.1–42.1%) co-occurred less frequently. There was only one case with a coinfection of all three pathogens (1/24, 4.2%, 95% CI: 0.1–21.1%; Table 1). _A. phagocytophilum_ was identified in 0.96% (5/522, 95% CI: 0.3–2.2%) of specimens. All positive samples were _I. ricinus_ (5/423, 1.2%, 95% CI: 0.4–2.9%; Table 1). Coinfections with _A. phagocytophilum_ and _B. burgdorferi_ s.l were not detected.

2.3. BLASTn Data Analysis

Comparative analysis with data registered in the GenBank database using BLASTn showed that all obtained sequences (Aph1-5: KY319143, KY828226, MK530241-MK530243) belonged to one haplotype and revealed 100% homology to the sequence of _A. phagocytophilum_ first described in a human patient from Wisconsin, USA (U02521) and the _A. phagocytophilum_ sequence from a tick removed from human skin in north-eastern Poland (DQ006828).
Table 1. Infection rates of *Ixodes ricinus* and *Dermacentor reticulatus* ticks removed from dogs in the Olsztyn-city agglomeration, north-eastern Poland, with the *Borrelia* genospecies and *Anaplasma phagocytophilum*.

| Pathogens                      | **NE** Females (% (95% CI)) | **E** Females (% (95% CI)) | **NE** Males (% (95% CI)) | **E** Males (% (95% CI)) | Total Ticks (% (95% CI)) |
|--------------------------------|-----------------------------|-----------------------------|---------------------------|--------------------------|--------------------------|
| *B. garinii*                   | 0/11 (0.0)                  | 113/402 (28.1)              | 0/10 (0.0)                | 0/34 (0.0)               | 1/37 (0.0–14.1)           |
|                                | (0.0–28.5)                  | (23.8–32.8)                 | (0.0–10.3)                | (0.0–12.3)               | (73.4–86.9)               |
| *B. afzelii*                   | 1/11 (9.1)                  | 13/402 (3.2)                | 0/10 (0.0)                | 0/34 (0.0)               | 3/37 (0.0–14.1)           |
|                                | (0.2–41.3)                  | (1.7–5.4)                   | (0.0–10.3)                | (0.0–12.3)               | (5.3–16.1)                |
| *B. burgdorferi s.s.*          | 0/11 (0.0)                  | 8/402 (2.0)                 | 0/10 (0.0)                | 0/34 (0.0)               | 5/37 (0.0–14.1)           |
|                                | (0.0–28.5)                  | (0.8–3.9)                   | (0.0–10.3)                | (0.0–12.3)               | (5.0–15.2)                |
| Borrelia Monoinfections        | 1/11 (9.1)                  | 134/402 (33.3)              | 0/10 (0.0)                | 0/34 (0.0)               | 6/37 (0.0–14.1)           |
|                                | (0.2–41.3)                  | (28.7–38.2)                 | (0.0–12.3)                | (0.0–12.3)               | (79.1–90.4)               |
| *B. garinii/B. afzelii*        | 0/11 (0.0)                  | 9/402 (2.2)                 | 0/10 (0.0)                | 3/34 (0.0)               | 12/24 (0.0–14.1)          |
|                                | (0.0–28.5)                  | (1.0–4.2)                   | (0.0–10.3)                | (8.8)                    | (50.0)                   |
| *B. garinii/B. burgdorferi s.s.* | 0/11 (0.0)               | 3/402 (0.75)                | 0/10 (0.0)                | 3/34 (0.0)               | 3/37 (0.0–14.1)           |
|                                | (0.0–28.5)                  | (0.15–2.1)                  | (0.0–12.3)                | (8.8)                    | (20.8)                   |
| *B. afzelii/B. burgdorferi s.s.* | 0/11 (0.0)               | 3/402 (0.75)                | 0/10 (0.0)                | 3/34 (0.0)               | 6/37 (0.0–14.1)           |
|                                | (0.0–28.5)                  | (0.15–2.1)                  | (0.0–12.3)                | (8.8)                    | (25.0)                   |
| *B. garinii/B. afzelii/B. burgdorferi s.s.* | 0/11 (0.0)               | 1/402 (0.25)                | 0/10 (0.0)                | 0/34 (0.0)               | 0/37 (0.0–14.1)           |
|                                | (0.0–28.5)                  | (0.006–1.4)                 | (0.0–12.3)                | (0.0)                    | (4.1)                    |
| Borrelia Coinfections          | 0/11 (0.0)                  | 16/402 (4.0)                | 0/10 (0.0)                | 3/34 (0.0)               | 11/24 (0.0–14.1)          |
|                                | (0.0–28.5)                  | (2.6–5.4)                   | (0.0–12.3)                | (8.8)                    | (14.5)                   |
| Borrelia Total                 | 1/151/423 (0.0–14.1)        | 150/436 (34.4)              | 3/34 (0.0–12.3)           | 4/51 (0.0–14.1)          |
|                                | (15.7) (31.1–40.4)          | (29.9–39.1)                 | (0.7) (0.14–2.0)          | (4.5–28.8)               |
| Borrelia Total **E**           | 1/214 (0.96)                | 11/65 (6.2)                 | 2/123 (1.9)               | 5/52 (0.96)              |
|                                | (4.7) (21.2–23.8)           | (16.9) (87.2–28.2)          | (16.9) (87.2–28.2)        | (16.9) (87.2–28.2)        |
| A. phagocytophilum              | 0/11 (0.0)                  | 5/402 (1.2)                 | 0/10 (0.0)                | 0/34 (0.0)               | 5/37 (0.0–14.1)           |
|                                | (0.0–28.5)                  | (0.4–2.9)                   | (0.0–10.3)                | (0.0–9.5)                | (3.2–2.2)                |

*NE—non-engorged ticks, **E—engorged ticks; \(^1\) \chi^2 = 350.2, p < 0.05; \(^2\) \chi^2 = 14.85, p < 0.05; \(^3\) \chi^2 = 214.91, p < 0.05.

3. Discussion

Tick infestation and the risk of tick-borne diseases are commonly connected with forested and rural areas. However, many reports indicate that ticks are well adapted to urban and suburban environments [24,26,43,44]. Ticks that inhabit urban localities originate from tick populations persisting in wild natural habitats around cities and towns, and environmental conditions in both localities, urban and natural, are suitable and promote the development of a tick population [45,46]. In our opinion, in green areas located within the administrative boundaries of large cities, not only are residents exposed to the ticks and the pathogens transmitted by them, but so are their pets, such as domestic dogs and cats.

Lyme disease also has veterinary importance, affecting dogs, cattle, horses, and cats. The most common clinical signs in domestic animals are lameness, loss of appetite, weight loss, and kidney...
17% of ticks. In Poland, a study was conducted on ticks collected in 2013 from domestic dogs and cats widespread in most of Europe, because the pathogen incidence in tick-carriers ranges from moderate to high, with the median prevalence of \textit{Borrelia} spp. spirochetes was 27.4% in Olsztyn, and the dominant genospecies was \textit{B. afzelii} (93.1%). It is noteworthy that they collected ticks by flagging while we specifically studied the ticks that were engaged (12%) compared to semi-engorged ticks (2%), explaining this phenomenon by loss of infectivity during the start of the feeding phase. According to De la Fuente et al. [53], pathogen transmission by ticks requires many unexplored tick–pathogen interactions, from the migration of these pathogens from the gut to their secretion in tick saliva. It is possible that the infection, although largely unrecognized, may be transmitted as quickly as the other two genospecies during tick feeding.

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The infestation rates of domestic animals with pathogen-infected ticks are poorly documented in Europe, including in Poland. Even fewer publications report on the proportions of pathogen-infected ticks removed from animal or human skin. As early as the 1990s, in Germany (North Baden), 22% of \textit{I. ricinus} and \textit{I. hexagonus} removed from domestic animals were infected by \textit{B. burgdorferi}, while in Lower Saxony, fewer than 10% of all human skin-attached ticks were \textit{Borrelia}-positive. The PCR method was used in both studies using 23S rRNA and 16S rRNA genes, respectively [48,49]. In the latest research from Germany (2013–2017), the overall \textit{Borrelia} infection rate of \textit{I. ricinus} ticks attached to human skin by real-time PCR was 20.02% [50]. In 2016 in Great Britain, molecular analysis of the \textit{ospA} gene confirmed that 1.8% of ticks collected from cat skin were \textit{Borrelia}-positive, and the most frequent species was \textit{B. garinii} [51]. In 2018 Geurden et al. [43] analyzed the prevalence of \textit{Borrelia} spp. and \textit{A. phagocytophilum} by the real-time PCR method. Ticks were collected from dogs and cats in Hungary, France, Italy, Belgium, and Germany. \textit{Borrelia} spp. were mainly identified in \textit{I. ricinus} collected from cats (18%) and to a lesser extent in dog-sourced ticks (1%); while \textit{A. phagocytophilum} was also found in 17% of ticks. In Poland, a study was conducted on ticks collected in 2013 from domestic dogs and cats in the Wroclaw Agglomeration (south-west Poland). Using nPCR, the authors revealed that 22.5% and 21.3% of the ticks were \textit{Borrelia}-positive, respectively. A comparable level of \textit{Borrelia} infection between \textit{I. ricinus} from pets and vegetation indicates that domestic animals may participate in the circulation of these pathogens, and that they do not have zooprophylactic competence [28]. Moreover, it is very well documented that there is a possibility of cross-infection (including \textit{B. burgdorferi} and \textit{A. phagocytophilum}) when multiple ticks, infected and non-infected specimens, are co-feeding on one animal [52].

We assumed that adult feeding ticks are characterized by the highest degree of infection due to the transstadial transmission of pathogens. It is known that engorged ticks cause problems in molecular testing. Substances present in mammalian blood can inhibit the PCR amplification. Beichel et al. [48] pointed out that ticks larger than 4 mm inhibit the PCR reaction. Therefore, in our study, only the anterior parts of engorged ticks were analyzed. We have shown that from 165 cases of \textit{Borrelia} spp., as many as 153 cases were from engorged ticks and only 12 came from non-engorged ticks. The study of Scott et al. [8] showed that 36% of \textit{I. scapularis} adults collected from 41 mammalian hosts (dogs, cats, humans) were positive for the Lyme disease bacterium. Actually, 35.7% of the examined \textit{I. ricinus} and 14.1\% \textit{D. reticulatus} collected in the urban area of Olsztyn tested positive for the DNA of \textit{Borrelia} spp. spirochetes. The dominant species of \textit{B. garinii} was detected in over 80% of cases. In some contrast to our results, Kubiak et al. [44] showed that the overall infection rate of questing \textit{I. ricinus} with \textit{Borrelia} spirochetes was 27.4% in Olsztyn, and the dominant genospecies was \textit{B. afzelii} (93.1%). It is noteworthy that they collected ticks by flagging while we specifically studied the ticks that were actively parasitizing dogs. Perhaps dogs in Olsztyn are mainly a reservoir of \textit{B. garinii}, but this should be confirmed by a city-wide dog blood test for the presence of DNA from the \textit{Borrelia} genospecies. This may be due to the different mechanism of transmission of the spirochete genospecies. Hovius et al. [38] observed a higher infestation of \textit{B. burgdorferi} s.s. in non-engorged (either questing or attached) \textit{I. ricinus} (12%) compared to semi-engorged ticks (2%), explaining this phenomenon by loss of infectivity during the start of the feeding phase. According to De la Fuente et al. [53], pathogen transmission by ticks requires many often unexplored tick–pathogen interactions, from the migration of these pathogens from the gut to their secretion in tick saliva. It is possible that \textit{B. garinii} is not transmitted as quickly as the other two genospecies during tick feeding.

HGA, a zoonotic acute febrile disease, is difficult to recognize because its symptoms are rather nonspecific. European studies warn that the infection, although largely unrecognized, may be widespread in most of Europe, because the pathogen incidence in tick-carriers ranges from moderate to high, with the median prevalence of \textit{A. phagocytophilum} in European \textit{I. ricinus} ticks at approximately 3\% [18,54,55]. In our study, only 5 out of 402 (1.2\%) engorged \textit{I. ricinus} females showed the presence of \textit{A. phagocytophilum} DNA. The prevalence of \textit{A. phagocytophilum} was 6\% in the DNA of adult \textit{I. ricinus} ticks collected from dogs in Latvia, whereas in \textit{I. persulcatus} and \textit{D. reticulatus}, the pathogen was not
identified [56]. Even though similar studies are scarce, our data did seem rather low for the country. This is because in the urban areas of Wroclaw, 21.3% I. ricinus and 8.1% I. hexagonus ticks isolated from dogs and cats were A. phagocytophilum-positive [29]. This was higher than our rates of tick infection with A. phagocytophilum from 8.7% to 16.0%. These were also recorded in the north-eastern regions of Poland at the beginning of the century, and an extremely rapid increase was observed over the next few years of research, in some locations from 6.6% to a stunning 73.3% [37]. It is also important to note that in Poland, A. phagocytophilum more frequently infects ticks in urban areas than in natural forests [57]. Similar findings were also reported by Grzeszczuk and Stańczak [58] who published extremely disturbing data that 23.7% of A. phagocytophilum-positive I. ricinus ticks were removed from the skin of Bialystok residents (north-eastern Poland).

Nucleotide analysis of the partial sequence of the Anaplasma 16S rRNA gene showed that all amplified sequences belong to one haplotype. BLASTn analysis revealed a 100% similarity with the sequence of A. phagocytophilum from the first described human patient in Wisconsin (USA) and also with A. phagocytophilum from ticks removed from human skin in north-eastern Poland [58,59]. These results indicate that the Aph1-5 haplotype detected in I. ricinus collected from dogs in Olsztyn represents A. phagocytophilum, and that HGA in urban dogs may be significant in the future.

We also addressed the coexistence of B. burgdorferi s.l. and A. phagocytophilum in ticks, because in Pomerania (Pomorze, northern Poland) 5% of examined I. ricinus ticks contained both pathogens. Moreover, research in the urban and suburban forests in the Tri-City area of Gdańsk, Gdynia, and Sopot (northern Poland) showed the simultaneous presence of both pathogens in 8.3% of adult ticks. It is possible that B. burgdorferi s.l. and A. phagocytophilum consolidate in the same foci and often co-infect the same tick vector, thus increasing the risk of a mixed infection [60,61]. At the present time in Olsztyn, we have not observed such a dependence, most likely due to the low incidence (0.96%) of A. phagocytophilum. However, the epidemiological situation should be closely monitored, especially in the areas considered as endemic for tick-borne diseases.

4. Materials and Methods

4.1. Study Area and Tick Collection

For the analysis we used ticks collected from domestic dogs visiting veterinary clinics in the Olsztyn agglomeration (53°47′ N 20°29′ E; 88.33 km²; 173,070 inhabitants) between 2016 and 2018. Olsztyn is the capital of the Warmia-Masuria Province in north-eastern Poland. Within the city there are a large number of parks, squares, and forested recreational areas, which occupy a quarter of the city. These areas also provide an excellent habitat for ticks. Ticks were collected from the dogs by veterinarians and were placed in tubes with 70% ethanol. In the laboratory, the species and sex of the ticks were identified, then, individuals were weighed, measured, and separated into non-engorged and engorged categories. To the non-engorged I. ricinus female group, those weighing on average 2.13 ± 1.15 mg and measuring 3.63 ± 0.50 mm were included; while the engorged group weighed on average 104.26 ± 112.29 mg and measured 7.84 ± 2.36 mm. Non-engorged I. ricinus males weighed and measured 0.94 ± 0.2 mg and 2.7 ± 0.48 mm accordingly, while non-engorged D. reticulatus included individuals weighing on average 4.94 ± 0.98 mg and 4.69 ± 1.45 mg, and measuring 4.64 ± 0.49 mm and 4.38 ± 0.46 mm, for females and males, respectively. Those classified as D. reticulatus engorged females were the largest of the analyzed ticks. They weighed and measured on average 220.29 ± 140.65 mg and 11.2 ± 2.68 mm.

4.2. DNA Extraction

To start, the ticks preserved in 70% ethanol were dried. Fully-engorged ticks were bisected, only the anterior end was used. Anterior parts or entire ticks were then crushed using a sterile mortar, moved to 2 mL tubes filled with lysis buffer (A&A Biotechnology, Gdynia, Poland) and were incubated
for 2 h at 50 °C. Total DNA was extracted according to the manufacturer’s tissue protocol (Micro AX Tissue Gravity, A&A Biotechnology, Gdynia, Poland) and stored at −70 °C.

4.3. PCR Conditions

The Borrelia region of the fla B gene (422 bp) was amplified using the primer BFL1/BFL2 [62] (Table 2) and PCR was run under the following thermal cycle conditions: an initial activation step of 2 min at 94 °C was followed by 40 cycles of 30 s at 94 °C, 1 min at 58 °C, and 1 min at 72 °C. Finally, an extension step of 1 min was performed at 72 °C. For A. phagocytophilum, the primer sets EHR521 and EHR747 [63] (Table 2) were used to amplify a 247 bp fragment from the 16S RNA gene under the following thermal cycle conditions: an initial activation step of 5 min at 94 °C was followed by 40 cycles of 45 s at 94 °C, 45 s at 54 °C, 45 s at 72 °C, and a final extension of 5 min at 72 °C. In both cases, 5 µL of the DNA extracted from each tick was added to 20 µL of reaction mixture comprised of 12.5 µL DreamTaq Green PCR Master MIX (Thermo Scientific, Waltham, MA, USA), 9.4 µL nuclease-free water, and 0.05 µL of each primer (100 µM). All reactions were carried out using a Mastercycler Nexus (Eppendorf, Hamburg, Germany). PCR products were visualized by electrophoresis on 1.5% agarose gel stained with Midori Green DNA dye (Nippon Genetics Europe GmbH, Düren, Germany). Positive tick samples were analyzed twice. Each PCR analysis included negative (nuclease-free water instead of DNA) and positive control samples. The positive control was commercial DNA from B. burgdorferi s.l. purchased from the DNA Gda´ńsk Company (Gda´ńsk, Poland). Positive control samples for the A. phagocytophilum 16S rRNA gene included purified and confirmed samples obtained by sequencing genomic DNA from previously positive samples.

Table 2. Primer sets and TasI restriction patterns of BFL1/BFL2 products generated from the fla gene fragment of Borrelia DNA.

| Primer Name | Primer Sequence 5′→3′ | Product Size [bp] | Species          | Reference |
|-------------|-----------------------|-------------------|------------------|-----------|
| BFL1        | GCTCAATATAACCAAATGCACATG | 442               | B. burgdorferi s.l. | [62]      |
| BFL2        | CAAGTCTATTTTGGAAAGCACCTAA |                   |                  |           |
| EHR521      | TGTAGGCGGTTCGGTAAGTTAAAG | 247               | A. phagocytophilum | [63]      |
| EHR747      | GCACCTCATCGTATACACGGTG |                   |                  |           |

4.4. PCR-RFLP Analysis

PCR-RFLP analysis was carried out to identify three genospecies containing the B. burgdorferi s.l. complex: B. garinii, B. afzelii, and B. burgdorferi s.s. The amplified DNA was digested with the TasI endonuclease (Fast Digest Tsp 509I, Thermo Fisher Scientific, Waltham, MA, USA) to obtain the restriction patterns of the different genospecies of the spirochetes. For each positive sample, 10 µL of amplified DNA were digested in a Mastercycler Nexus (Eppendorf, Hamburg, Germany) at 65 °C for 15 min. The reaction mixture contained 17 µL of nuclease-free water, 2 µL of 10 × Fast-Digest buffer, and 1 µL of TasI enzyme. PCR-RFLP products were separated by electrophoresis in a 3% agarose gel and stained with Midori Green dye. DNA fragments subjected to restriction analysis were visualized in the G-BOX Syngene transilluminator. Membership in a given genospecies was determined by TasI restriction fragment sizes (bp) according to restriction patterns (Table 2) [62].

4.5. DNA Sequencing and Data Analysis

PCR products of the positive Anaplasma samples and the chosen positive products for Borrelia spirochetes were purified using the Clean Up purification kit (A&A Biotechnology, Gdynia, Poland) according to the manufacturer’s protocol and bidirectionally sequenced at Genomed (Warsaw, Poland). The obtained nucleotide sequences were assembled and compared with data registered in the
GenBank database using BLASTn (https://blast.ncbi.nlm.nih.gov) to confirm the attachment to the A. phagocytophilum or B. burgdorferi s. l. complex. The consensus A. phagocytophilum 16S rRNA gene and the Borrelia fla gene sequences were deposited in the GenBank database and registered under the access numbers KY319143, KY828226, MK530241-MK530243 (Aph1-5; A. phagocytophilum 16 S rRNA subunit gene), MK834321-MK834322 (Borrelia sp. fla gene—B. garinii), MK834319-MK834320 (Borrelia sp. fla gene—B. afzelii), and MK834317-MK834318 (Borrelia sp. fla gene—B. burgdorferi s.s.).

4.6. Statistical Analysis

All ticks were analyzed individually, and the prevalence was expressed as a percentage. Statistical analysis of the results was carried out using the two-sided Fisher’s exact test (Prism 7 program, GraphPad Software, San Diego, CA, USA). Prevalence of pathogens was calculated with 95% confidence intervals (95% CI). In order to check whether there was a prevalence of Borrelia in both tested tick species or non-engorged and engorged ticks and a relationship between variables (the occurrence of B. garinii and the other two genospecies), the Chi-square test (χ²) was used. Values of $p < 0.05$ were considered statistically significant.

5. Conclusions

This study indicates that domestic dogs in north-eastern Poland are at risk of infection from Borrelia species and A. phagocytophilum. The results of this study demonstrate the potential danger from ticks feeding on dogs. Ticks represent a serious risk of LB for companion animals and for city residents. There is a high percentage of Borrelia-positive ticks in the city of Olsztyn (north-eastern Poland); A. phagocytophilum-positive ticks were identified less frequently. However, veterinarians and physicians should be aware of anaplasmosis among domestic animals and among patients with a tick-bite history.

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References

1. Bengis, R.G.; Leighton, F.A.; Fischer, J.R.; Artois, M.; Mörner, T.; Tate, C.M. The role of wildlife in emerging and re-emerging zoonoses. Rev. Sci. Tech. 2004, 23, 497–511. [CrossRef] [PubMed]
2. Dantas-Torres, F.; Chomel, B.B.; Otranto, D. Ticks and tick-borne diseases: A one health perspective. Trends Parasitol. 2012, 28, 437–446. [CrossRef]
3. Jongejan, F.; Uilenberg, G. The global importance of ticks. Parasitology 2004, 129, 3–14. [CrossRef] [PubMed]
4. Kubiak, K.; Sielawa, H.; Dziekorińska-Rynko, J.; Kubiak, D.; Rydzewska, M.; Dzika, E. Dermaeceptor reticulatus ticks (Acari: Ixodidae) distribution in north-eastern Poland: An endemic area of tick-borne diseases. Exp. Appl. Acarol. 2018, 75, 289–298. [CrossRef] [PubMed]
5. Venclová, K.; Betášová, L.; Šikutová, S.; Jedličková, P.; Hubálek, Z.; Rudolf, I. Human pathogenic borreliae in Ixodes ricinus ticks in natural and urban ecosystem (Czech Republic). Acta Parasitol. 2014, 59, 717–720. [CrossRef] [PubMed]
6. Rauter, C.; Hartung, T. Prevalence of Borrelia burgdorferi sensu lato genospecies in Ixodes ricinus ticks in Europe: A metaanalysis. Appl. Environ. Microbiol. 2005, 71, 7203–7216. [CrossRef]
7. Križ, B.; Malý, M.; Balátová, P.; Kodym, P.; Kurzová, Z.; Daniel, M.; Kybicová, K. A serological study of antibodies to *Anaplasma phagocytophila* and *Borrelia burgdorferi* sensu lato in the sera of healthy individuals collected two decades apart. *Acta Parasitol.* 2018, 63, 33–39. [CrossRef]

8. Scott, J.D.; Foley, J.E.; Anderson, J.F.; Clark, K.L.; Durden, L.A. Detection of Lyme disease bacterium, *Borrelia burgdorferi* sensu lato, in blacklegged ticks collected in the Grand River Valley, Ontario, Canada. *Int. J. Med. Sci.* 2017, 14, 150–158. [CrossRef]

9. Stanek, G.; Reiter, M. The expanding Lyme *Borrelia* complex—Clinical significance of genomic species? *Clin. Microbiol. Infect.* 2011, 17, 487–493. [CrossRef]

10. Gil, H.; Barral, M.; Escudero, R.; García-Pérez, A.L.; Anda, P. Identification of a new *Borrelia* species among small mammals in areas of Northern Spain where Lyme disease is endemic. *Appl. Environ. Microbiol.* 2005, 71, 1336–1345. [CrossRef]

11. Tälleklint, L.; Jaenson, T.G. Transmission of *Borrelia burgdorferi* s.l. from mammal reservoirs to the primary vector of Lyme borreliosis, *Ixodes ricinus* (Acari: Ixodidae), in Sweden. *J. Med. Entomol.* 1994, 31, 880–886. [CrossRef]

12. Hovius, K.E.; Stark, L.A.; Bleumink-Pluym, N.M.; Van de Pol, I.; Verbeek-de Kruif, N.; Rijpkema, S.G.; Schouls, L.M.; Houwers, D.J. Presence and distribution of *Borrelia burgdorferi* sensu lato species in internal organs and skin of naturally infected symptomatic and asymptomatic dogs, as detected by polymerase chain reaction. *Vet. Q.* 1999, 21, 54–58. [CrossRef] [PubMed]

13. Sprong, H.; Dieuwertje Hoornstra, A.A.; Nijhof, A.M.; Knorr, S.; Baarsma, M.W.; Hovius, J.W. Control of Lyme borreliosis and other *Ixodes ricinus*-borne diseases. *Parasites Vectors* 2018, 11, 145. [CrossRef]

14. Carrade, D.D.; Foley, J.E.; Borjesson, D.L.; Sykes, J.E. Canine granulocytic anaplasmosis: A review. *J. Vet. Int. Med.* 2009, 23, 1129–1141. [CrossRef]

15. Dumler, J.S.; Barbet, A.F.; Bekker, C.P.; Dasch, G.A.; Palmer, G.H.; Ray, S.C.; Rikihisa, Y.; Rurangirwa, F.R. Reorganization of genera in the families *Rickettsiaceae* and *Anaplasmataceae* in the order *Rickettsiales*: Unification of some species of *Ehrlichia* with *Anaplasma*, *Cowdria* with *Ehrlichia* and *Ehrlichia* with *Neorickettsia*, descriptions of six new species combinations and designation of *Ehrlichia equi* and ‘HGE agent’ as subjective synonyms of *Ehrlichia phagocytophila*. *Int. J. Syst. Evol. Microbiol.* 2001, 51, 2145–2165. [CrossRef] [PubMed]

16. Sainz, A.; Roura, X.; Miró, G.; Estrada-Peña, A.; Kohn, B.; Harrus, S.; Solano-Gallego, L. Guideline for veterinary practitioners on canine ehrlichiosis and anaplasmosis in Europe. *Parasites Vectors* 2015, 8, 75. [CrossRef] [PubMed]

17. Stirle, F. Human granulocytic ehrlichiosis in Europe. *Int. J. Med. Microbiol.* 2004, 293, 27–35. [CrossRef]

18. Bakken, J.S.; Dumler, J.S. Human granulocytic anaplasmosis. * Infect. Dis. Clin. N. Am.* 2015, 29, 341–355. [CrossRef] [PubMed]

19. Bakken, J.S.; Kreuth, J.; Wilson-Nordskog, C. Clinical and laboratory characteristics of human granulocytic ehrlichiosis. *JAMA* 1996, 275, 199–205. [CrossRef] [PubMed]

20. Kohn, B.; Galke, D.; Beelitz, P.; Pfister, K. Clinical features of canine granulocytic anaplasmosis in 18 naturally infected dogs. *J. Vet. Intern. Med.* 2008, 22, 1289–1295. [CrossRef]

21. Egenvall, A.E.; Hedhammar, A.A.; Bjöersdorff, A.I. Clinical features and serology of 14 dogs affected by granulocytic ehrlichiosis in Sweden. *Vet. Rec.* 1997, 140, 222–226. [CrossRef]

22. Poitout, F.M.; Shinozaki, J.K.; Stockwell, P.J.; Holland, C.J.; Shukla, S.K. Genetic variants of *Anaplasma phagocytophilum* infecting dogs in Western Washington State. *J. Clin. Microbiol.* 2005, 43, 796–801. [CrossRef]

23. Tarello, W. Canine granulocytic ehrlichiosis (CGE) in Italy. *Acta Vet. Hung.* 2003, 51, 73–90. [CrossRef] [PubMed]

24. Kowalczyk, M.; Szweczyk, T.; Welc-Fałęciak, R.; Siński, E.; Karbowiak, G.; Bajer, A. Ticks and the city—Are there any differences between city parks and natural forests in terms of tick abundance and prevalence of *spondiohaetae*? *Parasites Vectors* 2017, 10, 573. [CrossRef] [PubMed]

25. Michalski, M.M. Composition of tick species (Acari: Ixodidae) on dogs in the urban agglomeration—A multi-year study. *Med. Weter.* 2017, 73, 698–701. [CrossRef]

26. Uspensky, I. Tick pests and vectors (Acari: Ixodoidea) in European towns: Introduction, persistence and management. *Ticks Tick Borne Dis.* 2014, 5, 41–47. [CrossRef] [PubMed]

27. Rizzoli, A.; Silaghi, C.; Obiogala, A.; Rudolf, I.; Hubálek, Z.; Földvári, G.; Plantard, O.; Vayssier-Taussat, M.; Bonnet, S.; Špišalká, E.; et al. *Ixodes ricinus* and its transmitted pathogens in urban and peri-urban areas in Europe: New hazards and relevance for public health. *Front. Public Health* 2014, 2, 25. [CrossRef]
28. Król, N.; Kiewra, D.; Szymanowski, M.; Lonc, E. The role of domestic dogs and cats in the zoonotic cycles of ticks and pathogens. Preliminary studies in the Wrocław Agglomeration (SW Poland). Vet. Parasitol. 2015, 214, 208–212. [CrossRef]

29. Król, N.; Obiegała, A.; Pfeffer, M.; Lonc, E.; Kiewra, D. Detection of selected pathogens in ticks collected from cats and dogs in the Wrocław agglomeration, South-West Poland. ParasitES Vectors 2016, 9, 351. [CrossRef]

30. Kantar Public. 2017. Available online: http://www.tnsglobal.pl/archiwumraportow/files/2017/05/K.021_Zwierza domowe_O04a-17.pdf+&ecd=1&hl=pl&ct=clnk&gl=pl (accessed on 31 May 2017). (In Polish)

31. Nowak-Chmura, M.; Siuda, K. Ticks of Poland. Review of contemporary issues and latest research. Ann. Parasitol. 2012, 58, 125–155.

32. Zhioua, E.; Aeschlimann, A.; Gern, L. Infection of field-collected Ixodes ricinus (Acari: Ixodidae) larvae with Borrelia burgdorferi in Switzerland. J. Med. Entomol. 1994, 31, 763–766. [CrossRef] [PubMed]

33. Franke, J.; Fritsch, J.; Tomas, H.; Straube, E.; Dorn, W.; Hildebrandt, A. Coexistence of pathogens in host-seeking and feeding ticks within a single natural habitat in central Germany. Appl. Environ. Microbiol. 2010, 76, 6829–6836. [CrossRef]

34. Krčmar, S.; Ferizbegović, J.; Lon, E.; Kamberović, J. Hard tick infestation of dogs in the Tuzla area (Bosnia and Herzegovina). Vet. Archiv 2014, 84, 177–182.

35. Jouda, F.; Perret, J.-L.; Gern, L. Density of questing Ixodes ricinus nymphs and adults infected by Borrelia burgdorferi sensu lato in Switzerland. Spatio-temporal pattern at a regional scale. Vector Borne Zoonotic Dis. 2004, 4, 23–32. [CrossRef] [PubMed]

36. Grzeszczyk, A. Anaplasma phagocytophilum in Ixodes ricinus ticks and human granulocytic anaplasmosis seroprevalence among forestry rangers in Białystok region. Adv. Med. Sci. 2006, 51, 283–286. [PubMed]

37. Grzeszczyk, A.; Stańczak, J. Highly variable year-to-year prevalence of Anaplasma phagocytophilum in Ixodes ricinus ticks in northeastern Poland: A 4-year follow-up. Ann. N. Y. Acad. Sci. 2006, 1078, 309–311. [CrossRef]

38. Hovius, K.E.; Beijer, B.; Rijpkema, S.G.; Bleumink-Pluym, N.M.; Houwers, D.J. Identification of four Borrelia burgdorferi sensu lato species in Ixodes ricinus ticks collected from dutch dogs. Vet. Q. 1998, 20, 143–145. [CrossRef]

39. Földvári, G.; Široký, P.; Szekeres, S.; Majors, G.; Sprang, H. Dermacentor reticulatus: A vector on the rise. Parasites Vectors 2016, 9, 314. [CrossRef]

40. Reye, A.L.; Stegniy, V.; Mishaeva, N.P.; Velhin, S.; Hübschen, J.M.; Gnatyev, G.; Muller, C.P. Prevalence of tick-borne pathogens in Ixodes ricinus and Dermacentor reticulatus ticks from different geographical locations in Belarus. PLoS ONE 2013, 8, e54476. [CrossRef]

41. Mierzewska, E.J.; Pawelczyk, A.; Radkowski, M.; Welc-Faleciak, R.; Bajer, A. Pathogens vectored by the tick, Dermacentor reticulatus, in endemic regions and zones of expansion in Poland. Parasites Vectors 2015, 8, 490. [CrossRef] [PubMed]

42. Lee, S.-H.; Goo, Y.-K.; Geraldino, P.J.L.; Kwon, O.-D.; Kwak, D. Molecular detection and characterization of Borrelia garinii (Spirochaetales: Borreliaeidae) in Ixodes nipponensis (Ixodida: Ixodidae) parasitizing a dog in Korea. Pathogens 2019, 8, 289. [CrossRef]

43. Geurden, T.; Becskei, C.; Six, R.H.; Maeder, S.; Latrofa, M.S.; Otranto, D.; Farkas, R. Detection of tick-borne pathogens in ticks from dogs and cats in different European countries. Ticks Tick Borne Dis. 2018, 9, 1431–1436. [CrossRef]

44. Kubiak, K.; Dziekońska-Rynko, J.; Szymańska, H.; Kubiak, D.; Dmitrijuk, M.; Dzika, E. Questing Ixodes ricinus (Acari, Ixodidae) as a vector of Borrelia burgdorferi sensu lato and Borrelia miyamotoi in an urban area of north-eastern Poland. Exp. Appl. Acarol. 2019, 78, 113–126. [CrossRef]

45. Blanton, L.S.; Walker, D.H.; Bouyer, D.H. Rickettsiae and ehrlichiae within a city park: Is the urban dweller dwelling at risk? Vector Borne Zoo. Dis. 2014, 14, 168–170. [CrossRef]

46. Brites-Neto, J.; Brasil, J. Epidemiological monitoring of ticks in public woods in a risk area for Brazilian Spotted Fever. Bol. Epidemiol. Paul. 2014, 11, 7–15. [CrossRef]

47. Spolidorio, M.G.; Labruna, M.B.; Machado, R.Z.; Moraes-Filho, J.; Zago, A.M.; Donatele, D.M.; Pinheiro, S.R.; Silveira, J.; Caliani, K.M.; Yoshinari, N.H. Survey for tick-borne zoonoses in the State of Espirito Santo, Southeastern Brazil. Am. J. Trop. Med. Hyg. 2010, 83, 201–206. [CrossRef]

48. Beichel, E.; Petney, T.N.; Hassler, D.; Brückner, M.; Maiwald, M. Tick infestation patterns and prevalence of Borrelia burgdorferi in ticks collected at a veterinary clinic in Germany. Vet. Parasitol. 1996, 65, 147–155. [CrossRef]
49. Liebisch, G.; Sohns, B.; Bautsch, W. Detection and typing of *Borrelia burgdorferi* sensu lato in *Ixodes ricinus* ticks attached to human skin by PCR. *J. Clin. Microbiol.* 1998, 36, 3355–3358. [CrossRef]

50. Springer, A.; Raulf, M.-K.; Fingerle, V.; Strube, C.H. *Borrelia* prevalence and species distribution in ticks removed from humans in Germany, 2013–2017. *Ticks Tick Borne Dis.* 2020, 11, 101363. [CrossRef]

51. Davies, S.; Abdullah, S.; Helps, C.; Tasker, S.; Newbury, H.; Wall, R. Prevalence of ticks and tick-borne pathogens: *Babesia* and *Borrelia* species ticks infesting cats of Great Britain. *Vet. Parasitol.* 2017, 244, 129–135. [CrossRef]

52. Voordouw, M.J. Co-feeding transmission in Lyme disease pathogens. *Parasitology* 2015, 142, 290–302. [CrossRef]

53. De la Fuente, J.; Antunes, S.; Bonnet, S.; Cabezas-Cruz, A.; Domingos, A.G.; Estrada-Peña, A.; Johnson, N.; Kocan, K.M.; Mansfield, K.L.; Nijhof, A.M.; et al. Tick-pathogen interactions and vector competence: Identification of molecular drivers for tick-borne diseases. *Front. Cell. Infect. Microbiol.* 2017, 7, 114. [CrossRef]

54. Dumler, J.S.; Choi, K.-S.; Garcia-Garcia, J.C.; Barat, N.S.; Scorpio, D.G.; Garyu, J.W.; Grab, D.J.; Bakken, J.S. Human granulocytic anaplasmosis and *Anaplasma phagocytophilum*. *Emerg. Infect. Dis.* 2005, 11, 1828–1834. [CrossRef] [PubMed]

55. Heyman, P.; Cochez, C.; Hofhuis, A.; Van der Giessen, J.; Sprong, H.; Porter, S.R.; Losson, B.; Saegerman, C.; Donoso-Mantke, O.; Niedrig, M.; et al. A clear and present danger: Tick-borne diseases in Europe. *Expert Rev. Anti Infect. Ther.* 2010, 8, 33–50. [CrossRef]

56. Namina, A.; Capligina, V.; Seleznova, M.; Krumins, R.; Aleinikova, D.; Kivrane, A.; Akopjana, S.; Lazovska, M.; Berzina, I.; Ranka, R. Tick-borne pathogens in ticks collected from dogs, Latvia, 2011–2016. *BMC Vet. Res.* 2019, 15, 398. [CrossRef]

57. Welc-Falciak, R.; Kowalec, M.; Karbowiak, G.; Bajer, A.; Behnke, J.M.; Sinski, E. Rickettsiaceae and Anaplasmataceae infections in *Ixodes ricinus* ticks from urban and natural forested areas of Poland. *Parasites Vectors* 2014, 7, 121. [CrossRef]

58. Grzeszczyk, A.; Satarczak, J. High prevalence of *Anaplasma phagocytophilum* infection in ticks removed from human skin in north-eastern Poland. *Ann. Agric. Environ. Med.* 2006, 13, 45–48.

59. Chen, S.M.; Dumler, J.S.; Bakken, J.S.; Walker, D.H. Identification of a granulocytotropic *Ehrlichia* species as the etiologic agent of human disease. *J. Clin. Microbiol.* 1994, 32, 589–595. [CrossRef]

60. Satarczak, J.; Racewicz, M.; Kruminis-Lozowska, W.; Kubica-Biernat, B. Coinfection of *Ixodes ricinus* (Acari:Ixodidae) in northern Poland with the agents of Lyme borreliosis (LB) and human granulocytic ehrlichiosis (HE). *Int. J. Med. Microbiol.* 2002, 33, 198–201. [CrossRef]

61. Satarczak, J.; Gabre, R.M.; Kruminis-Lozowska, W.; Racewicz, M.; Kubica-Biernat, B. *Ixodes ricinus* as a vector of *Borrelia burgdorferi* sensu lato, *Anaplasma phagocytophilum* and *Babesia microti* in urban and suburban forests. *Ann. Agric. Environ. Med.* 2004, 11, 109–114.

62. Strzelczyk, J.K.; Gądźwicka, J.; Cuber, P.; Asman, M.; Trapp, G.; Gołabek, K.; Zalewska-Ziob, M.; Nowak-Chmura, M.; Siuda, K.; Wiczkowski, A.; et al. Prevalence of *Borrelia burgdorferi* sensu lato in *Ixodes ricinus* ticks collected from southern Poland. *Acta Parasitol.* 2015, 60, 666–674. [CrossRef]

63. Chmielewska-Badora, J.; Zwoliński, J.; Cisak, E.; Wójcik-Fatla, A. Prevalence of *Anaplasma phagocytophilum* in *Ixodes ricinus* ticks determined by polymerase chain reaction with two pairs of primers detecting 16S rRNA and ankA genes. *Ann. Agric. Environ. Med.* 2007, 14, 281–285.

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