UNIWERSYTET IM. ADAMA MICKIEWICZA W POZNANIU

WYDZIAŁ BIOLOGII

ROZPRAWA DOKTORSKA

Współwystępowanie *Babesia* spp. (Apicomplexa: Piroplasmida) i *Borrelia* spp. (Bacteria: Spirochaetes) w kleszczach pospolitych, *Ixodes ricinus*, na terenach zurbanizowanych na przykładzie Poznania

Co-occurrence of *Babesia* spp. (Apicomplexa: Piroplasmida) and *Borrelia* spp. (Bacteria: Spirochaetes) in common ticks, *Ixodes ricinus*, in urban areas on the example of Poznań.

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Poznań, luty 2024

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Podziękowania

...dowiadujemy się, że jedyne co się liczy to Miłość. Wszystko inne - dyplomy, pieniądze, przedmioty - nie ma większego znaczenia. Okazuje się też, że to, co robiliśmy, nie jest ważne. Ważne jest jak robiliśmy, a najistotniejsze jest to, że robiliśmy coś z miłością...

Elisabeth Kübler-Ross

Pani prof. UAM dr hab. Mirosławie Dabert, za danie mi szansy na realizację moich badań, przekazaną wiedzę, wszystkie nauki oraz lekcje.

Panu prof. UAM dr hab. Jerzemu Michalikowi, za wiarę we mnie i moją pracę, za pomoc, zaangażowanie, zrozumienie i wsparcie.

Pani prof. UAM dr hab. Agnieszce Ludwików, za pomoc, życzliwość i wsparcie.

Zespołowi Laboratorium Technik Biologii Molekularnej UAM w Poznaniu za przekazaną wiedzę i wzajemne wsparcie; Uli Sobczyńskiej i Julii Olechnowicz za wspierające rozmowy i pomoc; Małgosi Łośko, Ani Labrzyckiej-Dulămescu i Beacie Pater za pracę do późnych godzin, życzliwość i wspólne powroty do domów; Arturowi Trzebnemu za czas, kiedy podnosiliśmy się nawzajem na duchu i czerpaliśmy radość ze współpracy; Sylwii Jedut i panu Witoldowi Nowakowi za ich obecność, dobre słowo i życzliwość.

Śp. Panu prof. UAM dr hab. Czesławowi Błaszakowi za wyjazdy na Międzynarodowe Sympozja "Stawonogi pasożytnicze, alergogenne i jadowite - znaczenie medyczne i sanitarne" inspirujące rozmowy, dopingowanie i podnoszenie na duchu.

Panu Prof. dr hab. Andrzejowi Kaźmierskiemu za umożliwienie realizacji 'kleszczowej' pracy magisterskiej i pokazanie mi drzwi, którymi mogłam wejść, by dalej odkrywać niezwykły świat kleszczy.

Osobom zaangażowanym w zbiór kleszczy: Łukaszowi Brodzie, Gniewkowi Gruszczyńskiemu, Weronice Pawlik, Anecie Łyś, lekarzom weterynarii i pracownikom: (1) Centrum Zdrowia Małych Zwierząt M. Majka, R. Starczewski s.c., (2) Gabinetu Weterynaryjnego Katarzyny Gryglewskiej, (3) Gabinetu Weterynaryjnego lek. wet. Dariusza Filipińskiego, (4) Gabinetu Weterynaryjnego Marcina Konieczнego, (5) Gabinetu weterynaryjnego - Piotra Winieckiego, (6) Kliniki Weterynaryjnej Juszczak & Gorzelańczyk, (7) Praktyki Weterynaryjnej A. Golec M. Rybski, (8) Gabinetu Weterynaryjnego Pluto Vet lek. wet. Krzysztofa Kaniewskiego, (9) Gabinetu Weterynaryjnego Zwierzak A. Adamskiej, (10) Gabinetu Weterynaryjnego Amicus Grzegorza Macioch, (11) Usług Weterynaryjnych Jakuba Golec, (12) Przychodni Weterynaryjnej Na Polance Katarzyny Marciniak, (13) Specjalistycznego Gabinetu Weterynaryjnego lek. wet. Żanety Sokołowskiej, (14) Przychodni Weterynaryjnej lek. wet. Adama Łowczaka, (15) Przychodni Weterynaryjnej Kociak lek. wet. Sylwii Łata, (16) Kliniki Weterynaryjnej dr. Grzegorza Wąsiatycza, (17) Specjalistycznego Gabinetu Weterynaryjny Juszczak & Gorzelańczyk.

Przyjaciołom i Rodzinie; Ewie Gawińskiej, Ewelinie Łuczka, Dominice Seroka, Małgosi Wolskiej, Natalii Radacz, Marlenie Kopackiej, za bycie obok ilekroć tego potrzebowałam, za Wasze wsparcie i przestrzeń na emocje.

Kasi Kaszewskiej-Gilas za wsparcie, za wznoszenie się nawzajem, za wiarę we mnie, inspirujące rozmowy, muzykę i dotlenianie;), za przyjaźń i wspólną drogę.

Agacie Drwięga za wsparcie i dodawanie sobie nawzajem siły, głęboko wierzę, że to wszystko jest po Coś.

Małgosi Kolickiej, za stanie obok, wsparcie, kleszczowe i brzuchorzęskowe fiksacje, miłość do kotów i zrozumienie.

Julii Durkiewicz, Małgosi Wolskiej, Mateuszowi Deker i Ani Olszewskiej za tworzenie bezpiecznych przestrzeni Domu Kultury Jogi, Yoga & Ayurweda i Yoga Academy gdzie zawsze mogłam znaleźć i znajduję ukojenie.

Magdzie Widłak- Langer za nasza współpracę, gdyby nie ogrom wykonanej z Tobą pracy, nie pisałabym teraz tych wyrazów wdzięczności, która obecnie mnie przepełnia, dziękuję, że nauczyłaś mnie jak wychodzić z mroku.

Maciejowi Nowiczowi, za nasze lekcje i atmosferę którą stworzyłeś, za pomoc i wsparcie.

Wszystkim Osobom, które przychodziły i przychodzą na moje zajęcia jogi, każde spotkanie z Wami dodaje sił w różnych obszarach życia; to niesamowita wymiana.

Kotykowi, za Twoją radosną niezależną naturę, za wyczuwanie moich emocji i kojące mruczenie, za każde 'stanie na straży', kiedy zwinięty w kłębek stacjonowałeś gdzieś w pobliżu, kiedy kolejne godziny dni noce, pracowałam.

Rodzicom &
Tacie Leszkowi Liberskiemu za rozbudzenie we mnie ciekawości, gdziekolwiek jesteś, czuję jak bardzo jesteś ze mnie dumny i ciągle podziwiasz mój upór, determinację i wytrwałość, bardzo za Tobą tęsknię,
Mamci Gabrysi Liberskiej za „zarażenie mnie" miłością do życia, dziękuję że uczymy się nawzajem i radujemy się z małych – wielkich – zwykłych rzeczy.

Dziękuję, że Jesteście, bez Was Wszystkich, To nigdy nie byłoby możliwe,
Dziękuję też Sobie za Siebie.
Z wdzięcznością Justyna 'Justice'

Spis treści

1. Streszczenie

Ixodes ricinus, wektor patogenów odkleszczowych (ang. TBPs, *tick borne pathogens*) jest najważniejszym epidemiologicznie kleszczem w Europie. Nadal brakuje kompleksowej wiedzy na temat enzootycznych cykli krążenia TBPs z udziałem *I. ricinus* w ekosystemach miejskich, które ze względu na wysokie zagęszczenie ludzi, zwierząt towarzyszących i naturalnych żywicieli kleszczy, stanowią unikalne warunki do szerzenia się zoonoz transmisyjnych. Oprócz krętków z kompleksu *Borrelia burgdorferi* s.l. będących przyczyną boreliozy z Lyme (LB), kleszcze *I. ricinus* zaangażowane są w wektorowanie *B. miyamotoi*, krętka z grupy gorączek powrotnych (ang. RFG, *relapsing fever group*), czynnika tzw. boreliozy miyamotoi. Niektóre TBPs, m.in. *B. burgdorferi* s.l. i *Babesia microti*, mogą współwystępować u kleszczy i żywicieli rezerwuarowych, co oznacza, że istnieje prawdopodobieństwo ich jednoczesnego przeniesienia na ludzi lub zwierzęta domowe. W Europie trzy gatunki *Babesia*, *Ba. microti*, *Ba. divergens* i *Ba. venatorum*, mogą powodować ludzką babeszjozę, niebezpieczną dla pacjentów z obniżoną odpornością. Z kolei *Ba. canis* to jedyny w Polsce gatunek powodujący babeszjozę psów, którego wektorem jest kleszcz łąkowy, *Dermacentor reticulatus*. Celem rozprawy była analiza występowania bakterii *Borrelia* spp. i protistów *Babesia* spp. wraz z oceną częstości ich współwystępowania (koinfekcji) w populacjach miejskich kleszczy *I. ricinus,* na przykładzie miasta Poznania.

Materiał do badań stanowiły kleszcze: (i) poszukujące żywiciela (tj. z roślinności), zebrane z 5 terenów rekreacyjnych Poznania (n=1059) oraz (ii) pozyskane od psów i kotów w ramach 3 letniej współpracy z 17 gabinetami weterynaryjnymi w Poznaniu (n=1268 samic). Do detekcji TBPs zastosowano sekwencjonowanie markerowych fragmentów DNA.

Obecność DNA *B. burgdorferi* s.l. potwierdzono u 8,7% kleszczy poszukujących żywiciela, w tym u 11,8% samic, 9,6% samców oraz 6.3% nimf. Blisko 2-krotnie niższy odsetek zakażeń (4,7%) wykryto u samic zebranych ze zwierząt. W obu grupach kleszczy zidentyfikowano gatunki należące do krętków powodujących LB: *B. afzelii*, *B. garinii*, *B. lusitaniae*, *B. valaisiana*, a wśród kleszczy zebranych ze psów dodatkowo *B. spielmanii*. Występowanie DNA *B. miyamotoi*, wykazano u 2,1% kleszczy poszukujących żywicieli oraz łącznie u 1,8% kleszczy z psów i kotów. Obecność DNA *Babesia* spp. wykryto u 4,4% (45/1029) kleszczy zebranych z roślinności, w tym u 7,3% samic (21/289), 4,6% samców (13/280) oraz 2,4% nimf (11/460). DNA *Babesia* spp. stwierdzono także u 6% (76/1268) samic *I. ricinus* żerujących na zwierzętach towarzyszących. W obu grupach kleszczy zidentyfikowano dwa gatunki potencjalnie chorobotwórcze dla człowieka: *Ba. microti* i *Ba. venatorum,* ponadto

Ba. canis. Wśród zakażeń dominowały *Ba. canis* (61,2%) i *Ba. microti* (34,7%). Łącznie, współwystępowanie zakażeń *Borrelia* spp. i *Babesia* spp. odnotowano u 1% (24/2297) kleszczy. Wśród zakażonych kleszczy z roślinności, *Ba. canis* dominowała w koinfekcjach (72,7%; 8/11), występując z *B. afzelii* lub *B. garinii*. W kleszczach ze zwierząt, przeważała *Ba. microti* (92,3%; 12/13) głównie z *B. afzelii*.

Podsumowując, wyraźna dominacja *Ba. canis* uprawdopodabnia funkcjonowanie *I. ricinus* jako wektora w cyklach enzootycznych na obszarach środkowo-zachodniej Polski, gdzie *D. reticulatus* jest nieobecny lub występuje rzadko. W takim przypadku miejskie tereny mogą stwarzać potencjalne ryzyko nabycia babeszjozy przez psy. Stwierdzenie dominacji dwóch patogennych gatunków krętków *B. afzelii* i *B. garinii* oraz wykrycie w obu grupach kleszczy sekwencji *Ba. microti* i *Ba. venatorum*, identycznych ze szczepami chorobotwórczymi dla człowieka, wskazują na ryzyko zachorowania osób korzystających z obszarów zieleni miejskiej na LB i/lub babeszjozę. Z kolei obecność *B. miyamotoi,* czyni możliwym nabycie boreliozy miyamotoi, która powinna być objęta diagnostyką różnicującą. Współwystępowanie potencjalnie chorobotwórczych gatunków z rodzajów *Borrelia* i *Babesia* wśród miejskich populacji kleszczy może modyfikować symptomy kliniczne, przebieg i nasilenie jednostek chorobowych powodowanych przez te patogeny. Koinfekcje te powinny być zatem brane pod uwagę przez lekarzy i diagnostów na etapie diagnozowania ludzi i psów z symptomami gorączki po kontakcie z kleszczem.

Słowa kluczowe: *Ixodes ricinus, Babesia, Borrelia* LB*,* borelioza z Lyme, *Borrelia* RF borelioza miyamotoi, babeszjoza, patogeny odkleszczowe, koinfekcje

2. Abstract

Ixodes ricinus, the vector of tick-borne pathogens (TBPs), is the most epidemiologically important tick in Europe. Comprehensive knowledge of enzootic circulation cycles of TBPs transmitted by *I. ricinus* in urban ecosystems is still lacking. Due to the dense population of humans, companion animals, and natural tick hosts, those environments provide unique conditions for tick-borne zoonotic infections to occur. In addition to spirochetes from the *Borrelia burgdorferi* s.l. complex, which are the cause of Lyme borreliosis (LB), *I. ricinus* ticks are involved in the transmission of *B. miyamotoi*—a spirochete from the relapsing fever group (RF), the causative agent of *B. miyamotoi* disease. Some TBPs may occur as coinfections in ticks and reservoir hosts, indicating the likelihood of their simultaneous transmission to humans or domestic animals. It has been demonstrated that coinfections with *B. burgdorferi* s.l. and *Babesia microti* protists in ticks and small rodents can locally intensify the expansion of *Ba. microti*. In Europe, three species—*Ba. microti*, *Ba. divergens*, and *Ba. venatorum*—can cause human babesiosis, posing a threat to immunocompromised patients. In the case of such dual infections, the course of the disease may be more severe, with persisting symptoms. Furthermore, *Ba. canis* is the sole species responsible for canine babesiosis in Poland; its competent vector is the meadow tick, *Dermacentor reticulatus*. This dissertation aimed to analyze the occurrence of *Borrelia* spp. bacteria and *Babesia* spp. protists, with an assessment of the frequency of their coexistence (coinfections) within urban populations of *I. ricinus* ticks, using the city of Poznań as an example.

The research material comprised host-seeking ticks (i.e., from vegetation) collected from five recreational areas in Poznań (n=1059) along with specimens obtained from companion animals (dogs and cats) in collaboration with 17 veterinary clinics in Poznań (n=1268 females). TBPs detection was performed by sequencing DNA marker fragments.

The presence of *B. burgdorferi* s.l. DNA was confirmed in 8.7% (90/1029) of host-seeking ticks, including 11.8% of females (34/289), 9.6% of males (27/280), and 6.3% of nymphs (29/460). A nearly two times lower infection rate (4.7%) was detected in females collected from animals. In both groups of ticks, species belonging to the spirochetes causing LB were identified: *B. afzelii*, *B. garinii*, *B. lusitaniae*, *B. valaisiana*, and among ticks collected from dogs, additionally, *B. spielmanii*. The presence of *B. miyamotoi* DNA, a species representing the *Borrelia* RF group, was detected in 2.1% of hosts-seeking ticks and 1.8% of ticks collected from dogs and cats.

The presence of *Babesia* spp. protists was detected in 4.4% of ticks collected from vegetation, including 7.3% of females, 4.6% of males, and 2.4% of nymphs. *Babesia* spp. DNA was also identified in 6% of female *I. ricinus* ticks feeding on companion animals. In both groups of ticks, two species potentially pathogenic to humans were identified: *Ba. microti* and *Ba. venatorum*, along with *Ba. canis*. Among the infections, *Ba. canis* (61.2%) and *Ba. microti* (34.7%) prevailed. In total, the co-occurrence of infections with *Borrelia* spp. and *Babesia* spp. was noted in 1% (24/2297) of ticks. Among the infected ticks collected from vegetation, *Ba. canis* predominated in coinfections (72.7%; 8/11), occurring alongside *B. afzelii* or *B. garinii*. In ticks from animals, *Ba. microti* prevailed (92.3%; 12/13), mainly with *B. afzelii*.

In summary, the clear dominance of *Ba. canis* suggests the functioning of *I. ricinus* as a vector in enzootic cycles in the central-western regions of Poland, where *Dermacentor reticulatus* is absent or rare. Under these circumstances, dogs in urban areas may face a potential risk of contracting babesiosis. Observing the dominance of two pathogenic spirochete species, *B. afzelii* and *B. garinii*, and detecting sequences that correspond to the strains causing babesiosis in humans (*Ba. microti* and *Ba. venatorum*) in both tick groups, points to the risk of acquiring LB and/or babesiosis by people using urban green areas. Moreover, the presence of *B. miyamotoi* infections makes it possible to contract *B. miyamotoi* disease, which should be considered in differential diagnosis. The coexistence of potentially pathogenic species from the *Borrelia* and *Babesia* genera within urban tick populations may modify clinical symptoms, the course, and severity of diseases caused by the pathogens. Therefore, in the examination of humans and dogs with presenting fever symptoms after tick exposure, physicians and diagnosticians should carefully consider the possibility of coinfections.

Keywords: *Ixodes ricinus*, *Babesia*, *Borrelia* LB, Lyme borreliosis, *Borrelia* RF, *B. miyamotoi* disease, babesiosis, tick-borne pathogens, coinfections.

3. Wykaz publikacji składających się na rozprawę doktorską

Podstawą niniejszej rozprawy doktorskiej są przedstawione w porządku chronologicznym trzy prace obejmujące: dwa artykuły (A.1 i A.2) opublikowane w czasopismach z listy JCR o łącznym wskaźniku IF 7,634 oraz jeden manuskrypt (M. 1) przygotowany do wysłania do czasopisma.

A.1. Liberska J, Michalik J, Pers-Kamczyc E, Wierzbicka A, Lane RS, Rączka G, Opalińska P, Skorupski M, Dabert M. 2021. Prevalence of *Babesia canis* DNA in *Ixodes ricinus* ticks collected in forest and urban ecosystems in west-central Poland. Ticks and Tick-borne Diseases.12, 101786; https://doi.org/10.1016/j.ttbdis.2021.101786

IF= 3,817, Q1, 91 perc., 100 pkt. MNiSW

A.2. **Liberska J**, Michalik J, Dabert M. 2023. Exposure of dogs and cats to *Borrelia miyamotoi* infected *Ixodes ricinus* ticks in urban areas of the city of Poznań, west-central Poland. Ticks and Tick-borne Diseases, 14(102188). https://doi.org/10.1016/j.ttbdis.2023.102188

IF= 3,817, Q1, 91 perc., 100 pkt. MNiSW

M.1. **Liberska J**, Michalik J, Olechnowicz J, Dabert M. Co-occurrence of *Borrelia burgdorferi* sensu lato and *Babesia* spp. DNA in Ixodes ricinus ticks collected from vegetation and pets in the city of Poznań, Poland. 2024.

Manuskrypt przygotowany do wysłania do czasopisma Pathogens, IF= 4,531, Q2, 74 perc., 100 pkt. MNiSW

4. Wprowadzenie i główne cele rozprawy doktorskiej

Około 75% nowo pojawiających się patogenów (ang. *new emerging pathogens*) ma pochodzenie odzwierzęce (zonotyczne), a około 60% wszystkich czynników zakaźnych będących przyczyną chorób człowieka, jest związana ze zwierzętami. Aktualnie zoonozy stanowią największe globalne zagrożenie dla zdrowia publicznego (Bueno-Marí i wsp.,2015). Rolnictwo, hodowla zwierząt, globalny handel i turystyka, masowe migracje oraz urbanizacja tworzą niespotykane wcześniej możliwości dla transmisji patogenów odkleszczowych (ang TBPs, *tick-borne pathogens*,) i ich wektorów na obszary, w których dotychczas nie występowały. Ryzyko nabycia chorób odkleszczowych (ang. *tick-borne disease*) jest możliwe także w aglomeracjach miejskich. Dlatego badania dotyczące rozpowszechnienia TBPs zakażających populacje kleszczy w miastach są konieczne dla identyfikacji czynników warunkujących istnienie endemicznych ognisk tych chorób. Na ich potrzebę wskazują szacunki the World Bank, według których około 56% światowej ludzkiej populacji żyje obecnie w miastach, a w wyniku urbanizacji do 2050 r wskaźnik ten może osiągnąć 70% https://www.worldbank.org/en/topic/urbandevelopment).

Obszary w obrębie miast, to wysoce rozdrobnione ekosystemy różniące się sposobem użytkowania oraz zwykle zubożoną florą i fauną. Istotnym elementem krajobrazu miejskiego są tereny zielone tj. parki miejskie, bulwary, zieleńce, cmentarze, ogrody botaniczne i prywatne oraz lasy komunalne. Stwarzają one korzystne warunki dla lokalnych populacji kleszczy oraz zwierząt będących ich żywicielami (Rizzoli i wsp., 2014). Ważną rolę w układzie żywiciel-kleszcz-patogen stanowią licznie reprezentowane na obszarach miejskich psy i koty (Uspensky 2014). W rezultacie tereny, w których obecne są kleszcze w połączeniu z wysokim zagęszczeniem ludzi oraz psów i kotów, tworzą specyficzne warunki dla transmisji TBPs. Wiedza na temat enzootycznych cykli determinujących krążenie tych patogenów w miastach pozostaje nadal niekompletna (Rizzoli i wsp., 2014; Noden i wsp., 2022).

Wśród ponad 980 gatunków kleszczy (Ixodida), około 10% uczestniczy w przenoszeniu czynników zakaźnych stanowiących zagrożenie dla człowieka i zwierząt domowych (Dantas-Torres i Otranto, 2022; Guglielmone i wsp., 2023). Po komarach, stanowią drugą najważniejszą grupę hematofagicznych stawonogów będących wektorami zoonoz transmisyjnych i w szczególności odpowiadają za szerzenie większości z nich w strefie klimatu umiarkowanego Ameryki Północnej, Europy i Azji. Kleszczom przypisuje się także kluczową rolę wektora chorób zwierząt gospodarskich (Bueno-Marí i wsp., 2015). Ponadto, w porównaniu do

komarów, spektrum przenoszonych TBPs jest bardziej różnorodne i obejmuje wirusy, bakterie, protisty, grzyby i nicienie (Rochlin i Toledo, 2020).

Kleszcz pospolity, *Ixodes ricinus*, to najważniejszy i najbardziej rozpowszechniony wektor TBPs w Europie (Rizzoli i wsp., 2014). Jest gatunkiem poza-gniazdowo-norowym, niespecyficznym żywicielsko pasożytującym na blisko 300 gatunkach lądowych kręgowców (ssaków, ptaków i gadów) (Gern i Humair, 2002). Kleszcze ulegają zakażeniu patogenami podczas pobierania krwi od gatunków rezerwuarowych pełniących rolę naturalnego źródła infekcji. Człowieka atakują nimfy i samice, stąd w epidemiologii chorób odkleszczowych tylko te dwa stadia są zagrożeniem dla ludzi i zwierząt domowych (Kahl i Gray, 2023).

Spośród chorobotwórczych gatunków bakterii wektorowanych przez *I. ricinus*, najważniejszą grupę stanowią krętki z kompleksu *Borrelia burgdorferi* s.l. będące przyczyną boreliozy z Lyme (LB). Jest to najczęstsza zoonoza odkleszczowa na półkuli północnej. Aktualnie kompleks ten nazwany także grupą LB (*Lyme borreliosis group*), obejmuje co najmniej 24 gatunki, z których 14 wykryto u *I. ricinus*, a sześć (*B. afzelii, B. garinii, B. burgdorferi* sensu stricto (s.s.), *B. spielmanii, B. bavariensis* i *B. bissettiae*) uważa się za patogenne dla człowieka (Steinbrink i wsp., 2022; Wodecka i Kolomiiets, 2023). W Europie corocznie zgłaszanych jest ponad 230 000 przypadków LB i większość spowodowana jest przez *B. afzelii* i *B. garinii*. (Mendoza-Roldan i wsp. 2019). W Polsce w 2019 roku odnotowano ponad 20 000 zachorowań (Czarkowski i wsp., 2021). Na boreliozę chorują także psy. Rozwija się ona najczęściej w postaci artretycznej, ze stanem zapalnym kończyn, zwłaszcza stawów nadgarstkowych lub stępowych, z powiększeniem węzłów chłonnych i może prowadzić do kulawizny (Jankauskaitė i wsp. 2023). U starszych psów pojawiają się dysfunkcje neurologiczne (Skotarczak, 2002). Zapadalność na boreliozę ludzi i psów zależy od odsetka zakażonych kleszczy, dlatego dane te stanowią podstawowy wskaźnik do oszacowania ryzyka nabycia tej choroby. Także badania serologiczne psów są pomocne dla oceny sytuacji epidemiologicznej (Hovius i wsp., 2000).

Borrelia miyamotoi to przedstawiciel krętów z grupy gorączek powrotnych (ang. RFGB, *Relapsing fever group Borrelia*), powodujących, nawracające zwykle po tygodniu remisji, epizody gorączkowe trwające do 3 dni. Podczas tych ataków występuje wysoka gorączka do 39°C, bóle głowy, mięśni i stawów. Wektorami krętków RFGB są głównie kleszcze miękkie (Argasidae) (Schwan i Piesman, 2002), stąd te gatunki notowane są w regionach tropikalnych i subtropikalnych. *Borrelia miyamotoi* to jedyny przedstawiciel

RFGB, który podobnie jak gatunki z grupy LB, jest związany z kleszczami twardymi (Ixodidae) z rodzaju *Ixodes*; w Europie z *I. ricinus*. Obecnie znane są trzy genotypy występujące w Ameryce Północnej, Europie oraz Azji, które powodują chorobę (boreliozę) miyamotoi (ang. BMD, *B. miyamotoi disease*) (Siński i wsp., 2016). Dotychczas zdiagnozowano ją u ponad 560 pacjentów, w tym ponad 60 przypadków pochodzi z Europy (Cleveland i wsp., 2023). W Polsce opisano jeden przypadek u pacjenta z objawami neuroboreliozy ocznej (Fiecek i wsp., 2019). U ponad 10% chorujących obserwuje się od dwóch do trzech epizodów gorączki dochodzącej nawet do 40ºC i nieswoiste objawy grypopodobne (Siński i wsp., 2016; Cleveland i wsp., 2023). W przypadku psów i kotów, częstość zakażeń i patogeniczność tego krętka pozostają nieznane.

Prostisty z rodzaju *Babesia* to pasożyty erytrocytów kręgowców, które należą do rzędu Piroplasmida i typu Apicomplexa. Rodzaje *Babesia* i *Theileria* nazwano „piroplazmami" ze względu na gruszkowatą morfologię pasożyta (ang. *pear shaped*) namnażającego się w erytrocytach (Uilenberg, 2006). Dotychczas opisano ponad 100 gatunków *Babesia*, a wśród nich chorobotwórcze dla ludzi i psów, które wywołują babeszjozę z objawami gorączki i anemii podobnymi do malarii (Beugnet i Moreau, 2015). *Ixodes ricinus* uczestniczy w transmisji *Ba. divergens, Ba. microti, Ba. venatorum, Ba. capreoli*, z których trzy pierwsze gatunki powodują babeszjozę u ludzi (Gray i wsp., 2010; Bajer i Dwużnik-Szarek, 2021). W Europie udokumentowano ponad 60 przypadków tej choroby, z których większość wywołana była przez *Ba. divergens*. Pięć przypadków przypisano *Ba. venatorum*, a kolejne jedenaście *Ba. microti* (Hildebrandt i wsp., 2021). Zakażenie zwykle przebiega bezobjawowo, jednak może wystąpić zmęczenie, gorączka, bóle głowy, mięśni, stawów, nudności, kaszel (Krause i wsp., 1991). U pacjentów z obniżoną odpornością i chorobami współistniejącymi może rozwinąć się ciężka postać z niebezpiecznymi powikłaniami obejmującymi zespół ostrej niewydolności oddechowej, ARDS (ang. *acute respiratory distress syndrome*) (Krause, 2019).

Babesia canis to czynnik etiologiczny babeszjozy psów notowanej na obszarach Europy w strefie klimatu umiarkowanego, której wektorem jest kleszcz łąkowy, *Dermacentor reticulatus* (Beugnet i Moreau, 2015). Objawami są gorączka, apatia, anoreksja, bladość błon śluzowych, hemoglobinuria, żółtaczka, anemia hemolityczna (Solano-Gallego i wsp., 2016). Chorobę tę mogą powodować dwa typy szczepów *Ba. canis*: typ A wywołujący łagodniejszą postać i typ B o większej patogeniczności (Adaszek and Winiarczyk, 2008).

Ixodes ricinus to gatunek trójżywicielowy i dlatego w rozwoju osobniczym jest eksponowany trzykrotnie (jako larwa, nimfa i samica) na zakażenie się jednym lub kilkoma

patogenami od gatunku rezerwuarowego, który może być równocześnie źródłem kilku patogenów. W konsekwencji mieszane infekcje kleszczy uprawdopodobniają wspólną transmisję patogenów do organizmu człowieka lub zwierząt domowych. Badania pokazują, że infekcje dwoma lub więcej patogenami u kleszczy i ich żywicieli występują częściej niż sądzono (Moutailler i wsp., 2016). Modyfikują one objawy kliniczne, przebieg i nasilenie choroby odkleszczowej u ludzi w porównaniu z chorobą spowodowaną pojedynczą infekcją (Jahfari i wsp., 2016, Dunaj i wsp., 2018; Cutler i wsp., 2020). Istnieją doniesienia, że bakterie *B. burgdorferi* s.l. i *Ba. microti* często występują w koinfekcjach przenoszonych przez kleszcze z kompleksu *I. ricinus* (Scott i Scott, 2018). Ich współwystępowanie może oddziaływać synergistycznie i wspierać ekspansję *Ba. microti* w lokalnych cyklach enzootycznych, zwiększając poziom zakażeń wśród kleszczy i ich żywicieli (Dunn i wsp., 2014; Diuk-Wasser i wsp., 2016). Pacjenci zakażeni przez oba patogeny cierpią na bardziej zróżnicowane i dłużej utrzymujące się objawy (Krause i wsp., 1996; Martínez-Balzano i wsp., 2015). Występowanie takich koinfekcji potwierdzono także u pacjentów w Polsce (Jabłońska i wsp., 2016; Pańczuk i wsp., 2016). Dlatego badania uwzględniające współwystępowanie gatunków z rodzajów *Borrelia* i *Babesia* w populacjach kleszczy są istotne dla rozpoznania sytuacji epidemiologicznej i mogą być pomocne dla lekarzy i diagnostów na etapie rozpoznawania i leczenia pacjentów z symptomami gorączki po kontakcie z kleszczem.

Głównym celem niniejszej rozprawy doktorskiej była analiza poziomu zakażeń spowodowanych bakteriami z rodzaju *Borrelia* i protistami z rodzaju *Babesia* w populacjach kleszczy *Ixodes ricinus* występujących w warunkach ekosystemów miejskich na przykładzie miasta Poznania.

W szczególności wyznaczono następujące cele:

1. określenie częstości występowania gatunków z rodzaju *Babesia*,
2. określenie częstości występowania krętków *Borrelia miyamotoi* z grupy gorączek powrotnych (RFGB),
3. określenie częstości występowania krętków *Borrelia burgdorferi* s.l. z grupy boreliozy z Lyme (LB),
4. określenie częstości współwystępowania gatunków z rodzajów *Babesia* oraz *Borrelia* z grupy LB.

Materiał do badań stanowiły dwie grupy kleszczy: **(i)** kleszcze z roślinności będące w fazie niepasożytniczej (n=1059) oraz **(ii)** w fazie pasożytniczej żerujące na psach i kotach (n=1268). Pierwszą grupę zebrałam z roślinności metodą flagowania na pięciu stanowiskach wykorzystywanych przez mieszkańców Poznania dla celów rekreacjii wypoczynku (tabela 1). Zbiór przeprowadzałam od maja do września 2017 r. i w kwietniu 2018 r. Drugą grupę kleszczy zbierałam w trakcie trzyletniej współpracy (od kwietnia do października 2015 r., od marca do listopada 2016 r. oraz od marca do września 2017 r.) z lekarzami z 17 lecznic weterynaryjnych w Poznaniu. Łącznie ze zwierząt towarzyszących zebrałam 1268 samic kleszczy *I. ricinus* w różnym stanie opicia krwią, w tym 711 od 609 psów, 153 od 117 kotów oraz 404 od 389 czworonogów, które potraktowano łącznie bez podziału na psy i koty (Tabela 2). Kleszcze przechowywałam w 96% etanolu. Ich identyfikację gatunkową przeprowadzałam za pomocą metod morfologicznych, przy użyciu klucza do oznaczania gatunków kleszczy (Siuda, 1993). W przypadku stadiów młodocianych przynależność gatunkową potwierdzałam metodami molekularnymi, opartymi na amplifikacji i sekwencjonowaniu fragmentu genu podjednostki I oksydazy cytochromu *c* (COI).

Detekcję DNA krętków *Borrelia* przeprowadziłam poprzez amplifikację i sekwencjonowanie hiperzmiennego regionu V4 genu 16S rRNA (V4 16S). Szczegóły dotyczące starterów, konstrukcji biblioteki, sekwencjonowania następnej generacji (NGS) i analizy danych sekwencji zostały opisane w artykule A.2. Dodatkowo izolaty, w których zidentyfikowałam DNA *Borrelia* sp. na podstawie V4 16S oraz izolaty kleszczy ze zwierząt towarzyszących poddałam amplifikacji i sekwencjonowaniu wykorzystując fragment genu *flaB* przy użyciu dwóch par zestawów starterów 132f/905r i 220f/823r (Wodecka i wsp., 2010). Protokoły reakcji PCR i sekwencjonowania Sangera zawarłam w artykule A.2.

DNA *Babesia* spp. wykrywałam poprzez amplifikację i sekwencjonowanie fragmentu genu 18S rRNA przy użyciu nested PCR i sekwencjonowania Sangera lub poprzez sekwencjonowanie NGS tego samego docelowego DNA. Do zagnieżdżonej reakcji PCR użyłam zestawu starterów RIB-19/RIB-20; do pierwszego etapu amplifikacji (Zahler i wsp., 2000) oraz zestawu starterów P3/BabR3; do drugiego etapu amplifikacji (Li i wsp., 2013; Liberska i wsp., 2021). Sekwencje starterów i protokoły zagnieżdżonej reakcji PCR i sekwencjonowania Sangera opisałam w artykule A.1. Drugie podejście do wykrywania *Babesia* spp. DNA przeprowadziłam przy użyciu zestawu starterów P3/BabR3 połączonych z podwójnie indeksowanymi adapterami Ion Torrent w celu wygenerowania amplikonów

do sekwencjonowania NGS. Szczegóły dotyczące konstrukcji biblioteki, sekwencjonowania Ion Torrent i analizy danych sekwencji opisałam w artykule A.2.

Dokładność chromatogramów sekwencji Sangera sprawdzałam w programie GeneiousR11.1.5 (Biomatters Ltd.), kontigi składałam w programie do edycji sekwencji GeneDoc 2.7 (Nicholas i Nicholas, 1997). Warianty sekwencji amplikonu (ASV) i operacyjne jednostki taksonomiczne (OTU) uzyskane z danych NGS porównałam z dostępnymi w GenBank przy użyciu BLASTN (https://blast.ncbi.nlm.nih.gov) i algorytmu megablast. Do określenia gatunków z rodzajów *Borrelia* i *Babesia* zastosowałam próg identyfikacji 99%, ponieważ określono je na podstawie sekwencji rDNA. Dla określenia gatunku kleszcza próg obniżyłam do 97% z racji na wewnątrzgatunkową zmienność markera COI.

Tabela 1. Wykaz 5 stanowisk będących miejscem wypoczynku i rekreacji na terenie miasta Poznania, z których metodą flagowania zebrano kleszcze *Ixodes ricinus* w okresie od maja do września 2017 r. i w kwietniu 2018 r.

Miejsce	Stadia				
	Larwy	Nimfy	Samice	Samce	Łącznie
1. Kampus UAM Morasko	0	17	15	9	41
2. Jezioro Rusałka*	1	313	170	153	637
3. Sołacki Park	13	17	15	14	59
4. Cytadela Park	0	4	24	41	69
5. Jezioro Maltańskie*	16	109	65	63	253
Łącznie	30	460	289	280	1059

*zadrzewione i zalesione tereny w sąsiedztwie obu sztucznych zbiorników wodnych.

Tabela 2. *Samice *Ixodes ricinus* zebrane ze zwierząt towarzyszących w 17 lecznicach weterynaryjnych w Poznaniu między IV - X 2015 r., III - XI 2016 r. oraz III - IX 2017 r.

Zwierzęta	Psy (n= 609)	Koty (n=117)	Nieokreślone (n=389)	Suma (n=1115)
Kleszcze	711	153	404	1268

* w publikacji A.2, opisano wyniki dotyczące 837 kleszczy zebranych z psów lub kotów; do manuskryptu M.1, dodatkowo włączono kleszcze pozbawione dokładnej informacji czy pochodziły od psów lub kotów

5. Rezultaty i wnioski rozprawy doktorskiej

5.1. Występowanie gatunków z rodzaju *Babesia*

W artykule A.1 badałam na obecność *Babesia canis*, kleszcze zebrane w dwóch odmiennych ekosystemach: (i) leśnym i (ii) miejskim. Pierwsza grupę kleszczy zebrano z 6 osób ubranych w kombinezony pszczelarskie, uczestniczących w eksperymencie mającym określić zachowania ludzi nasilające ekspozycję na kleszcze podczas pobytu w lasach. Eksperyment przeprowadzono na dwóch stanowiskach w drzewostanach mieszanych na terenie Wielkopolski (szczegóły: Wierzbicka i wsp., 2016). Grupę „miejskich kleszczy" zebrałam z pięciu terenów rekreacyjnych miasta Poznania (Tabela 1). Wśród „leśnych kleszczy" zakażenie *Ba. canis* wyniosło 10,2%. Odsetek zakażeń był podobny na obu stanowiskach (zakres od 9,9% do 12,5%). Najwyższy poziom infekcji sięgający 13% stwierdziłam u larw. W miejskich populacjach *I. ricinus*, frekwencja zakażeń okazała się czterokrotnie niższa i wyniosła 2,5%. Zakażenia były dwukrotnie częstsze u kleszczy dorosłych niż u nimf (3,3% *vs.* 1,5%). U trzech kleszczy wykryłam koinfekcję z *Ba. venatorum,* a u nimfy z *Ba. divergens* lub *Ba. capreoli* (oba gatunki mają tę samą sekwencję nukleotydów we fragmencie markerowym 18S rRNA). Analiza sekwencji 81 amplikonów ujawniła 20 wariantów genetycznych, które różniły się w jednej lub dwóch pozycjach nukleotydowych we fragmentach o długości 272 pz. Dominujący wariant stwierdzony u 74% (60/81) kleszczy wykazał 100% identyczności z izolatami *Ba. canis* zidentyfikowanymi u psów z Polski (np. KT844903). Analiza filogenetyczna wszystkie uzyskane sekwencje przyporządkowała do typu A (*Ba. canis* type A) grupującego szczepy powodujące łagodniejszą postać babeszjozy w porównaniu ze szczepami typu B (Adaszek i Winiarczyk, 2008). Ten szczep wykryto na północy Polski, jednak poziomy zakażeń *Ba. canis* były wielokrotnie niższe i wynosiły 0,2% u dorosłych (Cieniuch i wsp., 2009) oraz 0,4% u nimf i 1,0% u dorosłych *I. ricinus* (Stańczak i wsp., 2015).

Do tej pory wszystkie przypadki babeszjozy psów w Europy przypisuje się kleszczowi łąkowemu, *Dermacentor reticulatus*, którego uważa się za kompetentny wektor *Ba. canis* (Solano-Gallego i wsp., 2016). Dlatego w Polsce obszary uznane za endemiczne dla tej choroby są jednocześnie obszarami występowania tego kleszcza, który rozprzestrzenia zwykle bardziej wirulentne szczepy *Ba. canis* typ B (Adaszek i Winiarczyk, 2008; Łyp i wsp., 2016). Według Mierzejewskiej i wsp. (2015) częstość występowania tego patogenuu *D. reticulatus* w regionach endemicznych północno-wschodniej i środkowej Polski waha się pomiędzy 2,3%

i 8%. Najwyższy odsetek zakażeń (21,3%) stwierdzono w południowo-wschodniej Polsce (Dzięgiel i wsp., 2014). Ostatnie badania potwierdziły istnienie rozproszonych stanowisk *D. reticulatus* na obszarze województwa wielkopolskiego, jednak jak dotąd u żadnego z kleszczy nie potwierdzono *Ba. canis* (Mierzejewska i wsp., 2015b; Opalińska i wsp., 2016). Udział *I. ricinus* w przenoszeniu *B. canis* wydaje się być zatem prawdopodobny, co sugeruje wykrycie sekwencji *Ba. canis* typ B u samicy *I. ricinus* żerującej na psie na Słowacji (Rehackova i wsp., 2016). *Ixodes ricinus* mógłby potencjalnie pełnić rolę alternatywnego wektora przynajmniej dla mniej wirulentnych szczepów typu A, zwłaszcza, że jest najczęściej stwierdzanym kleszczem na psach na zachodzie Polski (Król i wsp., 2016). Na możliwość takiej roli wskazują badania (Foldvari i Farkas, 2005), w których stwierdzono, że chociaż babeszjoza była częściej diagnozowana u psów infestowanych przez *D. reticulatus*, to zdiagnozowano ją także u zwierząt infestowanych wyłącznie przez *I. ricinus* (61% vs. 26%).

Podsumowując, praca dokumentuje najwyższy w Europie poziom zakażeń *Ba. canis* wśród *I. ricinus* (10,2%). Potwierdzenie *Ba. canis* u 13% larw z ekosystemów leśnych, wskazuje na kluczowe znaczenie transmisji transowarialnej (wertykalnej) tego gatunku. Ten transfer jest charakterystyczny dla przedstawicieli z grupy *Babesia* sensu stricto (klad X), do której należą także *Ba. divergens, Ba. venatorum* (Azagi i wsp., 2021). Wykrycie u „miejskich kleszczy" co najmniej dwukrotnie wyższego odsetka zakażeń u dorosłych osobników w porównaniu z nimfami (3,3% vs. 1,5%) wskazuje na transstadialny transfer tego pasożyta. Należy podkreślić, że uzyskane wyniki sugerują możliwość funkcjonowania *I. ricinus* jako alternatywnego wektora *Ba. canis* na obszarach, gdzie *D. reticulatus* jest nieobecny lub rzadki. W konsekwencji psy mające regularny kontakt z *I. ricinus* mogą być eksponowane na *Ba. canis*, co powinno być brane pod uwagę przez weterynarzy, zwłaszcza, że kleszcz pospolity nie jest łączony z psią babeszjozą. Ostateczne określenie statusu *I. ricinus* jako wektora *Ba. canis* wymaga badań eksperymentalnych.

W pracy M.1. przedstawiłam rezultaty dotyczące pozostałych wykrytych gatunków z rodzaju *Babesia*. Łącznie DNA *Babesia* spp. wykryłam u 4,4% kleszczy z roślinności (7.3% samic, 4.6% samców i 2,4% nimf). Zakażone kleszcze występowały na 4 z 5 stanowisk (zakres: 0.8% - 9.8%). Podobne poziomy zakażeń opisano w Trójmieście (4,5%) (Stańczak i wsp., 2015) oraz w parkach miejskich Warszawy (6,5%) (Sytykiewicz i wsp., 2012). W przypadku samic *I. ricinus* zebranych ze zwierząt u 6,0% okazów zidentyfikowałam DNA *Babesia* spp., przy czym kleszcze z psów były częściej zakażone niż z kotów (6,8% vs. 3,9%). Podobny odsetek zakażeń (4,7%) opisano u kleszczy z psów na Litwie (Namina i wsp., 2019). Najwyższe

frekwencje zakażonych kleszczy z psów (66,8%) i kotów (15,4%) pochodzą z południowej Polski (Asman i wsp., 2015). W obu grupach kleszczy stwierdziłam dwa gatunki chorobotwórcze dla człowieka: *Ba. microti* i *Ba. venatorum,* ponadto *Ba. canis*. *Babesia canis* i *Ba. microti* dominowały zarówno w kleszczach z roślinności (2,6% i 1,4%) jak i ze zwierząt (2,8% i 2,2%), natomiast *Ba. venatorum* była najrzadszym gatunkiem. Doniesienia o *Ba. canis* u *I. ricinus* pochodzą z północy Polski (zakres: 0,2% - 5,8%) (Stańczak i wsp., 2015; Kubiak i wsp., 2022) oraz z Wielkopolski, gdzie wykazałam najwyższy jak dotąd odsetek (10,2%) u „leśnych kleszczy" (od 9,9% do 12,5%) opisany w artykule A.1. Prewalencja *Ba. canis* w kleszczach z psów (3,2%) okazała się podobna do opisanej na Litwie (3,8%) (Namina i wsp., 2019). Wszystkie uzyskane sekwencje *Ba. canis* należały do typu A reprezentującego szczepy o mniejszej chorobotwórczości (Adaszek i Winiarczyk, 2008). Z kolei w przypadku *Ba. microti* uważanej za najbardziej rozpowszechniony gatunek w populacjach *I. ricinus* (Onyiche i wsp., 2021), sekwencje (n=42) były w 100% identyczne z genotypem "Jena/Germany" uważanym za patogenny dla ludzi. Ponadto sekwencje *Ba. venatorum* (n=17) wykazały 100% identyczność ze szczepami u pacjentów we Włoszech, Austrii (Herwaldt i wsp., 2003) i Polsce (Rozej-Bielicka i wsp., 2017).

5.2. Występowanie krętków *Borrelia miyamotoi* z grupy gorączek powrotnych (RFGB)

W artykule A.2. opublikowałam rezultaty dotyczące częstości zakażeń *Borrelia miyamotoi,* bakterii z grupy gorączek powrotnych (RFGB) wśród miejskich populacji kleszczy *I. ricinus*. Poza kleszczami z roślinności (Tab. 1) stanowiących bezpośrednie zagrożenie dla ludzi, badałam także kleszcze z psów i kotów celem oceny częstości ich ekspozycji na kontakt z zakażonymi kleszczami. Łącznie przebadałam 837 okazów *I. ricinus* (831 samic i 6 larw) pochodzących od 680 zwierząt (567 psów i 113 kotów). Dodatkowo pozyskałam 33 okazy *Ixodes hexagonus,* w tym 31 (1 larwa, 13 nimf i 17 samic) zebrane z 3 kotów oraz 1 larwę i 1 nimfę z 2 psów, ponadto 1 okaz samicy *Dermacentor reticulatus* z psa. Należy zauważyć, że w odróżnieniu od ludzi, nie istnieją żadne publikacje o zakażeniach/chorobie wśród tej grupy zwierząt. Jedyne doniesienie o wykryciu *B. miyamotoi* u dwóch kotów bez symptomów chorobowych pochodzi z USA (Shannon i wsp., 2017). Może to oznaczać, że zwierzęta towarzyszące są odporne i nie dochodzi u nich do rozwoju spirochetemi (pojawienia się krętków we krwi) typowej dla *B. miyamotoi*. W Europie, brak danych o występowaniu zakażeń

B. miyamotoi u psów i kotów może wynikać z nieuwzględniania tego gatunku na etapie diagnozowania chorób odkleszczowych przez weterynarzy.

DNA *B. miyamotoi* wykryłam u 2,1% kleszczy zebranych z roślinności. Zakażone kleszcze występowały na wszystkich pięciu stanowiskach (zakres: 1,4% - 2,4%). Obecność patogenu wykazałam także we wszystkich stadiach rozwojowych, przy czym zakażenia były ponad dwukrotnie częstsze u nimf niż u postaci dorosłych (2,8% *vs*. 1,2%), a najczęstsze u larw (6,7%). Spośród 22 zakażonych kleszczy 27,3% wykazało koinfekcję z *B. burgdorferi* s.l., czyli łącznie, współwystępowanie obu patogenów dotyczyło 0,6% wszystkich kleszczy. W Słowacji 24% kleszczy *I. ricinus* zakażonych *B. miyamotoi* było w koinfekcji z *B. burgdorferi* s.l. (Hamsikova i wsp., 2017). W przypadku kleszczy *I. ricinus* ze zwierząt zakażenie *B. miyamotoi* wyniosło 1,8% i występowało wyłącznie u samic. Zakażone kleszcze infestowały 2,2% zwierząt (13 psów i 2 koty). Patogen wykryłam także u 9,1% kleszczy *I. hexagonus* (1 samica i 2 nimfy), natomiast nie potwierdziłam go u samicy *D. reticulatus*.

Analiza sekwencji (253 pz) regionu V4 genu 16S rRNA (V4 16S), wykrytych w zakażonych kleszczach z roślinności wykazała obecność dwóch typów. Typ 1 dominował (95%; 21/22) i był w 100% identyczny z wariantem *B. miyamotoi* wykrytym w jajach *I. ricinus* na terenie Czech (GenBank CP046389). Typ 2 wystąpił tylko u jednej larwy. Z kolei sekwencje genu *flaB* wykryte w kleszczach ze zwierząt wykazały 100% identyczność z izolatami opisanymi u *I. ricinus* żerujących na sarnie w Polsce (np., GenBank FJ874925).

Spośród uzyskanych wyników na szczególną uwagę zasługuje wykrycie zakażonych kleszczy na wszystkich 5 stanowiskach, co potwierdza istnienie aktywnych enzootycznych cykli *B. miyamotoi* w miejskich populacjach *I. ricinus* w Poznaniu. Łączny odsetek zakażeń (2,1%) jest zbliżony do wartości odnotowanych w miejskich ekosystemach w Holandii (2,5%), Niemczech (2,1%) czy Litwie (1,1%) (Wagemakers iwsp., 2017; Blazejak i wsp., 2018; Capligina i wsp., 2020). Na uwagę zasługuje wykrycie infekcji u 6,7% larw, co potwierdza transowarialny transfer tego patogenu. Dlatego, chociaż larwy *I. ricinus* stwierdza się zaledwie u 1,2-4,2% zaatakowanych ludzi (Duplaix i wsp., 2021), mogą one stanowić potencjalne zagrożenie infekcją *B. miyamotoi* dla człowieka. Brak różnic w poziomie zakażeń wykrytych w obu grupach kleszczy (2,1% i 1,8%), sugeruje, ze systematyczny monitoring miejskich psów i kotów pod kątem obecności *B. miyamotoi* w żerujących kleszczach, może być również wykorzystany dla oceny ryzyka nabycia tych krętków przez człowieka. Z kolei, obecność zakażonych samic *I. ricinus* u 2,2% (15/680) zwierząt badanych w okresie 3 lat

sugeruje, że ryzyko ekspozycji na tę bakterię jest stosunkowo niskie, co jednak nie powinno być argumentem dla nieuwzględniania przez weterynarzy możliwości wystąpienia infekcji *B. miyamotoi*. W tym kontekście przyszłe badania powinny być rozszerzone także na dzikie gatunki zwierząt drapieżnych, których rola pozostaje nieznana.

5.3. Występowanie krętków *Borrelia burgdorferi* s.l. z grupy boreliozy z Lyme (LB)

W pracy M1. analizowałam występowanie krętków z kompleksu *B. burgdorferi* s.l. oraz protistów z rodzaju *Babesia* w populacjach kleszczy *I. ricinus* wraz z ich identyfikacją gatunkową oraz oszacowaniem frekwencji współwystępowania w postaci koinfekcji. Wyniki dotyczące występowania pojedynczych infekcji *Babesia* spp. opisałam w podrozdziale 5.1. Poza kleszczami z roślinności – grupa I (Tab. 1) badałam osobniki z psów i kotów – grupa II, do których dodatkowo włączyłam 404 kleszcze bez danych o ich żywicielach (Tab. 2).

Ogółem DNA *B. burgdorferi* s.l. wykryłam u 8,7% kleszczy z roślinności (11,8% samic, 9,6% samców oraz 6.3% nimf), które występowały na 4 z 5 stanowisk (zakres: 3% - 40,3%). W Europie, średni poziom zakażeń *B. burgdorferi* s.l. u *I. ricinus* z terenów zieleni miejskiej wynosi 17,3% (zakres: 3,1% - 38,1%) (Hansford i wsp., 2022). W Polsce zbliżone wartości notowano w parkach Warszawy (10,9%) i Trójmiasta (12,4%) (Kowalec i wsp., 2017; Stańczak i wsp., 2004), a najwyższą wartość (27,4%) w Białymstoku (Grochowska i wsp., 2021). U kleszczy ze zwierząt stwierdziłam blisko dwukrotnie niższy procent infekcji (4,7% vs. 8,7%). Był on zbliżony do wartości opisanych w Holandii (4,8%) i Austrii (5,2%) (Leschnik i wsp., 2012; Kooyman i wsp., 2022). W Polsce wielokrotnie wyższe odsetki zakażeń u *I. ricinus* z psów notowano we Wrocławiu (21,7%) oraz Olsztynie (34.4%) (Król i wsp., 2015; Michalski i wsp., 2020). W obu grupach wykryłam gatunki należące do krętków powodujących LB: *B. afzelii*, *B. garinii*, *B. lusitaniae*, *B. valaisiana*, a u kleszczy z grupy II dodatkowo *B. spielmanii*. Dwa pierwsze gatunki przeważały w grupie I (3,7% i 3,7%), jak i II (3,7% i 0,6%), co potwierdza ich dominującą pozycję wśród gatunków z kompleksu *B. burgdorferi* s.l. występujących w populacjach *I. ricinus* (Strnad i wsp., 2017) oraz u psów z objawami boreliozy w Europie (Skotarczak, 2018).

5.4. Współwystępowanie gatunków z rodzajów *Babesia* oraz *Borrelia* z grupy LB

Łącznie, spośród 120 kleszczy, u których stwierdziłam DNA *Babesia* spp. prawie 20% było równocześnie zakażone *B. burgdorferi* s.l. Poziomy koinfekcji były podobne w obu grupach kleszczy (1,1% - grupa I oraz 1,0% - grupa II). W Polsce współzakażenia tymi patogenami u kleszczy wahają się w przedziale 0,3% - 2,8% (Stańczak i wsp., 2004; Grochowska i wsp., 2022). *Babesia canis* dominowała w koinfekcjach (72,7%; 8/11), występując z *B. afzelii* lub *B. garinii*, natomiast w kleszczach ze zwierząt, przeważała *Ba. microti* (92,3%; 12/13) głównie z *B. afzelii*.

Za istotne osiągnięcie pracy M.1 uważam wykazanie, że w koinfekcjach występowały patogenne dla ludzi sekwencje protistów *Ba. microti* i *Ba. venatorum* wespół z krętkami *B. afzelii* i *B. garinii* powodującymi LB. Informacje o takich podwójnych infekcjach obecnych w kleszczach mają istotne znaczenie dla epidemiologii obu jednostek chorobowych, ponieważ mogą one modyfikować ich symptomy kliniczne, przebieg oraz czasokres leczenia. Na podkreślenie zasługuje stwierdzenie zakażeń *Ba. canis* w kleszczach z psów (3,2%) i z roślinności (2,6%), co należy uznać za kolejną sugestię o potencjalnej roli *I. ricinus* jako alternatywnego wektora dla tego gatunku i związane z nim ryzyko nabycia babeszjozy przez psy na terenach miast, gdzie *D. reticulatus* jest rzadki lub nieobecny.

Wnioski

1. Populacje kleszczy *Ixodes ricinus* występujące na terenach rekreacyjnych Poznania uczestniczą jako wektory w transmisji *Borrelia miyamotoi* z grupy gorączek powrotnych, 5 gatunków krętków z grupy LB oraz co najmniej 2 gatunków protistów z rodzaju *Babesia*. Wykrycie tych zakażeń na większości badanych stanowisk oraz dodatkowo u kleszczy ze zwierząt towarzyszących, dowodzi istnienia aktywnych enzootycznych cykli trzymujących krążenie tych patogenów w warunkach miejskich.

2. Wyraźna dominacja *Babesia canis* (61%) w obu grupach kleszczy, u których wykryto DNA *Babesia* spp, uprawdopodabnia funkcjonowanie *I. ricinus* jako alternatywnego wektora na obszarze środkowo-zachodniej Polski, gdzie biologiczny wektor, *Dermacentor reticulatus* jest nieobecny lub występuje w rozproszeniu. Wykrycie wyłącznie sekwencji mniej wirulentnych szczepów typu A, sugeruje możliwość ich

powiązań z *I. ricinus*, który infestuje psy w zachodniej Polsce. W rezultacie psy mające regularny kontakt z tym kleszczem mogą być eksponowane na warianty *Ba. canis* powodujące łagodniejszą postać babeszjozy, co powinno być wzięte pod rozwagę przez weterynarzy, zwłaszcza że kleszcz pospolity nie jest łączony z tą chorobą.

3. Pomimo stosunkowo niskich poziomów zakażeń *Ba. microti* i *Ba. venatorum* u przebadanych kleszczy (1,8% i 0,7%), wykrycie sekwencji identycznych z wariantami występującymi u pacjentów z objawami babeszjozy wskazuje, że na terenach rekreacyjnych w obrębie miast istnieje potencjalnie ryzyko nabycia tej choroby.

4. Brak różnic w poziomie zakażeń *B. miyamotoi* w obu grupach kleszczy (2,1% i 1,8%), sugeruje, ze monitoring miejskich psów i kotów pod kątem obecności tego krętka w żerujących kleszczach, mógłby być wykorzystany dla oszacowania ryzyka nabycia boreliozy miyamotoi. Obecność *B. miyamotoi* u żerujących *I. ricinus* (2,2%) w ciągu 3 lat badań, pokazuje, że ryzyko ekspozycji jest niskie. Brak opisanych przypadków tej choroby u psów i kotów, nie wyklucza możliwości wystąpienia infekcji *B. miyamotoi*, dlatego zwierzęta te powinny być objęte poszerzoną diagnostyką przez weterynarzy zwłaszcza w przypadku zwierząt z symptomami gorączki po kontakcie z *I. ricinus*.

5. Wykrycie koinfekcji *Borrelia* spp. i *Babesia* spp. tylko u 1% (24/2297) kleszczy pokazuje, że są one stosunkowo rzadkie w miejskich populacjach *I. ricinus*. Dominacja dwóch patogennych gatunków krętków: *B. afzelii* i *B. garinii* w koinfekcjach z potencjalnie chorobotwórczymi szczepami *Ba. microti* i *Ba. venatorum* wskazuje na możliwość równoczesnego zakażenia i rozwoju obu chorób odkleszczowych. Dlatego lekarze i diagności w rejonach, gdzie wykrywane są takie mieszane infekcje u *I. ricinus*, powinni brać pod uwagę ich modyfikujący wpływ na objawy kliniczne, przebieg i leczenie takich podwójnych zakażeń u pacjentów.

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6. Publikacja A.1

Ticks and Tick-borne Diseases 12 (2021) 101786

Contents lists available at ScienceDirect

Ticks and Tick-borne Diseases

journal homepage: www.elsevier.com/locate/ttbdis

Prevalence of *Babesia canis* DNA in *Ixodes ricinus* ticks collected in forest and urban ecosystems in west-central Poland

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ARTICLE INFO

Keywords:
Babesiae
Canine babesiosis
Piroplasmorida
Piroplasms
Castor bean tick
DNA barcoding

ABSTRACT

Babesia canis, a widely distributed European tick-borne protozoan haemoparasite, causes canine babesiosis, the most important tick-borne disease afflicting dogs worldwide. The meadow tick, *Dermacentor reticulatus*, is considered to be the primary vector of this parasite in central Europe. Females of the more broadly distributed and medically important castor bean tick, *Ixodes ricinus*, also commonly feed upon dogs, but their role in the enzootic transmission cycle of *B. canis* is unclear. Here, we screened 1,598 host-seeking *I. ricinus* ticks collected from two different ecosystems, forest stands *vs*. urban recreational forests, for the presence of *B. canis* DNA. Ticks were sampled during their two seasonal peaks of activity, spring (May/June) and late summer (September). *Babesia* species were identified by amplification and sequencing of a hypervariable 18S rRNA gene fragment. *Babesia canis* was the only piroplasm detected in 13% of 200 larvae and 8.2% of 324 nymphs in the forest ecosystems. In urban recreational areas, *B. canis* DNA was found in 1.5% of 460 nymphs, 3.5% of 289 females and 3.2% of 280 males. Additionally, three samples, including one female, one male, and one nymph, were co-infected with *B. venatorum* and one nymph with *B. divergens* or *B. capreoli*. Our findings implicate that *B. canis* can be transmitted transovarially and maintained transstadially within populations of *I. ricinus*, but the vector competence of *I. ricinus* for transmitting *B. canis* remains to be investigated.

1. Introduction

Babesiosis is a globally distributed, tick-borne protozoan disease caused by hematotropic parasites of the genus *Babesia* that affect mammals and occasionally humans (Chauvin et al., 2009). These intraerythrocytic protists, which belong to the phylum Apicomplexa, order Piroplasmorida, can cause malaria-like clinical manifestations. To date, more than 100 species of *Babesia* have been described worldwide (Beugnet and Moreau, 2015). They infect the erythrocytes of wild mammalian reservoir hosts and are second only in importance to trypanosomes as hemoparasites of mammals (Chauvin et al., 2009; Yabsley and Shock, 2013). It is believed that transovarial transmission from infected female ticks to their larvae via the eggs plays an important role in maintaining many *Babesia* species in vector ticks (Bonnet et al., 2007; Mierzejewska et al., 2018).

In Europe, babesiae are transmitted mainly by *Dermacentor reticulatus, Rhipicephalus sanguineus* sensu lato, and *Ixodes ricinus* (Beugnet and Moreau, 2015; Gray et al., 2019; Nava et al., 2015). The most prevalent *I. ricinus* is involved in the transmission cycles of *B. divergens* (e.g., Blaschitz et al., 2008; Skotarczak and Cichocka, 2001), *B. microti* (Gray et al., 2002), *B. venatorum* (formerly *Babesia* sp. EU1) (e.g., Overzier et al., 2013; Silaghi et al., 2012), and *B. capreoli* (Venclikova et al., 2015). The first three species are responsible for cases of human babesiosis (Gray et al., 2010). Further, *I. ricinus* have been shown to be occasionally infected with *B. gibsoni* (Schorn et al., 2011), *B. major* (García-Sanmartín et al., 2008; Hilpertshauser et al., 2006), *B. odocoilei*

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https://doi.org/10.1016/j.ttbdis.2021.101786
Received 3 November 2020; Received in revised form 8 May 2021; Accepted 15 June 2021
Available online 12 July 2021

(Hamšíková et al., 2016), *B. bovis, B. caballi*, and *B. ovis* (García--Sanmartín et al., 2008). Furthermore, babesiae have been detected in four nest or burrow-dwelling *Ixodes* species, namely, *I. canisuga, I. hexagonus, I. rugicollis*, and *I. trianguliceps*. The latter one, parasitizing rodents, participates in the enzootic cycle of *B. microti* (Randolph, 1995). Three remaining tick species belonging to the subgenus *Pholeoixodes* feed upon Canidae, Mustelidae and domestic carnivores (Karbowiak et al., 2020). *Ixodes hexagonus*, exhibiting a strong host specificity to hedgehogs, has been shown to harbor DNA of *B. vulpes* (Checa et al., 2018) and *B. microti* (Kocoń et al., 2020). The latter piroplasm was identified in *I. canisuga* which has also been reported to harbor *B. odocoilei* (Najm et al., 2014) and the badger associated *Babesia* sp. Meles-Hu1 (Hornok et al., 2018). The Meles-Hu1 genotype was also identified in *I. rugicollis* (Hornok et al., 2017).

Canine babesiosis is an emerging infectious disease in Europe. The course of the disease depends on which *Babesia* species infects a dog (Solano-Gallego and Baneth, 2011). Five canine piroplasms are recognized to be pathogenic for dogs: the large-sized (3.0–5.0 μm) *B. canis, B. rossi* and *B. vogeli*, and the small-sized (1.5–2.5 μm) *B. gibsoni* and *B. vulpes*. The last-named piroplasm formerly was known as *Theileria annae, B. microti*-like species or the *Babesia* "Spanish dog isolate" (Baneth et al., 2015). *Babesia vogeli* and *B. gibsoni* are distributed globally, whereas *B. canis* and *B. vulpes* are restricted to Europe (Bilić et al., 2018). *Babesia rossi*, causing the most severe form of the disease, is confined to sub-Saharan Africa (Penzhorn, 2020).

The meadow tick, *D. reticulatus,* is regarded as the primary vector for *B. canis*, particularly in central Europe (Matijatko et al., 2012). This tick is the second most common hard-tick (ixodid) species in central Europe (Karbowiak, 2014; Mierzejewska et al., 2015a). It is broadly, albeit patchily, distributed across Europe from northern Portugal and Spain to Kazakhstan and the region of Yenisei River (western Siberia) (Rubel et al., 2016). However, in some European countries, the prevalence of *B. canis* in *D. reticulatus* is very low (0-0.7%) despite numerous confirmed cases of canine babesiosis (Bonnet et al., 2013; Cochez et al., 2012; Król et al., 2016; Rybářová et al., 2017; Seleznova et al., 2020; Wójcik-Fatla et al., 2015). This discrepancy between the increasing numbers of canine babesiosis in Europe and the low prevalence of *B. canis* in *D. reticulatus* may be explained by the presence of a new, widespread vector for this piroplasm. Available data on the distribution of *D. reticulatus* and *I. ricinus* indicate that these species increasingly share the same areas and may parasitize the same hosts, i.e., horses, sheep, wild boar, deer, domestic cattle, dogs, and red foxes (Mierzejewska et al., 2015a; Nowak-Chmura and Siuda, 2012). There are few reports, however, showing that *I. ricinus* could be positive for *B. canis* DNA (Cieniuch et al., 2009; Hamšíková et al., 2016; Rybářová et al., 2017; Stańczak et al., 2015). Therefore, the aim of our study was to determine the prevalence of *B. canis* in host-seeking *I. ricinus* ticks to test the hypothesis that the castor bean tick might be involved in the transmission cycle of *B. canis*.

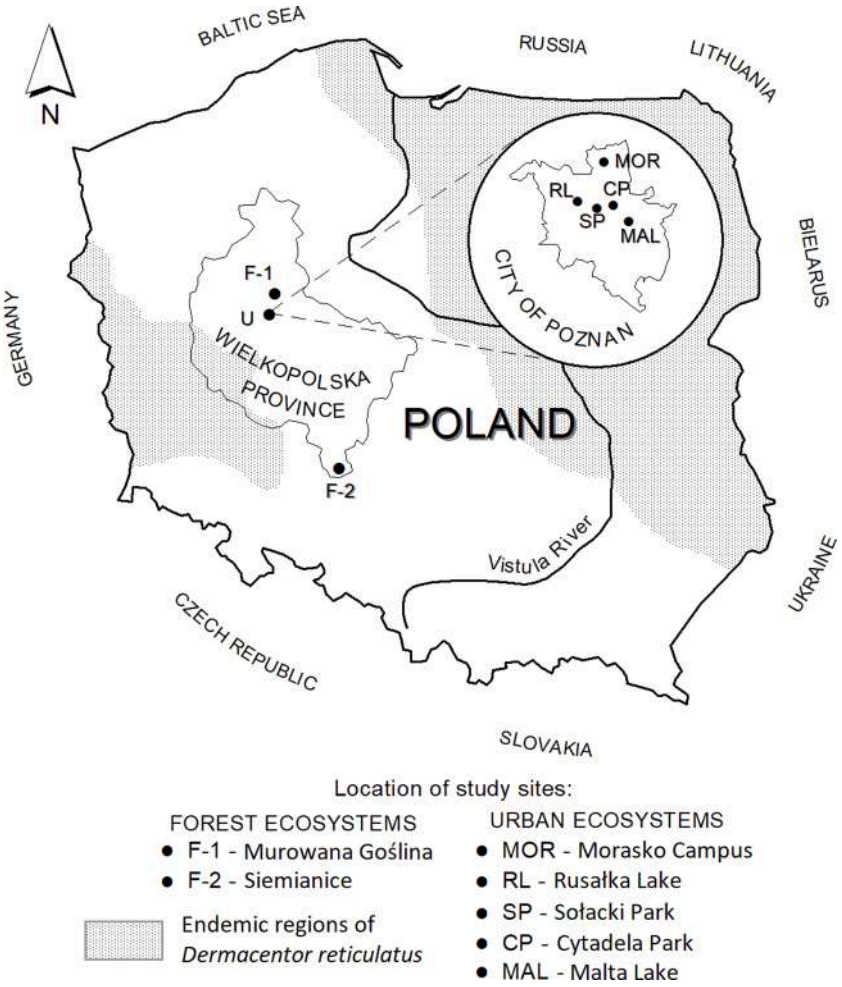

Figure 1. Location of study sites in west-central Poland. Forest ecosystems: (F-1) Murowana Goślina and (F-2) Siemianice marked on a map of Poland showing regions endemic for *Dermacentor reticulatus* ticks (shaded areas) according to Mierzejewska et al., (2015a). Five urban ecosystems within the city of Poznań: Morasko Campus (MOR), Rusałka Lake (RL), Sołacki Park (SP), Cytadela Park (CP), and Malta Lake (MAL).

2. Materials and methods

2.1. Study areas and tick sampling

Questing ticks analyzed in this study were collected in two different ecosystems: large forest stands, and urban recreational forested areas in the Wielkopolska Province of west-central Poland. The large forest stands (hereinafter referred to as "forest ecosystem") comprised two widely spaced (about 200 km) coniferous forest stands dominated mainly by Scots pine (*Pinus sylvestris*) in Murowana Goślina and Siemianice (Fig. 1, Table 1). At those sites, ticks were collected from human subjects dressed in white suits (for details see Wierzbicka et al., 2016). Sampling was conducted twice during the bi-annual peak activity periods of *I. ricinus*: May/June (spring) and September (late summer) 2013.

Ticks inhabiting urban parks and/or forests (hereinafter referred to as "urban ecosystem") were collected by dragging a 1-m^2 cotton cloth over low-lying vegetation within five forested recreational areas in the city of Poznań from May to September 2017 and in April 2018: Morasko Campus (MOR) of Adam Mickiewicz University, Rusałka Lake (RL), Sołacki Park (SP), Cytadela Park (CP), and Malta Lake (MAL) (Fig. 1, for details see supplementary Table A.1).

All ticks were preserved in 96% ethanol until DNA extraction. Adult ticks were identified to species using morphological characters (Siuda, 1993), whereas nymphs and larvae for which morphological identification is more laborious were identified using DNA barcoding based on cytochrome *c* oxidase subunit I (COI) amplification and next generation sequencing (NGS) of the amplicons.

2.2. DNA extraction

All ticks were processed individually; however, total genomic DNA was extracted in two laboratories using different methods. Ticks from the forest ecosystem were placed separately in 2-ml tubes containing Lysing Matrix A (MP Biomedicals, USA). After the addition of 360 µl of ATL lysis buffer (Qiagen, Germany), samples were homogenized twice for 30 s at 6.0 ms-1 using a FastPrep-24 homogenizer (MP Biomedicals, USA). Proteinase K (Bio Basic, Canada) was added to samples to the final concentration of 0.2 mg/ml, then samples were incubated for 48 h at 56°C with shaking. Next, 100 µl of the lysate were applied for DNA purification using the DNA ZR-96 Quick-gDNA kit (Zymo Research). Ticks collected in the urban ecosystem were subjected to DNA extraction using the ammonium hydroxide method (Rijpkema and Bruinink, 1996). All DNA extracts were stored at -20°C until analysis.

Table 1
Babesia canis detected in questing *Ixodes ricinus* ticks collected in two forest complexes (Murowana Goślina and Siemianice), and in five urban recreational forested areas in the city of Poznań (MOR, RL, SP, CP, MAL). Forest and urban ecosystems are located in the Wielkopolska Province, west-central Poland.

Sampling sites	No. infected/tested (%)				
	Larvae	Nymphs	Females	Males	Total
Murowana Goślina (F-1)	20/156 (12.8)	27/312 (8.7)	0/5	0/2	47/475 (9.9)
Siemianice (F-2)	6/44 (13.6)	2/12 (16.7)	0/5	0/3	8/64 (12.5)
Subtotal	26/200 (13)	29/324 (9.0)	0/10	0/5	55/539 (10.2)
Morasko Campus (MOR)	0/0	0/17	0/15	1/9 (11.1)	1/41 (2.4)
Rusałka Lake (RL)	0/1	6/313 (1.9)	10/170 (5.9)	2/153 (1.3)	18/637 (2.8)
Sołacki Park (SP)	0/13	0/17	0/15	0/14	0/59
Cytadela Park (CP)	0/0	0/4	0/24	5/41 (12.2)	5/69 (7.2)
Malta Lake (MAL)	0/16	1/109 (0.9)	0/65	1/63 (1.6)	2/253 (0.8)
Subtotal	0/30	7/460 (1.5)	10/289 (3.5)	9/280 (3.2)	26/1059 (2.5)
Total	26/230 (13)	36/784 (4.6)	10/299 (3.3)	9/285 (3.2)	81/1598 (5.1)

2.3. Screening for Babesia DNA by nested PCR

Babesia DNA was detected using nested PCR. The first round reaction was performed using RIB-19 (CGGGATCCAACCTGGTTGATCCTGC) and RIB-20 (CCGAATTCCTTGTTACGACTTCTC) primers that are specific for about 1700 bp of the piroplasm 18S rRNA gene including *Babesia* and *Theileria* spp. (Zahler et al., 2000). The second round PCR, targeting about 360 bp of the first round amplicon, was performed using the primer pair P3 (GTCTTGTAATTGGAATGATG) (Li et al., 2013) and BabR3 (CCTCTGACAGTTAAATACG) (developed in this study). Sequence comparison with GenBank sequence data using BLASTN 2.10.1+ program (Altschul et al., 1997) demonstrated that this primer set enables successful amplification in various apicomplexan groups, including small- and large-sized *Babesia* spp. and species belonging to the genera *Theileria, Hepatozoon* and *Sarcocystis* (data not shown).

First round PCR was prepared in a total volume of 6 µl containing HOT FIREPol Blend Master Mix with 2.5 mM final concertation of $MgCl_2$ (Solis BioDyne, Estonia), 0.25 µM of each primer and 1 µl of template DNA. The amplification program included 12 min at 95°C, followed by 40 cycles of 15 s at 95°C, 30 s at 58°C and 1.5 min at 72°C, with a final extension step at 72°C for 5 min. Second round PCR was prepared in a total volume of 10 µl containing HOT FIREPol Blend Master Mix, 0.25 µM of each primer and 1 µl of the first round PCR reaction. Control reactions were used in each experiment: a blank PCR reaction containing all PCR components except template DNA and the positive control from PK25N *Babesia* and *Theileria* detection kit (BLIRT S.A., Poland).

The amplicons were sequenced in one direction using the P3 or BabR3 primers. Sequencing was performed with BigDye Terminator v3.1 on an ABI Prism 3130XL Analyzer (Applied Biosystems, USA). Sequence chromatograms were checked for accuracy in Geneious R11.1.5 (Biomatters Ltd.) and contigs were assembled manually in GeneDoc sequence editing tool version 2.7 (Nicholas and Nicholas, 1997).

Seven samples that generated unreadable sequence chromatograms in direct Sanger sequencing were used for PCR with the P3 and BabR3 primers fused with Ion Torrent indexed adapters and sequenced using Ion Torrent PGM system (Thermo Fisher Scientific). PCRs were performed in two technical replicates, each in a total volume of 10 µl containing HOT FIREPol Blend Master Mix, 0.25 µM of each primer and 1 µl of template DNA. The amplification program included 12 min at 95°C, followed by 40 cycles of 15 s at 95°C, 30 s at 48°C and 45 s at 72°C, with a final extension step at 72°C for 7 min. After the PCR, amplicons were pooled and stored at 4°C until analysis.

2.3. DNA metabarcoding of ticks

In case of larvae and nymphs, the tick species was determined using sequence data from the COI gene fragment that covers about 370 bp from the 5′ end of the standard DNA-barcode (Hebert et al., 2003). The shortened COI was amplified using the primer pair bcdF01 (CATTTTCHACTAAYCATAARGATATTGG) (Dabert et al., 2010) and bcdR06 (GGDGGRTAHACAGTYCAHCCNGT) (Trzebny et al., 2020) fused with Ion Torrent double-indexed adapters. This primer set allows COI amplification of various metazoan lineages, including mites and ticks (M. Dabert, unpubl. data). PCR amplification was performed in a volume of 5 µl containing HOT FIREPol Blend Master Mix, 0.25 µM of each fused primer and 1 µl of template DNA. The amplification program included 12 min at 95°C, followed by 35 cycles of 15 s at 95°C, 30 s at 50°C and 45 s at 72°C, with a final extension step at 72°C for 5 min. PCR

samples were pooled, and stored at 4°C until analysis.

2.4. Library construction and NGS sequencing

The pooled amplicons were separated from non-specific PCR products using 2% E-Gel SizeSelect II Agarose Gels system (Invitrogen, USA), according to manufacturer's instructions. Library concentration and its fragment length distribution were established using the 2200 Tape Station system (Agilent Technologies, USA) and High Sensitivity D1000 Screen Tape assay, according to manufacturer's instructions. Clonal template amplification and sequencing were performed using the Ion Torrent One Touch System II and the Ion PGM Hi-Q View OT2 Kit. Sequencing was carried out using the Hi-Q View Sequencing Kit and Ion PGM system on Ion 314 and Ion 318 chips. All steps of the NGS sequencing were performed according to the manufacturer's instructions (Thermo Fisher Scientific). Libraries were sequenced to get at least 500 reads per sample.

2.5. NGS data analysis

Raw sequence data were pre-filtered by Ion Torrent Suite software version 5.10.1 (Life Technologies, USA) to remove polyclonal and low quality sequences. Further bioinformatic analysis was conducted using fastq data and custom workflow. Sequence reads shorter than 180 bp were removed from the dataset. Quality filtered sequences were separated into individual combinations of indexes in Geneious R11.1.5. Next, the sequences were trimmed at 5' and 3' ends to exclude PCR primers. Operational taxonomic unit (OTU) clustering was done in USEARCH version 11.0.667 (Edgar, 2010). The OTU consensus sequences were compared to GenBank using BLASTN (Zhang et al., 2000) optimized for highly similar sequences (megablast algorithm) (Morgulis et al., 2008) with 97% identity threshold to determine tick species.

2.6. Phylogenetic and statistical analyses

For phylogenetic analysis, the sequences identified were aligned with sequences representing different *B. canis* isolates identified in dogs (n = 9), bats (n = 2), *D. reticulatus* (n = 18), *I. ricinus* (n = 4) and selected species representing Babesiidae sensu stricto (n = 8) (supplementary Table A.2). *Anthemosoma garnhami* was used to root the tree, according to Chavatte et al. (2018). Sequence alignment was done using MAFFT v7.450 (Katoh and Standley, 2013) as implemented in Geneious R11.1.5 and adjusted manually in GeneDoc 2.7. Phylogenetic tree was constructed using FastTree 2.1.11 and Mr Bayes 3.2.6 (Huelsenbeck and Ronquist, 2001) with GTR+G model as implemented in Geneious Prime 2020.2.3 (Biomatters Ltd.). Statistical supports for branches were estimated by the Shimodaira-Hasegawa test (SH) (Shimodaira and Hasegawa, 1999) for the FastTree tree and posterior probability (PP) for the Bayesian tree. Each tree was edited in MEGA7 (Kumar et al., 2016) and further in Corel Draw X5. The chi-square ($\chi 2$) test was used to evaluate any significant differences among the variables, and probability values (p) less than 0.05 were considered significant.

3. Results

3.1. Collection and identification of questing ticks

A total of 1,598 ticks was collected and analyzed individually including 539 (200 larvae, 324 nymphs, 10 females, and five males) from the forest ecosystem and 1,059 (30 larvae, 460 nymphs, 289 females, and 280 males) from the urban recreational areas (Table 1). In urban localities, most ticks were derived from more wooded areas surrounding Rusałka and Malta lakes, which belong to communal forests of the city of Poznań. Based on morphological characteristics and COI sequence data, all ticks collected in our study were identified as *I. ricinus*.

3.2. Prevalence of B. canis in ticks

Babesia canis DNA was found in 5.1% (81/1,598) of the *I. ricinus* ticks tested (Table 1). Ion Torrent sequencing of the seven amplicons that were unreadable using Sanger method revealed that four ticks collected in different urban areas were positive for *B. canis* and co-infected with another *Babesia* species. We found one female (JR158), one nymph (JR464), and one male (PC43) co-infected with *B. venatorum*, and one nymph (MAL71) additionally positive for *B. divergens* or *B. capreoli* (both babesiae share the same nucleotide sequence in the marker fragment of 18S rRNA). All sequences generated were deposited in GenBank under accession numbers MF797815-830, MT981800-826, MW090682-720, MW012665-668 (for details, see Table A.2).

Overall, the prevalence of *B. canis* infection in *I. ricinus* obtained from two forested areas was 10.2% (55/539). *Babesia* DNA was detected only in the immature stages (10.5%; 55/524); none of 15 adult ticks was positive. The prevalence of *B. canis* infection was similar in both study sites (range, 9.9% to 12.5%). In Siemianice, 13.6% of larvae and 16.7% of nymphs yielded *B. canis* DNA, whereas in Murowana Goślina, 12.8% of larvae and 8.7% of nymphs were infected. The overall infection prevalence was similar in spring (10.4%, 45/432) and late summer (7.6%, 7/92).

In the urban ecosystem, the overall prevalence of *B. canis* DNA was 2.5% (26/1,059), a four-fold lower prevalence compared to the forest ecosystems ($\chi 2$ test, $p < 0.001$) (Table 1). Similarly, and in marked contrast to the forest ecosystems, *B. canis* infection was detected more often in adult ticks *vs*. nymphs (3.3% = 19/569 *vs*. 1.5% = 7/460), but this difference was not significant ($\chi 2$ test, $p = 0.059$). None of the 30 larvae tested was infected.

3.3. Diversity of B. canis 18S rRNA sequences

Analysis of nucleotide sequences of 81 *B. canis*-specific amplicons revealed 20 variants of 18S rRNA gene-fragment sequences that differed in either one (n = 19) or two (n = 1) nucleotide positions in the 272 bp sequence fragment. These sequence variants were found in *I. ricinus* collected from both forested and urban ecosystems. The dominating sequence variant, found in 74% (60/81) of the *B. canis*-positive ticks, demonstrated 100% nucleotide identities to *B. canis* isolates from dogs from Poland (e.g., GenBank acc.no. KT844903).

3.4. Phylogenetic analyses

The 18S rRNA phylogenetic tree revealed *B. gibsoni* as a sister group to *B. canis* with moderate support in both analyses (0.61 PP, 66% SH) (Fig. 2). *Babesia canis* sequences formed two well-supported clades corresponding to *B. canis* types A and B. All sequences found in this study grouped in the same clade as *B. canis* type A (type E18, GenBank acc. no. KP835549). Different 18S rRNA sequence variants were unstructured, i. e., the tree showed a polytomous relationship in this clade (data not shown).

4. Discussion

In central Europe, dogs are infested almost exclusively by adults of two ticks, *I. ricinus* and *D. reticulatus* (e.g., Beck et al., 2014; Eichenberger et al., 2015; Król et al., 2016). The relative abundance of both ticks varies geographically and depends upon many biotic and abiotic factors, such as availability and the composition of their preferred hosts, differences in their seasonality, habitat quality, and meteorological conditions (Bartosik et al., 2011; Buczek et al., 2014; Karbowiak 2014; Medlock et al., 2013). In Hungary, *D. reticulatus* and *I. ricinus* tend to parasitize dogs with comparable frequency, but this ratio varies from year-to-year (Földvári and Farkas, 2005; Földvári et al., 2007). A similar infestation trend was observed among dogs living in the Berlin/-Brandenburg area, Germany, where *D. reticulatus* and *I. ricinus*

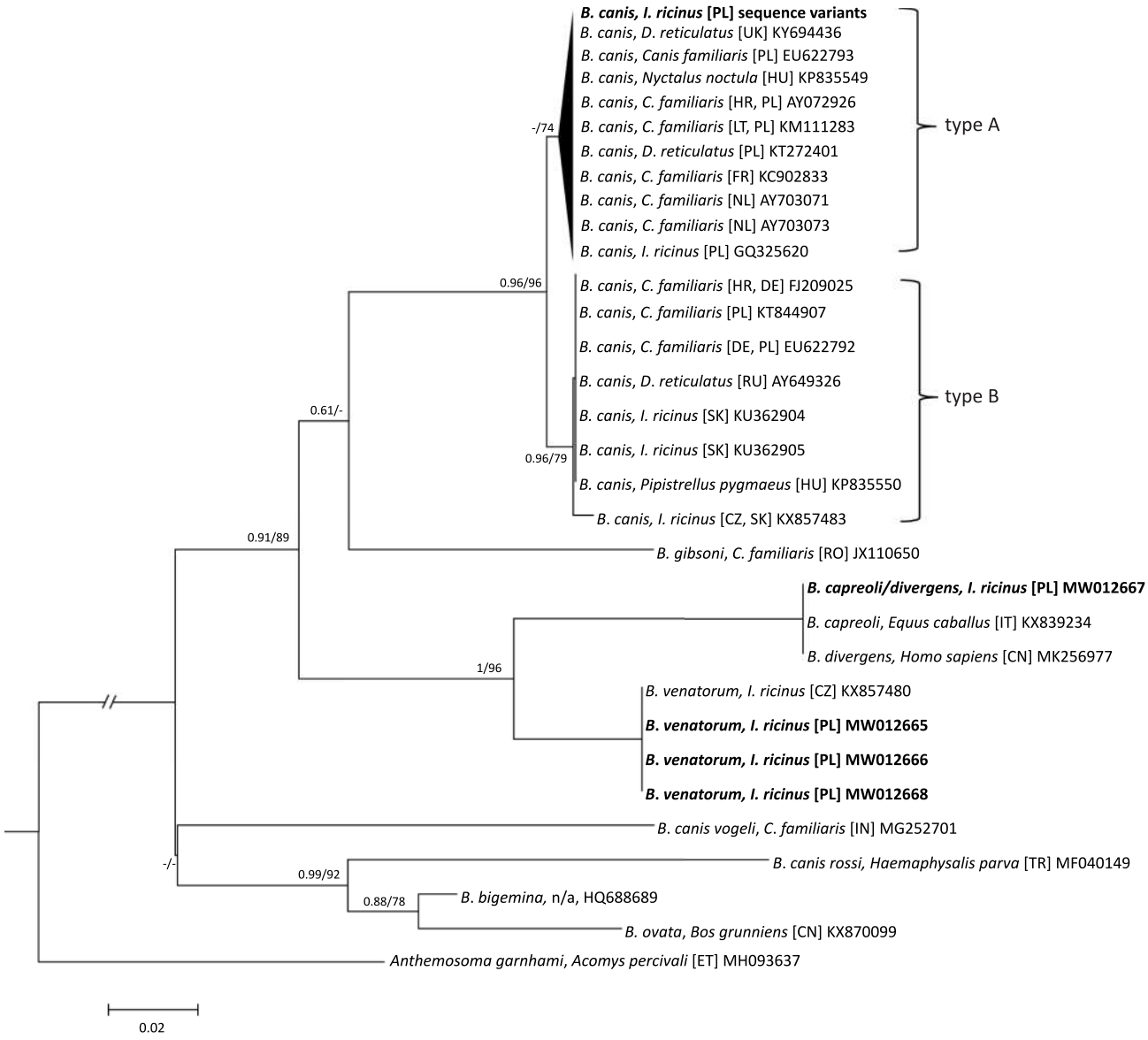

Figure 2. Phylogenetic tree reconstructed using 18S rRNA sequence fragments and BI/FastTree methods. Bayesian tree was polytomous in the clade grouping *B. canis* type A sequences (data not shown). Numbers near branches show support values (PP/SH); for SH only values >70% are present. *Babesia canis* type A clade is condensed. Sequences obtained in this study are shown in boldface. Sequences are described as follows: *Babesia* species, host species [country code], GenBank acc. no. The *Ixodes ricinus* [PL] sequence variants – 20 variants of *B. canis* 18S rRNA sequences found in this study. For details see supplementary Table A.1.

comprised 45% and 46% of 1,728 ticks, respectively (Beck et al., 2014).

In Poland, *D. reticulatus* occurs mainly in north-eastern and east-central parts of the country (Fig. 1), except for several recently reported range extensions into the mid-western and western areas (Mierzejewska et al., 2015a). In east-central Poland, considered a typical endemic area for meadow ticks, *D. reticulatus* composed 81% of all individuals collected from dogs (Mierzejewska et al., 2015a). A reverse trend occurs in west-central Poland, including our study area, i.e., the Wielkopolska Province. By example, *I. ricinus* and *D. reticulatus* respectively comprised 89% and 4% of 1,133 ticks removed from dogs in the Wrocław area of southwestern Poland (Król et al., 2016). The predominance of *I. ricinus* over *D. reticulatus* seems to occur in many central European countries implying that the former tick is the dominant species associated with companion animals (e.g., Duscher et al., 2013; Eichenberger et al., 2015).

To date, all reported cases of canine babesiosis throughout central Europe are attributed to the bite of *D. reticulatus*. Not surprisingly, it has been the only tick species examined for the presence of *B. canis* (Adaszek et al., 2011; Solano-Gallego et al., 2016). According to Mierzejewska et al. (2015b), the prevalence of this pathogen in *D. reticulatus* in endemic regions of northeastern and central Poland ranged between 2.3% and 8%. The highest mean prevalence of 21.3% was found in ticks (105/506) from southeastern Poland (Dzięgiel et al., 2014). In Poland generally, endemic areas for canine babesiosis are inhabited by *D. reticulatus* (Adaszek and Winiarczyk, 2008; Adaszek et al., 2011; Łyp et al., 2016). The highest canine infection prevalence reported so far, 25.3% of 79 animals, was found in dog kennels in the Mazovia Province (Welc-Falęciak et al., 2009).

The Wielkopolska Province was believed to be free of *D. reticulatus* until recent studies revealed its presence in several scattered localities in the west-central region. Intriguingly, none of meadow ticks collected from vegetation in western Poland were infected with *B. canis* (Mierzejewska et al. 2015b; Opalińska et al., 2016). Since the distribution of *D. reticulatus* appears to be restricted to those few isolated sites, involvement of other tick species in transmitting *B. canis* merits evaluation. Indeed, putative canine babesiosis cases have been observed in

several veterinary clinics in the city of Poznań, the capital of the Wielkopolska Province (J. Liberska, unpubl. data). We posited that *I. ricinus* might be an alternative vector therein because it is the most prevalent tick associated with dogs in this region (Król et al., 2016). This assumption is supported by a Hungarian study (Földvári and Farkas, 2005) in which dogs presenting with clinical symptoms of babesiosis prevailed among animals infested with *D. reticulatus*, though other dogs infested solely with *I. ricinus* also were diagnosed with the disease (61% and 26% of dogs, respectively).

The initial European evidence for the presence of *B. canis* in questing *I. ricinus* was reported in urban and rural recreational areas in north Poland. In the first report, this piroplasm was identified in only 0.2% of 1,078 adult ticks, while none of 314 nymphs was infected (Cieniuch et al., 2009). In the next report, 0.4% of 791 nymphs and 1.0% of 1,084 adult ticks were PCR-positive for *B. canis* (Stańczak et al., 2015). Likewise, merely 0.2% of 2,799 host-seeking *I. ricinus* ticks sampled in Bratislava, Slovakia contained *B. canis* DNA (Hamšíková et al., 2016). Its presence also was confirmed in 1.4% of 1,408 ticks collected from 59 sites in the Czech Republic and Slovakia (Rybářová et al., 2017). In the current study, about 5% of questing *I. ricinus* ticks sampled in west-central Poland were positive for *B. canis* DNA, the highest prevalence ever reported in this tick. Moreover, *B. canis* seems to be widely distributed in *I. ricinus* populations in the Wielkopolska Province. In fact, *B. canis* was found in both forest and urban ecosystems and four out of the five locations surveyed in the city of Poznań. Circumstantial evidence therefore suggests that *I. ricinus* could act locally as a vector of *B. canis* to dogs; however, its competence remains to be determined by transmission experiments.

European *B. canis* isolates have been classified into groups designated A and B types or as *B. canis* 18S RNA-A and *B. canis* 18S RNA-B genotypes, respectively (Adaszek and Winiarczyk, 2008), This grouping was based on the AG/GA nucleotide arrangement in the 91/92 nucleotide positions of the KP835549 and KP835550 sequences (Hornok et al., 2015). *Babesia canis* strains belonging to the type A or type B induce different clinical symptoms of babesiosis in dogs. Babesiae belonging to type B cause increased thrombocytopenia, increases in internal body temperature, rapid heart rate and discoloration of urine (Adaszek et al., 2009). In the current study, all *B. canis* sequences identified in *I. ricinus* from west-central Poland belong to the milder type A. This *B. canis* genotype also has been found in *I. ricinus* from Croatia, northern Poland, Slovakia, and the Czech Republic (Cacciò et al., 2002; Cieniuch et al., 2009; Rybářová et al., 2017). Detection of the type A within forest and urban ecosystems of west-central Poland suggests that *I. ricinus* maintains natural enzootic foci in that region. Type A has also been identified in unfed *D. reticulatus* in eastern Poland, Hungary, the Netherlands, and the United Kingdom (Hornok et al., 2016, Jongejan et al., 2015, Wójcik-Fatla et al., 2015). In central Europe, however, the more virulent type B prevails in *D. reticulatus,* the only experimentally proven vector of *B. canis*. The presence of this virulent type in unfed adult *D. reticulatus* ticks has been reported from eastern Poland, Germany, and Hungary (Beelitz et al., 2012; Hornok et al., 2016; Mierzejewska et al., 2015b). In Slovakia, Řeháčková et al. (2016) reported that one of 21 *I. ricinus* ticks removed from dogs was infected with *B. canis* type B; that isolate was identical to the dog-derived isolate EU622792 from Poland (Adaszek and Winiarczyk, 2008).

Taken together, our data from forest and urban ecosystems confirmed the presence of *B. canis* DNA in all parasitic stages of *I. ricinus*. However, we are aware of the fact that the combined prevalence values of *B. canis* calculated for larval and adult stages could also have been caused by differences in the numbers of these developmental stages collected in the two ecosystems. The unexpectedly high prevalence of *B. canis* detected in *I. ricinus* larvae (13%) from forest ecosystems demonstrates unequivocally that this parasite is transmitted transovarially, and its detection in questing nymphs suggests that nymphs can acquire babesiae by transstadial passage (stage-to-stage) from blood-fed larvae. In urban ecosystems, the fact that female and male ticks were at least two-fold more frequently infected as compared with nymphs (3.5% and 3.2% *vs*. 1.5%, respectively) substantiates transstadial passage of *B. canis* in populations of *I. ricinus*. Hence, two-thirds of the principal criteria, i.e., acquisition and transstadial passage, establishing that a tick species is a vector for a particular pathogen seems to be fulfilled during this study. However, we are aware that the detection of pathogen DNA in unfed ticks may not be considered as evidence of their vector competence (Uilenberg et al., 2018; Gray et al., 2019). Therefore, the final criterion, the active transmission of *B. canis* by the bite of infected *I. ricinus* larvae, nymphs, or females to naïve vertebrate hosts, requires experimental studies.

We conclude that *I. ricinus* is likely to be a vector of *B. canis* in certain forests and urban recreational areas of west-central Poland, a region where *D. reticulatus* is either absent or uncommon. Consequently, such habitats may pose a risk for dogs to become infected with this parasite. Hence, we recommend that *I. ricinus* ticks obtained subsequently from companion animals, humans or vegetation throughout Europe be tested for presence of piroplasms to further clarify the importance of this tick in the eco-epidemiology of *B. canis* and related babesiae.

CRediT authorship contribution statement

Justyna Liberska: Conceptualization, Investigation, Data curation, Formal analysis, Writing – original draft, Writing – review & editing, Funding acquisition. **Jerzy Michalik:** Conceptualization, Writing – review & editing. **Emilia Pers-Kamczyc:** Conceptualization, Investigation, Data curation, Writing – review & editing. **Anna Wierzbicka:** Conceptualization, Investigation, Resources, Writing – review & editing. **Robert S. Lane:** Conceptualization, Writing – review & editing. **Grzegorz Rączka:** Investigation, Writing – review & editing. **Patrycja Opalińska:** Investigation, Writing – original draft, Writing – review & editing. **Maciej Skorupski:** Project administration, Writing – review & editing, Funding acquisition. **Miroslawa Dabert:** Methodology, Validation, Supervision, Writing – review & editing.

Declaration of Competing Interest

None

Funding

This work was supported by the project Passport to the Future - Interdisciplinary doctoral studies at the Faculty of Biology, AMU, POWR.03.02.00-00-I006/17 (JL), Poznan University of Life Sciences project no. MN 507.216.44 (MS), and a statutory research program of the Institute of Dendrology Polish Academy of Sciences (EPK).

Acknowledgements

We thank Urszula Sobczyńska, Łukasz Broda, Weronika Pawlik and Aneta Łyś, Adam Mickiewicz University in Poznan, for their assistance in collecting ticks from urban ecosystems.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ttbdis.2021.101786.

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7. Publikacja A.2

Ticks and Tick-borne Diseases 14 (2023) 102188

Contents lists available at ScienceDirect

Ticks and Tick-borne Diseases

journal homepage: www.elsevier.com/locate/ttbdis

Original article

Exposure of dogs and cats to *Borrelia miyamotoi* infected *Ixodes ricinus* ticks in urban areas of the city of Poznań, west-central Poland

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ARTICLE INFO

Keywords:
Borrelia RF-group
TBRF
BMD
Ixodes ricinus
Ixodes hexagonus
Dermacentor reticulatus
Canis familiaris
Felis catus

ABSTRACT

Borrelia miyamotoi is an emerging human pathogen that causes a relapsing fever-like disease named *B. miyamotoi* disease. The bacterium belongs to the relapsing fever borreliae, and similar to spirochetes of the *Borrelia burgdorferi* sensu lato group, it is transmitted only by hard ticks of the *Ixodes ricinus* complex. To date, *B. miyamotoi* has not been demonstrated to cause illness in dogs or cats, and is poorly documented in veterinary medicine. The aim of this study was to determine the *B. miyamotoi* presence in (i) host-seeking ticks and (ii) engorged *Ixodes* sp. ticks collected from dogs and cats during their inspection in veterinary clinics of the city of Poznań, west-central Poland. Host-seeking ticks were sampled in dog walking areas localized in urban forested recreational sites of the city. In this study, 1,059 host-seeking and 837 engorged *I. ricinus* ticks collected from 680 tick-infested animals (567 dogs and 113 cats) were screened. Additionally, 31 *I. hexagonus* ticks (one larva, 13 nymphs, and 17 females) were collected from three cats; one larva and one nymph were collected from two dogs; and one dog was infested with a single *Dermacentor reticulatus* female.

Borrelia DNA was identified by the amplification and sequencing of the V4 hypervariable region of the 16S rRNA gene and *flaB* gene fragments. DNA of *B. miyamotoi* was detected in 22 (2.1%) of the host-seeking ticks (in all developmental tick stages and in all study areas). In addition, the engorged *I. ricinus* ticks exhibited a similar *B. miyamotoi* presence (1.8%). Fifteen *I. ricinus* ticks collected from animals tested positive for the presence of *B. miyamotoi* DNA, and the DNA of *B. miyamotoi* was observed in three (9.1%; one female and two nymphs) *I. hexagonus* ticks. The single *D. reticulatus* female collected from a dog tested PCR-negative for the bacterium. The results of this study demonstrated the establishment and broad presence of the bacterium in tick populations from different urban ecosystems of the city of Poznań. The lack of difference in the mean infection presence of animal-derived and host-seeking *I. ricinus* ticks suggests that the systematic surveillance of pets may be useful for the evaluation of human exposure to *B. miyamotoi* infected ticks in urban areas. Additional studies are required to further elucidate the role of domestic and wild carnivores in the epidemiology of *B. miyamotoi*, which remains unknown.

1. Introduction

Borrelia miyamotoi – a relapsing fever (RF)-group spirochete – is an emerging tick-borne pathogen, which affects humans and can cause *B. miyamotoi* disease (BMD). Compared to Lyme disease (LD), which causes an episodic erythema migrans, BMD causes recurrent bacteriemia with a high relapsing fever reaching up to 39 °C, accompanied with flu-like symptoms, such as headache, chills, and arthralgia/myalgia. Similar to other RF borreliae, *B. miyamotoi* multiplies efficiently in blood, which can lead to high spirochaetemia, and the transportation of spirochetes to

Abbreviations: RF, a relapsing fever; BMD, *B. miyamotoi* disease; LD, Lyme disease; HTBRF, hard-tick-borne relapsing fever; TBRF, tick-borne relapsing fever; BSL, *Borrelia burgdorferi* sensu lato complex; RL, urban forest localized around Rusałka lake; ML, urban forest localized around Malta lake; SP, Sołacki City Park; CP, Citadel City Park; MOR, Morasko Adam Mickiewicz University Campus; RA, the ticks from resident animals; TA, the ticks from travelling animals; NGS, next generation sequencing, COI, Cytochrome c oxidase subunit I gene; *flaB*, partial DNA sequences of *flaB* gene, encoding flagellin; V4 16S, V4 hypervariable region of the 16S rRNA gene; OTU, operational taxonomic unit.

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https://doi.org/10.1016/j.ttbdis.2023.102188
Received 8 November 2022; Received in revised form 19 April 2023; Accepted 23 April 2023
Available online 10 May 2023

any blood perfused tissue (Talagrand-Reboul et al., 2018). Furthermore, this bacterium has been demonstrated to be the causative agent of meningoencephalitis in immunocompromised patients (Hovius et al., 2013). Cases of BMD, also designated as hard-tick-borne relapsing fever (HTBRF), have been reported in Russia, the United States, Europe, and Japan (Platonov et al., 2011; Krause et al., 2015). Most of the cases were described with a nonspecific clinical picture, including fever and malaise, which suggests the underestimation of the disease and its causative agent (Hovius et al., 2013; Wagemakers et al., 2015). There are suggestions that LD-endemic areas may be falsely free from *B. miyamotoi*, and this may have an impact on the misdiagnosis of BMD. Therefore, the identification of this spirochete in such regions should be included in the current approach to the diagnosis in febrile patients without an erythema migrans rash suspected of a tick-borne infection (Telford III et al., 2015). Furthermore, BMD can be misdiagnosed as human granulocytic anaplasmosis caused by *Anaplasma phagocytophilum* infection or human monocytic ehrlichiosis induced by *Ehrlichia* spp. (Chowdri et al., 2013; Telford III et al., 2015).

Borrelia miyamotoi was originally isolated from unfed adult *Ixodes persulcatus* ticks and the rodent *Apodemus argenteus* in Japan (Fukunaga et al., 1995). Phylogenetically, *B. miyamotoi* clusters with the tick-borne relapsing fever (TBRF) borreliae, which are primarily transmitted to vertebrate hosts by soft ticks (Argasidae) of the genus *Ornithodoros* (Schwan and Piesman, 2002). The geographic distribution of the vector and reservoir hosts indicates that the TBRF borreliae group is genetically and ecologically distinct from the LD borreliae. The latter comprises of at least 24 species within the *B. burgdorferi* sensu lato complex (BSL), nine of which have been detected in European *I. ricinus* (Michalski et al., 2020). *Borrelia miyamotoi* has a global distribution in the northern hemisphere, and similarly as all recognized BSL species, it is vectored by the *Ixodes* spp. (Cutler et al., 2019). The global incidence of *B. miyamotoi* spirochete in host-seeking *Ixodes* species ranges from 0.2 to 10% (Kubiak et al., 2021). To date, the highest infection rates have been reported in *I. pacificus* (15.4%) sampled in California and in *I. persulcatus* (16%) in Russia (Cutler et al., 2019).

Similarly to the other spirochetes of the BSL group, *B. miyamotoi* is vectored by tick species from the *I. ricinus* complex feeding on a wide range of vertebrate species; thus, its reservoir hosts are believed to be primarily the same as those of *B. burgdorferi* s.l. (Krause et al., 2015). In Europe, small rodents (*Apodemus* spp. mice and *Myodes glareolus* voles) were demonstrated as reservoirs for this spirochete (Burri et al., 2014; Cosson et al., 2014; Gryczyńska et al., 2021). In addition, the DNA of the bacterium has been observed in European hedgehogs (*Erinaceus europaeus*), Eurasian red squirrels (*Sciurus vulgaris*), roe deer (*Capreolus capreolus*), and passerine birds (Passeriformes), e.g., blackbirds (*Turdus merula*), robins (*Erithacus rubecula*) (Talagrand-Reboul et al., 2018; Majerová et al., 2020). Domestic ruminants do not seem to eliminate the bacterium in moulted *I. ricinus* ticks previously engorged on ruminants; thus, they may not support its circulation (Richter and Matuschka, 2010).

In Europe, dogs and cats are frequently infested with *I. ricinus* ticks (Król et al., 2015). Studies on *Borrelia* spp. infection in dog-derived ticks revealed that these animals are exposed to at least four BSL species: *B. burgdorferi* sensu stricto, *B. afzelii, B. garinii,* and *B. valaisiana* (Hovius et al., 1999; Skotarczak and Wodecka, 2005; Michalski et al., 2020), which may induce canine LD (Skotarczak, 2018). *Borrelia burgdorferi* s.l. seropositive cats rarely display clinical symptoms (neither after natural nor experimental infections) (Littman et al., 2018). In addition, only two cases of Lyme carditis have been reported in cats in the UK (Tørnqvist-Johnsen et al., 2020). In contrast, dogs and cats have been demonstrated to be susceptible to *B. turicatae* and *B. hermsii* vectored by soft ticks in the United States (Kelly et al., al.,2014), and a novel species, *B. persica*, has been described in companion animals in Israel and Iran (Baneth et al., 2016; Shirani et al., 2016). Furthermore, Margos et al. (2020) reported two cases of canine TBRF caused by *B. hispanica* in Spain. However, there is only one report on *B. miyamotoi* infection in two healthy cats in the United States (Shannon et al., 2017). Nevertheless, the susceptibility of dogs and cats to this bacterium remains unknown.

The aim of this study was to assess the presence of *B. miyamotoi* in ixodid ticks collected from vegetation, as well as from dogs and cats in urban areas, of the city of Poznań, west-central Poland.

2. Materials and methods

2.1. Study areas, tick collection

Host-seeking ticks were collected by flagging a 1 m^2 woolen blanket over the vegetation along the paths in five forested green areas used for recreational activities and as walking areas for dogs in the city of Poznań. The samples were collected from May to September 2017 and in April 2018 (Fig. 1). The five study sites included: (i) two urban forests localized around Rusałka (RL) and Malta (ML) lakes, (ii) Sołacki (SP) and Citadel (CP) city parks, and (iii) Morasko Adam Mickiewicz University Campus (MOR), which includes teaching buildings adjacent to farmland and forest stands dominated mostly by Scots pine (*Pinus sylvestris*). Rusałka Lake is surrounded mainly by mixed and deciduous forests with numerous paths for strolling, cycling, and a public summer bath. Malta Lake is the largest artificial water reservoir in Poznań and is surrounded by mixed communal forest stands. Sołacki Park, with two ponds and a Bogdanka Stream running through it, is covered by deciduous tree stands with large numerous lawns. Citadel Park is the largest municipal park localized in the center of the city adjacent to residential areas. Deciduous trees and lawn areas are dominated with numerous promenades, large lawns and military cemeteries, and remains of fortifications.

Ticks from infested dogs and cats were collected in 17 veterinary clinics in Poznań (Fig. 1; suppl. Tab. S1). The tick collection was performed during three years: from April to October 2015, from March to November 2016, and from March to September 2017. Based on information from questionnaires regarding the travel history of the animal (with departure and return dates) outside the city at least two weeks prior to tick collection, the host-derived ticks were split into two groups: (i) resident animals (RA) and (ii) travelling animals (TA). Ticks were preserved in 96% ethanol until DNA extraction. Adult ticks were identified to the species level using morphological characters (Siuda, 1993). The *Ixodes* nymphs and larvae were identified using DNA-barcoding based on cytochrome *c* oxidase subunit I (COI) amplification and next generation sequencing (NGS) of the amplicons (Trzebny et al., 2020; Liberska et al., 2021).

2.2. DNA extraction from ticks

All ticks were tested individually. The host-seeking ticks were subjected to DNA extraction using the ammonium hydroxide method (Rijpkema and Bruinink, 1996), whereas the engorged ticks were homogenized using a FastPrep-24 homogenizer (MP Biomedicals, USA) and subjected to DNA extraction using ZR-96 Quick-gDNA kit (Zymo Research), as previously described by Liberska et al. (2021). All DNA isolates were stored at −20 °C until analysis.

2.3. DNA metabarcoding of ticks

In detail, the metabarcoding method was based on a previously described method (Trzebny et al., 2020) involving ticks (Liberska et al., 2021). Briefly, the COI gene fragment was amplified using the primer pair bcdF01 (CATTTTCHACTAAYCATAARGATATTGG) (Dabert et al., 2010) and bcdR06 (GGDGGRTAHACAGTYCAHCCNGT) (Trzebny et al., 2020) fused with Ion Torrent double-indexed adapters. PCR amplification was performed in a 5 μL mixture containing HOT FIREPol Blend Master Mix, 0.25 μM of each fused primer, and 1 μL of template DNA. The amplification program was performed for 12 min at 95 °C, followed by 35 cycles of 15 s at 95 °C, 30 s at 50 °C, and 45 s at 72 °C, with a final extension step at 72 °C for 5 min. PCR samples were pooled and stored at

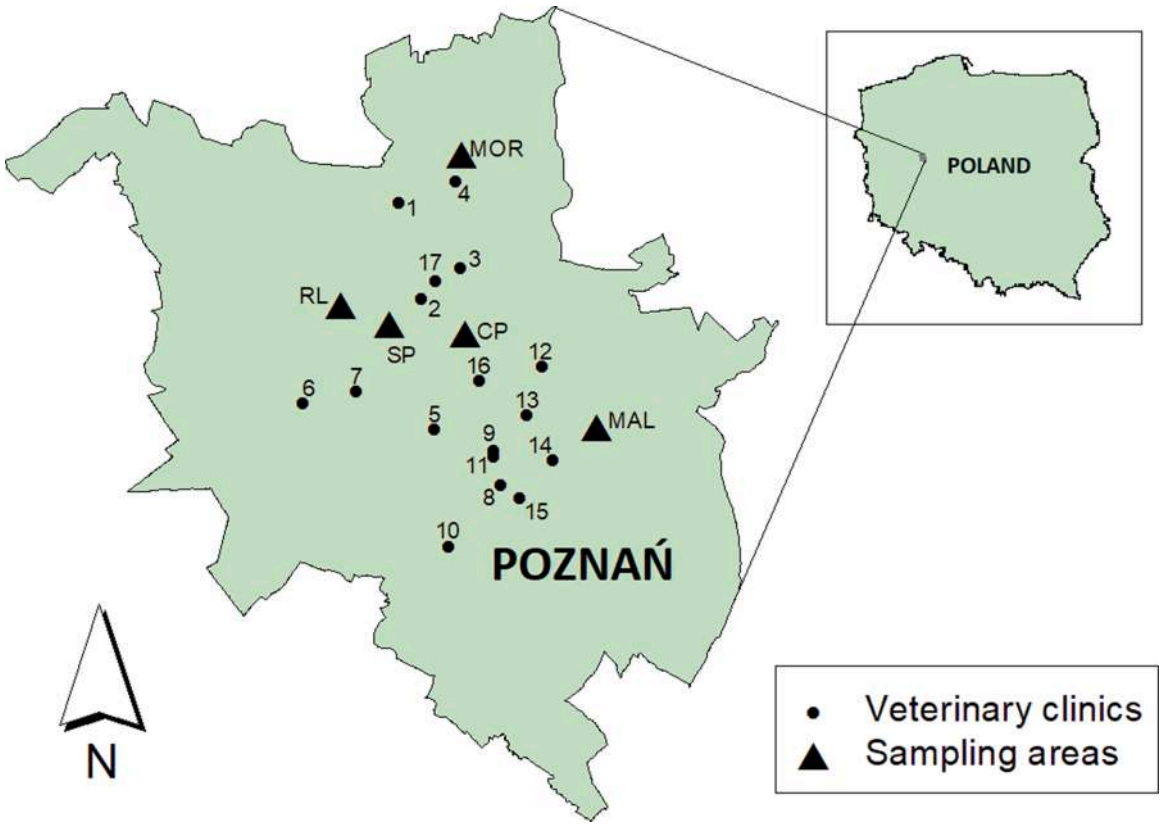

Fig. 1. Location of the five urban sampling areas (MOR - Morasko Campus, RL - Rusałka Lake, SP - Sołacki Park, CP - Cytadela Park, and MAL - Malta Lake) and 17 veterinary clinics within the city of Poznań, west-central Poland. The map was created using licensed software ArcMap ver. 9.3 (copyright ESRI Inc.). Names of veterinary clinics: (1) Jagiełły, (2) Piątkowska, (3) Wichrowe Wzgórze, (4) Batorego, (5) Chwiałkowskiego, (6) Bułgarska, (7) Marcelińska, (8) Armii Krajowej, (9) Jagiellońskie, (10) Św. Antoniego, (11) Rzeczypospolitej, (12) Bnińska, (13) Katowicka, (14) Lecha, (15) Orła Białego, (16) Chwaliszewo, (17) Księcia Mieszka I.

4 °C until library preparation and NGS sequencing. Negative controls from null DNA extraction and PCR reagents were amplified and sequenced together with the samples tested in this study.

2.4. Screening for Borrelia spp. DNA using nested PCR

Engorged ticks were screened for the presence of *Borrelia* DNA using the *flaB* gene amplified by nested PCR with two primer sets 132f/905r and 220f/823r (Wodecka et al., 2010). First round of the PCR was performed in a 6 μL mixture containing Type-it Microsatellite PCR Master Mix 2x (Qiagen), 0.25 μM of each 132f (TGGTATGGGAGTTTCTGG) and 905r (TCTGTCATTGTAGCATCTTT) primer, and 1 μL of the template DNA. Amplification program was performed as follows: 5 min at 95 °C, followed by 40 cycles of 30 s at 95 °C, 45 s at 50 °C, and 1 min at 72 °C, with a final extension step at 72 °C for 7 min. Second round PCR was prepared in a total volume of 10 μL containing Type-it Microsatellite PCR Master Mix, 0.2 μM of each fla220f (CAGACAACAGAGGGAAAT), and 823r (TCAAGTCTATTTTGGAAAGCACC) primer, and 1 μL of the 10 times diluted first round PCR reaction. Amplification program was performed as follows: 5 min at 95 °C, followed by 40 cycles of 30 s at 95 °C, 45 s at 50 °C, and 1 min at 72 °C, with a final extension step at 72 °C for 7 min. PCR products of the second round of amplification were diluted 2 times with water and 5 μL of the sample was analysed by electrophoresis on a 1% agarose gel. Samples containing visible bands were purified using thermosensitive Exonuclease I and FastAP Alkaline Phosphatase (Thermo Scientific, USA). In addition to negative controls, DNA extracted from a tick infected by *B. afzelii* was used to control unspecific detection of *Borrelia* DNA by flagellin gene amplification.

The amplicons (~500 bp) were sequenced in both directions using the 220f and 823r primers. Sequencing was performed using BigDye Terminator v3.1 on an ABI Prism 3130XL Analyser (Applied Biosystems, USA). The accuracy of the sequence chromatograms was examined in FinchTV 1.4.0 (Geospiza Inc., USA) and contigs were assembled manually in GeneDoc (Nicholas et al., 1997). The assembled sequences were aligned using ClustalW (Larkin et al., 2007). Obtained sequences were compared to those available in the GenBank using BLASTN (Zhang et al., 2000) (https://blast.ncbi.nlm.nih.gov) and the megablast algorithm.

2.5. Screening for Borrelia spp. DNA using 16S rRNA gene-profiling

Host-seeking ticks were screened for *B. miyamotoi* DNA by amplification and sequencing of the V4 hypervariable region of the 16S rRNA gene (V4 16S). This region is commonly used to screen environmental samples for bacterial taxa, and may be used to detect different *Borrelia* species. In this study, PCR primers V4F (GATCAGCAGCCGCGGTAATA) (Glowska et al., 2020) and V4R (GGACTACCAGGGTATCTAA) (Therese et al., 1998) fused with indexes and Ion Torrent adapters were used. PCRs were performed in a 10 μL mixture containing 2 μL HOT FIREPol Blend Master Mix (Solis BioDyne, Estonia), 0.25 μM of each primer, and approximately 1 ng of template DNA. Each sample was amplified in two technical replicates, which were pooled after PCR. The PCR program was performed as follows: 12 min at 95 °C, 30 cycles of 15 s at 95 °C, 30 s at 50 °C, 30 s at 72 °C, and a final step for 5 min at 72 °C.

2.6. Library preparation and NGS sequencing

The pooled amplicons (COI and V4 16S) were separated from non-specific PCR products using 2% E-Gel SizeSelect II Agarose Gels system (Invitrogen, USA), according to the manufacturer'sinstructions. Library concentration and its fragment length distribution were

established using the 2200 Tape Station system (Agilent Technologies, USA) and High Sensitivity D1000 Screen Tape assay, according to the manufacturer's instructions. Clonal template amplification and sequencing were performed using the Ion Torrent One Touch System II and the Ion PGM Hi-Q View OT2 Kit. Sequencing was performed using the Hi-Q View Sequencing Kit and Ion PGM system on Ion 314 and Ion 318 chips. All steps of the NGS sequencing were performed according to the manufacturer's instructions (Thermo Fisher Scientific). Libraries were sequenced to obtain at least 500 reads per sample for COI gene fragment and 50,000 for V4 16S.

2.7. NGS data analysis

Raw sequence data were pre-filtered using Ion Torrent Suite software version 5.10.1 (Life Technologies, USA) to remove polyclonal and low-quality sequences. Further bioinformatic analysis was conducted using fastq data and custom workflow. Sequence reads shorter than 180-bp were removed from the dataset. Quality filtered sequences were separated into individual combinations of indexes in Geneious R11.1.5 (Biomatters Ltd.). Next, the sequences were trimmed at 5′ and 3′ ends to exclude PCR primers. Operational taxonomic unit (OTU) clustering was performed in USEARCH version 11.0.667 (Edgar, 2010). The OTU consensus sequences were compared to those available in the GenBank using BLASTN and the megablast algorithm; we used 100 and 97% identity thresholds to determine *Borrelia* and immature *Ixodes* tick species, respectively.

2.8. Statistical analysis

Data analysis was performed using STATISTICA software version 6.0 (StatSoft Inc., Tulsa, OK, USA). Rates of infection were analysed using Chi-squared test $\chi 2$ and Fisher exact test. A $p < 0.05$ was considered statistically significant.

2.9. Phylogenetic analysis

For phylogenetic analysis, we used 19 sequences representing V4 16S of all Borreliaceae species for which the genome sequence has been published in GenBank to date, two different types of V4 16S sequences found in this study and a *Spirochaeta lutea* sequence used to root the tree (suppl. Tab. S2). Sequences were aligned in MUSCLE v. 3.8.425 and a phylogenetic tree was built using FastTree 2.1.11 as implemented in Geneious Prime 2022.1.1 (Biomatters Ltd.). Statistical supports for branches were estimated by Shimodaira-Hasegawa test (Shimodaira and Hasegawa, 1999). The tree was edited in Mega7 (Kumar et al., 2016) and Corel Draw v. X5.

3. Results

3.1. Collection and identification of ticks

A total of 1059 host-seeking *I. ricinus* ticks (30 larvae, 460 nymphs, 289 females, and 280 males) were collected from five urban green areas (Table 1). Among the sampled areas, ticks were most abundant in the two forested recreational areas localized around lakes (RL and ML), and accounted for 84% of the total tick population sampled in this study.

In addition, a total of 837 engorged *I. ricinus* ticks (831 females and 6 nymphs) were removed from 680 tick-infested companion animals (567 dogs and 113 cats) in the 17 veterinary clinics (Table 2). Females distinctly prevailed over nymphs (99.3% vs. 0.7% of total tick numbers, respectively), and were partially engorged. Furthermore, 14 non-parasitic males were collected. The number of ticks attached on the dogs and cats hosts were comparable (1.2 and 1.3 per animal, respectively), and most animals were infested with only one tick at the time of examination. Moreover, 31 *I. hexagonus* ticks (one larva, 13 nymphs and 17 females) were collected from three cats. Two dogs hosted one larva and one nymph of *I. hexagonus*, and one dog was infested with a single female *D. reticulatus*. Each of the three cats and the two dogs infested with *I. hexagonus* was concurrently parasitized by a single *I. ricinus* female.

Table 1
Borrelia miyamotoi-positive host-seeking *Ixodes ricinus* ticks collected in urban recreational areas of the city of Poznań, west-central Poland. Table 1. *Borrelia miyamotoi*-positive host-seeking *Ixodes ricinus* ticks collected in urban recreational areas in Poznań, Poland.

Sampling area	No. ticks infected/tested (%)				
	Larvae	Nymphs	Females	Males	Total
Rusałka Lake (RL)	0/1	11/313 (3.5)	2/170 (1.2)	1/153 (0.7)	14/637 (2.2)
Malta Lake (MAL)	2/16 (12.5)	1/109 (0.9)	1/65 (1.5)	1/63 (1.6)	5/253 (2.0)
Citadel Park (CP) 0/40/241/41 (2.4)1/69 (1.4)		0/4	0/24	1/41 (2.4)	1/69 (1.4)
Sołacki Park (SP)	0/13	0/17	0/15	1/14 (7.1)	1/59 (1.7)
Morasko Campus (MOR)		1/17 (5.9)	0/15	0/9	1/41 (2.4)
Total	2/30 (6.7)	13/460 (2.8)	3/289 (1.0)	4/280 (1.4)	22/1059 (2.1)

Table 2
Borrelia miyamotoi-positive engorged *I. ricinus* ticks collected from animals (567 dogs and 149 cats) surveyed in 17 veterinary clinics localized in the city of Poznań (names of subsequent clinics are given in Fig. 1 and Tab. S1). Table 2. Presence of Borrelia miyamotoi DNA in I. ricinus ticks feeding on dogs and cats examined in 17 veterinary clinics localized in the city of Poznań. The names of the enumerated clinics are shown in Fig. 1; N – number of dogs and cats tested in each clinic.

Clinics	Dogs			Cats			Total ticks
	N	Ticks infected/tested (%)		N	Ticks infected/tested (%)		
		Nymphs	Females		Nymphs	Females	
1	23		23	15		18	41
2	10		21				21
3	72	0/2	1/76 (1.3)	5		1/7 (14.3)	2/85 (2.3)
4	12	0/1	12				13
5	24		23	1		1	24
6	39		1/39 (2.6)	5		5	1/44 (2.3)
7	11		18	2		2	20
8	31		32	3		3	35
9	40		3/56 (3.6)	5		5	3/61 (4.9)
10	3		1/3 (33.3)	4		7	1/10 (10.0)
11	104		4/138 (2.9)	9		10	4/148 (2.7)
12	43		1/53 (1.9)	6		1/6 (16.7)	2/59 (3.4)
13	26		26	13		13	39
14	30		38	12	0/2	24	64
15	25		30	3		3	33
16	30		39	11		13	52
17	44	0/1	2/55 (3.6)	19		32	2/88 (2.3)
Total	567	0/4	13/682 (1.9)	113	0/2	2/149 (1.3)	15/837 (1.8)

3.2. Presence of B. miyamotoi in host-seeking ticks

Borrelia miyamotoi DNA was detected in 22 (2.1%) of the 1059 host-seeking *I. ricinus* ticks. The bacterium was found in all developmental

tick stages and in all study areas (Table 1). The *B. miyamotoi* DNA presence in female and male ticks were comparable (1.0%; 3/289 and 1.4%; 4/280, respectively). Additionally, compared to adult ticks (1.2%; 7/569), nymphs (2.8%; 13/460) were more frequently infected, but the difference was statistically insignificant ($\chi 2$ test, $P = 0.061$).

In the two forested recreational areas localized around lakes (RL and ML), the bacterium was identified in nymphs, females, and males. In ML area, an infection rate of 12.5% (2/16) was confirmed in the larvae. In two city parks, CP and SP, *B. miyamotoi* was observed only in *I. ricinus* males (2.4 and 7.1%, respectively), whereas in MOR Campus, only nymphs (5.9%) were confirmed to be infected with the bacterium. The infection presence in ticks collected in forested areas situated around lakes (RL and ML) and in MOR campus were comparable (2.2, 2.0, and 2.4%, respectively). In addition, the infection rates in ticks from two strictly city parks (CP and SP) were similar (1.4 and 1.7%, respectively).

Of the 22 host-seeking ticks infected with *B. miyamotoi*, six (27.3%) ticks (four nymphs, one female and one male tick) yielded concurrently DNA of *B. burgdorferi* s.l.

3.3. Presence of B. miyamotoi in ticks removed from dogs and cats

Borrelia miyamotoi DNA was identified in 15 (1.8%) of the 831 *I. ricinus* ticks, all of which were females. The bacterium was not observed in any of the six nymphs (Table 2). These *B. miyamotoi*-positive females were collected from 13 dogs and two cats examined in seven veterinary clinics. The proportion of cats and dogs exposed to infected ticks varied among the clinics (Table 2). There was no statistically significant difference in mean infection presence of engorged and host-seeking *I. ricinus* ticks (1.8% versus 2.1%, $\chi 2$ test, $P = 0.656$). None of the fifteen *B. miyamotoi* positive *I. ricinus* ticks removed from animals was co-infected with the *B. burgdorferi* s.l. spirochetes.

Among the 31 *I. hexagonus* cat-derived ticks, the DNA of *B. miyamotoi* was identified in two (6.5%), including one female and one nymph. In addition, the bacterium was observed in one *I. hexagonus* nymph removed from a dog. However, the single *D. reticulatus* female collected from a dog tested PCR-negative for the bacterium.

Assuming that the average feeding time of *I. ricinus* females can take up to 11 days (Balashov (1967, cited after Siuda 1991), we used a two-week period to compare potential differences in the risk of exposure to infected ticks between two group of animals: resident and travelling outside the city. Based on the questionnaire, this risk among dogs which travelled outside of the city of Poznań, was slightly higher compared to resident animals that did not travel (3.3% vs. 1.4%, $\chi 2$ test, $P = 0.106$); however, this difference was statistically insignificant. A similar trend was noted among travelling and resident cats (3.6% vs. 0.8%, $\chi 2$ test, $P = 0.250$; suppl. Tab. S3).

3.4. Diversity of B. miyamotoi V4 16S and flaB sequences

Among 22 *B miyamotoi* V4 16S sequences identified in the PCR-positive host-seeking ticks, two variants differing in one nucleotide position within the 290 bp sequence fragment were identified. The prevailing sequence variant, which was observed in 95% (21/22) of the *B. miyamotoi*-positive ticks, exhibited 100% nucleotide identities with the *B. miyamotoi* found in *I. ricinus* eggs from the Czech Republic (i.e., GenBank CP046389). The second variant of the V4 16S sequence (GenBank MZ918969) was found only in *I. ricinus* larva collected around ML lake.

Borrelia miyamotoi-specific amplicons detected from companion animals exhibited the same variant of *flaB* gene, presenting 100% nucleotide identity with *B. miyamotoi* isolates from ticks feeding on roe deer from Poland (i.e., GenBank FJ874925).

3.5. Phylogenetic analysis of V4 16S

Results of the phylogenetic analysis revealed that the V4 16S sequence enabled the differentiation of most of the *Borrelia* species (14/19) in both the LD- and RF-group spirochetes (Fig. 2). Only *B. bissettiae* and *B. burgdorferi* sensu stricto in the LD-group and *B. crocidurae, B. duttonii* and *B. recurrentis* in the RF-group were undifferentiated in this marker. However, this problem did not affect *B. miyamotoi*. Both sequence variants observed in this study formed one well-supported clade with *B. miyamotoi* observed in *I. persulcatus* collected from *Apodemus argenteus* in Japan (GenBank CP004217). Ticks containing different variants of the V4 16S sequence (represented by isolates mal51 and mal241) contained the same *flaB* gene sequence, identical to those identified in ticks collected from dogs and cats (GenBank OQ106981 and OQ106982).

3.6. Availability of the sequences observed in this study

Forty-three partial *B. miyamotoi* sequences were generated in this study and deposited in GenBank (suppl. Tab. S4). The V4 16S rRNA gene sequences ($n = 22$) obtained from host-seeking *I. ricinus* ticks (2 larvae, 13 nymphs, 3 females, and 4 males) were deposited under the following accession numbers: MZ918966–MZ918987. The *flaB* gene sequences ($n = 21$) identified in feeding females of *Ixodes* ticks are listed as follows: ON000073–ON000074, ON000076–ON000081, ON000084–ON000090 (*I. ricinus* from dogs); ON000075, ON000082 (*I. ricinus* from cats), ON000091 (*I. hexagonus* from a cat).

4. Discussion

In Europe, the primary biological vector of *B. miyamotoi*, the newly confirmed human pathogenic tick-borne relapsing fever spirochete, is the most widespread and abundant *I. ricinus*. In this study, we confirmed the presence of *B. miyamotoi* DNA in two groups of *I. ricinus* ticks

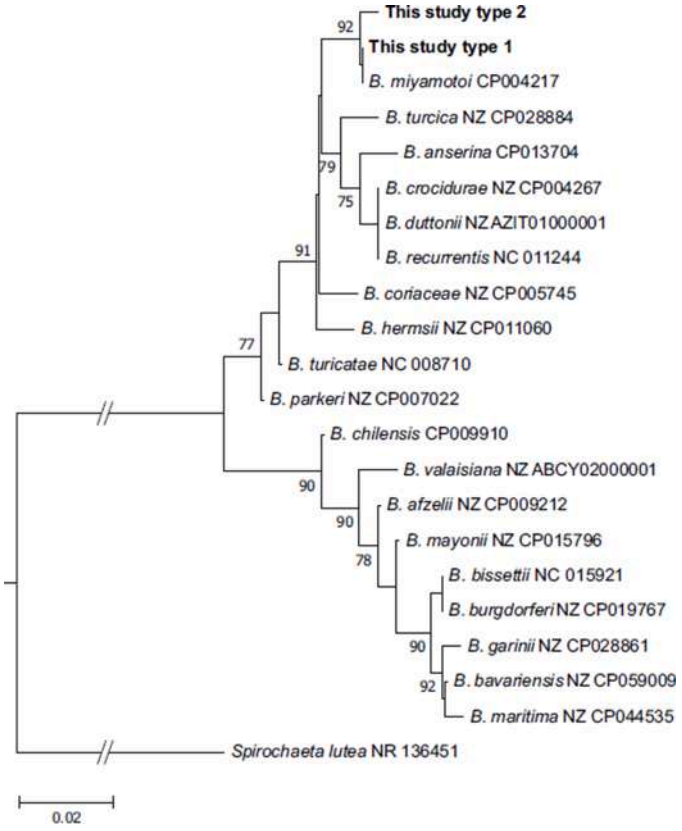

Fig. 2. Phylogenetic tree (FastTree) of the *Borreliaceae* species based on the fragment V4 16S sequences. Values near branches show Shimodaira-Hasegawa local supports.

occurring in urban areas of the city of Poznań, west-central Poland: (i) host-seeking ticks collected in forested recreational sites, and (ii) engorged ticks obtained from dogs and cats in veterinary clinics of the city. The mean infection presence of the bacterium found in the host-seeking ticks was 2.1% (range: from 1.4 to 2.4%). This level corresponds to the infection rates previously described in Netherlands, Switzerland, Germany, and Latvia (2.5, 2.5, 2.1, and 1.1%, respectively) (Oechslin et al., 2017; Wagemakers et al., 2017; Blazejak et al., 2018; Capligina et al., 2020). In Europe, the overall prevalence of *B. miyamotoi* in host-seeking *I. ricinus* ticks varies from 0.16% in Portugal (Nunes et al., 2015) to 4.8% in Hungary (Szekeres et al., 2015), and is typically approximately ten times lower than that of BSL spirochetes (Cutler et al., 2019). The infection presence determined in our study was comparable to those reported from two other Polish urban agglomerations: the city of Warsaw (1.3%) in central Poland (Kowalec et al., 2017) and the city of Olsztyn (3.5%) in north-eastern Poland (Kubiak et al., 2019). In Poland, the highest *B. miyamotoi* prevalences (6.4–8.4%) were observed in suburban recreational areas of the city of Białystok in North-eastern Poland (Grochowska et al., 2021). However, to date, no endemic or risk sites for *B. miyamotoi* have been identified in Poland. A recent Polish study by Gryczyńska et al. (2021), which was conducted in the Warsaw agglomeration, highlights the contribution of two *Apodemus* mice species (*A. flavicolis* and *A. agrarius*) to the spread of *B. miyamotoi*. The authors observed that the frequency of the bacterium in rodents captured in suburban forests, where the anthropogenic disturbances are relatively small, was significantly higher than that in animals examined in parks situated strictly in the city center (6.7 versus 4.3%). The higher mean infection presence observed in ticks collected in three urban forests (Rusałka lake, Malta lake, and MOR University Campus) compared to ticks collected in Sołacki and Citadel city parks (2.1% versus 1.6%) could be attributed to ecological differences in urban habitats; however, the difference was not statistically significant. In the case of forest stands localized at Rusałka lake, it has been demonstrated that both *Apodemus* species may act as significant reservoir hosts to BSL spirochetes (Michalik et al., 2003).

Our finding that 6.7% (2/30) of host-seeking *I. ricinus* larvae yielded the bacterium confirms the well-documented transovarial (vertical) transmission strategy from infected tick females to their offspring, which is utilized by most of the RF borreliae, including *B. miyamotoi* (Rollend et al., 2013). Furthermore, this finding highlights the differences in the transmission dynamics of BSL group and RF borreliae. The transovarial transmission of the spirochaetes from the first group is believed to be rather inefficient (Burgdorfer and Varma, 1967), whereas species belonging to the second group (e.g., *B. hermsi, B. turicata, B. crocidurae,* and *B. duttoni*) can be maintained by this type of transmission for as many as five generations (van Duijvendijk et al., 2016). Therefore, although *I. ricinus* larvae are responsible for only 1.3 to 4.2% of human tick bites (Duplaix et al., 2021), they may pose a potential risk of acquiring *B. miyamotoi* infections.

Most engorged *I. ricinus* ticks removed from pets were adult females, which is consistent with the findings of previous studies (Król et al., 2015; Michalski et al., 2020). We found *B. miyamotoi* DNA in ticks collected from 2.2% (15/680) of *I. ricinus* infested animals (567 dogs and 113 cats). A similar proportion of dogs and cats exposed to *B. miyamotoi* infected *I. scapularis* ticks was observed during a four-year study conducted in Canada (≈1.2%, range: 0.3–7.2%, and ≈1.3%, range: 0.2–8.0%, respectively) (Duplaix et al., 2021). The overall infection presence in the engorged ticks (1.8%) tested in our study was comparable to that of *I. ricinus* ticks removed from dogs in Germany (1.4%) and slightly higher than that of dog-derived ticks in Latvia (1.0%) (Schreiber et al., 2014; Namina et al., 2019). We did not observe any differences in the mean infection presence of engorged and host-seeking *I. ricinus* ticks (1.8 versus 2.1%). This finding may indicate that the risk of exposure of dogs or cats to *B. miyamotoi* is associated with infection rates among local populations of host-seeking *I. ricinus*, particularly female ticks. The lack of difference in the mean infection presence of animal-derived and host-seeking *I. ricinus* ticks, suggests that the systematic surveillance of pets may be useful for the evaluation of human exposure to *B. miyamotoi* infected ticks in urban areas. We identified *B. miyamotoi* DNA in 9.0% of the 33 engorged *I. hexagonus* ticks, which mainly infested cats ($n = 31$) and occasionally dogs ($n = 2$). This infection rate was higher than those of dog-derived *I. hexagonus* ticks (2.0%) tested in Germany (Schreiber et al., 2014) and ticks removed from European hedgehogs (1.5%) examined in Belgium (Jahfari et al., 2017). Although the nidicolous *I. hexagonus* tick associated primarily with hedgehogs has been demonstrated to act as the competent vector for BSL spirochetes (Gern et al., 1991), there is still no data regarding its role in the enzootic cycle of *B. miyamotoi*. In this study, the bacterium was not detected in the single dog-derived *D. reticulatus* female tick. To date, the only evidence of *B. miyamotoi* infection in host-seeking *D. reticulatus* ticks (0.5%; 1/2000) was reported in Berlin, Germany (Kohn et al., 2019). Presumably this tick species, which supports the transmission of *Babesia canis* (Dwużnik-Szarek et al., 2021), is an incompetent vector in enzootic cycles of *B. miyamotoi*.

We observed that dogs and cats that travelled outside of the city of Poznań (prior to tick collection) exhibited slightly higher risk of exposure to *B. miyamotoi*-infected ticks compared to resident dogs and cats that did not travel (3.3 and 3.6% vs. 1.4 and 0.8%, respectively); however, the differences were statistically insignificant. According to Duplaix et al. (2021), the travelling of dogs and cats outside the province of Quebec, Canada, was a risk factor for exposure to *I. scapularis* ticks infected with *B. burgdorferi* spirochetes compared to resident animals that did not travel outside their municipality of residence. However, they failed to identify such correlation for *B. miyamotoi* positive ticks.

Compared to BSL spirochetes, *B. miyamotoi* has not been identified as a bacterial agent causing illness in dogs or cats to date, and is poorly documented in veterinary medicine. Furthermore, there are no reports of either suspicion or confirmation of HTBRF infections in other domestic animals (Elelu, 2018). There is also no information on the role of wild carnivore species in the transmission cycles of *B. miyamotoi*. To date, the only report documenting the bacterium in one *I. ricinus* female collected from a raccoon dog (*Nyctereutes procyonoides*) (Wodecka et al., 2016). Limitation of epidemiological information can partially result from similarity in hematological and clinical symptoms of TBRF diseases and LD (Piccione et al., 2016). Detection of *B. miyamotoi* DNA in host-seeking *I. ricinus* ticks in all developmental tick stages and in all study areas indicates the good establishment and broad presence of the bacterium in tick populations from different urban ecosystems of the city of Poznań. The lack of difference in the mean infection presence between animal-derived and host-seeking *I. ricinus* ticks suggests that the systematic surveillance of pets may be useful in the evaluation of the risk involved in being infected with BMD in urban areas. Therefore, veterinarians and physicians need to be aware of this potential threat to the health of pets and their owners. In our opinion, knowledge on *B. miyamotoi* reservoir species, including domestic and wild carnivores species, needs to be completed to understand the enzootic transmission cycles of the bacterium in urban environments.

Funding

This work was supported by the project Passport to the Future-Interdisciplinary doctoral studies at the Faculty of Biology, AMU, POWR.03.02.00-00-I006/17 (JL).

CRediT authorship contribution statement

Justyna Anna Liberska: Conceptualization, Investigation, Data curation, Formal analysis, Writing – original draft, Writing – review & editing, Funding acquisition. **Jerzy Franciszek Michalik:** Conceptualization, Writing – review & editing. **Mirosława Dabert:** Methodology, Validation, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no competing interest.

Data availability

Data will be made available on request.

Acknowledgements

We would like to thank Urszula Sobczyńska, Łukasz Broda, Weronika Pawlik, and Aneta Łyś of Adam Mickiewicz University in Poznan for their assistance in the collection of ticks from urban ecosystems. We would like to thank all the veterinary clinics involved in the study: Centrum Zdrowia Małych Zwierząt M. Majka, R. Starczewski s.c., Gabinet Weterynaryjny Katarzyna Gryglewska, Gabinet Weterynaryjny lek. wet. Dariusz Filipiński, Gabinet Weterynaryjny Marcin Konieczny, Gabinet weterynaryjny - Piotr Winiecki, Klinika Weterynaryjna Juszczak & Gorzelańczyk, Praktyka Weterynaryjna A. Golec M. Rybski, Gabinet Weterynaryjny PLUTO VET lek wet Krzysztof Kaniewski, Zwierzak Gabinet Weterynaryjny A. Adamska, AMICUS Gabinet Weterynaryjny Grzegorz Macioch, Usługi Weterynaryjne Jakub Golec, Przychodnia Weterynaryjna Na Polance Katarzyna Marciniak, Specjalistyczny Gabinet Weterynaryjny lek. wet. Żaneta Sokołowska, PRZYCHODNIA WETERYNARYJNA lek. wet. Adam Łowczak, Przychodnia Weterynaryjna Kociak lek. wet. Sylwia Łata, Klinika Weterynaryjna dr. Grzegorza Wąsiatycza. This research was supported partly by PLGrid Infrastructure.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ttbdis.2023.102188.

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8. Manuskrypt M.1

Co-occurrence of *Borrelia burgdorferi* sensu lato and *Babesia* spp. DNA in *Ixodes ricinus* ticks collected from vegetation and pets in the city of Poznan, Poland

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Abstract

Green spaces localized in cities create favourable environmental conditions for *Ixodes ricinus*, the most widespread and important vector of tick-borne pathogens in Central Europe. In this study, we described the prevalence of *Borrelia burgdorferi* s.l. and *Babesia* species found in mono- and double infections among *I. ricinus* ticks occurring in urban areas of the city of Poznań, west-central Poland. Two tick groups were examined: (i) a group of 1,029 host-seeking ticks (460 nymphs, 289 females, and 280 males and (ii) a group of 1,268 engorged female ticks removed from 1,115 tick-infested animals, dogs and cats. The overall prevalence of *B. burgdorferi* s.l. found in host-seeking ticks was almost two-fold higher in comparison to ticks derived from pets (8.7 *vs*. 4.7%, respectively, $\chi 2$ test, $P = 0.001$). *Borrelia afzelii* and *B. garinii* were the most prevalent species both in ticks from vegetation (3.7% and 3.7%, respectively) and from pets (3.7% and 0.6%, respectively). *Babesia* infections were slightly more common in feeding ticks compared to those from vegetation (6.0 *vs*. 4.7%, respectively), however, the difference was not significant. Three *Babesia* species including *Ba. microti, Ba. venatorum* and *Ba. canis* were identified. *Babesia canis* and *Ba. microti* were the most prevalent in host-seeking (2.6% and 1.4%, respectively) and feeding ticks (2.8% and 2.2%, respectively), whereas *Ba. venatorum* was the rarest piroplasm. All *Ba microti* obtained sequences (n=42) were identical to *Ba. microti* genotype "Jena/Germany" that is considered as pathogenic to humans. Furthermore, the sequences of *Ba. venatorum* (n=17) were identical with those isolated from patients in Italy, Austria and Poland. Altogether, out of 121 *Babesia*-positive ticks, 19.8% were simultaneously infected with *B. burgdorferi* s.l. spirochetes. We found similar prevalences of co-infections in host-seeking (1.1%) as well as in feeding ticks (1.0%). In the first group, *Ba. canis* prevailed in coinfections (73%), mostly with *B. afzelii* and *B. garinii*. In the second group, *Babesia microti* dominated with *B. afzelii* (92.7%). This is the first finding documenting the co-occurrence of both pathogens in ticks from companion

animals. The detection of *Ba. microti* and *Ba. venatorum* sequences identical to strains pathogenic to humans as well as predominance of LB spirochetes, *B. afzelii* and *B. garinii* in *I. ricinus* from vegetation and animals indicates that there is a potential risk of acquiring human babesiosis and/or Lyme borreliosis in urban areas of the city of Poznań. Although, the overall risk of encountering a *Ba. microti* and *B. burgdorferi* s.l. co-infected tick was low (1.0%), the potential for more severe clinical symptoms and complications deserves notice. A relatively high infection rates of *Ba. canis* in dog-derived ticks (3.2%) and in host-seeking ticks (2.6%), suggests that *I. ricinus* could potentially be involved in the circulation of this piroplasm in areas, where the competent vector, *Dermacentor reticulatus* is absent or rare.

Keywords: *Ixodes ricinus*, *Borrelia burgdorferi* s.l., *Babesia microti, Babesia venatorum, Babesia canis, Borrelia miyamotoi,* coinfections, babesiosis, urban green areas

1. Introduction

Green spaces inside European towns such as parks, leisure-time areas for hiking and biking, botanic gardens, private properties with gardens, cemeteries, and urban forests, create favourable environmental (e.g., temperature and humidity) conditions for *Ixodes ricinus,* the most widespread and important vector of tick-borne pathogens (TBPs) in Central Europe. One of the key factors influencing the survival and maintenance of local tick populations is their access to appropriate and abundant tick bloodmeal hosts [1]. Within urban green areas, immature stages of this primarily a forest-dwelling tick species, feed mostly on small rodents, ground-feeding passerines, and hedgehogs which are additionally important hosts of adult female ticks. The latter feed also on urban pet populations represented by dogs and cats, including stray animals. The observed ongoing increase in the number of pets in towns, suggests that this group of mammals appears to be increasingly important for the persistence and size of tick populations in these ecologically altered habitats. Some of vertebrate species acting as maintenance hosts for *I. ricinus*, may concurrently serve as reservoir hosts of TBPs. Their high or low abundance and species composition in urban habitats, influence the level of infection of local tick populations and is critical for public health importance [1]. Therefore, investigations regarding the prevalence of TBPs infecting ticks, are necessary to establish or predict the emergence of active endemic foci of tick-borne diseases. It is particularly crucial because, there is still a lack of comprehensive knowledge on the eco-epidemiology of these infections in urban ecosystems and our understanding of how urbanization affects pathogen-host-vector relationships [2].

Ticks can carry two or more pathogenic microorganisms with a subsequent high likelihood of co-transmission to humans or animals [3,4]. *Ixodes ricinus* ticks infected with various bacteria, i.e., spirochetes of the *Borrelia burgdorferi* sensu lato (s.l.) complex, the agent of Lyme borreliosis (LB) or *Anaplasma phagocytophilum*, the agent of human

anaplasmosis, are regularly found in urban and suburban areas across Europe [5-7]. Furthermore, *B. burgdorferi* s.l. spirochetes and intraerythrocytic parasites of the protozoan genus *Babesia* (Apicomplexa: Piroplasmida), including *Ba. microti, Ba. divergens, Ba. venatorum,* and *Ba. duncani* (present only in North America), can co-occur and be co-transmitted by ticks of the *Ixodes ricinus* species complex [8]. These protozoan pathogens are responsible for human babesiosis causing a febrile hemolytic anemia, that is generally asymptomatic or self-limiting in healthy humans, however, is a serious health concern in splenectomised, immunocompromised patients [9]. In North America, the major agent of human babesiosis is *Ba. microti*, a parasite associated with small mammals serving as primary reservoir hosts. There are eco-epidemiological evidence indicating that coinfections *B. burgdorferi* s.l. and *Ba. microti* among ticks and *Peromyscus leucopus* mice may contribute to the emergence and expansion of *Ba. microti* in the enzootic cycle. Ecological models demonstrated the strongest effects when the prevalence of *B. burgdorferi* in mice was high [10]. Recent research clearly demonstrated that infection of *I. scapularis* ticks with *B. burgdorferi* s.l. spirochetes increases the likelihood of infection with *Ba. microti* as well as *A. phagocytophilum* compared with borreliae-free ticks [11]. Furthermore, according to Zembsch et al. [12] host-seeking *I. scapularis* ticks that are infected with *Ba. microti,* are more likely to be coinfected with *B. burgdorferi* than expected if the pathogens were transmitted independently. This implies that such positive pathogen-vector-host interactions of both tick-borne agents may favour their emergence and maintenance in local tick populations [13]. Co-infections may also change clinical symptoms, course, severity of tick-associated disease in humans and animals compared to those induced by a single infection [14-16]. Patients co-infected with *B. burgdorferi* s.l. and *Ba. microti* suffer from significantly more diverse, intense, and persisting disease symptoms compared to those infected with each pathogen separately [17,18]. It has been shown that *Ba. microti* weakens adaptive immunity and

increases the severity of LD [19]. Furthermore, human coinfection with *Ba. microti* and *B. burgdorferi* s.l. seems to be serious clinical problem because of the difficulties in diagnosis and treatment, since the antibiotics used to treat borreliae are ineffective against *Ba. microti* [20]. In Europe, most cases of human babesiosis are attributed to *Ba. divergens*, usually a cattle parasite or less frequently to *Ba. venatorum* for which the roe deer is the main reservoir host. Interestingly, European genotypes of *Ba. microti* infecting humans are not as infectious or pathogenic than those in the USA [21].

The aim of our study was to ascertain the co-occurrence of *B. burgdorferi* s.l. spirochetes and *Babesia* piroplasms in *I. ricinus* ticks collected from vegetation as well as from dogs and cats in urban areas of the city of Poznań, west-central Poland.

2. Materials and methods

2.1. Tick collection and identification, and DNA extraction

Host-seeking ticks were collected by sweeping up the vegetation up to 1 m with a 1 m^2 flannel flag along the paths in five forested green areas used for recreational activities and as walking areas for dogs in the city of Poznań. Ticks were collected from May to September 2017 and in April 2018. The five study sites included: Morasko Adam Mickiewicz University Campus, Sołacki and Citadel city parks, two urban forests localized around Rusałka and Malta lakes, for details see [22].

Ticks feeding on dogs and cats, were collected during a three-year survey (April to October 2015, March to November 2016, and March to September 2017) in 17 veterinary clinics in Poznań (for details see [22]). In this study, we tested ticks derived from animals which did not travel outside the city, based on information from questionnaires.

All ticks were preserved in 96% ethanol until DNA extraction. Adult ticks were identified to the species level using morphological characters [23]. *Ixodes* nymphs were identified using DNA-barcoding based on cytochrome *c* oxidase subunit I (COI) amplification and next generation sequencing (NGS) of the amplicons [22,24]. Ticks were tested individually. DNA extraction from host-seeking ticks was performed with the ammonium hydroxide method, whereas DNA from pet-derived ticks were isolated using a silica-column method. Details concerning DNA extractions are described by Liberska et al. [22].

2.2. Screening for Borrelia burgdorferi s.l. DNA

Host-seeking ticks were initially screened for *B. burgdorferi* s.l. DNA by amplification and sequencing of the V4 hypervariable region of the 16S rRNA gene (V4 16S). Details concerning PCR primers, library construction, NGS sequencing, and sequence data analysis were described previously [25]. Host-seeking and feeding ticks positive for *B. burgdorferi* s.l. V4 16S were retested by amplification and sequencing the *flaB* gene fragment using two primer sets 132f/905r and 220f/823r [26]. Protocols for PCR reactions and Sanger sequencing followed [25].

2.3. Screening for Babesia spp. DNA

Babesia spp. DNA was detected by amplification and sequencing of the 18S rRNA gene fragment using nested PCR and Sanger sequencing or by next generation sequencing (NGS) of the same target DNA. For the nested PCR, we used the RIB-19/RIB-20 primer set for the first-round reaction [27], and the P3/BabR3 primer set for the second-round reaction [22, 28]. Primer sequences and protocols for nested PCR and Sanger sequencing are described by Liberska et al. [22].

The second approach for the detection of *Babesia* DNA was conducted with the use of the P3/BabR3 primer set fused with dual-indexed Ion Torrent adapters to generate *Babesia*-specific amplicons for NGS sequencing. Details concerning library construction, Ion Torrent sequencing, and sequence data analysis were described previously [25].

2.4. Statistical analysis

Data analysis was performed using STATISTICA software version 6.0 (StatSoft Inc., Tulsa, OK, USA). Rates of infection were analysed using Chi-squared test $\chi 2$ and Fisher exact test. A $p < 0.05$ was considered statistically significant.

2.5. Sequence analysis

Sanger sequence chromatograms were checked for accuracy in GeneiousR11.1.5 (Biomatters Ltd.) and contigs were assembled manually in GeneDoc sequence editing tool version 2.7 [29]. Amplicon sequence variants (ASVs) and operational taxonomic units (OTUs) obtained from NGS data were compared to those available in the GenBank using BLASTN (https://blast.ncbi.nlm.nih.gov) and the megablast algorithm. We used 100% identity threshold to determine *Borrelia* and *Babesia* species because they were determined from rDNA sequences. The identity threshold for tick species determination was lowered to 97% due to intra-species variability of the COI marker.

2.6. Phylogenetic analysis

For phylogenetic analysis, we used 49 V4 16S sequences (Table S1) including ASVs found in this study (n=18), V4 16S sequences identical to the ASVs found in this study and sourced from reference *B. burgdorferi* s.l. genomes deposited in GenBank (n=18), and representants of *Borrelia* RF group (n=13) used to root the tree. Sequences were aligned manually in GeneDoc and a phylogenetic tree was built using FastTree 2.1.11 as implemented

in Geneious Prime 2023.2.1 (Biomatters Ltd.). The tree was edited in Mega7 [30] and Corel Draw v. X5.

3. Results

3.1. Collection and identification of ticks

A total of 2297 I. ricinus ticks including 1029 host-seeking individuals (460 nymphs, 289 females, and 280 males) and 1268 feeding female ticks removed from 1115 tick-infested companion animals, were collected during this study (Tables 1, 2). In the case of pet-derived ticks sampled in veterinary clinics, 711 were removed from 609 dogs, 153 from 117 cats, and 404 from 389 undefined animals, i.e., without giving information about host species (Table 2).

3.2. Detection of Borrelia burgdorferi s.l. DNA

Based on V4 16S and *flaB* gene fragments, DNA of *B. burgdorferi* s.l. was detected in 90 (8.7%) of the 1,059 host-seeking *I. ricinus* ticks. The bacterium was found in nymphs, males, and females with prevalences of 6.3%, 9.6% and 11.8%, respectively (Table 1). Four out of the five collection sites yielded ticks that tested positive for *Borrelia* spirochetes (range between 3.0% and 40.6%). Sequencing of the amplified *flaB* gene fragments revealed four *Borrelia* species: *B. afzelii, B. garinii*, *B. lusitaniae*, and *B. valaisiana* (Table 1). The first two prevailed and reached the same prevalence of 3.7%, followed by *B. lusitaniae* (0.9%), *B. valaisiana* (0.3%) and *Borrelia* undetermined species (0.2%).

Among the 1,268 feeding *I. ricinus* females, DNA *B. burgdorferi* s.l. was detected in 4.7% (n=59) individuals, and this prevalence was almost two-fold lower in comparison to host-seeking ticks (8.7%, $\chi 2$ test, P = 0.001). Infected ticks were found in all animal groups: dogs (4.8%), cats (3.3%) and undefined pets (5.0%). Five spirochete species were identified with a clear predominance of *B. afzelii* (3.7%; n=47) followed by *B. garinii* (0.6%; n=7), *B.*

spielmani (0.2%; n=3), *B. lusitaniae* (0.1%; n=1) and *B. valaisianae* (0.1%). Except of *B. valaisiana*, the remaining four species were detected in dog-derived ticks. The five infected ticks collected from cats, harboured only *B. afzelii*, whereas 20 individuals from the group of undefined pets were infected with *B. afzelii* (n=16)*, B. garinii* (n=3) and *B. valaisiana.*

3.3. Genetic diversity of B. burgdorferi s.l. V4 16S amplicon sequence variants

In total, 18 different *B. burgdorferi* s.l. V4 16S ASVs were found in questing ticks (Supplementary table with GenBank acc. nos.). For most ASVs, identical sequences in species reference genomes published in GenBank could be assigned (Figure V4 Tree). Phylogenetic analysis enabled the assignment of one of the previously unknown variants, ASV05, to *B. afzelii* with a relatively high support (85%). Another new variant, ASV17, was reconstructed as a sister to the V4 16S sequence from the *B. maritima* reference genome, although with moderate support (76%). The remaining four new variants (ASV10-12 and ASV14) were reconstructed by the phylogenetic analysis as a separate, well supported clade (90%). These ASVs tended to co-occur with other *B. burgdorferi* s.l. V4 16S ASVs either in a relatively large number of ticks (ASV10 and ASV 14 in nine positive samples each) or in a predominant number of reads (ASV11). Amplicon sequence variant 12 was found in only one sample and in co-occurrence with ASV14; however, both sequences differed at two variable nucleotide positions, indicating that ASV12 cannot be considered an NGS artifact of the ASV14 amplicon.

Analysis of the *flaB* gene sequence was unable to unambiguously assign most of the V4 16S ASVs to the species. Samples positive for *B. burgdorferi* s.l. V4 16S DNA that clustered basally at our phylogenetic tree (ASV01-03 and ASV18), failed in PCR amplification of the *flaB* gene fragment. Moreover, most of the ASVs occurred in co-infections, making it impossible to assign a specific *flaB* gene sequence to a specific V4 16S ASV. However, in

these cases, the *flaB* gene results did not exclude the presence of the species identified in by the V4 16S analysis in the analyzed samples. This also applied to the clade grouping of the previously unknown V4 16S variants (ASV10-12, ASV14). In this case, the *flaB* gene sequences indicated the presence of *B*. *garinii* and *B*. *lusitaniae*. In this regard, due to the presence of *B. burgdorferi* s.l. V4 16S sequence variants that could not be unambiguously assigned to the species, further analyses were based on sequencing data from *flaB* amplicons.

3.3. Presence of Babesia spp. DNA in Ixodes ricinus

The overall prevalence of *Babesia* spp. in host-seeking *I. ricinus* ticks was 4.4% (45/1,029), and the infection was noticed in all developmental stages (Table 1), with the highest prevalence found in females and the lowest in nymphs (7.3% *vs*. 2.4%; respectively; $\chi 2$ test: P = 0.001). *Babesia* infected ticks occurred in four out of five locations (range between 0.8% and 9.8%). Among three identified *Babesia* species, *Ba. canis* (2.6%) followed by *Ba. microti* (1.4%) were the most prevalent, and *Ba. venatorum* (0.4%) was the rarest species. These three species were noted in adult females and males, whereas *Ba. venatorum* was absent in nymphs.

In total, 6.0 % (76/1,268) feeding female *I. ricinus* ticks were positive for the presence of *Babesia* DNA (Table 2). The highest infection rate was noted in ticks collected from dogs (6.8%) and undefined animals (5.7%), and the lowest, in cat-derived ticks (3.9%), but the difference was statistically insignificant. Three *Babesia* species were identified: two of them, *Ba. canis* and *Ba. microti,* reached similar infection rates (2.8% and 2.2%, respectively), whereas *Ba. venatorum,* was the rarest species (1.0%). Ticks infected with these three species were confirmed in all animal groups. The infection levels of *Ba. canis* and *Ba. microti* in ticks derived both from dogs and undefined pets were similar (3.2%, 2.5% and 2.7%, 2.0%, respectively), with the lowest level for *Ba. venatorum* (1.0% and 0.7%, respectively). The

latter prevailed (2.0%) in cat-derived ticks, followed by *Ba. microti* (1.3%) and *Ba. canis* (0.7%) that was found only in one female tick.

All obtained *Ba. canis* sequences demonstrated 100% similarity to *Ba. canis* isolates detected in the blood of infected dogs from Poland (e.g., GenBank: EU622793, KT844903), as well as in questing *I. ricinus* ticks (GenBank: MF797820). *Babesia microti* sequences were identical to the zoonotic *Ba. microti* "Jena/Germany" genotype (GenBank acc. no. EF413181), whereas *Ba. venatorum* sequences showed 100% homology to isolates found in patients in Italy and Austria (AY046575), and also in an asymptomatic patient in Poland (KP072001).

3.4. Co-occurrence of Borrelia burgdorferi s.l. and Babesia spp. DNA in Ixodes ricinus

Overall, of the 2,297 *I. ricinus* ticks, 10.7% (n=246) tested positive for a single pathogen, and 1.0% (n=24) yielded dual infections. Of the 149 positive for *B. burgdorferi* s.l. DNA ticks, mono-infections were identified in 83.8% (n=125) and co-occurrence with *Babesia* spp. DNA in 16.1% ticks. Of the 121 ticks infected with *Babesia* species, 80.2% (n=97) were single infections, and 19.8% were co-infected with *B. burgdorferi* s.l. Among host-seeking ticks, co-occurrence of *Borrelia* and *Babesia* was identified in 1.1% (11/1,029) of adult ticks. None of the 460 nymphs was concurrently infected with both pathogens (Tab. 1). *Babesia canis* was the most prevalent species in co-infected ticks (72.7%; 8/11), and was confirmed in four (0.4%) co-infections with *B. afzelii* and in four (0.4%) with *B. garinii*. In the remaining three female ticks, *B. lusitaniae* co-occurred with *Ba. venatorum* (n=2; 0.2%) and *Ba. microti* (n=1; 0.1%).

Among feeding female ticks, 1.0% (13/1,268) were co-infected with both pathogens. *Babesia microti* was the most prevalent species in all co-infections (92.3%; 12/13). Most co-infections of this species was recorded with *B. afzelii* (10/13). Furthermore, *Ba. microti* co-

occurred with *B. spielmanii* and *B. garinii* in two other ticks. *Babesia canis* co-occurred with *B. garinii* only in one female tick.

3.5. Co-occurrence of Borrelia burgdorferi s.l. and Borrelia miyamotoi in Ixodes ricinus

Of the 90 positive for *B. burgdorferi* s.l. host-seeking ticks, six (6.7%) were co-infected with *B. miyamotoi* spirochetes identified in our previous study [25]. *Borrelia afzelii* was the most common and occurred in three coinfections (2 nymphs and one male), followed by *B. garinii* (2 nymphs) and *B. lusitaniae* (one female). None of 59 *B. burgdorferi* s.l. positive feeding ticks, yielded concurrently *B. miyamotoi.*

4. Discussion

In this study, we described prevalence of *B burgdorferi* s.l. and *Babesia* spp. found in mono- and double infections among I. ricinus ticks occurring in urban areas of the city of Poznań. We focused on two tick groups: (i) a group of host-seeking collected from vegetation, and (ii) a group of feeding ticks removed from pet animals, dogs and cats.

According to a review by Hansford et al. [31], the mean *Borrelia* prevalence in questing ticks in urban green areas across Europe was 17.3% (range: 3.1% to 38.1%). In our study, 8.7% of ticks (range: 0.0% to 40.6%) yielded borreliae. Comparable or higher mean infection rates were found in urban *I. ricinus* populations of several European cities from neighboring countries. For example, in Slovakia, the prevalence of *B. burgdorferi* s.l. ranged from 6.8% to 15.3% in parks of Bratislava, [32,33], and in agglomerations of Košice and Bardejov reached 10.2% [34]. In the Czech Republic, 12.1% of ticks collected in parks in Brno and 13.2% in Ostrava city, yielded spirochetes [35,36]. Furthermore, in the city parks of Vilnius, Lativa, and in recreational areas of Hanover, Germany, 25% of ticks yielded *B. burgdorferi* s.l. [37,38]. Comprehensive studies on host-seeking ticks infected with *B. burgdorferi* s.l. in strictly urban

agglomerations in Poland, are relatively rare. In city forests and parks of Warsaw, the mean prevalence of *Borrelia* spirochaetes was 10.9% [6], and in the Tri-City agglomeration area of Gdańsk, Gdynia, and Sopot, reached 12.4% [39]. To date, the highest levels of *Borrelia* infections have been described in green areas of the city Białystok (25.7%) and Olsztyn (27.4%) [40,41]. In our study, the highest prevalence of 40.3% (28/69) was recorded in ticks of the Citadel Park, the largest municipal park strictly in the center of the city. Although, the number of tested ticks was limited, this high infection level, may result from the lack of roe deer, that is considered to eliminate *B. burgdorferi* s.l. in feeding ticks [42].

The overall prevalence of *B. burgdorferi* s.l. found in ticks from pet animals (4.7%), was almost two-fold lower than in host-seeking ticks (8.7%). Comparable prevalences were described in the Netherlands [43,44] and Austria (4.8% and 5.2%, respectively) [45]. Higher infection rates in *I. ricinus* mainly from dogs, were recorded in Lativa (10.7%), Germany (11.6%), Finland (11.8%), Norway (14%), and Denmark (15%) [46-50]. So far, the highest prevalences in *I. ricinus* from dogs have been found in two city agglomerations: Wrocław (21.7%), in the southwest [51] and Olsztyn (34.4%), in the northeast of Poland [52].

Borrelia afzelii and *B. garinii* proved to be the most prevalent species among infected ticks both from vegetation and pets. Our results are in agreement with the frequency of main *Borrelia* species infecting host-seeking *I. ricinus* ticks in central Europe [53] and with a report by Skotarczak [54], documenting that *B. afzelli* and *B. garinii* are the most common species in dogs. Dogs can develop infection of *B. burgdorferi* s.l. and exhibit the presence of antibodies, but unlike humans, they rarely get sick [55]. The distribution of *Borrelia* species in our study, may be explained by species composition of wild hosts. The predominant *B. afzelii*, together with *B. spielmanii*, are maintained in enzootic cycles associated with small rodents and medium-sized mammals, including hedgehogs [56-58]. We found B. spielmanii only in three feeding females, what confirms the rarity and highly focal distribution of this spirochete. The

mentioned groups of mammals were observed in our study locations, with predominance of small rodents. In the present research, *B. afzelii* prevailed in feeding ticks (3.7%). This spirochete was also the most prevalent (3.6%) followed by *B. garinii* (1.7%), *B. valaisiana* (1.4%) and *B. spielmanii* (1.4%) in ticks from dogs in Lativa [49]. A reverse pattern, with predominance of *B. garinii* over *B. afzelii* (28.1% vs. 3.2%), was observed in ticks from dogs in north-eastern Poland [52]. Avian associated *B. garinii* and *B. valaisiana* [59] were present in both infected tick groups, with almost a fourfold higher prevalence of *B. garinii* in ticks from vegetation in comparison to feeding ticks (42.2% vs. 11.2%). The lizard-associated *B. lusitaniae* [60] prevailed in host-seeking ticks compared to pet-derived ticks (10% vs. 1.7%). The finding of this spirochete only at the Rusałka lake, suggests its highly focal distribution.

In this study, the method of amplification and sequencing of the V4 region in 16S rRNA was not specific enough to clearly identify the obtained *B. burgdorferi* s.l. sequences to species as displayed on the phylogenetic tree. The 16S rRNA region had different sequences of V4 for some species identified by flaB gene, therefore the complex analysis, including other marker genes is necessary to correctly assign 16S rDNA sequences to the species.

We detected *Babesia* DNA in 4.4% of host-seeking ticks, which were recorded in four out of five locations (range: 0.8% to 9.8%). The meta-analysis by Onyiche et al. [61] estimated the overall prevalence of babesiae in questing *I. ricinus* in Europe at 2.1%. Our results agree with infection rates found in the city Białystok (3.7%) [41] and within the Tri-City agglomeration (4.5%) [62]. Lower prevalences (range: 0.4% to 0.5%) were reported in ticks tested in Bavarian public parks [63] or in urban Bielański Forest (0.8%), in Warsaw [64].

Babesia DNA was identified in 6.0% of feeding ticks, with a higher prevalence in ticks from dogs (6.8%) compared to those from cats (3.9%). Stensvold et al. [48] documented a prevalence of 8.0% in dog-derived ticks in Denmark. Lower prevalences of *Babesia* spp. in pet-derived ticks (usually from dogs) were 0.8% in the Netherlands [44], 1.0% in Finland

[50], 1.4% (62/4316) in the United Kingdom [65], 2.5% in Germany [47], and 4.7% in Latvia [49]. The highest infection rates in ticks feeding upon dogs (66.8%) and cats (15.4%) were reported in southern Poland [66].

In Europe, *I. ricinus* is involved in the transmission cycles of *Ba. divergens, Ba. venatorum, Ba. microti,* and *Ba. capreoli,* of which the first three are considered as human pathogens [8]. In our study, three species including *Ba. microti, Ba. venatorum* and *Ba. canis* were identified in both groups of ticks. The finding of the first two species, might be explain the availability of ticks to suitable reservoir hosts of both parasites. In Europe, *Ba. microti* infects small rodents [67]. The reservoir host for *Ba. venatorum,* is the roe deer, and this species was observed in at least four out of five study sites. However, we did not find *Ba. divergens*, the main etiological agent of human babesiosis in European patients [21]. Since cattle are regarded as the reservoir hosts for *Ba. divergens*, their absence in urban areas of Poznań, may explain that we failed to find the pathogen. In our study, *Ba. canis* and *Ba. microti* were the most prevalent species in feeding (2.6% and 1.4%, respectively) and host-seeking ticks (2.8% and 2.2%, respectively), whereas *Ba. venatorum* was the rarest piroplasm. Although, the role of *I. ricinus* in the transmission of *Ba. canis* remains still unclear, this parasite has already been found in questing ticks in northern Poland (range: 0.2% to 5.8%) [40,62,68], in Slovakia and the Czech Republic (range; 0.2% to 1.4%) [69,70]. In our previous study, we found that 10.2% of ticks in forest ecosystems in west-central Poland were positive for *Ba. canis* DNA, the highest prevalence ever reported for this tick [22]. European *Ba. canis* strains belong to the type A or type B, which induce different clinical symptoms of canine babesiosis [71]. In this previous and the current study, all *Ba. canis* obtained sequences represented the milder type A. In Europe, *Ba. canis* infections in *I. ricinus* from dogs were previously documented only in Latvia and Russia, with prevalences of 1.0% and 3.8%, respectively [49,72], and a prevalence of 3.2% in our study is comparable with those data. In

Europe, most *Babesia* infections in *I. ricinus* collected from companion animals are caused by *Ba. microti* and *Ba. venatorum*. The overall prevalence of 2.2% of *Ba. microti* in feeding ticks in our study was lower compared to a 5.7% infection rate in dog-derived ticks in Warsaw which yielded *Ba. microti* Gray strain pathogenic to humans (GenBank: AY693840) [73]. In Europe, the highest prevalences of *Ba. microti* in this group of ticks, were reported in southeastern (42.6%) and southern (24.3%) Poland [66, 74]. In the case of *Ba. venatorum,* the highest prevalence of 1.6% was recorded in dog-derived ticks in Lativa [49]. According to Onyiche et al. [61], *Ba. microti* is considered to be the most common *Babesia* species in questing *I. ricinus* in the eastern Europe. In our study, *Ba. microti* (1.4%) was the second most prevalent species following *Ba. canis*. Much higher infection rates of *Ba. microti* in ticks from urban areas were 2.6% in northern [39], 6.5% in central [75], and 26.4% in southern Poland [76]. *Babesia venatorum* that infected only 0.4% of host-seeking ticks in our study as well as in Bavarian public parks [63], reached also low infection rates of 0.8% in central [64] and 0.9% in northern Poland [68].

In Europe, more than 60 cases of human babesiosis have been caused mainly by *Ba. divergens*, with five cases attributed to *Ba. venatorum* and eleven autochthonous cases to *Ba. microti*, most of which were identified in Poland [21,77]. Two European genotypes of *Ba. microti* can infect humans, but are less infectious or pathogenic to humans as those in the United States. The first *Ba. microti* genotype (strain) "Jena/Germany" (EF413181) is closely related to the USA genotypes (Clade 1) including e.g., the Gray strain isolated from a patient in Massachusetts (AY693840), responsible for most cases of human babesiosis worldwide. The second *Ba. microti* genotype, the ‘Munich’ type (AB071177) is widely distributed in Europe and belongs to Clade 3 [78]. This genotype was originally thought to be non-zoonotic, however, it has recently been identified in six patients with mild and asymptomatic infections in Poland [79] and in one from Spain [80]. However, its pathological potential remains still

uncertain [77]. It should be noted, that *Ba microti* sequences identified in our study, were identical to *Ba. microti* genotype "Jena/Germany" that is considered as pathogenic to humans [77]. In Poland, Welc-Falęciak et al. [81] found the same genotype in two asymptomatic patients. Furthermore, among 1.3% of *Babesia* infected *I. ricinus* removed from humans in Poland, most showed a high similarity (> 99%) to the *Ba. microti* strain Jena followed by *Ba. venatorum* [82]. Of note, that the sequences of *Ba. venatorum* found in the two mentioned study as well as in our, were identical with those isolated from splenectomised patients in Italy and Austria [83], and with an asymptomatic patient from Poland [84].

Altogether, out of 121 *Babesia*-positive ticks, 19.8% were simultaneously *Borrelia*-positive. We found almost identical prevalences of co-infections with *B. burgdorferi* s.l. and *Babesia* spp. in host-seeking (1.1%) as well as in feeding ticks (1.0%). To the best of ourknowledge, this is the first report documenting the co-occurrence of both pathogens in ticks from companion animals in Europe. In Poland, coinfection rates of both pathogens in host-seeking ticks were described in the north (range; 0.3% to 0.6%) [39,85], in the east (1.6%) [86], and in the northeast (2.8%) [41]. In the present study, *Ba. canis* prevailed in coinfections mostly with *B. afzelii* and *B. garinii* in host-seeking ticks, whereas *Ba. microti* dominated predominantly with *B. afzelii,* in ticks from the pets. The prevalence of 0.9% (n=9) *Ba. canis* and *B. burgdorferi* s.l. coinfections in host-seeking ticks in our study, is consistent with a previous Polish study, in which 1.0% of 104 ticks showed a coinfection of *Ba. canis* with *B. afzelii* [87]. Recently, a fatal case of a dog co-infected with *Ba. canis* and *B. burgdorferi* s.l. was diagnosed in Romania [88]. Hildebrand et al. [89], found 1.6% of coinfections *Ba. microti* followed by *Ba. divergens,* mostly with *B. afzelii* and *B. garinii* in questing ticks in Middle Germany, whereas *Ba. microti* and *B. afzelii* prevailed in co-infected ticks from humans [82].

Taking into account the mentioned studies, in host-seeking *I. ricinus*, *Ba. microti* seems to be the most prevalent species in co-infections with *B. afzelii* and *B. garinii*. Co-occurrence of these pathogens is of significant importance from a medical point of view. In humans, they may affect the clinical course of disease, especially in non-immunocompetent patients, and might be difficult to differentiate since both infections induce often nonspecific symptoms including fever, fatigue, and flu-like illness [90,91]. Jabłońska et al. [92] described a symptomatic case of babesiosis and LB in a Polish immunocompetent patient after travelling to Canada and the USA. Furthermore, in a study of 24 tick-exposed individuals from southeastern Poland, a piroplasm 98.9% homologous with *Ba. divergens* and *Ba. venatorum* was detected in one person concurrently seropositive for *B. burgdorferi* s.l. [93]. In another Polish report, Pańczuk et al. [94] also found a single *B. burgdorferi* s.l. and *Ba. microti* coinfection among foresters with significant levels of IgG anti-*B. burgdorferi* antibodies.

Using our previous results regarding the presence of *B. miyamotoi* spirochetes [25] in the same group of host-seeking ticks, we found that 0.6% of 1,029 individuals yielded concurrently DNA of *B. burgdorferi* s.l., with predominance of *B. afzelii* and *B. garinii*. Co-occurrence of both pathogens was also documented in 0.4% and 1.4% of adult *I. ricinus* collected from vegetation in France [95] and eastern Poland [86], respectively.

In conclusion, the finding of a predominance of two pathogenic species of spirochetes, *B. afzelii and B. garinii*, and the detection in both groups of ticks of the sequences *Ba. microti* and *Ba. venatorum,* which are identical to human pathogenic strains, indicate that people using urban green spaces are at risk of contracting LB and/or babesiosis. The occurrence of double infections, even though with a low prevalence of 1.0%, is both clinically and epidemiologically significant and indicates that they pose a challenge for differential diagnosis in patients with acute febrile disease after contact with a tick. Furthermore, the finding of *Ba. canis* in both groups of *I. ricinus* suggests that this species could potentially be

involved in the circulation of this piroplasm in areas of western central Poland, where the competent vector, *Dermacentor reticulatus* is absent or rare. If this is the case, urban areas may pose a potential risk of dogs acquiring babesiosis.

Acknowledgements

We would like to thank Urszula Sobczyńska, Łukasz Broda, Weronika Pawlik, and Aneta Łyś of Adam Mickiewicz University in Poznań for their assistance in the collection of ticks from urban ecosystems. We would like to thank all the veterinary clinics involved in the study: Centrum Zdrowia Małych Zwierząt M. Majka, R. Starczewski s.c., Gabinet Weterynaryjny Katarzyna Gryglewska, Gabinet Weterynaryjny lek. wet. Dariusz Filipiński, Gabinet Weterynaryjny Marcin Konieczny, Gabinet weterynaryjny - Piotr Winiecki, Klinika Weterynaryjna Juszczak & Gorzelańczyk, Praktyka Weterynaryjna A. Golec M. Rybski, Gabinet Weterynaryjny PLUTO VET lek wet Krzysztof Kaniewski, Zwierzak Gabinet Weterynaryjny A. Adamska, AMICUS Gabinet Weterynaryjny, Usługi Weterynaryjne Jakub Golec, Przychodnia Weterynaryjna Na Polance Katarzyna Marciniak, Specjalistyczny Gabinet Weterynaryjny lek. wet. Żaneta Sokołowska, Przychodnia Weterynaryjna lek. wet. Adam Łowczak, Przychodnia Weterynaryjna Kociak lek. wet. Sylwia Łata, Klinika Weterynaryjna dr. Grzegorza Wąsiatycza.

This work was partially supported by the COST Action CA21170 “Prevention, anticipation and mitigation of tick-borne disease risk applying the DAMA protocol (PRAGMATICK)”.

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Table 1. The prevalence of *Borrelia burgdorferi* s.l. and *Babesia* spp. in single and double infections found in 1029 host-seeking *I. ricinus* ticks collected in urban areas of the city of Poznań.

	Females	Males	Nymphs	TOTAL
***Borrelia* spp.**				
B. afzelii	10	17	11	38 (3.7)
B. garinii	19	8	11	38 (3.7)
B. lusitaniae	2	0	7	9 (0.9)
B. valaisianae	2	1	0	3 (0.3)
Borrelia spp.	1	1	0	2 (0.2)
Total	34/289 (11.8)	27/280 (9.6)	29/460 (6.3)	90/1029 (8.7)
***Babesia* spp.**				
Babesia microti	10	9	8	27 (2.6)
Babesia canis	8	3	3	14 (1.4)
Babesia venatorum	3	1	0	4 (0.4)
Total	21/289 (7.3)	13/280 (4.6)	11/460 (2.4)	45/1029 (4.4)
co-infections				
B. afzelii + *Ba. canis*	1	3	0	4 (0.4)
B. garinii + *Ba. canis*	1	3	0	4 (0.4)
B. lusitaniae + *Ba. venatorum*	2	0	0	2 (0.2)
B. lusitaniae + *Ba. microti*	1	0	0	1 (0.1)
Total	5	6	0	11/1029 (1.1)

Table 2. The prevalence of *Borrelia burgdorferi* s.l. and *Babesia* spp. in single and double infections found in 1268 feeding *I. ricinus* female ticks removed from three groups of pet animals surveyed in veterinary clinics of the city of Poznań.

	Dogs (n= 609)	Cats (n=117)	Undefined hosts (n=389)	TOTAL
***Borrelia* spp.**				
B. afzelii	26 (3,7)	5 (3,3)	16 (4,0)	47 (3,7)
B. garinii	4 (0,6)	0	3 (0,7)	7 (0,6)
B. spielmanii	3 (0,4)	0	0	3 (0,2)
B. lusitaniae	1 (0,1)	0	0	1 (0,1)
B. valaisianae	0	0	1	1 (0,1)
Total	34/711 (4.8)	5/153 (3,3)	20/404 (5,0)	59/1268 (4,7)
***Babesia* spp.**				
Babesia canis	23 (3.2)	1 (0.7)	11 (2.7)	35 (2.8)
Babesia microti	18 (2.5)	2 (1.3)	8 (2.0)	28 (2.2)
Babesia venatorum	7 (1.0)	3 (2.0)	3 (0.7)	13 (1.0)
Total	48/711 (6,8)	6/153 (3,9)	22/404 (5,7)	76/1268 (6,0)
co-infections				
B. afzelii + *Ba. microti*	5	0	5	10 (0.8)
B. spielmanii + *Ba. microti*	1	0	0	1 (0.1)
B. garinii + *Ba. microti*	0	0	1	1 (0.1)
B. garinii + *Ba. canis*	0	1	0	1 (0.1)
Total	6	1	6	13/1268 (1.0)

Table S1. Sequences of the V4 16S rDNA region used in this study.

Species	**GenBank acc. no.**
ASV01	this study
ASV02	this study
ASV03	this study
ASV04	this study
ASV05	this study
ASV06	this study
ASV07	this study
ASV08	this study
ASV09	this study
ASV10	this study
ASV11	this study
ASV12	this study
ASV14	this study
ASV15	this study
ASV16	this study
ASV17	this study
ASV18	this study
ASV19	this study
B. afzelii	CP075440
B. afzelii	NZ_CP009212
B. anserina	CP013704
B. bavariensis	NC_6156
B. bissettiae	CP002746
B. carolinensis	NZ_CP132445
B. carolinensis	NZ_CP132465
B. chilensis	CP009910
B. coriaceae	NZ_CP005745
B. crocidurae	NZ_CP004267
B. duttonii	NZ_AZIT01000001
B. garinii	CP075451
B. garinii	NZ_CP028861
B. garinii subsp. *bavariensis*	NR_178227
B. hermsii	NZ_CP011060
B. lusitaniae	NZ_CP124050
B. maritima	NZ_CP044535
B. miyamotoi	this study
B. miyamotoi	this study
B. miyamotoi	this study
B. miyamotoi	CP004217
B. parkeri	NZ_CP007022
B. recurrentis	NC_11244

B. spielmanii	NZ_CP124042
B. turcica	NZ_CP028884
B. turicatae	NC_8710
B. valaisiana	NZ_ABCY02000001
B. valaisiana	U78154
B. valaisiana	U78155
B. yangtzensis	CP124002
Borrelia sp.	AY570512

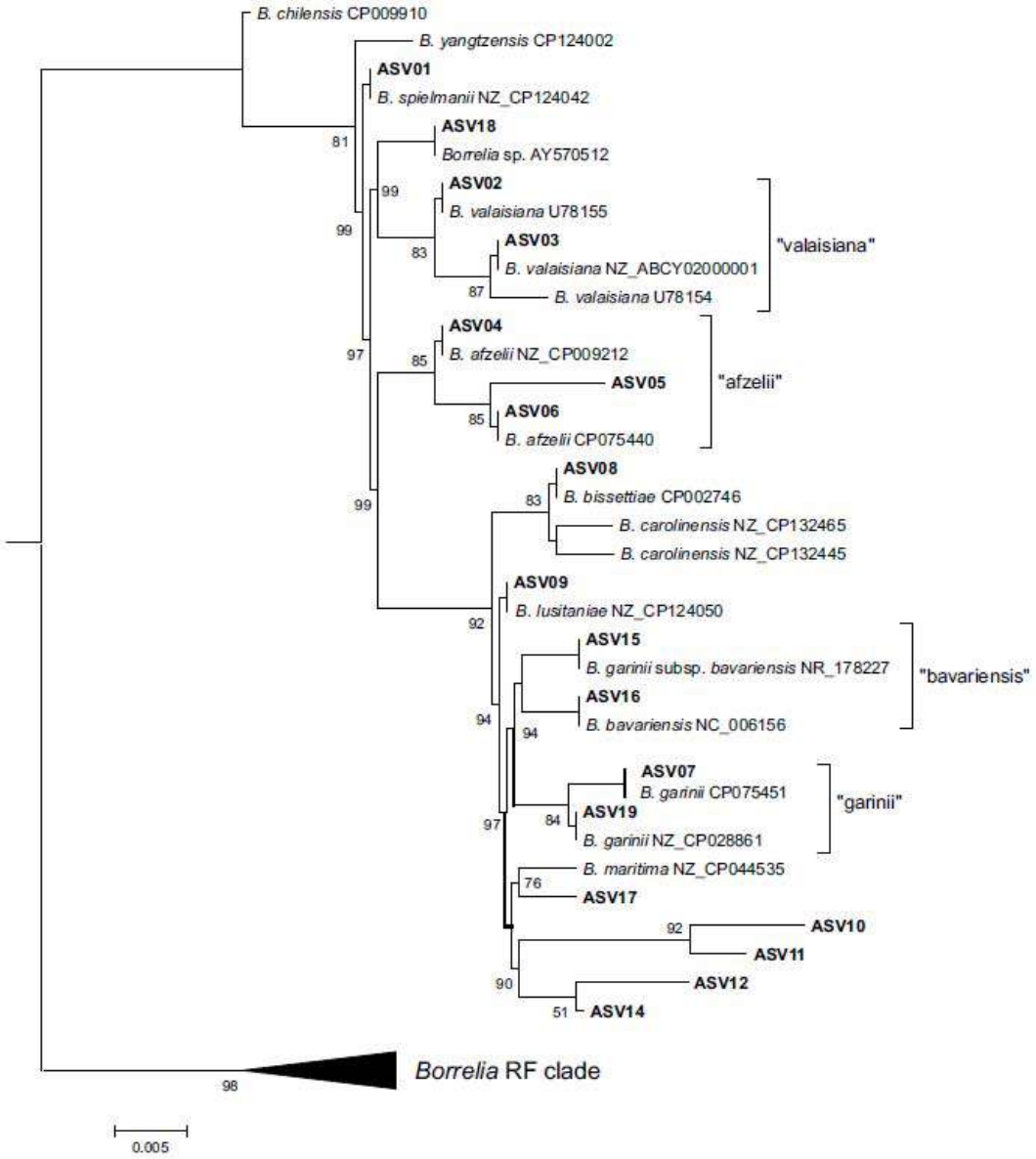

Figure 1. Phylogenetic analysis of amplicon sequence variants (ASV) of the V4 16S rRNA gene found in this study (ASV1-ASV19). The tree was constructed by FastTree method as described in Materials and Methods section.

9. Oświadczenia o wkładzie w powstawanie artykułów

OŚWIADCZENIE OKREŚLAJACE WKŁAD W POWSTANIE ARTYKUŁU

Poznań, dnia 19.02.2024

Mgr Justyna Liberska
Laboratorium Technik Biologii Molekularnej
Wydział Biologii
Uniwersytet im. Adama Mickiewicza w Poznaniu
Ul. Uniwersytetu Poznańskiego 6
61-614 Poznań

Niniejszym oświadczam, że mój wkład merytoryczny w pracę:

Liberska J, Michalik J, Pers-Kamczyc E, Wierzbicka A, Lane RS, Rączka G, Opalińska P, Skorupski M, Dabert M. Prevalence of *Babesia canis* DNA in *Ixodes ricinus* ticks collected in forest and urban ecosystems in west-central Poland. Ticks Tick Borne Dis. 2021 Sep;12(5):101786. doi: 10.1016/j.ttbdis.2021.101786. Epub 2021 Jul 12. PMID: 34280697

obejmował:

Sformułowanie hipotezy badawczej, pozyskiwanie materiału z terenów rekreacyjnych Poznania, założenie i prowadzenie bazy danych, przeprowadzenie identyfikacji gatunkowej kleszczy metodami morfologicznymi oraz molekularnymi oraz izolacji DNA z kleszczy pozyskanych z terenów rekreacyjnych Poznania, testowanie i dostosowanie warunków detekcji *Babesia* sp. za pomocą metod molekularnych, przeprowadzenie identyfikacji *Babesia* sp. z uzyskanych kleszczy, dokonanie interpretacji wyników, pozyskanie funduszy na badania i przygotowanie manuskryptu.

Mój całkowity wkład w pracę wynosił 60%.

Justyna Liberska
.....
Czytelny podpis

Potwierdzenie przez Promotorów:

.....

Jerzy Michalik

.....

OŚWIADCZENIE OKREŚLAJACE WKŁAD W POWSTANIE ARTYKUŁU

Poznań, dnia 19.02.2024

Prof. UAM dr hab. Jerzy Michalik
Zakład Morfologii Zwierząt
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Ul. Uniwersytetu Poznańskiego 6
61-614 Poznań

Niniejszym oświadczam, że mój wkład merytoryczny w pracę:

Liberska J, Michalik J, Pers-Kamczyc E, Wierzbicka A, Lane RS, Rączka G, Opalińska P, Skorupski M, Dabert M. Prevalence of *Babesia canis* DNA in *Ixodes ricinus* ticks collected in forest and urban ecosystems in west-central Poland. Ticks Tick Borne Dis. 2021. 5, 101786. doi: 10.1016/j.ttbdis.2021.101786. Epub 2021 Jul 12. PMID: 34280697

obejmował:

Zaproponowanie koncepcji, wykonanie analiz statystycznych, branie udziału w przygotowaniu manuskryptu.

Mój całkowity wkład w pracę wynosił 5%.

…….
Czytelny podpis

OŚWIADCZENIE OKREŚLAJACE WKŁAD W POWSTANIE ARTYKUŁU

Poznań, dnia 20.02.2024

Prof. UAM dr hab. Mirosława Dabert
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Uniwersytet im. Adama Mickiewicza w Poznaniu
Ul. Uniwersytetu Poznańskiego 6
61-614 Poznań

Niniejszym oświadczam, że mój wkład merytoryczny w pracę:

Liberska J, Michalik J, Pers-Kamczyc E, Wierzbicka A, Lane RS, Rączka G, Opalińska P, Skorupski M, Dabert M. Prevalence of *Babesia canis* DNA in *Ixodes ricinus* ticks collected in forest and urban ecosystems in west-central Poland. Ticks Tick Borne Dis. 2021 Sep;12(5):101786. doi: 10.1016/j.ttbdis.2021.101786. Epub 2021 Jul 12. PMID: 34280697

obejmował:

Opracowanie metodyki oraz walidację badań, zaproponowanie koncepcji oraz branie udziału w przygotowaniu manuskryptu (autor korespondencyjny).

Mój całkowity wkład w pracę wynosił 5%.

.....
Czytelny podpis

OŚWIADCZENIE OKREŚLAJACE WKŁAD W POWSTANIE ARTYKUŁU

Poznań, dnia 20.02.2024 r.

Dr inż. Patrycja Opalińska
Katedra Łowiectwa i Ochrony Lasu
Wydział Leśny
Uniwersytet Przyrodniczy w Poznaniu
Wojska Polskiego 71 D
60-625 Poznań

Niniejszym oświadczam, że mój wkład merytoryczny w pracę:

Liberska J, Michalik J, Pers-Kamczyc E, Wierzbicka A, Lane RS, Rączka G, Opalińska P, Skorupski M, Dabert M. Prevalence of *Babesia canis* DNA in *Ixodes ricinus* ticks collected in forest and urban ecosystems in west-central Poland. Ticks Tick Borne Dis. 2021 Sep;12(5):101786. doi: 10.1016/j.ttbdis.2021.101786. Epub 2021 Jul 12. PMID: 34280697

obejmował:

Pozyskiwanie materiału badawczego z terenów leśnych oraz branie udziału w przygotowaniu manuskryptu.

Mój całkowity wkład w pracę wynosił 5%.

Opalińska
.....
Czytelny podpis

OŚWIADCZENIE OKREŚLAJACE WKŁAD W POWSTANIE ARTYKUŁU

Poznań, dnia 19 lutego 2024 r.

Dr hab. inż. Emilia Pers-Kamczyc
Instytut Dendrologii Polskiej Akademii Nauk
ul. Parkowa 5
62-035 Kórnik

Niniejszym oświadczam, że mój wkład merytoryczny w pracę:

Liberska J, Michalik J, Pers-Kamczyc E, Wierzbicka A, Lane RS, Rączka G, Opalińska P, Skorupski M, Dabert M. Prevalence of *Babesia canis* DNA in *Ixodes ricinus* ticks collected in forest and urban ecosystems in west-central Poland. Ticks Tick Borne Dis. 2021 Sep;12(5):101786. doi: 10.1016/j.ttbdis.2021.101786. Epub 2021 Jul 12. PMID: 34280697

obejmował:

Przeprowadzenie izolacji DNA z kleszczy pozyskanych z terenów leśnych, pozyskanie funduszy na badania oraz branie udziału w przygotowaniu ostatecznej wersji manuskryptu.

Mój całkowity wkład w pracę wynosił 5%.

Emilia Pers-Kamczyc

.....

Czytelny podpis

OŚWIADCZENIE OKREŚLAJACE WKŁAD W POWSTANIE ARTYKUŁU

Poznań, dnia 19.02.24

Dr inż. Anna Wierzbicka
Katedra Łowiectwa i Ochrony Lasu
Wydział Leśny i Technologii Drewna
Uniwersytet Przyrodniczy w Poznaniu
Wojska Polskiego 71 D
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Niniejszym oświadczam, że mój wkład merytoryczny w pracę:

Liberska J, Michalik J, Pers-Kamczyc E, Wierzbicka A, Lane RS, Rączka G, Opalińska P, Skorupski M, Dabert M. Prevalence of *Babesia canis* DNA in *Ixodes ricinus* ticks collected in forest and urban ecosystems in west-central Poland. Ticks Tick Borne Dis. 2021 Sep;12(5):101786. doi: 10.1016/j.ttbdis.2021.101786. Epub 2021 Jul 12. PMID: 34280697

obejmował:

Pozyskiwanie materiału badawczego z terenów leśnych oraz branie udziału w przygotowaniu ostatecznej wersji manuskryptu.

Mój całkowity wkład w pracę wynosił 5%.

Czytelny podpis

OŚWIADCZENIE OKREŚLAJACE WKŁAD W POWSTANIE ARTYKUŁU

Poznań, dnia 5.01.24

Dr inż. Grzegorz Rączka
Katedra Urządzania Lasu
Wydział Leśny i Technologii Drewna
Uniwersytet Przyrodniczy w Poznaniu
Wojska Polskiego 71 D
60-625 Poznań

Niniejszym oświadczam, że mój wkład merytoryczny w pracę:

Liberska J, Michalik J, Pers-Kamczyc E, Wierzbicka A, Lane RS, Rączka G, Opalińska P, Skorupski M, Dabert M. Prevalence of *Babesia canis* DNA in *Ixodes ricinus* ticks collected in forest and urban ecosystems in west-central Poland. Ticks Tick Borne Dis. 2021 Sep;12(5):101786. doi: 10.1016/j.ttbdis.2021.101786. Epub 2021 Jul 12. PMID: 34280697

obejmował:

Pozyskiwanie materiału badawczego z terenów leśnych oraz branie udziału w przygotowaniu ostatecznej wersji manuskryptu.

Mój całkowity wkład w pracę wynosił 5%.

.....
Czytelny podpis

OŚWIADCZENIE WSPÓŁAUTORA PUBLIKACJI

Poznań, dnia ..19.02.2024..

Mgr Justyna Liberska
Laboratorium Technik Biologii Molekularnej
Wydział Biologii
Uniwersytet im. Adama Mickiewicza w Poznaniu
Ul. Uniwersytetu Poznańskiego 6
61-614 Poznań

Niniejszym oświadczam, że mój wkład merytoryczny w pracę:

Liberska, J.A., Michalik, J.F., Dabert, M. 2023. Exposure of dogs and cats to *Borrelia miyamotoi* infected *Ixodes ricinus* ticks in urban areas of the city of Poznań, west-central Poland. Ticks Tick Borne Dis. Jul;14(4):102188. https://doi.org/10.1016/j.ttbdis.2023.102188.

obejmował:

Sformułowanie hipotezy badawczej, pozyskiwanie materiału z terenów rekreacyjnych Poznania, założenie i prowadzenie bazy danych, przeprowadzenie identyfikacji gatunkowej kleszczy metodami morfologicznymi oraz molekularnymi oraz wykonanie izolacji DNA z kleszczy pozyskanych z terenów rekreacyjnych Poznania oraz ze zwierząt towarzyszących, testowanie i dostosowanie warunków detekcji *Borrelia* sp. za pomocą metod molekularnych, przeprowadzenie identyfikacji *Borrelia miyamotoi* w zebranych kleszczach, dokonanie interpretacji wyników oraz przygotowanie manuskryptu (autor korespondencyjny).

Mój całkowity wkład w pracę wynosił 70%.

Czytelny podpis:

.....Justyna Liberska.....

Potwierdzenie przez Promotorów:

.....

.....

OŚWIADCZENIE OKREŚLAJACE WKŁAD W POWSTANIE ARTYKUŁU

Poznań, dnia 19.02.2024

Prof. UAM dr hab. Jerzy Michalik
Zakład Morfologii Zwierząt
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Ul. Uniwersytetu Poznańskiego 6
61-614 Poznań

Niniejszym oświadczam, że mój wkład merytoryczny w pracę:

Liberska, J.A., Michalik, J.F., Dabert, M. 2023. Exposure of dogs and cats to *Borrelia miyamotoi* infected *Ixodes ricinus* ticks in urban areas of the city of Poznań, west-central Poland. Ticks Tick Borne Dis. 4, 102188. https://doi.org/10.1016/j.ttbdis.2023.102188.

obejmował:

Zaproponowanie koncepcji, wykonanie analiz statystycznych, branie udziału w przygotowaniu manuskryptu.

Mój całkowity wkład w pracę wynosił 15%.

…….
Czytelny podpis

OŚWIADCZENIE OKREŚLAJACE WKŁAD W POWSTANIE ARTYKUŁU

Poznań, dnia 20.02.2024

Prof. UAM dr hab. Mirosława Dabert
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Ul. Uniwersytetu Poznańskiego 6
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Niniejszym oświadczam, że mój wkład merytoryczny w pracę:

Liberska, J.A., Michalik, J.F., Dabert, M. 2023. Exposure of dogs and cats to *Borrelia miyamotoi* infected *Ixodes ricinus* ticks in urban areas of the city of Poznań, west-central Poland. Ticks Tick Borne Dis. Jul;14(4):102188. https://doi.org/10.1016/j.ttbdis.2023.102188.

obejmował:

Opracowanie metodyki oraz walidację badań, pozyskanie funduszy na badania oraz branie udziału w przygotowaniu manuskryptu.

Mój całkowity wkład w pracę wynosił 15%.

Mirosława Dabert
.....
Czytelny podpis

OŚWIADCZENIE OKREŚLAJACE WKŁAD W POWSTANIE ARTYKUŁU

Poznań, dnia 26.02.24

Mgr Justyna Liberska
Laboratorium Technik Biologii Molekularnej
Wydział Biologii
Uniwersytet im. Adama Mickiewicza w Poznaniu
Ul. Uniwersytetu Poznańskiego 6
61-614 Poznań

Niniejszym oświadczam, że mój wkład merytoryczny w pracę:

Liberska J., Michalik J., Olechnowicz J., Dabert M. Co-occurrence of *Borrelia burgdorferi* sensu lato and *Babesia* spp. DNA in *Ixodes ricinus* ticks collected from vegetation and pets in the city of Poznan, Poland.
obejmował:

Sformułowanie hipotezy badawczej, pozyskiwanie materiału z terenów rekreacyjnych Poznania, założenie i prowadzenie bazy danych, przeprowadzenie identyfikacji gatunkowej kleszczy metodami morfologicznymi oraz molekularnymi oraz izolację i DNA z kleszczy pozyskanych z terenów rekreacyjnych Poznania oraz ze zwierząt towarzyszących, testowanie i dostosowanie warunków detekcji *Babesia* sp. oraz *Borrelia burgdorferi* s.l. za pomocą metod molekularnych, przeprowadzenie identyfikacji *Babesia* sp. oraz *Borrelia burgdorferi* s.l. w zebranych kleszczach, dokonanie interpretacji wyników i przygotowanie manuskryptu (autor korespondencyjny).

Mój całkowity wkład w pracę wynosił 70%.

Czytelny podpis:

.....Justyna Liberska.....

Potwierdzenie przez Promotorów:

Mirosława Dabert

.....

Jerzy Michalik

.....

OŚWIADCZENIE OKREŚLAJACE WKŁAD W POWSTANIE ARTYKUŁU

Poznań, dnia, 20.02.2024

Prof. UAM dr hab. Jerzy Michalik
Zakład Morfologii Zwierząt
Wydział Biologii
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Ul. Uniwersytetu Poznańskiego 6
61-614 Poznań

Niniejszym oświadczam, że mój wkład merytoryczny w pracę:

Liberska J., Michalik J., Olechnowicz J., Dabert M. Co-occurrence of *Borrelia burgdorferi* sensu lato and *Babesia* spp. DNA in *Ixodes ricinus* ticks collected from vegetation and pets in the city of Poznan, Poland.

obejmował:

Zaproponowanie koncepcji, wykonanie analiz statystycznych, branie udziału w przygotowaniu ostatecznej wersji manuskryptu.

Mój całkowity wkład w pracę wynosił 15%.

Jerzy Michalik

.....
Czytelny podpis

OŚWIADCZENIE OKREŚLAJACE WKŁAD W POWSTANIE ARTYKUŁU

Poznań, dnia 27.02.24r

Prof. UAM dr hab. Mirosława Dabert
Laboratorium Technik Biologii Molekularnej
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Uniwersytet im. Adama Mickiewicza w Poznaniu
Ul. Uniwersytetu Poznańskiego 6
61-614 Poznań

Niniejszym oświadczam, że mój wkład merytoryczny w pracę:

Liberska J., Michalik J., Olechnowicz J., Dabert M. Co-occurrence of *Borrelia burgdorferi* sensu lato and *Babesia* spp. DNA in *Ixodes ricinus* ticks collected from vegetation and pets in the city of Poznan, Poland.

obejmował:

Opracowanie metodyki oraz walidację badań, zaproponowanie koncepcji, pozyskanie funduszy na badania oraz branie udziału w przygotowaniu manuskryptu.

Mój całkowity wkład w pracę wynosił 10%.

Mirosława Dabert
.....
Czytelny podpis

OŚWIADCZENIE OKREŚLAJĄCE WKŁAD W POWSTANIE ARTYKUŁU

Poznań, dnia 27.02.202...

Mgr Julia Olechnowicz
Laboratorium Technik Biologii Molekularnej
Wydział Biologii
Uniwersytet im. Adama Mickiewicza w Poznaniu
Ul. Uniwersytetu Poznańskiego 6
61-614 Poznań

Niniejszym oświadczam, że mój wkład merytoryczny w pracę:

Liberska J., Michalik J., Olechnowicz J., Dabert M. Co-occurrence of Borrelia burgdorferi sensu lato and Babesia spp. DNA in Ixodes ricinus ticks collected from vegetation and pets in the City of Poznan, Poland.

obejmował:

Dokonanie re-analiz, przy użyciu metod molekularnych, potwierdzając obecność DNA Babesia sp. oraz Borrelia sp. w wytypowanym materiale.

Mój całkowity wkład w pracę wynosił 5%.

Olechnowicz
.....

Czytelny podpis

10. Materiały dodatkowe

Supplementary Table A.1. List of 18S rRNA gene sequences of *Babesia* spp. used in the phylogenetic analysis.

Species	Host species	Location	Isolate	GenBank	References
B. canis	*I. ricinus*	Poland	EPKp2C06	MF797821	this study
B. canis	*I. ricinus*	Poland	EPKp2D01	MF797829	this study
B. canis	*I. ricinus*	Poland	EPKp2D09	MF797816	this study
B. canis	*I. ricinus*	Poland	EPKp2H01	MF797815	this study
B. canis	*I. ricinus*	Poland	EPKp3A04	MF797819	this study
B. canis	*I. ricinus*	Poland	EPKp3A12	MF797820	this study
B. canis	*I. ricinus*	Poland	EPKp3D12	MF797826	this study
B. canis	*I. ricinus*	Poland	EPKp3F06	MF797818	this study
B. canis	*I. ricinus*	Poland	EPKp3H07	MF797817	this study
B. canis	*I. ricinus*	Poland	EPKp4A08	MF797822	this study
B. canis	*I. ricinus*	Poland	EPKp4H09	MF797823	this study
B. canis	*I. ricinus*	Poland	EPKp5A06	MF797825	this study
B. canis	*I. ricinus*	Poland	EPKp5E07	MF797824	this study
B. canis	*I. ricinus*	Poland	EPKp5G04	MF797827	this study
B. canis	*I. ricinus*	Poland	EPKp5H04	MF797830	this study
B. canis	*I. ricinus*	Poland	EPKp6G05	MF797828	this study
B. canis	*I. ricinus*	Poland	EPKp1A01	MW090682	this study
B. canis	*I. ricinus*	Poland	EPKp1E09	MW090683	this study
B. canis	*I. ricinus*	Poland	EPKp1E10	MW090684	this study
B. canis	*I. ricinus*	Poland	EPKp1F09	MW090685	this study
B. canis	*I. ricinus*	Poland	EPKp1H09	MW090686	this study
B. canis	*I. ricinus*	Poland	EPKp2A06	MW090687	this study
B. canis	*I. ricinus*	Poland	EPKp2B02	MW090688	this study
B. canis	*I. ricinus*	Poland	EPKp2C09	MW090689	this study
B. canis	*I. ricinus*	Poland	EPKp2C12	MW090690	this study
B. canis	*I. ricinus*	Poland	EPKp2D06	MW090691	this study

B. canis	*I. ricinus*	Poland	EPKp2D10	MW090692	this study
B. canis	*I. ricinus*	Poland	EPKp2E06	MW090693	this study
B. canis	*I. ricinus*	Poland	EPKp2E12	MW090694	this study
B. canis	*I. ricinus*	Poland	EPKp2F10	MW090695	this study
B. canis	*I. ricinus*	Poland	EPKp2H02	MW090696	this study
B. canis	*I. ricinus*	Poland	EPKp2H06	MW090697	this study
B. canis	*I. ricinus*	Poland	EPKp3A02	MW090698	this study
B. canis	*I. ricinus*	Poland	EPKp3B07	MW090699	this study
B. canis	*I. ricinus*	Poland	EPKp3C02	MW090700	this study
B. canis	*I. ricinus*	Poland	EPKp3E07	MW090701	this study
B. canis	*I. ricinus*	Poland	EPKp3F05	MW090702	this study
B. canis	*I. ricinus*	Poland	EPKp3H10	MW090703	this study
B. canis	*I. ricinus*	Poland	EPKp3H11	MW090704	this study
B. canis	*I. ricinus*	Poland	EPKp4A02	MW090705	this study
B. canis	*I. ricinus*	Poland	EPKp4A05	MW090706	this study
B. canis	*I. ricinus*	Poland	EPKp4C01	MW090707	this study
B. canis	*I. ricinus*	Poland	EPKp4C04	MW090708	this study
B. canis	*I. ricinus*	Poland	EPKp4C07	MW090709	this study
B. canis	*I. ricinus*	Poland	EPKp4D05	MW090710	this study
B. canis	*I. ricinus*	Poland	EPKp4D08	MW090711	this study
B. canis	*I. ricinus*	Poland	EPKp4E01	MW090712	this study
B. canis	*I. ricinus*	Poland	EPKp4E02	MW090713	this study
B. canis	*I. ricinus*	Poland	EPKp4F12	MW090714	this study
B. canis	*I. ricinus*	Poland	EPKp5H03	MW090715	this study
B. canis	*I. ricinus*	Poland	EPKp5H10	MW090716	this study
B. canis	*I. ricinus*	Poland	EPKp6nr16	MW090717	this study
B. canis	*I. ricinus*	Poland	EPKp6nr28	MW090718	this study
B. canis	*I. ricinus*	Poland	EPKp6nr39	MW090719	this study
B. canis	*I. ricinus*	Poland	EPKp6nr41	MW090720	this study
B. canis	*I. ricinus*	Poland	JR158	MT981825	this study
B. canis	*I. ricinus*	Poland	JR237	MT981803	this study
B. canis	*I. ricinus*	Poland	JR240	MT981804	this study
B. canis	*I. ricinus*	Poland	JR241	MT981805	this study
B. canis	*I. ricinus*	Poland	JR249	MT981806	this study

B. canis	*I. ricinus*	Poland	JR283_285	MT981807	this study
B. canis	*I. ricinus*	Poland	JR317	MT981808	this study
B. canis	*I. ricinus*	Poland	JR320	MT981809	this study
B. canis	*I. ricinus*	Poland	JR332	MT981810	this study
B. canis	*I. ricinus*	Poland	JR338	MT981811	this study
B. canis	*I. ricinus*	Poland	JR464_466	MT981812	this study
B. canis	*I. ricinus*	Poland	JR467_469	MT981813	this study
B. canis	*I. ricinus*	Poland	JR470_472	MT981800	this study
B. canis	*I. ricinus*	Poland	JR491	MT981814	this study
B. canis	*I. ricinus*	Poland	JR492	MT981815	this study
B. canis	*I. ricinus*	Poland	JR518	MT981816	this study
B. canis	*I. ricinus*	Poland	JR539	MT981817	this study
B. canis	*I. ricinus*	Poland	MAL130_133	MT981818	this study
B. canis	*I. ricinus*	Poland	MAL71	MT981819	this study
B. canis	*I. ricinus*	Poland	MOR26	MT981802	this study
B. canis	*I. ricinus*	Poland	PC40	MT981820	this study
B. canis	*I. ricinus*	Poland	PC43	MT981821	this study
B. canis	*I. ricinus*	Poland	PC44	MT981822	this study
B. canis	*I. ricinus*	Poland	PC51	MT981823	this study
B. canis	*I. ricinus*	Poland	PC54	MT981824	this study
B. canis	*I. ricinus*	Poland	JR76	MT981826	this study
B. canis	*Canis familiaris*	Croatia, Poland	Bc1	AY072926	Caccio i wsp. 2002
B. canis	*C. familiaris*	Croatia, Germany	BCC2	FJ209025	Beck i wsp. 2009
B. canis	*I. ricinus*	Czech Republic, Slovakia	5488	KX857483	Rybarova and Siroky 2016
B. canis	*C. familiaris*	France	Dog#135	KC902833	Rene-Martellet i wsp. 2015
B. canis	*Nyctalus noctula*	Hungary	E18_typeA	KP835549	Hornok i wsp. 2015
B. canis	*Pipistrellus pygmaeus*	Hungary	E21_typeB	KP835550	Hornok i wsp. 2015
B. canis	*C. familiaris*	Lithuania, Poland	KA13-1	KM111283	Paulauskas i wsp. 2014
B. canis	*C. familiaris*	Netherlands	2	AY703073	Matjila i wsp. 2005

B. canis	*C. familiaris*	Netherlands	1	AY703071	Matjila i wsp. 2005
B. canis	*C. familiaris*	Poland, Germany	1	EU622792	Adaszek and Winiarczyk 2008
B. canis	*C. familiaris*	Poland	2	EU622793	Adaszek and Winiarczyk 2008
B. canis	*C. familiaris*	Poland	K035	KT844907	Rozej-Bielicka i wsp. 2015
B. canis	*Dermacentor reticulatus*	Poland	331D	KT272401	Mierzejewska i wsp. 2015
B. canis	*I. ricinus*	Poland	20-04	GQ325620	Cieniuch i wsp. 2009
B. canis	*I. ricinus*	Slovakia	N30B	KU362904	Hamsikova i wsp. 2016
B. canis	*I. ricinus*	Slovakia	M398B	KU362905	Hamsikova i wsp. 2016
B. canis	*Dermacentor reticulatus*	Russia	D2	AY649326	Rar i wsp. 2005
B. canis	*D. reticulatus*	United Kingdom	UK 2016	KY694436	Fernandez de Marco et al 2017
B. bigemina	n/a	Argentina	n/a	HQ688689	Thompson i wsp. 2010
B. canis rossi	*Haemaphysalis parva*	Turkey	Ba-704	MF040149	Orkun and Karaer 2017
B. canis vogeli	*C. familiaris*	India	n/a	MG252701	Roopesh i wsp. 2017
B. caperoli/divergens	*I. ricinus*	Poland	MAL71	MW012667	this study
B. capreoli	*Equus caballus*	Italy	B5/B6	KX839234	Bassano i wsp. 2016
B. gibsoni	*C. familiaris*	Romania	11	JX110650	Imre i wsp. 2013
B. divergens	*Homo sapiens*	China	clone x36-1	MK256977	Wang i wsp. 2018
B. ovata	*Bos grunniens*	China	Tiantang 5	KX870099	Liu i wsp. 2017
B. sp. venatorum	*I. ricinus*	Poland	JR170	MW012665	this study
B. sp. venatorum	*I. ricinus*	Poland	JR466	MW012666	this study
B. sp. venatorum	*I. ricinus*	Poland	PC43	MW012668	this study
B. sp. venatorum	*I. ricinus*	Czech Republic	6345	KX857480	Rybarova and Siroky 2016
Anthemosoma garnhami	*Acomys percivali*	Ethiopia	675CC	MH093637	Chavatte i wsp. 2018

n/a not available

Table S1. List of veterinary clinics sampled in this study.

Nr	ID	Veterynary Clinic Name	Adress
1	A	Centrum Zdrowia Małych Zwierząt M. Majka	os. W. Jagiełły 33
2	AA	Gabinet Weterynaryjny Katarzyna Gryglewska	ul. Piątkowska 105
3	C	Gabinet Weterynaryjny lek. wet. Dariusz Filipiński	os. Wichrowe Wzgórze paw. 1
4	E	Gabinet Weterynaryjny Marcin Konieczny	os. S. Batorego 82N/L6
5	F	Gabinet Weterynaryjny Piotr Winiecki	ul. Chwiałkowskiego 24
6	G	Klinika Weterynaryjna Juszczak & Gorzelańczyk	ul. Bułgarska 68A
7	H	Praktyka Weterynaryjna A. Golec M. Rybski	ul. Marcelińska 100/ 202
8	J	Specjalistyczny Gabinet Weterynaryjny Juszczak & Gorzelańczyk	ul. Armii Krajowej 85A
9	K_L	Gabinet Weterynaryjny PLUTO	os. Jagiellońskie 77
10	M	Gabinet Weterynaryjny ZWIERZAK lek. wet. A. Adamska	ul. Św. Antoniego 32
11	N	Gabinet Weteynaryjny AMICUS	os. Rzeczypospolitej 3
12	O	Usługi Weterynaryjne lek. wet. J. Golec	ul. Bnińska 40
13	P	Przychodnia Weterynaryjna NA POLANCE	ul. Katowicka 11 lokal U1-2
14	S	Specjalistyczny Gabinet Weterynaryjny lek. wet. Ż. Sokołowska	os. Lecha 92
15	T	Przychodnia Weterynaryjna	os. Orła Białego 85A
16	U	Przychodnia Weterynaryjna Kociak lek.wet. Sylwia Łata	Chwaliszewo 12
17	W	Klinika Weterynaryjna dr. Grzegorza Wąsiatycza	ul. Księcia Mieszka I 18

Table S2. List of V4 16S sequences of Borreliaceae used in the phylogenetic analysis.

	***Borrelia* species**	**GenBank acc. no.**
1	*B. afzelii*	NZ_CP009212
2	*B. anserina*	CP013704
3	*B. bavariensis*	NZ_CP059009
4	*B. bissettii*	NC_015921
5	*B. burgdorferi*	NZ_CP019767
6	*B. chilensis*	CP009910
7	*B. coriaceae*	NZ_CP005745
8	*B. crocidurae*	NZ_CP004267
9	*B. duttonii*	NZ_AZIT01000001
10	*B. garinii*	NZ_CP028861
11	*B. hermsii*	NZ_CP011060
12	*B. maritima*	NZ_CP044535
13	*B. mayonii*	NZ_CP015796
14	*B. miyamotoi*	CP004217
15	*B. parkeri*	NZ_CP007022
16	*B. recurrentis*	NC_011244
17	*B. turcica*	NZ_CP028884
18	*B. turicatae*	NC_008710
19	*B. valaisiana*	NZ_ABCY02000001
20	This study V4 16S type 1	
21	This study V4 16S type 2	
22	*Spirochaeta lutea*	NR_136451

Table S4. List of *flaB* gene fragment sequences of *Borrelia miyamotoi* detected in *Ixodes* spp. ticks collected from dogs or cats in 17 veterinary clinics in Poznań, and deposited in GenBank.

Tick species	Tick stage	Host	Location	Isolate	GenBank acc. no.
Ixodes ricinus	female	dog	ul. Księcia Mieszka I 18,	W067.1	ON000073
Ixodes ricinus	female	dog	ul. Księcia Mieszka I 18,	W023	ON000074
Ixodes ricinus	female	cat	ul. Bnińska 40,	O010	ON000075
Ixodes ricinus	female	dog	ul. Św. Antoniego 32,	M017	ON000076
Ixodes ricinus	female	dog	os. Jagiellońskie 77,	L050.3	ON000077
Ixodes ricinus	female	dog	os. Jagiellońskie 77,	L030	ON000078
Ixodes ricinus	female	dog	os. Jagiellońskie 77,	L026	ON000079
Ixodes ricinus	female	dog	ul. Armii Krajowej 85A,	JL637	ON000080
Ixodes ricinus	female	dog	os. Wichrowe Wzgórze paw. 104,	C056	ON000081
Ixodes ricinus	female	cat	os. Wichrowe Wzgórze paw. 104,	C040	ON000082
Ixodes ricinus	female	unknown	os. Wichrowe Wzgórze paw. 104,	C001	ON000083
Ixodes ricinus	female	dog	ul. Bnińska 40, Poznań	O030	ON000084
Ixodes ricinus	female	dog	ul. Armii Krajowej 85A, Poznań	JL593	ON000085
Ixodes ricinus	female	dog	os. Rzeczypospolitej 3, Poznań	N001	ON000086
Ixodes ricinus	female	dog	ul. Bułgarska 68A, Poznań	G024	ON000087
Ixodes ricinus	female	dog	os. Rzeczypospolitej 3, Poznań	N0300.2	ON000088
Ixodes ricinus	female	dog	os. Rzeczypospolitej 3, Poznań	N017.2	ON000089
Ixodes ricinus	female	dog	os. Rzeczypospolitej 3, Poznań	N014	ON000090
Ixodes hexagonus	female	cat	os. Lecha 92, Poznań	S006.8	ON000091
Ixodes hexagonus	nymph	cat	os. Lecha 92, Poznań	S006.9	ON000092
Ixodes hexagonus	nymph	dog	os. Lecha 92, Poznań	S007	ON000093

Table S5. List of V4 16S sequences of *Borrelia miyamotoi* detected in host-seeking *Ixodes ricinus* ticks collected in urban areas of the city of Poznań, and deposited in GenBank.

Tick stage	Location	Isolate	GenBank acc. no.
nymph	urban forest around Malta Lake,	mal47	MZ918966
larva	urban forest around Malta Lake,	mal51	MZ918967
female	urban forest around Malta Lake,	mal153_155	MZ918968
larva	urban forest around Malta Lake,	mal241	MZ918969
male	urban forest around Malta Lake,	mal165_167_212	MZ918970
male	Sołacki Park,	ps23_24	MZ918971
male	Citadel Park,	pc46	MZ918972
female	urban forest around Rusałka Lake,	jr146_149	MZ918973
nymph	Morasko Adam Mickiewicz University Campus,	mor27	MZ918974
nymph	urban forest around Rusałka Lake,	jr286_288	MZ918975
nymph	urban forest around Rusałka Lake,	jr289_291	MZ918976
nymph	urban forest around Rusałka Lake,	jr374_376	MZ918977
nymph	urban forest around Rusałka Lake,	jr458_460	MZ918978
nymph	urban forest around Rusałka Lake,	jr479_481	MZ918979
nymph	urban forest around Rusałka Lake,	jr538_545_546	MZ918980
female	urban forest around Rusałka Lake,	jr234	MZ918981
male	urban forest around Rusałka Lake,	jr321	MZ918982
nymph	urban forest around Rusałka Lake,	jr571_573	MZ918983
nymph	urban forest around Rusałka Lake,	jr586_589	MZ918984
nymph	urban forest around Rusałka Lake,	jr590_592	MZ918985
nymph	urban forest around Rusałka Lake,	jr607_609	MZ918986
nymph	urban forest around Rusałka Lake,	jr613_615	MZ918987