

The Occurrence of Tick-Borne Pathogens in Samples Collected from the West Pomeranian Voivodeship

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In this work, ticks were collected from the military area of the West Pomeranian Voivodeship in Poland, and the most important pathogens were detected using the real-time PCR method. The ticks were collected in mid-spring (April and May) using the flagging technique. They were collected in five places: on the shoreline of Drawa River and Konotop Lake, as well as in forest and meadow areas near Karwice, Konotop and Oleszno Villages. During the work, only one species of ticks was caught – *Ixodes ricinus*. All the collected specimens were assessed in terms of their species, sex and developmental stage by zoological specialists. The largest number of specimens was caught in Karwice (35%, n = 666). The highest number of ticks in each test site were nymphs (57%, n = 1106), except at Konotop. The reaction of RT-PCR was tested in 111 pools of ticks. As a result of the PCR reactions (VIASURE Tick-Borne Diseases Real-Time PCR Detection Kit), *Rickettsia* spp. (n = 70) and *Borrelia burgdorferi* s.l. (n = 35) were determined to be the most frequently identified pathogens in the tick samples. Other pathogens such as *Anaplasma phagocytophilum* and *Ehrlichia chaffeensis* were detected in a smaller number of samples. The results in this paper confirm the epidemiological data collected and analysed prior to now.

Key words: ticks, *Ixodes ricinus*, tick-borne diseases, North-West Poland.

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From an epidemiological point of view, next to mosquitoes and fleas, ticks are one of the most important groups of arthropods that convey pathogens to animals and humans. With the present fast pace of globalisation and progressive climate changes, these organisms are increasingly becoming the vectors of many infectious diseases. Warmer winters, with prolonged spring and autumn, favour the spread of many tick species to new areas. Global warming may also affect many factors, such as the development of the vector itself, changes in the geographical distribution of the vectors and hosts, as well as the interactions be-

tween the vector, host and the pathogens (PARHAM *et al.* 2015).

Currently, ticks are extremely important in relation to public health, due to their ability to transmit many dangerous pathogenic agents. The efforts made by public health institutions are focused on monitoring tick populations, as well as detecting and treating diseases caused by the pathogens transmitted by ticks. Knowledge of the species of microorganisms that can be transmitted by ticks in a particular geographic region will enable better diagnostics and allow for the implementation of appropriate treatment following the occurrence of disease symptoms (MADISON-ANTENUCCI

et al. 2020). It is therefore extremely important to monitor the presence of ticks and their microbiota composition, especially in places that are visited intensively by people. Currently, this subject – along with the supervision of the zoonoses connected with those vectors – is widely addressed in medical, environmental and veterinary research. Monitoring the distribution of ticks is necessary, in order to control the risk of human infection from those diseases (KIEWRA *et al.* 2017).

Environmental studies of ticks include an analysis of the frequency of the occurrence of these arthropods in selected areas. To date, 896 species of ticks have been identified in the world belonging to three families: *Amblyomidae*, *Ixodidae* and *Argasidae*. 77 of them exist in Europe and 19 are part of the Polish fauna. The most common, and simultaneously the most important ticks in the epidemiology of infectious diseases, are those belonging to two species: *Ixodes ricinus* and *Dermacentor reticulatus*. Habitats for the ticks belonging to *I. ricinus* encompass mainly wet areas covered with grassy vegetation and deciduous forests (KIEWRA 2014). However, the number of these arthropods is also influenced by factors such as the presence of hosts, humidity, air temperature, vegetation, photoperiod and insolation (KIEWRA *et al.* 2017). Over 300 vertebrates may be hosts for specimens from the *I. ricinus* species, including domestic and wild animals, birds and even reptiles. The tick populations occur in microclimates and their density may change even within small areas (KIEWRA 2014). *I. ricinus* is the best-studied tick species as a vector of pathogens due to its widespread occurrence, as is evidenced by records of its presence in many places throughout Poland (NOWAK-CHMURA & SIUDA 2012). The occurrence of *D. reticulatus* is not as common as *I. ricinus*, and until the 1990s, the areas where specimens belonging to this species could be found covered only the north-east and eastern terrain of Poland. However, during the last few years, its presence has also been recorded in an area that was considered to be free of this species (KUBIAK *et al.* 2018).

Ticks can carry a wide range of pathogenic endosymbiotic microorganisms, and the currently available methods of molecular analysis have significantly increased the ability to identify those pathogens. A great deal of research has been undertaken to detect the pathogens living in ticks including the following genera: *Babesia*, *Coxiella*, *Borrelia*, *Anaplasma*, *Ehrlichia*, *Rickettsia*, *Francisella*, *Neohrlichia* and *Bartonella* – which are responsible for such diseases as babesiosis, Q fever, Lyme disease, human granulocytic anaplasmosis, rickettsiosis and tularemia. *Babesia microti* and *Babesia divergens* are protozoa belonging to the Piroplasmida order, which can infect the red blood cells. These organisms require the presence of two hosts during their life cycle: the indirect host belongs to vertebrates, while the tick is the direct host (ALBERTYŃSKA *et al.* 2017). Another patho-

genic agent, *Coxiella burnetii* is a gram-negative rod from the Coxiellaceae family and is polymorphic, which influences the Gram staining, i.e. the bacteria does not always stain pink. Moreover, it does not have the ability to reproduce in bacteriological media, so it can only be cultured using animal cell lines or chicken embryos. Infection with this bacterium can occur through a tick vector, but also without the tick's participation via an aerosol route (TRUSZCZYŃSKI 2010). *Borrelia burgdorferi*, gram-negative spirochetes belonging to the Spirochaetales order, are the most common tick-borne pathogens. Three of the eighteen *Borrelia* species belonging to the *B. burgdorferi* s.l. complex are pathogenic to humans; these are *B. afzelii*, *B. burgdorferi* and *B. garinii*. Additionally, the main vector for Lyme disease in Europe is *I. ricinus* (STANEK & REITER 2011). Three more of the previously mentioned dangerous pathogens belong to the Rickettsiales order. *Anaplasma phagocytophilum* which is from the Anaplasmataceae family, a gram-negative aerobic bacterium, has an affinity for granulocytes, specifically neutrophils, where it multiplies. From a clinical point of view, it is important to start the appropriate treatment as soon as possible, if an infection caused by that agent is suspected. An additional factor, which increases the probability of becoming infected, are reservoirs which include both wild and domestic animals (GUZMAN 2021). Other bacteria such as *Ehrlichia chaffeensis* and *Ehrlichia muris* belong to the same family as *A. phagocytophilum*, and together with such species as *Ehrlichia canis*, *Ehrlichia ewingii* and *Ehrlichia ruminantium* they present a significant threat to human and animal health. Furthermore, all of these species are characterised by a similarity in the 16S rRNA gene sequence at the level of 97.7% and all of them can be transmitted by ticks (PADDOCK & CHILDS 2003). *Rickettsiae* are closed groups related to the genus *Ehrlichia*. These gram-negative bacilli from the Rickettsiaceae family spread through the blood and, like most of the previously-mentioned pathogens, cause diseases with nonspecific symptoms. This makes a proper diagnosis, as well as the treatment more difficult. People are exposed to infection by spending time in meadows, forests or gardens, where the probability of a tick bite increases dramatically (ASMAN *et al.* 2018). The possibility of infection with the aforementioned agents strictly depends on factors such as the number of infected ticks in the population, their density and the number of tick hosts (TOKARSKA-RODAK *et al.* 2016).

The presence of microorganisms in ticks may arise from several reasons, including transovarial or transstadial transmission, or through a tick infection through food taken from the host. In addition, many bacterial species naturally exist on the surface of these arthropods and in their tissues. The currently available methods of molecular analysis do not allow for the acquisition of detailed knowledge about the origins of a particular microorganism (DWUŻNIK *et al.* 2019; PALOMAR *et al.* 2019).

This work was aimed at determining the rate of infection among ticks collected in the West Pomeranian Voivodeship with the following pathogens: *B. microti/divergens*, *B. burgdorferi* s.l./*miyamotoi/hermsii*, *A. phagocytophilum*, *E. chafeensis/muris* and *Rickettsia* spp., with their detection performed by the real-time PCR method.

Material and Methods

Place of the research

The research material was comprised of specimens collected in the spring of 2017 in the West Pomeranian Voivodeship in Poland, in the administrative district of Drawsko Pomorskie at five locations: the shorelines of Drawa River and Konotop Lake, as well as in forest and meadow areas near Karwice, Konotop and Oleszno Villages. The flora was characterised mainly by mid-forest bogs, which are rich habitats for animals such as Capreolinae, moose and wolves. The fauna favours the occurrence of numerous animal species that participate in the life cycle of ticks as a reservoir for pathogens. The fauna of military training grounds, together with the flora, is conducive to the presence of ticks due to the presence of large wet and grassy areas. The sampling near Konotop Lake and Drawa River was carried out on high coastal vegetation. Konotop Lake is located on the Torfowisko Konotop nature reserve. This area is a natural habitat for wild animals. In the case of Konotop Village, the sampling was performed at a military camp near the buildings. The samples from Karwice and Oleszno were collected from the proving grounds, which are places where the professional soldiers perform exercises. The main aim of this study was an analysis of the risk level among the professional soldiers to the presence of infectious biological agents. For this reason, the research was focused mainly on places where the soldiers are most often present during their service.

Sampling

The selection of the test sample was simple and random. The samples were collected in several places in each given location. The ticks were collected in the spring (April and May) using the flagging method, which involves wiping low-growing vegetation with a white flannel cloth measuring 70 x 80 cm. The specimens were collected in sterile Eppendorf tubes, and the species, developmental stage and sex were determined. The samples were then frozen to be stored until subsequent analyses.

Genetic material isolation

The specimens were pooled according to their type. The pooled samples were washed with sterile injec-

tion water and were homogenised with 80 µl of PBS using steel spheres in a Bead Ruptor 12 homogeniser until the tissues were completely disintegrated. Genetic material was isolated from the homogenate using the QIAamp DNA Mini Kit (Qiagen), according to the manufacturer's instructions. The genetic material was then subjected to a quantitative and qualitative analysis using the NanoDrop 2000 (ThermoScientific).

PCR analysis

A real-time PCR analysis was performed to determine the infection rate among the ticks. The reaction was carried out using the VIASURE Tick Borne Diseases Real-Time PCR Detection Kit (Certest Biotec), according to the manufacturer's instructions. In this study, the aim was to identify the genes specific for: *B. microti/divergens* (CCT/hsp70), *C. burnetii* (IS1111), *B. burgdorferi* s.l./*miyamotoi/hermsii* (23S rRNA), *A. phagocytophilum* (msp2), and *E. chafeensis/muris* (GroEl), *Rickettsia* spp. (23S rRNA). The analysis was carried out on the CFX96 (BioRad).

Pools and statistics analysis

Single samples were pooled taking into account the sampling site criteria and the developmental stage of 20 specimens. For an estimation of the prevalence, EpiTools Epidemiological Calculators were used.

Results

Number of ticks in the study areas

During the research, 1933 specimens were collected and all of them belonged to the *I. ricinus* species. 108 larvae, 2 pairs, 59 females and 52 males were caught near the Drawa River. 220 nymphs, 6 couples, 93 females and 79 males were caught on the shoreline of Konotop Lake. In Karwice, the number of larvae was 4, nymphs 348, pairs 2, females 173 and males 139. In Konotop, 78 larvae, 1 pair, 17 females and 22 males were caught. Finally, in Oleszno, the number of larvae was 3, with 430 nymphs, 3 pairs, 54 females and 40 males. The highest number recorded in each test site were nymphs (57%, n = 1106), except at Konotop, where no specimens in the nymph stage were collected. The *I. ricinus* ticks were the least frequent in the larvae (4%, n = 85) and pairs (1%, n = 14) stages. The largest number of specimens was caught in Karwice (35%, n = 666), while the smallest numbers were recorded in Konotop (6%, n = 118) and at the Drawa River (11%, n = 221). The percentage share was calculated taking into account the number of ticks at a given development stage in each of the examined sites compared to the total number of collected specimens.

The infection rate in the collected specimens

In this research 111 pools were tested. The most frequently identified pathogen in the tick samples was *Rickettsia* spp. (n = 70), while the next most frequently detected microorganism was spirochetes *B. burgdorferi* s.l. (n = 35). The infection rates for *B. microti/divergens* and *E. chaffeensis/muris* were at the same level (n = 12). In 7 samples, no genetic material belonging to the tested pathogens was detected. The largest number of ticks infected with *Rickettsia* spp. and *B. burgdorferi* s.l. were recorded in Karwice and Oleszno. The specific results are shown in Table 1. In three locations, i.e. the shoreline of Konotop Lake, Drawa River and at Karwice, the presence of all the diagnosed pathogens was observed in the nymph development stage. The research results may be a result of the fact that the highest number of collected ticks were in the nymph stage. However, in the case of Konotop and Oleszno, no *B. microti/divergens* and *E. chaffeensis/muris* infections were recorded, except

for one sample of male ticks infected with *Babesia*. Using the EpiTools Epidemiological Calculators, the highest prevalence level was estimated for the nymph pool, at 0.062, for *B. burgdorferi* s.l. The estimated prevalence for *Rickettsia* spp. was in range of 0.016 to 0.057, for *B. microti/divergens* it was 0.008 to 0.037, for *E. chaffeensis/muris* it was from 0.0096 to 0.02, for *B. burgdorferi* s.l. it was from 0.008 to 0.062, and for *A. phagocytophilum* it was from 0.0013 to 0.037 [SERGEANT, ESG. 2018].

Discussion

The analysis of epidemiological reports collected from the West Pomeranian Voivodeship confirmed that ticks are a real threat to human health and life. Since 2014, there has been an increase in the number of reported cases of Lyme disease: in 2014, 513 cases were reported; while over the next four years, the number

Table 1

Number of positive ticks pools in research position for *Rickettsia* spp., *B. microti/divergens*, *E. chaffeensis/muris*, *B. burgdorferi* s.l., *A. phagocytophilum*

Number of positive pools	<i>Rickettsia</i> spp.	<i>B. microti/divergens</i>	<i>E. chaffeensis/muris</i>	<i>B. burgdorferi</i> s.l.	<i>A. phagocytophilum</i>
Couple	1	1			1
Karwice	1				
Oleszno					
Shoreline Drawa river		1			
Shoreline Konotop Lake					1
Female	12	1	3	9	8
Karwice	5		2	4	4
Konotop	1				
Oleszno	3			3	1
Shoreline Drawa river					1
Shoreline Konotop Lake	3	1	1	2	2
Larvae	1				
Oleszno	1				
Male	8	4	1	3	6
Karwice	3	3	1	1	1
Konotop		1		1	
Oleszno	2			1	2
Shoreline Drawa river	1				1
Shoreline Konotop Lake	2				2
Nymph	48	6	8	23	8
Karwice	16	3	4	11	1
Konotop	2			2	1
Oleszno	20			5	1
Shoreline Drawa river	3	1	1	3	1
Shoreline Konotop Lake	7	2	3	2	4

increased to 768 cases in 2018 (CZARKOWSKI *et al.* 2015; CZARKOWSKI *et al.* 2019). The obtained research shows that soldiers are a professional group that is exposed to the bites of ticks infected with biological pathogenic agents. The analyses showed that the ticks in the study area transmit pathogens such as *Rickettsia* spp., *B. microti/divergens*, *E. chafeensis/muris*, *B. burgdorferi* s.l., and *A. phagocytophilum*. All of these microorganisms are pathogenic for humans; therefore, information campaigns should be carried out among professional soldiers to make them aware of the existing threat, as well as indicating the available preventive methods, which include the use of repellents or routine body inspections after spending time in the field, whether there has been a tick bite. The natural conditions in the area's training ground foster the development of ticks that transmit pathogenic microorganisms (STĘPIEŃ-MATUSZCZYK 2014). As soldiers are not the only professional group exposed to tick bites, similar actions should be implemented in the case of foresters, farmers and even people spending time in the forest for recreational purposes. The necessity of routine screening for the presence of infections caused by tick-borne agents, the symptoms of which are very often non-specific, should also be considered. It is noteworthy that the nymphs, in which the most pathogens were also identified, were type of ticks most often found – which is particularly important because their bite may go unnoticed. Such tests will help to monitor the degree of threat to human life and health that is caused by the presence of these vectors. The current sanitary and epidemiological situation shows how important it is to constantly monitor pathogenic vectors, so that doctors and public health institutions can quickly and effectively implement the correct treatment and diagnosis of pathogens present in the environment. The threat of human tick-borne diseases has increased dramatically in recent years, which is largely influenced by climate changes that favour the development of ticks, as well as increased public activity in forest areas. The available data in the literature on the presence of vectors (ticks) in the Pomeranian Voivodeship show that they coincide with the species analysis obtained as part of this research (KMIECIAK *et al.* 2016; MUCHA *et al.* 2012).

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Author Contributions

Research concept and design: P.W., B.W.-S., D.Ż., A.M.-P.; Collection and/or assembly of data: P.W., B.W.-S., D.Ż., A.M.-P.; Data analysis and interpreta-

tion: P.W., D.Ż., A.M.-P., P.R.; Writing the article: P.W., D.Ż., A.M.-P.; Critical revision of the article: B.W.-S., P.R., G.G.; Final approval of article: B.W.-S., P.R., G.G.

Conflict of Interest

The authors declare no conflict of interest.

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