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Serological screening of antibodies to *Borrelia burgdorferi* in stray and pet dogs populations in Ukraine

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This study describes for the first time in Ukraine the results of serological screening for antibodies to *Borrelia burgdorferi sensu lato (s.l.)* in the population of pet and stray dogs. The study was conducted in the city of Bila Tserkva, Kyiv region, in 2021 and 2022. A total of 351 serum samples were examined, of which 168 were collected from pet dogs and 183 from stray dogs. The study included the use of serological methods, including immunochromatographic analysis, enzyme-linked immunosorbent assay and Line blot is a simplified form of Western Blot.

The results of the enzyme-linked immunosorbent assay showed that 14 positive samples from stray dogs had specific antibodies to *B. burgdorferi s.l.* In addition, 10 questionable serum samples were received, all from stray dogs. Further testing of the questionable samples using a Line blot assay showed that five of them contained antibodies to highly specific surface proteins of *B. burgdorferi s.l.*, in particular to p100, VlsE and p18, confirming the presence of a natural infection. The remaining five samples were negative, indicating nonspecific immunoassay reactions. In contrast, the results of immunochromatographic analysis did not reveal any positive serum samples, which casts doubt on the possibility of using this method as a rapid screening tool for seroprevalence studies and requires further investigation.

In summary, the study showed that the seroprevalence of Lyme borreliosis among the studied population of stray dogs remained stable between 2021 and 2022, with rates of 10.2% and 10.7%, respectively, without a statistically significant difference ($p=0.9164$). In contrast, the studied population of pet dogs was consistently seronegative for *B. burgdorferi s.l.* in both years. Statistical analysis revealed a significant difference in seroprevalence between the stray and pet dog populations studied ($p<0.00001$).

The stability of the seroprevalence of Lyme borreliosis in the population of stray dogs indicates the constant presence and persistence of this zoonosis in the study region. This emphasizes the need to implement long-term surveillance to better understand the dynamics of the disease and the possibility of using dogs, especially stray dogs, as a «marker» species for predicting the risks associated with the spread of Lyme borreliosis. Continuous surveillance is crucial for the development of evidence-based strategies to combat vector-borne diseases within the framework of the One Health concept.

Key words: Lyme borreliosis, seroprevalence, dogs, antibodies, *B. burgdorferi sensu lato*, Ukraine.

Problem statement and analysis of recent research. Lyme borreliosis (LB), caused by the spirochete *B. burgdorferi s.l.*, is a significant public health and animal health problem [1]. The incidence of LB in Ukraine tends to increase annually. This is evidenced by the open data of the Public Health Center of the Ministry of Health of Ukraine on infectious diseases of the population, which indicates that over the past 5 years in Ukraine (2018-2022), 18,962 officially diagnosed cases of LB (9.03 $^{0}/_{0000}$) have been registered, which is 1.4 times more than in 2013-2017 – 13,682 (6.3$^{0}/_{0000}$) [2]. Although knowledge of the incidence and spatial distribution of LB is growing worldwide, comprehensive data on the epidemiology and regional trends in the endemic areas of Ukraine remain limited.

The main vectors of *B. burgdorferi s.l.* are *Ixodes* spp. ticks, in particular *Ixodes ricinus* in Europe and Ukraine [3-5]. Also, *Dermacentor reticulatus* ticks, which are also common in Ukraine, play a significant role in the transmission of LB pathogens [6]. Ticks become infected with the pathogen while feeding on the blood of small mammals, which are the main reservoirs of *B. burgdorferi s.l.*, and can subsequently transmit the infection to humans and other accidental hosts, including dogs [7].

Dogs are considered competent reservoirs for *B. burgdorferi s.l.* [8]. LB in these species tends to be subclinical, but in about 5-10% of seropositive dogs, the disease can manifest itself with nonspecific clinical signs such as lameness, fever, lethargy and lymphadenopathy [9].

In the context of Ukraine, special attention should be paid to the population of stray dogs, which is a significant problem within the framework of the One Health concept [10]. Stray dogs are at increased risk of exposure to ixodid ticks and the pathogens they carry. A number of studies have shown that when the seroprevalence in dogs exceeds 5%, it serves as a sensitive indicator of the risk of LB in humans [11]. Given the synanthropic lifestyle of stray dogs in the same environment as humans, dogs can act as important «markers» of the endemic situation of LB in different areas and serve as «sentinel hosts» between the circulation of *B. burgdorferi s.l.* in the wild and the introduction of the pathogen into the human population. In addition, it is believed that pet dogs can also mechanically transfer infected ticks from the natural environment to human dwellings, thereby increasing the risk of human infection with *B. burgdorferi s.l.* [12].

Thus, LB is a complex public health problem, and dogs, both stray and pet, play a role in its epidemiology. Therefore, the study of their seropositivity to *B. burgdorferi s.l.* can be a source of information on the prevalence of LB in certain areas and identify potential risks of human infection.

The aim of the study. The aim of this study was to assess the prevalence of antibodies to *B. burgdorferi s.l.* in the population of pet and stray dogs. Also, to study the possibility of using dogs as «markers» for predicting and monitoring the risks associated with the spread of LB.

Materials and methods. In 2021-2022, 351 blood serum samples were collected from pet (n=168) and stray (n=183) dogs in Bila Tserkva, Kyiv region, Ukraine. In 2021, 102 serum samples from pet dogs and 108 samples from stray dogs were collected, and in 2022, 66 samples from pet dogs and 75 samples from stray dogs were collected.

Serum samples from stray dogs were collected during a sterilization campaign for stray animals conducted by the Bila Tserkva City State Hospital of Veterinary Medicine. The animals were vaccinated only against rabies, had not been treated for other infectious or parasitic diseases, and were clinically healthy.

Samples from pet dogs were collected during treatment and preventive procedures at the Interdepartmental Veterinary Clinic of Bila Tserkva National Agrarian University. The pet dogs sampled were not vaccinated against Lyme disease, but were vaccinated against other infectious diseases, including rabies, plague, infectious hepatitis, parainfluenza, adenovirus, coronavirus, parvovirus, and leptospirosis. According to the owners, the dogs were systematically treated for endo- and ectoparasites. The selected dogs were either clinically healthy or had pathologies of non-contagious etiology and were not treated with antibiotics.

All samples were collected using disposable sterile instruments. Sera obtained from the dogs were stored in Espendorf tubes in a freezer at -15 °C until serological testing. Each serum sample was split into multiple samples to ensure testing with all serologic methods used in this study.

The serological study included the use of three serological methods for the detection of antibodies to LB pathogens in dogs. The IgG antibodies directed against *B. burgdorferi s.l.* and specifically targeting the antigens of *B. burgdorferi sensu stricto*, *B. afzelii* and *B. garinii* genotypes were detected by semi-quantitative enzyme-linked immunosorbent assay (ELISA) using a commercial

Anti-Borrelia ELISA Dog (IgG) reagent kit (Euroimmun Medical Laboratory Diagnostics AG, Lübeck, Germany). ELISA was performed using the following equipment: Stat Fax 2200 thermostat incubator, Stat Fax 2600 plate washer, and Stat Fax 2100 enzyme-linked immunosorbent assay plate analyzer. ELISA color reaction was measured at a wavelength of 450 nm and a reference wavelength of 650 nm. The results were interpreted by determining the ratio of the optical density of the samples to the optical density of the calibrator. If the ratio was <0.8, the result was negative; from ≥0.8 to <1.1, the result was questionable, and if the ratio was ≥1.1, the result was positive (according to the manufacturer's instructions).

The next step was to distinguish between specific and nonspecific reactions for samples that had a questionable result for the presence of antibodies to *B. burgdorferi s.l.* using a linear enzyme-linked immunosorbent assay – Line Blot (MegaLine *Borrelia* IgG, Hoerbranz, Austria). Line blot is a simplified form of Western Blot. The principle of the test is that the antibodies of the sample bind to the antigens (proteins) labeled with a conjugate on the test strips. Line blot analysis has been used to detect IgG antibodies to a number of highly specific *B. burgdorferi s.l.* surface proteins. In particular, to the proteins: p100, VlsE, C6 peptide, p39 (BmpA), p31 (OspA – vaccination marker), p23 (OspC), p18 (DbpA), specific but not yet characterized p58, and the endoflagellin protein p41 (Flagellin), whose role is not yet defined. Reactions were performed and read according to the kit manufacturer's instructions. Reaction results were visualized and scored using the template on the kit insert. Samples were considered positive if they showed VlsE and/or C6 bands or a combination of VlsE/C6 and positive bands for one or more proteins p100, p58, p41, p39, OspC, p18. Such a combination of bands corresponding to highly specific surface proteins indicates a natural infection of the animal and confirms nonspecific reactions obtained by ELISA.

Also, all dog sera were tested for the presence of antibodies to *B. burgdorferi s.l.* using a rapid immunochromatographic assay (ICA) – a set of Lym Ab Canine Lyme Disease Test Paper Antibody test cards (Ysenmed, China). After applying the diluted sample to the test card, if antibodies to *B. burgdorferi s.l.* are present in the sample, they should specifically bind to the antigen. The formed antigen-antibody complex moves along the chromatographic membrane and is captured by the pre-coated protein on the chromatographic membrane, forming wine-red lines. The reaction results were evaluated visually by the presence of bands opposite the corresponding letters on the cartridge, in particular: bands opposite the letters T and C – positive result; C – negative result; T – unreliable result (according to the manufacturer's instructions).

To statistically process the data and test the assumption of a difference in the seroprevalence of *B. burgdorferi s.l.* in each of the dog populations between 2021 and 2022, the chi-square statistical test was used. The Fisher's exact test statistical calculator was used to test the statistical difference in the seroprevalence of antibodies to LB pathogens between stray and pet dog populations. The results were considered statistically significant if the p-value was less than 0.05. For statistical analysis, the Jamovi computer program (Australia, 2023, version 2.4) was used [obtained from https://www.jamovi.org].

Results. The ELISA results of 351 dog serum samples revealed 14 positive samples for antibodies *B. burgdorferi sensu stricto*, *B. afzelii*, and *B. garinii* genotypes. The ratio of the optical density of positive samples to the calibrator exceeded 1.1 and ranged from 1.236 to 2.825. There were also 10 questionable serum samples with optical densities ranging from 0.814 to 0.946. All positive and questionable serum samples were obtained from stray dogs. Of the 108 serum samples collected from stray dogs in 2021, there were 10 positive and 6 questionable sample, and of the 75 samples collected from stray dogs in 2022, there were 4 positive and 4 questionable samples. According to the ELISA results, all 168 blood serum samples collected from pet dogs were negative for antibodies to *B. burgdorferi s.l.*

The strips with the color reaction of the semi-quantitative ELISA are shown in Figure 1, and the calculated values of the ratio of the optical density of the calibration and samples are given in Table 1.

At the next stage, 10 samples that showed a questionable ELISA reaction were tested by Line Blot. According to the results of the Line Blot, antibodies to highly specific surface proteins of *B. burgdorferi s.l.* were detected in 5 of the 10 preliminary questionable samples. In particular, antibodies to the borrelia surface proteins p100 and VlsE were detected in one sample, and to the surface proteins p100, VlsE and p18 in four samples, respectively. In the remaining 5 out of 10 samples, the presence of antibodies to *B. burgdorferi s.l.* was not confirmed, indicating nonspecific ELISA reactions. The results of the Line Blot analysis are shown in Figure 2.

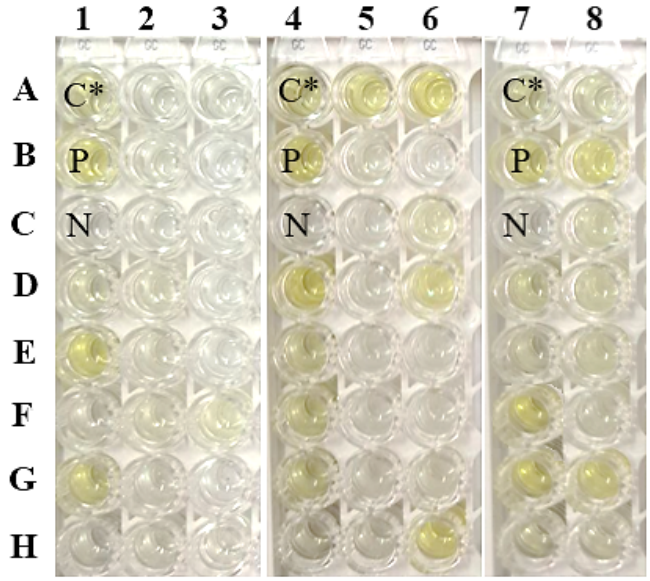

Fig. 1. **Appearance of strips at the color reaction stage of ELISA:**
C* – Calibration, P – Positive control, N – Negative control.

Table 1 – **Interpretation of the results of enzyme-linked immunosorbent assay of stray dog sera for the detection of IgG antibodies directed against *B. burgdorferi s.l.***

	Calculated ratios of the optical density of the calibration and samples							
	1	2	3	4	5	6	7	8
A	0.320 [C]	0.175	0.247	0.326 [C]	1.236*	2.653*	0.295 [C]	0.839**
B	0.654 [P]	0.197	0.088	0.683 [P]	0.227	0.107	0.644 [P]	2.247*
C	0.025 [N]	0.144	0.134	0.023 [N]	0.199	0.819**	0.023 [N]	0.826**
D	0.887**	0.858**	0.131	2.825*	0.328	1.939*	0.822**	0.946**
E	2.197*	0.241	0.122	1.436*	0.107	0.169	0.468	0.917**
F	0.200	0.826**	0.814**	1.834*	0.126	0.215	1.417*	0.302
G	1.653*	0.096	0.106	1.724*	0.172	0.276	2.603*	1.834*
H	0.197	0.288	0.266	0.267	0.218	1.721*	0.492	0.390

***Notes*:** [C] Calibration; [P] Positive control; [N] Negative control; * Positive samples (optical density ratio ≥1.1); ** Questionable samples (optical density ratio from ≥0.8 to <1.1); all other unlabeled samples are negative (optical density ratio <0.8).

In contrast to ELISA, ICA testing of the same serum samples (n=351) for antibodies to *B. burgdorferi s.l.* did not yield positive results. All test cards had stripes opposite the letter C, indicating negative immunochromatographic results, as shown in Figure 3.

The results of serological screening showed that in 2021, the seroprevalence of LB among stray dogs was 10.2%, with 11 out of 108 samples being positive. In 2022, the seroprevalence was 10.7%, with 8 out of 75 samples positive. The seroprevalence of *B. burgdorferi s.l.* in the stray dog population was not statistically different between 2021 and 2022 (p=0.9164). In contrast, the prevalence of antibodies to *B. burgdorferi s.l.* in the pet dog population was 0.0% in both 2021 and 2022. Statistical comparison of seroprevalence between stray and pet dog populations revealed a statistically significant difference between the two groups of animals (p<0.00001).

The summary results of serological screening using different serological methods for the detection of antibodies to *B. burgdorferi s.l.* among the populations of pet and stray dogs in Bila Tserkva, Kyiv region, in 2021 and 2022 are presented in Table 2.

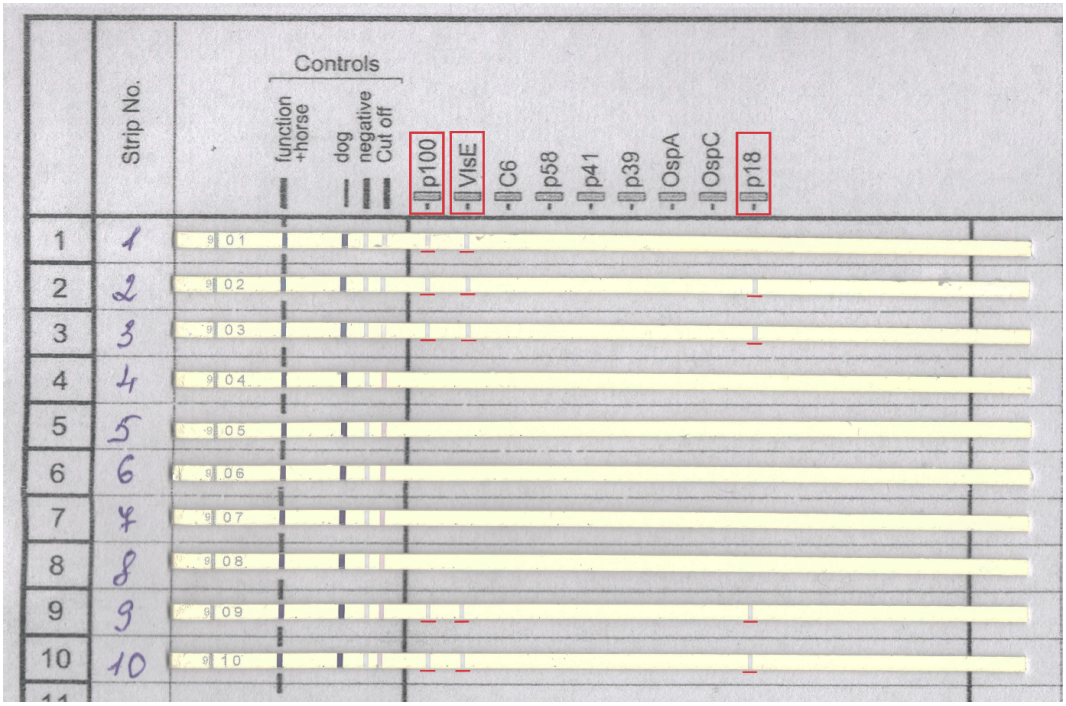

Fig. 2. **Results of Line Blot analysis of 10 questionable serum samples from stray dogs:** red rectangles circle the names of highly specific proteins to which the samples showed a positive reaction; red lines under the test strips indicate positive reactions; positive samples are numbered 1, 2, 3, 9 and 10; negative samples are numbered 4, 5, 6, 7 and 8.

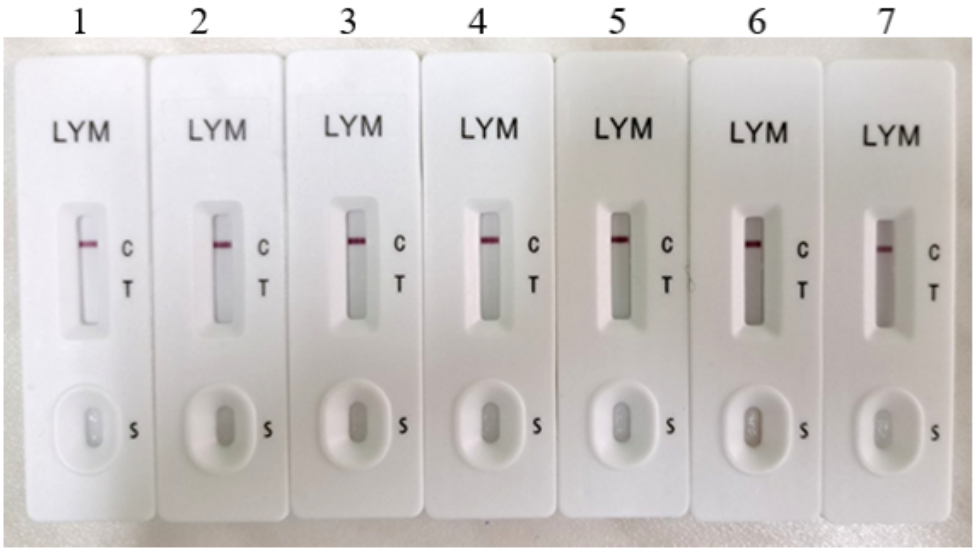

Fig. 3. **Results of ICA for antibodies to *B. burgdorferi s.l.* antigens in dog sera:** the wine-red band opposite the letter C indicates a negative result.

Table 2 – **Results of serological screening for antibodies to *B. burgdorferi s.l.* in dogs,** 2021-2022, Bila Tserkva, Kyiv region, Ukraine

Dog populations studied	ICA results		ELISA results		Line blot results		Combined seroprevalence, %	
	2021	2022	2021	2022	2021	2022	2021	2022
Pet dogs	0/102*	0/66	0/102	0/66	-	-	0.0	0.0
Stray dogs	0/108	0/75	10/108	4/75	1/6	4/4	10.2	10.7

***Note*: *** number of positive samples / total number of serum samples tested.

Discussion. This study is an important contribution to the understanding of the seroprevalence of LB in pet and stray dog populations in Ukraine. The results of the study add to the knowledge of the potential role of dogs, especially stray dogs, as a «marker» species in predicting the risk of Lyme disease.

This first serologic screening conducted in Ukraine revealed significant differences in the prevalence of antibodies to *B. burgdorferi s.l.* between stray and pet dog populations in the study area. In 2021 and 2022, the prevalence of antibodies to *B. burgdorferi s.l.* among stray dogs was more than 10%, while pet dogs remained seronegative. This marked difference emphasizes the potential role of stray dogs as competent hosts and indicators of the prevalence of LB in certain areas.

Stray dogs pose a serious veterinary, health and socio-environmental problem in many parts of the world [13]. Dogs living on the street as stray animals are at an increased risk of contracting vector-borne diseases, as they are often the first to come into contact with ticks and various infectious agents in an urbanized environment. Pet dogs living in human homes have a relatively lower risk of infection. In our study, it was reported that owners systematically treated their pet dogs for ectoparasites and frequently examined them for ticks after walking outside. These prerequisites may have contributed to the high level of difference between the seroprevalence of LB between the stray and pet dog populations in our study.

According to various scientific sources, the seroprevalence among dogs to LB pathogens differs depending on the geographical area. Among European countries, the highest serological rates of Lyme borreliosis in different time periods were recorded in Switzerland (57.5%) and Germany (43.3%) [14, 15]. In the countries bordering Ukraine, in particular, in Slovakia, 33.5% of seropositive dogs were detected by ELISA and only 2.8% by rapid ELISA; in Poland, the highest rates were registered at 40.2% (ELISA); in Hungary – 0.8% (rapid ELISA), respectively [16-18]. Similar seroprevalence rates to B. burgdorferi s.l. in dogs were reported in the Czech Republic – 9.2% (ELISA), France – 10.4% (ELISA and Western blot), and Eastern Poland – 11.0% (ELISA) [15,19-20]. Although we obtained seroprevalence data from different scientific sources, they all differ significantly in terms of the time period of the studies, the testing methods used and do not cover the territories of different countries sufficiently to draw conclusions about the overall prevalence of LB in dogs, and also indicate significant gaps in this area of research.

Data on the seroprevalence of antibodies to *B. burgdorferi s.l.* in dog populations from other regions and cities of Ukraine are not available. Therefore, we cannot confirm whether the results of our study are consistent with the seroprevalence among dogs from other regions of Ukraine or comparable to other periods.

It should also be noted that the ELISA method detected both positive and questionable results, and subsequent linear blot confirmed the presence of specific antibodies in some of the initially questionable samples. However, the rapid ICA tests did not yield any positive results when tested on the same serum samples, indicating potential limitations of their sensitivity for screening for LB seroprevalence and requiring further study of the sensitivity levels of ICA and the feasibility of its use for monitoring LB seroprevalence in dogs. Earlier it was reported that veterinarians have successfully used rapid tests to diagnose clinical LB in dogs, positive results of which could be associated with high antibody titers in the clinical course of the disease [22]. Instead, in one of our previous studies, we found no antibodies to *B. burgdorferi s.l.* in the clinical course of LB in dogs with signs of Lyme arthritis, which may seem contradictory, but most likely indicates the peculiarities of the pathogenesis of the disease and requires further study [23]. Thus, our results emphasize the importance of selecting appropriate serologic methods for specific study purposes and the need to confirm questionable ELISA results with linear blot analysis to provide a more accurate estimate of the seroprevalence of LB.

Conclusions. 1. An enzyme-linked immunosorbent assay revealed 14 positive serum samples, all from stray dogs, indicating the presence of antibodies to *B. burgdorferi sensu stricto*, *B. afzelii* and *B. garinii* genotypes. In addition, 10 questionable serum samples were obtained, all also from stray dogs. Line Blot confirmation of the 10 previous questionable samples by ELISA showed that 5 of them did indeed have antibodies to highly specific surface proteins of *B. burgdorferi s.l.*, while the other 5 showed nonspecific ELISA reactions.

2. Immunochromatographic analysis did not detect positive serum samples from both stray and pet dogs, which may indicate a low sensitivity of the test and requires further investigation of the specificity of using rapid tests for seroprevalence screening studies.

3. The seroprevalence of LB among stray dogs in 2021 and 2022 was similar and amounted to 10.2% and 10.7%, respectively. These figures were not statistically different (p=0.9164).

4. The pet dog population was seronegative for *B. burgdorferi s.l.* in 2021 and 2022.

5. A statistical comparison of the seroprevalence of antibodies to *B. burgdorferi s.l.* in stray and pet dog populations revealed a statistically significant difference between them ($p<0.00001$).

Information on compliance with bioethical standards. All manipulations with animals were carried out as part of treatment or preventive treatments of animals and did not involve additional impact on animals, and all bioethical standards were observed in accordance with bioethical rules.

Conflict of interest disclosure. The authors declare that they have no conflict of interest.

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Серологічний скринінг антитіл до *Borrelia burgdorferi* у популяціях безпритульних та домашніх собак в Україні

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У дослідженні вперше в Україні описано результати серологічного скринінгу на наявність антитіл до *Borrelia burgdorferi sensu lato* (*s.l.*) у популяціях домашніх та безпритульних собак. Дослідження проводили у м. Біла Церква Київської області у 2021 та 2022 роках. Всього було досліджено 351 зразок сироватки крові, з яких 168 були відібрані у домашніх собак та 183 – у безпритульних. Дослідження включало використання серологічних методів, зокрема імунохроматографічного аналізу, імуноферментного аналізу та лінійного блоту – спрощеної форми вестерн-блоту.

Результати імуноферментного аналізу показали, що 14 позитивних зразків від безпритульних собак мали специфічні антитіла до *B. burgdorferi s.l.* Крім того, було отримано 10 сумнівних зразків сироватки, всі від безпритульних собак. Подальше тестування сумнівних зразків методом лінійного блот-аналізу показало, що п'ять з них містили антитіла до високоспецифічних поверхневих білків *B. burgdorferi s.l.*, зокрема до p100, VlsE та p18, що підтверджує наявність природного інфікування. Решта п'ять зразків були негативними, що свідчить про неспецифічні реакції імуноферментного аналізу. На відміну від цього, результати імунохроматографічного аналізу не виявили жодного позитивного зразка сироватки, що ставить під сумнів можливість використання цього методу як інструменту швидкого скринінгу для дослідження серопоширеності і потребує подальшого вивчення.

Підсумовуючи, дослідження показало, що серопоширеність Лайм-бореліозу серед досліджуваної популяції безпритульних собак залишалася стабільною в період між 2021 і 2022 роками, з показниками 10,2 і 10,7 % відповідно, без статистично значущої різниці ($p=0,9164$). На противагу цьому, досліджувана популяція домашніх собак була стабільно серонегативною щодо *B. burgdorferi s.l.* в обидва роки. Статистичний аналіз виявив достовірну різницю в серопоширеності між популяціями безпритульних і домашніх собак ($p<0,00001$).

Стабільність серопоширеності Лайм-бореліозу в популяції безпритульних собак свідчить про постійну присутність і персистенцію цього зоонозу в досліджуваному регіоні. Це підкреслює необхідність впровадження довготривалого нагляду для кращого розуміння динаміки розвитку хвороби та можливості використання собак, особливо безпритульних, як «маркерного» виду для прогнозування ризиків, пов'язаних з поширенням Лайм-бореліозу. Безперервний епіднагляд має вирішальне значення для розробки науково обґрунтованих стратегій контролювання трансмісивних хвороб в межах концепції «Єдине здоров'я».

Ключові слова: серопоширеність, домашні та безпритульні собаки, Лайм-бореліоз, антитіла, *B. burgdorferi s.l.*, Україна.

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