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Serologic Evidence of Exposure to *Leishmania infantum* in Captive and Free-Ranging European Bison (*Bison bonasus*) in Poland, 2017–23

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ABSTRACT: The European Bison (*Bison bonasus*) is the largest mammal in Europe and is classified as an endangered species. Leishmaniosis is a vector-borne disease caused by the protozoan *Leishmania infantum*. In general, this infection has been associated with dogs, cats, and humans. However, epidemiologic studies and reports confirm that the parasite is able to infect many other mammalian species. Recent evidence has demonstrated that ruminants in endemic areas are exposed to *L. infantum* infection. Moreover, climate change has allowed the northward spread of vector species, causing the expansion of *L. infantum* infection in regions traditionally classified as nonendemic in Europe. The aim of this study was to determine the presence or absence of anti–*L. infantum* antibodies in serum samples from 343 European bison in Poland, collected from 2017 to 2023. For this purpose, the presence of anti-*Leishmania* antibodies was analyzed using an in-house multispecies ELISA. Anti-*Leishmania* antibodies were detected in four animals, an overall seroprevalence of 1.17%. The results provide scientific evidence of serologic exposure to the parasite in Poland, a country previously considered nonendemic for *L. infantum* infection.

Key words: *Bison bonasus*, *Leishmania infantum*, serology, Poland.

Leishmaniasis is a vector-borne disease caused by *Leishmania* spp. protozoa and transmitted by phlebotomine sand flies (*Phlebotomus* spp., Diptera: Psychodidae). In continental Europe, *Leishmania infantum* is the only autochthonous species responsible for infection in animals, with the exception of sporadic human cases caused by *Leishmania tropica* in Greece (Christodoulou et al. 2012). In Europe, the domestic dog (*Canis familiaris*) is considered the main domestic reservoir of human *Leishmania infantum* infection (Maia et al. 2023). Other animals may also play an epidemiologic role in the urban cycle, including cats (*Felis catus*; Alcover et al. 2021) and ferrets (*Mustela putouris furo*; Alcover et al. 2022). Under certain circumstances, the sylvatic transmission cycle is closely interconnected with urban areas, and hares and rabbits (Lagomorpha) played a role as sylvatic reservoirs in the largest human leishmaniasis outbreak in Europe, in the southwestern region of Madrid, Spain, in 2012 (Molina et al. 2012; Jiménez et al. 2014). Recent evidence suggests that in endemic areas of *Leishmania* infection, it is possible to detect the presence of seropositive animals in various mammalian species other than dogs and cats, including livestock and wildlife.

Little is known about the presence of seropositive ruminants, as potential sylvatic reservoirs in Europe (Cardoso et al. 2021). For small ruminants, such as domestic goats (*Capra aegagrus hircus*) and domestic sheep (*Ovis aries*), there is very limited epidemiologic information, with only three published epidemiologic studies performed in Europe. A study performed in Thessaly, Greece, looked for the presence of anti-*Leishmania* antibodies in serum samples from goats and sheep; ELISA results indicated the absence of antibodies in livestock on several farms (Kantzoura et al. 2013). In contrast, the presence of low levels of anti-*Leishmania* antibodies were detected in two studies performed in Spain, including in sheep and goat samples (Portús et al. 2002), and a second study containing only serum samples from sheep (Villanueva-Saz et al. 2024). Portús et al. (2002) detected the presence anti-*Leishmania* antibodies in 18.85% of goats, while in sheep, the seroprevalence level ranged from 9.27%

(Villanueva-Saz et al. 2024) to 11.86% (Portús et al. 2002). Two clinically ill ruminants have been reported: a goat in Spain (Ruíz et al. 2023) and a Swiss cow (*Bos taurus*) in Switzerland (Lobsiger et al. 2010). No epidemiologic studies have been performed in cattle in Europe.

The European bison (*Bison bonasus*) is a member of the Bovidae family. Historically, this species was widely distributed throughout central Europe. After World War I, the species was extinct in the wild; the only remaining individuals resided in captivity (Pucek et al. 2004). The captive population experienced a modest recovery until the onset of the World War II, which led to a considerable decrease in the number of remaining bison (Pucek et al. 2004). In 1996, The International Union for Conservation of Nature classified the European bison as an endangered species. Since then, conservation efforts have succeeded to the point that currently this species is classified as near-threatened (Plumb et al. 2020).

An important consequence of climate change in Europe has been an impact on vector-borne diseases. As temperatures rise and weather patterns shift, there has been an expansion of the geographic distribution of disease vectors such as phlebotomine sand flies. These vectors may now thrive in regions where they were previously uncommon or traditionally classified as nonendemic areas of infection, including Northern Europe (Oerther et al. 2020). There is no previous information on the exposure of European bison to *L. infantum* infection under natural conditions in Poland, with no epidemiologic surveys regarding the presence of the parasite both in domestic and wild reservoirs in this country (Mihalca et al. 2019). The aim of our study was to investigate the seroprevalence of *L. infantum* exposure in European bison in Poland using an in-house ELISA.

From 2017 to 2023, 343 European bison (185 females, 152 males, and six not determined) were sampled. The age of the animals was assessed by veterinarians based on teeth eruption (Krasińska and Krasiński 2017). Information including geographic location, sex, age, and type of population (captive or free ranging) was recorded and subjected to statistical analysis. For some European bison, it was not possible to acquire all data; these animals were not considered when performing statistics.

No animal was culled or immobilized specifically for this study. Antemortem sampling was undertaken from animals immobilized in the course of routine veterinary care situations, such as putting on a telemetry collar or during translocation. Other individuals were sampled at necropsy. According to the Local Ethical Committee for Animal Experiments (Warsaw, Poland), the procedures did not require ethical approval. Most of the samples were collected as part of the Complex Project of European Bison Conservation by State Forests. Before initiating the study, a permit was obtained from the General Director of Environmental Protection.

Immobilization was performed as described (Krzysiak and Larska 2014). Briefly, etorphine hydrochloride (Captivon, 9.8 mg/mL, Wildlife Pharmaceuticals, White River, South Africa) and xylazine (Nerfasin, 100 mg/mL, Rio Saliceto, Livistio, Italy) were delivered by remote injection using various dart guns. Animals appeared healthy following immobilization. Blood was collected from the jugular vein using a 1.2-mm-diameter needle into 6- to 9-mL tubes containing a clot activator. The blood tubes were refrigerated and transported immediately to the laboratory, where they were centrifuged (3,000 × G, 10 min). Serum samples were then stored at −20 C until analysis. Before performing ELISA, samples were thawed and brought to room temperature.

Routine laboratory tests, such as a complete blood count and biochemistry profile, were not performed. An ELISA to detect antibodies against *Leishmania* spp. for multiple animal species was conducted on all sera, as described by Villanueva-Saz et al. (2024), with some modifications, using 100 μL of European bison sera diluted 1:100. The ELISA used a conjugate composed of protein A/G peroxidase. This interacts with immunoglobulin G in different mammal species, allowing the use of positive and negative controls from different species in the absence of controls for the species being serologically tested. Each plate included a panel of positive serum samples with a known antibody status from

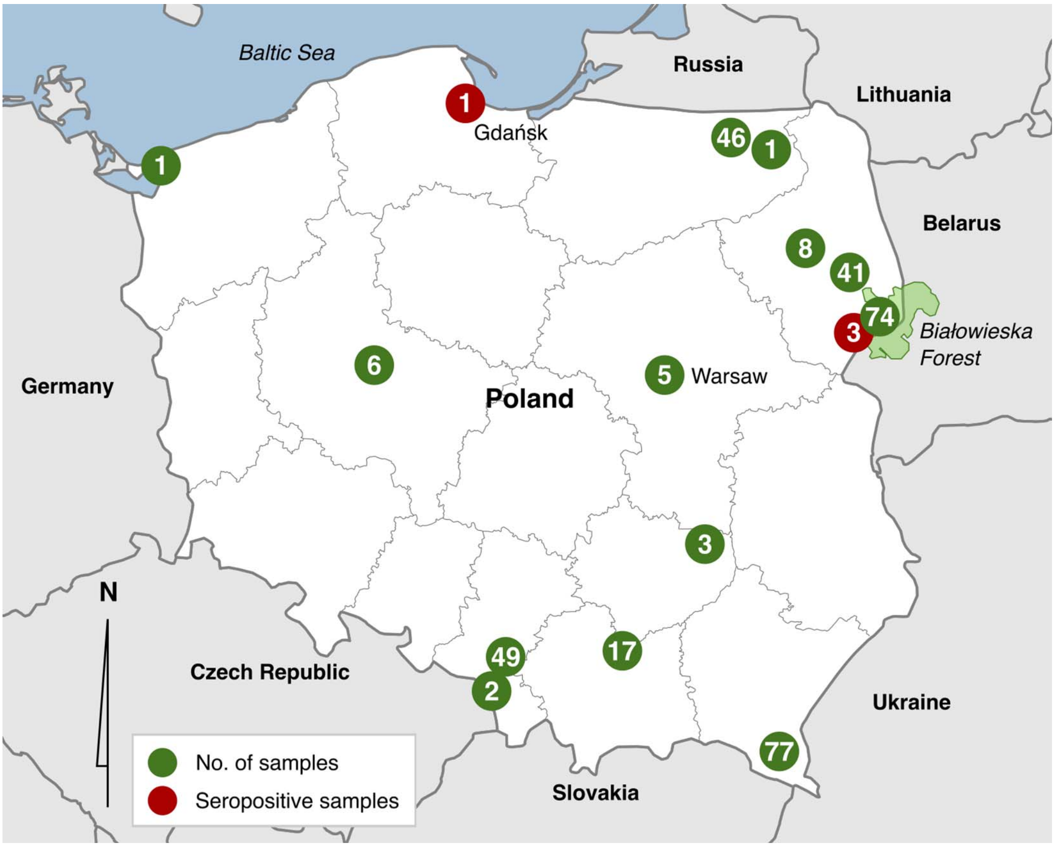

FIGURE 1. Map of Poland showing the locations where serum samples were collected from European bison (*Bison bonasus*). Red dots are specific places where seropositive *Leishmania infantum* European bison were confirmed.

different species such as goats, sheep, ferrets, minks, cats, and dogs with low levels of anti-*Leishmania* antibodies. As negative controls, serum samples from healthy, noninfected animals (classified based on negative serologic results and molecular tests and the absence of abnormal clinical and laboratory findings) from the same species were used. In the absence of a negative control, the cutoff was based on the *Leishmania* spp. antibody cutoff for sheep (0.38 optical density units [OD units]), as described (Villanueva-Saz et al. 2024). Moreover, this in-house ELISA test did not cross-react with antibodies to *Toxoplasma gondii* or *Neospora caninum*, both protozoa (Villanueva-Saz et al. 2022). Additional pathogens that share antigens and may produce cross-reactions are other *Leishmania* spp. species and *Trypanosoma cruzi*; however, *L. infantum* is the parasite responsible for leishmaniosis in animals from European countries, and *T. cruzi* is absent from Europe.

The data were analyzed using SPSS version 22 software (IBM Corp. 2022). Descriptive analysis of the variables was carried out, considering the proportion of qualitative variables. The Fisher exact test and a 95% confidence interval (CI) were used to compare proportions. In all analyses, the significance level was established at $P<0.05$.

Animals were from captive (n=157) or free-ranging (n=178) herds from different regions of Poland (Fig. 1): Bałtów (n=3), Białowieska Forest (n=77), Bieszczady Mountains (n=77), Borecka Forest (n=46), Gołuchów (n=6), Kiermusy (n=8), Międzyzdroje (n=1), Niepołomice (n=17), Pszczyna (n=49), Augustowska Forest (n=1), Knyszyńska Forest (n=41), Ustroń (n=2), and from Warsaw (n=5), and Gdańsk (n=1) zoologic gardens. Samples were collected in 2017 (n=12), 2018 (n=93), 2019 (n=43), 2020 (n=36), 2021 (n=68), 2022 (n=35), and 2023 (n=55). The mean age of the animals was 6.7 yr (range from 0.25 yr to 25 yr, with age not determined in 13 individuals), and they were classified as calves (≤1 yr old), juveniles (2–3 yr old), and adults (≥4 yr old).

Of the 343 European bison tested by ELISA, 339 were seronegative (mean±SD: 0.11±0.06 OD units). One of the most common approaches used in veterinary diagnosis to determine the cutoff for detecting pathogens is to calculate the mean value from a known negative animal population reference and add two, three, or four SDs to mean OD units to establish this reference. Based on the seronegative results we obtained, our calculated cutoffs of 3, 4, or 5 SD above the mean of the seronegative animals were 0.22, 0.27, and 0.33, respectively. Four animals tested positive for *L. infantum* antibodies, with OD units of 0.42, 0.49, 0.52, and 0.65 and mean±SD of 0.52±0.10 OD units. Thus, samples classified as seropositive by the multispecies ELISA were above the different cutoffs based on the mean plus 3–5 SDs, suggesting the seropositivity of these four animals. The seropositive rate was 1.17% (95% CI, 0.45–2.96%). No doubtful results were obtained with this test for any given animal. No significant association (P>0.05) was found between seropositivity for anti-*Leishmania* antibodies and the variables studied, including sex, age, and type of population.

Seropositive samples were collected in different years: 2018 (n=1), 2019 (n=2), and 2020 (n=1). Three of the seropositive samples were from the Białowieska Forest (two from a free-ranging herd and one from a 6-yr-old female in a captive herd), while the fourth seropositive sample was from the Gdansk Zoological Garden (a 1-yr-old female). The captive animals had been born captive.

The presence of phlebotomine sand flies, the main vectors of *L. infantum*, has not been confirmed in Poland; however, to our best knowledge, there have been no scientific studies focusing on this subject in this country. Leishmaniosis in Poland has been detected sporadically in dogs: in 2008, in a stray dog without prior clinical history (it was not possible to determine whether it was a case imported from abroad or whether the dog was infected in the country). In other cases of leishmaniosis affecting Polish dogs, animals had traveled abroad in the past (Sapierzyński et al. 2008). There have been no previous epidemiologic studies investigating wildlife in Poland for *L. infantum* seropositivity, nor has there been previous detection of antibodies against *L. infantum* in European bison.

One of the seropositive animals from the free-ranging herd in Białowieska Forest was a 14-yr-old bull. Necropsy revealed scar tissue and alopecia of the skin on the right side of the chest and the hip joint area, a healed fracture of the 10th rib on the right side, adhesions in the pleural cavity, pneumonia, focal hepatitis, fluke, adhesions of the liver to the parietal peritoneum, and atrophy of the right testicle. Some of the cutaneous and visceral lesions could potentially be related to *L. infantum* infection.

The main limitation of our study is its retrospective nature; its strengths include the large number of samples obtained from different regions of Poland, as well as the long study period. Most of the *Leishmania*-seropositive European bison in our study came from one area, Białowieska Forest, which may be related to the potential presence of the vector in that area. Nevertheless, the detection of seropositive animals in two different locations strengthens the hypothesis regarding the probable occurrence of the phlebotomine sand fly in Poland due to climate change and thus a higher risk of the occurrence of *L. infantum* infection in domestic and free-living animals.

Given the results obtained in our study, and the general lack of epidemiologic data on *L. infantum* infection in Poland, broader serologic testing of animals in Poland, including wild, free-living individuals, would be worthwhile. In addition, work is needed to look for the presence of phlebotomine sand flies in Poland, particularly in the Białowieska Forest.

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