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Is serological monitoring a fit-for-purpose tool to assess the epidemiological situation of tuberculosis in the sylvatic species of European bison (*Bison bonasus*) in Poland?

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Abstract

Introduction: Bovine tuberculosis is one of the most dangerous zoonotic diseases. Despite the near-complete elimination of the disease from cattle breeding in Poland achieved in 2009, its re-emergence is now observed. Globally, the number of human cases is underestimated and the importance of free-living animals as reservoirs of tuberculosis is growing. As a species highly susceptible to *Mycobacterium tuberculosis* complex infection, the European bison (Bison bonasus) has a role in the transmission of the disease in Poland. The purpose of the investigation was to assess the epidemiological situation of tuberculosis in Polish European bison serologically. **Material and Methods:** A total of 460 serum samples were collected from 436 European bison from 15 out of 26 national populations between 2013 and 2020. An *M. bovis* ELISA was used, and its sensitivity and specificity were assessed with an eyelid tuberculin skin test (TST) and interferon gamma release assay (IGRA). **Results:** *Mycobacterium bovis* antibodies were detected in nine serum samples. The presence of antibodies was found in two animals from the Białowieża Forest (1.2% of the population), and one each from the Borecka Forest (2.4%) and the Warsaw Zoo (14.3%). One European bison among the 14 sampled (7.1%) from Smardzewice was positive on five occasions. Other samples from Smardzewice and the Bieszczady Mountains, where tuberculosis had previously been reported, were negative. **Conclusion:** ELISA testing is an effective, easy and cost-efficient tool for monitoring of tuberculosis-naïve populations. Serological testing in tuberculosis control programmes can significantly improve the detection of infected herds. Antibody ELISAs may supplement TST and IGRA, but cannot replace them.

Keywords: bovine tuberculosis, European bison, *Mycobacterium bovis*, *M. caprae*, serology, ELISA, tuberculin skin test, interferon gamma release assay.

Introduction

Bovine tuberculosis (BTB) is one of the most dangerous zoonotic diseases (37). Despite the near-complete elimination of the disease from cattle breeding in Poland, the country becoming officially tuberculosis free in 2009, its re-emergence has recently been observed (33). While the number of cases in humans is considered to be underestimated globally (25), BTB eradication programs in domestic animals are mostly successful. The importance of free-living animals as reservoirs of BTB in the environment is increasing (29).

Bovine tuberculosis is a climate-sensitive disease, and its threat would be considered to increase with environmental changes as well as changes in human activity. Human migration, globalisation, the intensification of agriculture and the shrinkage of the human–wildlife interface zone are among the anthropogenic drivers of increased risk of zoonotic pathogen transmission. Unlike in the UK, in Poland the badger (*Meles meles*) is not considered an important reservoir of BTB (2). One of the species highly susceptible to *Mycobacterium tuberculosis* complex (MTBC) infection is the European bison (*Bison bonasus*), which plays an important role in transmission of the disease in the BTB-endemic sylvatic environment in Poland. This animal, the largest herbivore in Europe, went extinct in its native continent in the last century. The efforts of the last 90 years of conservation have resulted in the recovery of the species and its restitution into the wild. The worldwide population size reached more than 9,000 individuals in 2020. In some countries such as Germany, Spain, Romania and the UK, their populations are growing rapidly. The largest populations inhabit Poland, Belarus and Russia, where most of the animals live free. The free-ranging population is growing steadily and reached 74% of the total number of European bison in 2019 (32). One of the key components of European bison restitution is on-going health monitoring, which includes BTB, one of the diseases most threatening to the species. Between 1996 and 2013, 45 cases of BTB were reported among 81 tested European bison in the Bieszczady Mountains (in the south-eastern part of Poland) (1). In the four following years (1997–2001), tuberculosis was microbiologically confirmed in 18 culled European bison from the Bieszczady herd of "Brzegi Dolne" (39). In 2009, the entire herd was culled. The occurrence of tuberculosis cases in the eastern sub-population of European bison in the Bieszczady Mountains met the criteria for endemicity, threatening the efforts towards restitution of the European bison – a species noted as vulnerable on the IUCN Red List of Threatened Species. At the turn of 2012 and 2013, another herd from the Bieszczady Mountains, "Górny San", consisting of 26 European bison, was culled due to BTB (17). Generalised multiorgan tuberculosis was observed at necropsy in most of the animals and *Mycobacterium caprae* was isolated from tissue samples. The transmission of the infection to wild boars (*Sus scrofa*) from the same area was also confirmed (15). Additionally, *M. caprae* was isolated from wolves (*Canis lupus*) in 2014 (26) and later also from wild boar again and roe deer (*Capreolus capreolus*) (27). In recent years, BTB was diagnosed at the European Bison Breeding Centre in Smardzewice in central Poland (7, 9, 16), in the Bison Breeding Centre in Wolisko in north-eastern Poland and in the free-ranging herd from the Borecka Forest around Wolisko (8), as well as in the Warsaw Zoo (18).

The increasing number of wild European bison raises the likelihood of frequent contact with people and domestic animals. European bison have been shown to be exposed to many zoonotic agents (21), among which BTB is one of the most harmful. Since multidirectional transmission of infection between livestock and free-living animal populations has been reported (5), the problem was considered a safety risk affecting food products from cattle, pigs, and game species (roe deer, deer and wild boar) in Poland, especially in the Bieszczady Mountains. The first case of *M. caprae* infection in a human was recently described in Poland (14). To protect both humans and European bison, monitoring for BTB should be carried out not only in cattle but also in wildlife, as the One Health concept would suggest. The standard ante-mortem tuberculosis tests include the tuberculin skin test (TST), interferon gamma release assay (IGRA) and microbiological isolation. However, the technical limitations of the available tests, which may produce false results in non-domestic animal species, are still an issue (10). The TST and IGRA are nevertheless officially validated examinations required for international movement of European bison or in suspected cases of BTB. However, the low sensitivity and specificity of the diagnostic tests make it necessary for alternative methods or procedures to be developed.

So far, no serological monitoring studies of BTB have been carried out to make possible large-scale epidemiological analysis and determination of risk factors in the populations of European bison. The only studies in the literature include suspected BTB or clinical cases and present microbiological and molecular characterisation of the pathogen conducted on samples from a small number of animals (7, 9). Such methods are much more expensive than serological equivalents. Another surveillance study on BTB in European bison from the Borecka Forest was carried out using the gamma interferon test; however, the sample size was again not large (8). Serological tests to detect the presence of specific antibodies to MTBC are easy to perform, inexpensive and allow a representative number of animals to be tested to determine the spread of infections. Their usage is all the more justified since the antibody response in European bison naturally infected with *M. caprae* was confirmed recently (7). The purpose of the study was to use a tool such as serological monitoring to assess the epidemiological situation of tuberculosis in European bison in Poland.

Material and Methods

Study design and sample collection. A total of 460 serum samples were collected from 436 European bison from 15 out of the 26 existing different populations spread across the country, including the

largest free-living European bison herds from the Białowieża (including Białowieża National Park) (n = 173), Borecka (n = 42) and Knyszyńska (n = 62) forests in north-eastern Poland, a herd from the Bieszczady Mountains (n = 38) in the south of the country and several other captive herds between 2013 and 2020 (Fig. 1). The samples originated both from female (n = 242) and male (n = 194) European bison aged between 2 months and 29 years. The samples were collected from live European bison (n = 269) which had been pharmacologically immobilised as described by Krzysiak and Larska (20) for fitment of collars with telemetric transmitters or for diagnostic reasons (*e.g.* when transported to other populations or suspected of having infectious diseases such as tuberculosis), as well as from dead individuals found in that state (n = 20), killed in traffic accidents (n = 9), or euthanised (n = 138) due to poor health in accordance with the relevant resolutions of the Minister of the Environment and the General Director for Environmental Protection. Blood was most commonly collected from immobilised animals by puncture of the external jugular vein (*vena iugularis externa*) and less often from the tail vein (*v. caudalis mediana*). Blood from dead and necropsied animals was collected in the form of a clot from the heart or from body cavities, and from the external jugular vein or heart from euthanised animals. Blood was collected into sterile 7–9 mL tubes and centrifuged within 24 h, and the obtained serum was frozen at −70°C until analysis. The distribution and relevant characteristics of animals which provided positive samples are presented in Table 1. No clinical symptoms of BTB were observed in any of the European bison, except for those originating from the Smardzewice enclosure in the Kampinoski National Park, where BTB was reported (see below).

Another group of European bison were sampled in order to assess the sensitivity, specificity and agreement between the ELISA and tuberculin skin (TST) and gamma interferon tests. This group of 14 European bison from the Smardzewice population included in the epidemiological study were sampled on multiple occasions between 2 September 2013 and 11 December 2018, *i.e.* over a period of 63 months. A total of 33 samples were used. The first case of tuberculosis in European bison at the Smardzewice enclosure was detected in 2013 (16). The herd then consisted of 20 European bison, of which 6 were euthanised after positive TSTs in 2014. With the consent of the Ministry of the Environment, the outbreak was managed successfully by TB testing and elimination of suspected or diseased animals one by one. The last animals were culled in 2018 (7).

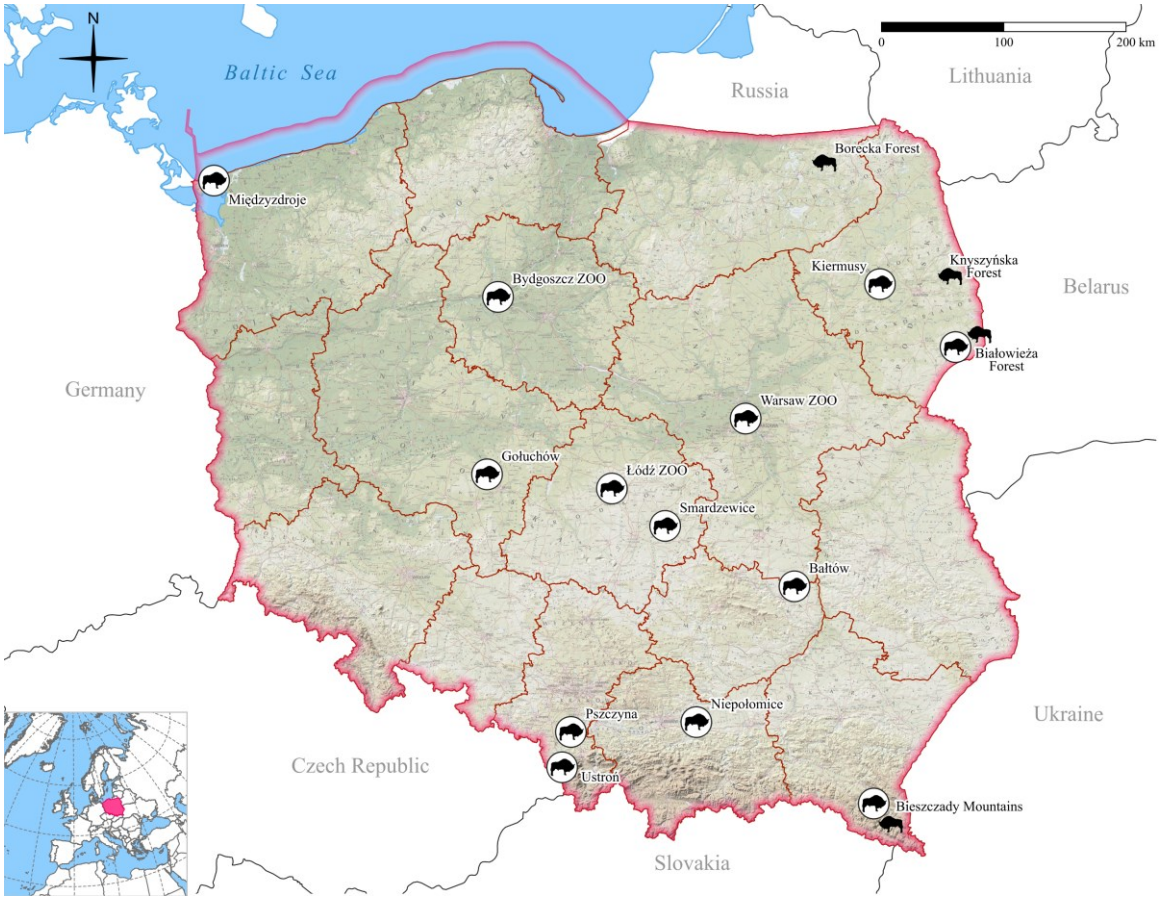

Fig. 1. Distribution of European bison populations included in the study. Herd locations are marked with European bison silhouettes. The captive herds are marked with encircled silhouettes

Serological test. The IDEXX *M. bovis* bovine-specific Ab test (IDEXX Laboratories, Westbrook, ME, USA) was used. The test detects the presence of antibodies to the *M. bovis* MPB83 and MPB70 proteins in bovine serum and plasma samples (38). The test was performed according to the manufacturer's instructions. Briefly, 100 µL of serum samples diluted 1:50 was added to 96-well microtitre plates coated with the two mycobacterial antigens (MPB83 and MPB70) and incubated at room temperature for 1 h. After the plate was washed, 100 µL of a monoclonal anti-bovine IgG–horseradish peroxidase conjugate was added and the solution was incubated for 30 min. The reaction was visualised by adding 100 µL of TMB substrate to each well and blocked after 15 min by an acidified stop. The optical density (O.D.) of colour intensity was measured at 450 nm. The results were calculated as sample-to-positive ratios (S/P) as follows:

$$\frac{\text{O.D. tested sample-mean O.D. negative control}}{\text{mean O.D. positive control-mean O.D. negative control}}$$

Samples with S/P ratios greater than or equal to 0.30 were considered positive for *M. bovis* antibodies. The cattle herd sensitivity of the test declared by IDEXX is 77.8%. The test is especially valuable in disease freedom studies as the specificity reaches 98% according to the manufacturer.

Eyelid tuberculin skin test (TST). The intradermal tuberculin test is the delayed hypersensitivity assay recommended for the detection of bovine tuberculosis by the World Organisation for Animal Health (34). In cattle and American bison (*Bison bison*), a purified protein derivative (PPD) of bovine tuberculin is applied intradermally into the neck or caudal skin fold. In European bison, the site used most commonly is the upper eyelid (10) due to the thickness, pigmentation and hair cover of the skin of this species (19). Additionally, it facilitates assessment of the reaction to the tuberculin injection without a second immobilisation of the wild animal. The procedure used resembles the one used in primates (31). The animal was pharmacologically immobilised as described previously (20). An injection of 2,800 IU of PPD of bovine (strain AN 5) (Bovitubal 28000; Bioveta, Ivanovice na Hané, Czech Republic) and avian tuberculin (Avitubal 28000; Bioveta) was given into the skin fold of the left and right upper eyelid, respectively. Prior to injection, the site was cleaned, the fold was measured with a calliper with an accuracy of 0.1 mm and the result was recorded. Then, 0.1 mL of the preparation was administered intradermally using a short sterile needle and a graduated syringe. Correct administration of the preparation led to the formation of a small palpable spherical bulge. The reaction was evaluated 72 (± 4) h after injection on the basis of thickening and oedema of the upper lid. The TST result was considered positive if oedema of the eyelid (also the tissue around the orbit) and/or seropurulent discharge from the conjunctival sac of the left eye were observed. Oedema or eye discharge observed in the right eye, where avian tuberculin was injected, was considered an inconclusive result. The reading was performed by binoculars (20×) from a maximum distance of 30 m from European bison. Where possible, photographic documentation was prepared.

Interferon gamma release assay (IGRA). The interferon gamma release assay (IGRA) was conducted with the use of a commercial Bovigam TB Kit (Prionics, Schlieren, Switzerland) in accordance with the manufacturer's instructions. Briefly, blood samples were collected into 6 mL lithium heparin tubes and transported to the laboratory at room temperature within 24 h. The blood sample was divided into three aliquots of 1.5 mL each, to which the appropriate antigen diluted in Roswell Park Memorial Institute 1640 medium (Sigma-Aldrich, St. Louis, MO, USA) was added. T-lymphocytes were either stimulated with bovine PPD or avian PPD or underwent addition of phosphate-buffered saline as a negative control antigen. After incubation at 37°C for 24 h, 500 µL of plasma was collected from each tube and the concentration of interferon gamma (INF-γ) was determined using the Bovigam test read with an EPOCH spectrophotometer (BioTek Instruments, Winooski, VT, USA). The test result was determined by comparing the INF-γ levels in the three plasma samples.

Statistical analysis. The sample size for the study was calculated as the required sample size and cut-point for testing to demonstrate population freedom from disease using imperfect tests in the Epitools application (2). The mean population size was determined from data in the European Bison Pedigree Books (2013–2019). European bison were categorised as calves (<1 year old), juveniles (2–3 years old) or adults (≥4 years) as described by Krasińska and Krasiński (19). The univariate associations between SBV seropositivity and exposure variables (origin, age group, sex, population type and sanitary status) were estimated using the χ^2 test. The analyses were performed using STATA v.13.0 software (StataCorp, College Station, TX, USA). A P value of ≤0.05 was considered significant in all the performed analyses.

The evaluation of ELISA was performed using MedCalc Version 19.2.0 (MedCalc, Ostend, Belgium). Sensitivity, specificity, positive and negative predictive values, sample size for receiver operating characteristic (ROC) curve and the area under the ROC curve (AUC) were determined.

Results

Serosurvey. *Mycobacterium bovis* antibodies were detected in 9 serum samples among the total of 460 (435 included in the epidemiological study and a further 25 included in the ELISA evaluation) (Table 1). The S/P values for the positive samples (S/P ≥ 0.3) ranged from 0.477 to 11.671; their distribution is presented in Fig. 2. Antibodies were found in two

European bison from the Białowieża Forest (1.2% of the population), one from the Borecka Forest (2.4%) and one from the Warsaw Zoo (14.3%). One of the European bison (animal D) among the 14 sampled (7.1%) from Smardzewice was positive on five occasions. Other samples from Smardzewice and the Bieszczady Mountains, where tuberculosis was reported previously, were negative in the test used. The locations of populations are shown in Fig. 1. The bison which yielded the 9 *Mycobacterium bovis* ELISA-positive samples among the 436 serum samples collected from the 15 different Polish populations are detailed in Table 1.

No significant associations were found in the univariate analysis between *M. bovis* seroprevalence and the year of sampling, origin, population type, sanitary status, sex or age group variables (Table 2).

Evaluation of ELISA for the detection of European bison exposed to *Mycobacterium tuberculosis*. The results of ELISA tests on 38 samples collected from European bison from the Smardzewice centre tested by ELISA were compared with the corresponding results of TST and IGRA tests (Table 3). Doubtful or inconclusive results of the latter two were excluded from the analysis. Since the TST and IGRA results were not in agreement (kappa = 0.167; 95% confidence interval: −0.315 to 0.649), they were also combined and compared with those of the ELISA. In the tested pool of 38 samples, the results of the TST were available for 28, 3 were inconclusive, and only 4 animals were positive. Nine samples out of 22 for which results of IGRA were available were positive. However, only one animal was positive in both tests at the same time.

Table 1. List of *Mycobacterium bovis* ELISA-positive samples among 436 serum samples collected from European bison from 15 different Polish populations

No.	Year of sampling	Date of sampling (dd/mm)	Animal	Year of birth	Age	Sex	Sanitary status	Origin	Population type	S/P value
1	2019	21/02	B	2018	7 mo	XY	culled	Białowieża Forest	free-ranging	2.311
2	2013	21/11	A	2001	12 yrs	XY	immobilised	Białowieża Forest	captive	0.477
3	2018	24/10	E	2013	5 yrs	XX	immobilised	Białowieża Forest	captive	0.530
4	2019	23/02	C	2012	7 yrs	XY	immobilised	Borecka Forest	free-ranging	1.068
5	2018	11/12	D	2008	10 yrs	XX	culled	Smardzewice	captive	11.671
6	2016	01/02	D	2008	8 yrs	XX	immobilised	Smardzewice	captive	1.240
7	2016	14/11	D	2008	8 yrs	XX	immobilised	Smardzewice	captive	3.891
8	2017	18/09	D	2008	9 yrs	XX	immobilised	Smardzewice	captive	4.137
9	2018	22/05	D	2008	10 yrs	XX	immobilised	Smardzewice	captive	4.365

S/P – sample-to-positive

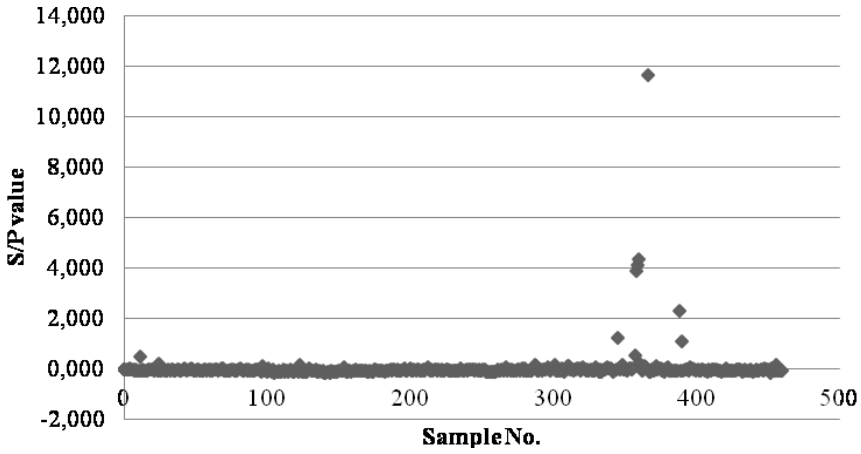

Fig. 2. Distribution of sample-to-positive (S/P) values ((O.D. value (optical density at 450 nm) of the test sample − mean O.D. value for negative controls)/(mean O.D. value for positive controls − mean O.D. value for negative controls)) of individual samples

Positive ELISA results corresponded to positive TST and IGRA results in two cases each. Five positive ELISA results were obtained for animals which were positive in TST or in IGRA (TST+IGRA), while no antibodies were found by the ELISA in the remaining eight TST+IGRA-positive animals. While the specificity of the ELISA against TST, IGRA and TST+IGRA was high, the sensitivity ranged between 22.2% and 50%. However, the low number of positive samples in both reference tests should be considered when evaluating. Therefore, even though the AUC values of ELISA against IGRA and TST+IGRA were acceptable, the optimal cut-off values designated by Youden's J statistics were unacceptably low. The ROC curve and detailed sensitivities and specificities for each cut-off point of ELISA against TST+IGRA are presented in Table 4 and Fig. 3.

Table 2. Descriptive statistics of 436 European bison tested for the presence of *Mycobacterium bovis* antibodies with respect to the year of sampling, origin, population type, sanitary status, sex and age group. Statistically significant differences were determined using Pearson's χ^2 test. A P value of ≤0.05 was considered significant

	Variable				
	n/*N*[a]	%	95% CI[b]	χ^2	P
Year				9.50	0.22
2013	1/13	7.7	0.2–36.0		
2014	0/47	0	0–7.5[c]		
2015	0/63	0	0–5.7		
2016	0/43	0	0–8.2		
2017	1/81	1.2	0.03–6.7		
2018	1/111	0.9	0.02–4.9		
2019	2/60	3.33	0.4–11.5		
2020	0/18	0	0–18.5		
Origin				18.08	0.26
Bałtów	0/7	0	0–41.0		
Białowieża Forest	3/173	1.7	0.4–5.0		
Bieszczady Mountains	0/38	0	0–9.2		
Gołuchów	0/11	0	0–28.5		
Kiermusy	0/5	0	0–52.2		
Międzyzdroje	0/5	0	0–52.2		
Niepołomice	0/22	0	0–15.4		
Pszczyna	0/45	0	0–7.9		
Borecka Forest	1/42	2.3	0.06–12.6		
Knyszyńska Forest	0/62	0	0–5.8		
Smardzewice	1/14	7.1	0.2–33.9		
Ustroń	0/2	0	0–84.2		
Bydgoszcz Zoo	0/1	0	0–97.5		
Łódź Zoo	0/3	0	0–70.7		
Warsaw Zoo	0/6	0	0–45.9		
Population type				0.11	0.736
free-ranging	2/207	0.9	0.1–3.4		
captive	3/229	1.3	0.3–3.7		
Sanitary status				0.45	0.93
potentially healthy (immobilised)	3/269	1.1	0.1–3.2		
eliminated	2/138	1.4	0.2–5.1		
fallen	0/20	0	0–16.8		
killed in accidents	0/9	0	0–33.6		
Sex				0.49	0.48
XX	2/242	0.8	0.1–2.9		
XY	3/194	1.5	0.3–4.4		
Age group				3.18	0.20
calves (≤1 year)	1/66	1.5	0.03–8.1		
juveniles (2–3 years)	0/161	0	0–2.2		
adults (≥4 years)	4/199	2.0	0.5–5.1		
no data	0/10	0	0–30.8		

[a] – number of ELISA seropositives/numer of all samples tested; [b] – confidence interval; [c] – one-sided 97.5% confidence interval

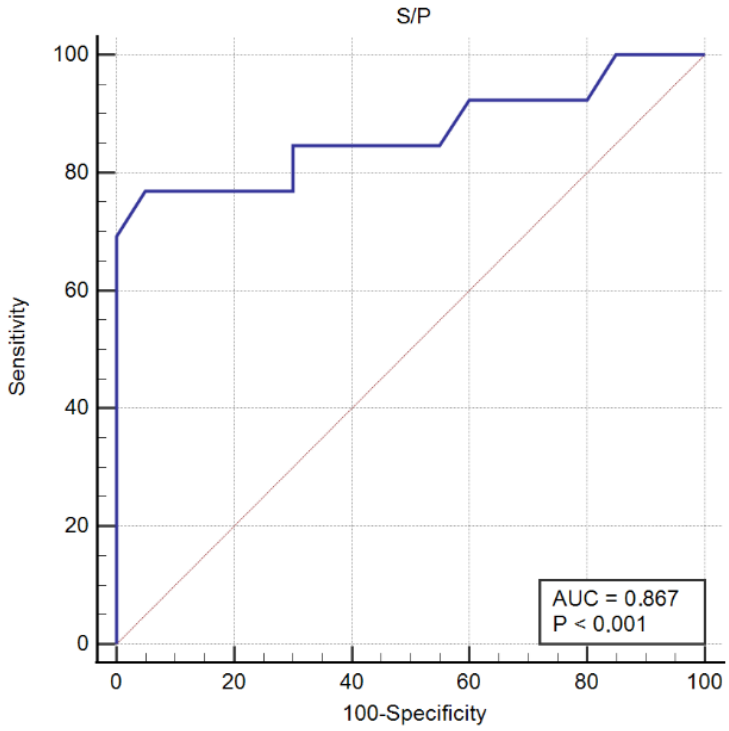

Fig. 3. Receiver operating characteristic curve of *M. bovis* antibody ELISA against a combined tuberculin skin test and interferon gamma release assay (TST+IGRA)

Table 3. Comparison of an antibody ELISA and tuberculin skin test (TST), interferon gamma release assay (IGRA), and TST and IGRA combined in identification of tuberculosis-affected European bison

Antibody ELISA *versus*	TST (n^a = 25; 4/21[b])	IGRA (n = 31; 9/22[b])	TST+IGRA (n^a = 33; 13/20[b])
	Positive/negative results		
Sensitivity (%)	50.00	22.22	38.46
95% CI[c]	6.16–93.24	2.81–60.01	13.86–68.42
Specificity (%)	95.24	90.91	100
95% CI	76.18–99.88	70.84–98.88	83.16–100
Positive predictive value (%)	9.59	2.41	100
95% CI	1.22–47.64	0.41–12.10	–
Negative predictive value (%)	99.47	99.14	99.38
95% CI	98.60–99.80	98.76–99.41	99.05–99.60
Accuracy	94.76	90.22	99.38
95% CI	77.86–99.72	74.12–97.91	88.27–100
Kappa	0.503	0.157	0.431
95% CI	0.019–0.987	−0.191–0.506	0.148–0.713
	S/P values		
Area under ROC curve	0.619	0.848	0.867
Standard error[d]	0.205	0.077	0.0773
95% CI	0.405–0.804	0.674–0.951	0.704–0.960
Significance level P	0.562	<0.0001	<0.0001
Youden's J statistics[e]	0.452	0.641	0.719
Optimum cut-off point	>0.002	>−0.02	>−0.016
Sensitivity	50.00	77.78	76.92
Specificity	95.24	86.36	95.00

[a] – sample size; [b] – positive/negative sample ratio; [c] – binominal exact; [d] – according to DeLong *et al.* (6); [e] – according to Schisterman *et al*. (35); CI – confidence interval

Table 4. Detailed sensitivity and specificity for each cut-off value of *M. bovis* antibody ELISA against a combined tuberculin skin test and interferon -gamma release assay (TST+IGRA)

Cut-off	Sensitivity (%)	Specificity (%)	Correctly classified (%)	LR+	LR−
≥0.124	100.00	0.00	39.39	1.0000	
≥0.120	100.00	5.00	42.42	1.0526	0.0000
≥0.111	100.00	10.00	45.45	1.1111	0.0000
≥0.103	100.00	15.00	48.48	1.1765	0.0000
≥0.080	92.31	20.00	48.48	1.1538	0.3846
≥0.079	92.31	30.00	54.55	1.3187	0.2564
≥0.075	92.31	40.00	60.61	1.5385	0.1923
≥0.075	84.62	40.00	57.58	1.4103	0.3846
≥0.074	84.62	45.00	60.61	1.5385	0.3419
≥0.068	84.62	50.00	63.64	1.6923	0.3077
≥0.066	84.62	55.00	66.67	1.8803	0.2797
≥0.060	84.62	60.00	69.70	2.1154	0.2564
≥0.050	84.62	65.00	72.73	2.4176	0.2367
≥0.039	84.62	70.00	75.76	2.8205	0.2198
≥0.033	76.92	70.00	72.73	2.5641	0.3297
≥0.030	76.92	75.00	75.76	3.0769	0.3077
≥0.027	76.92	80.00	78.79	3.8462	0.2885
≥0.019	76.92	85.00	81.82	5.1282	0.2715
≥0.016	76.92	90.00	84.85	7.6923	0.2564
≥0.012	76.92	95.00	87.88	15.3846	0.2429
≥0.012	69.23	95.00	84.85	13.8462	0.3239
≥0.002	69.23	100.00	87.88		0.3077
≥0.099	61.54	100.00	84.85		0.3846
≥0.125	53.85	100.00	81.82		0.4615
≥0.165	46.15	100.00	78.79		0.5385
≥1.240	38.46	100.00	75.76		0.6154
≥3.890	30.77	100.00	72.73		0.6923
≥4.137	23.08	100.00	69.70		0.7692
≥4.364	15.38	100.00	66.67		0.8462
≥11.670	7.69	100.00	63.64		0.9231
>11.670	0.00	100.00	60.61		1.0000

Discussion

The present study is the first attempt to perform a cross-sectional study of BTB in European bison in Poland. The epidemiological study was conducted by an assessment of a serological test allowing large-scale testing as an alternative to traditional *in vivo* cell-based BTB tests. Although these tests (TST and IGRA) can detect animals in the early stages of infection, they are not fit for purpose for BTB monitoring especially in wildlife and still fail to detect up to 20% of truly infected animals. In European bison, this sensitivity is even lower (7). The use of serological tests in tuberculosis control programmes may significantly improve the detection of infected herds. As demonstrated by the results, the TST is less effective in detecting tuberculosis-affected European bison than the IGRA (7, 10). Additionally, the results of both tests did not overlap. For the evaluation of the antibody ELISA, the results of both tests were combined as reference values for our ELISA results in the selected number of samples. The ELISA sensitivity was very low. This was also reported in earlier studies in which a multi-antigen print immunoassay (MAPIA) and dual-path platform (DPP) test showed a higher number of correctly detected positive European bison than the IDEXX *M. bovis* ELISA (7). It has been shown that testing the presence of antibodies with the IDEXX *M. bovis*

ELISA has a limited sensitivity of 50–65% with a high specificity of 98% in bovine serum (3, 12, 38). Furthermore, after testing 2,661 bovine sera, Koni *et al.* (13) showed that the ELISA test is not an effective tool for monitoring tuberculosis in cattle. The number of positive animals detected by the ELISA test was low (13). They suggested that the poor sensitivity of the test may be due to the animals not having previously been sensitised by TST. For more accurate results, the authors proposed sensitising the cattle by exposure to tuberculin *via* injection two weeks prior to blood sampling for serological tests (13, 30), which should increase the cut-off value and thus the specificity of the ELISA test (13).

African buffaloes were another serologically tested bovid species (4, 24, 36). The endemic presence of tuberculosis in African buffaloes in South Africa has serious consequences for the eradication and control of BTB in dairy cattle, buffaloes and wildlife conservation (36). Van der Heijden *et al.* (36) tested nearly 1,000 of the animals with the TST and TB ELISA test, and the overall performance of the TB ELISA test in the buffaloes was shown to be poor in this study. Interesting results were also obtained by Mhongovoyo (24), who examined 60 animals from the African buffalo population in Botswana recognised as BTB-free. All the animals tested were positive in the ELISA test and negative in the Bovigam assay (IGRA). It was concluded that the TB ELISA results were false positive, showing the extremely low specificity of the assay (24). The reason for these results is unknown. It may be partially attributed to cross-reaction with antibodies to nontuberculous mycobacterial antigens (24). Other non-specific reactions affecting the TB ELISA test results may also be due to the combination of drugs used to immobilise these buffaloes, other health issues, and inappropriate collection and storage conditions (36). It has recently been shown that the reactivity of the anti-bovine conjugate of the TB ELISA assay is significantly reduced in African buffalo serum compared to bovine serum (4). Consequently, Cloete (4) believes that tests should be developed using a species-specific conjugate or a broadly cross-reactive conjugate such as A/G protein.

In our opinion, by supplementing the IGRA results with the IDEXX Ab *M. bovis* ELISA results, a sensitivity of up to 95% can be achieved. For the purposes of monitoring BTB-free populations, ELISA testing may be an effective, easy and cost-efficient tool. Additionally, it should be remembered that a representative number of samples should be used for epidemiological studies. Perhaps verification of the cut-off value would increase the diagnostic sensitivity of the test without increasing the number of false positives (12). In this research, the Youden statistics failed to compute productive adjusted cut-off values. Further studies using a larger sample size would possibly allow cut-off value verification, however, the deficient availability of such material from European bison limits such estimations at the present time. In summary, an antibody ELISA may be used as a supplement to TST and IGRA but cannot replace it (13, 22). However, TST and IGRA have their technical restrictions, especially when used in wildlife species. The performance of TST is limited by the technical experience of the veterinarian and the test requires reading of its results on an occasion subsequent to the initial injection, during which the animals need to be maintained in the field of view or captured and held in captivity, which is not possible in free-living populations. The drawback of the IGRA is that to be valid, it requires timely delivery of samples in controlled conditions. By combining TST with ELISA in diagnostic practice, sensitivity of 83% in detection of cattle infected with *M. bovis* can be achieved (3).

The most important observation is the detection of antibodies in European bison in the Białowieża and Borecka forests (Table 1, nos 1–4), which proves that these populations could have been exposed to mycobacteria. The origin of these animals was fully documented. All the animals were asymptomatic and had not been subjected to tuberculosis testing before. So far, no cases of the disease have been reported in the Białowieża Forest. The 12-year-old bull identified with the letter A was brought to the Białowieża National Park Show Reserve from Smardzewice European Bison Breeding Centre in 2005, eight years before the first case of BTB was diagnosed in Smardzewice (16). The bull showed reduced reproductive fitness and was treated for respiratory illness in 2013 when the samples were collected. After improvement in its health, its state worsened and it was treated again. The animal died in 2015 with signs of cachexia and respiratory failure. Diffuse peritonitis, generalised interstitial pneumonia, nephritis, inflammatory foci in the liver, an enlarged gallbladder and a rumen filled with liquid were observed at the necropsy, while no tuberculous changes were found and no microbiological testing was performed. Another Białowieża case revealed in this study was more unexpected as it concerned a 7-month-old male calf born in the free-ranging population of the Białowieża Forest, euthanised due to an injury it suffered from a falling tree. The animal was in good condition, weighing 105 kg, and the only lesions observed at the necropsy involved those caused by the trauma (fracture of the left scapula, extensive haematoma, fractures of the ribs with displacement into the peritoneal cavity, and pneumothorax). Another case concerned a 7-year-old free-ranging European bison cow (animal C) from the Borecka Forest from which a sample was collected while the animal was immobilised in order to be fitted with a telemetric collar. At the time of the manuscript's preparation, the animal was still alive and showing no symptoms of tuberculosis. Further studies are recommended. However, tuberculosis was previously diagnosed in the Borecka Forest in 2016 (8). Again, one of the affected European bison had been translocated from the

Smardzewice population. Further testing of 22 European bison from the forest in 2017 by IGRA and a microbiological isolation test gave negative results (8). The last case of an animal not suspected of having tuberculosis but transpiring to be seropositive was a female European bison from Warsaw Zoo immobilised during a training course in wildlife anaesthesia for veterinarians in 2018.

The study shows that the primary factor in the transmission of tuberculosis in European bison is anthropogenic and associated with the translocation of subclinically infected individuals between herds, and indicates the sources of the disease to be the enclosures of zoological gardens and wildlife reserves. To minimise the risk of mycobacterium transmission, testing all European bison before transportation should be implemented, no exceptions being made for domestic translocations, despite no such obligation being mandated by law. The appearance of tuberculosis in the Smardzewice centre was the result of the introduction of a European bison from the Chorzów Zoo. The problem of tuberculosis in zoological gardens is significant. Zoo animals tend to live to a very long age, which exposes them to various pathogens from other animal species and humans for a longer time than their free-living counterparts. Moreover, mycobacteria are very resistant to environmental conditions, and the animals are kept in relatively small enclosures with darkened resting shelters, which are two factors which favour the survival of mycobacteria. It should be noted that the problem of tuberculosis transmission from cattle seems rather marginal, since only very few cases of the disease are reported in ruminants in some areas where European bison are reared and no cases at all are reported in the other areas. However, the rapidly growing population size and density extends the European bison habitat over farmland, which leads to more frequent inter-species contacts and possible spill-over or spill-back transmission events. Additionally, the transmission of tuberculosis between European bison and wild boar and carnivores in the Bieszczady Mountains has already been reported (15, 17, 26). Although most human cases of tuberculosis are caused by *Mycobacterium tuberculosis*, human *M. bovis* infections are also reported every year and the real number is considered to be underestimated. The European Food Safety Authority and the European Centre for Disease Prevention and Control report published in 2021 provides information on human infections caused by bovine bacilli (*M. bovis* and *M. caprae*) (28). As in previous years, the greatest number of cases was identified in three countries: Germany (48 people), Great Britain (35 people) and Spain (32 people). Twenty-two people died of *M. bovis* or *M. caprae* infection. In Poland, two cases of tuberculosis in humans due to *M. bovis* have been described to date in patients from the southern region of Poland (40). The first Polish case of *M. caprae* infection in a human was reported three years after the country's attainment of tuberculosis-free status in cattle (14). This shows that the monitoring of BTB in European bison is also an element of public health protection, in particular the health of breeders and veterinarians who have contact with those animals and the health of visitors to zoos and enclosed breeding centres.

Therefore, it is important to continue research giving attention to the determination of the source of infection and the route of transmission. The research should also include other ruminants, wild boar and predators from the Białowieża and Borecka forests as the main reservoirs of tuberculosis in the sylvatic environment. Further optimisation of the ELISA and evaluation of other available serological tests in order to increase the sensitivity of detecting European bison infected with *Mycobacterium* would also be valuable (3). Interestingly, successful attempts at ante-mortem isolation of mycobacteria or their genetic material were carried out in European bison (11). This might be a helpful diagnostic tool in the case of extremely valuable or tuberculosis-suspected individuals but is not suitable for epidemiological or retrospective studies, for which serological methods seem to be the easiest to perform. It should be noted that false-positive results are also possible in the case of infections with atypical mycobacteria. However, initial serological studies of antibody response in European bison have shown that infection with atypical mycobacteria did not lead to other serological tests such as MAPIA or DPP giving positive results (7).

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