Praca oryginalna *Original paper*

Seroprevalence of *Brucella* spp. in sheep (*Ovis aries*) in Małopolskie Voivodeship, southern Poland

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Summary

Brucellosis is a major public health threat worldwide. The disease is caused by bacteria belonging to the Brucella genus and is associated with reproductive disorders. Poland is classified as an area free from infection caused by *B. abortus, B. melitensis* and *B. suis* in cattle populations across the entire territory. Little is known about the occurrence of brucellosis in other domestic and wild animal species. The aim of the study was to assess the seroprevalence of *Brucella* sp. in sheep from Małopolskie Voivodeship. In 2021-2024, serum samples were collected from 241 sheep from four herds. For serological tests, the commercial ELISA PrioCHECK™ (Thermo Fisher Scientific, Waltham, MA, USA) and Rose Bengal Tests (ID.VET, Grabels, France) were used. Antibodies against *Brucella* sp. were detected in 41/241 (17%; 95% CI: 12.6-22.36) of sheep; Rose Bengal-positive reaction was observed in only 7/241 (2.9%; 95% CI: 1.38-5.94). Controlling brucellosis in sheep is highly important not only because of the potential economic losses to farmers and occupational risks, but also because of the consumption of unprocessed dairy products. More detailed studies should be performed to identify the epidemiological situation in Małopolskie Voivodeship.

Keywords: *Brucella* spp., zoonoses, public health

Brucellosis is an endemic zoonosis considered a major public health threat worldwide (7, 11, 15). Brucellosis is caused by bacteria of the genus *Brucella*. In animals, it causes mainly abortion and infertility (1). There are 12 species within the genus recognized for their host specificity (1, 17). *Brucella abortus* is responsible for brucellosis in cattle; *Brucella suis* in pigs; *Brucella ovis*, *Brucella melitensis* in sheep and goats (3, 11). Additionally, *B. abortus* and *B. suis* have been isolated from various wildlife species, including moose, deer, ferrets, rabbits, wolves, and others (12, 16, 19, 22).

B. melitensis causes mainly abortion and stillbirths (14) and is the most virulent zoonotic species (6). In contrast, *Brucella ovis* is non-pathogenic for humans, and the main clinical sign is epididymitis in rams (14). In sheep and goats, brucellosis is mainly caused by *B. melitensis*; however, other *Brucella* species can also infect small ruminants (8, 18, 24). It should be noted, however, that in Poland, *B. ovis* infections occurred in sheep, but *B. melitensis* infections did not occur.

In accordance with Commission Implementing Regulation (EU) 2021/620, Poland is classified as an area free of infection caused by *B. abortus*, *B. melitensis*, and *B. suis* in cattle populations across the entire territory. This is because 99.8% of all cattle herds have been officially declared bovine brucellosis-free in the last 5 years since obtaining the status. In Poland, there has been no case of *B. abortus* being reported since its eradication in 1980. However, cases of infections

by other *Brucella* species have been reported. In cattle with positive serological results, *B. suis* biovar 2 has been reported (20). The same biovar has been confirmed in Polish wildlife (21). Recently, anti-*Brucella* spp. antibodies were detected in 36% of tested European bison (*Bison bonasus*) (9). Considering the existence of common pastures for sheep, the excessive movement of the herds due to summer-winter migration, and possible wildlife-livestock disease transmission, it is highly important to control the prevalence of *Brucella* sp. in Polish sheep populations. Moreover, a rising problem in Europe is canine brucellosis (10, 23). Even though *Brucella canis* has not been confirmed in sheep, they have close contact with shepherd dogs, so transmission cannot be excluded.

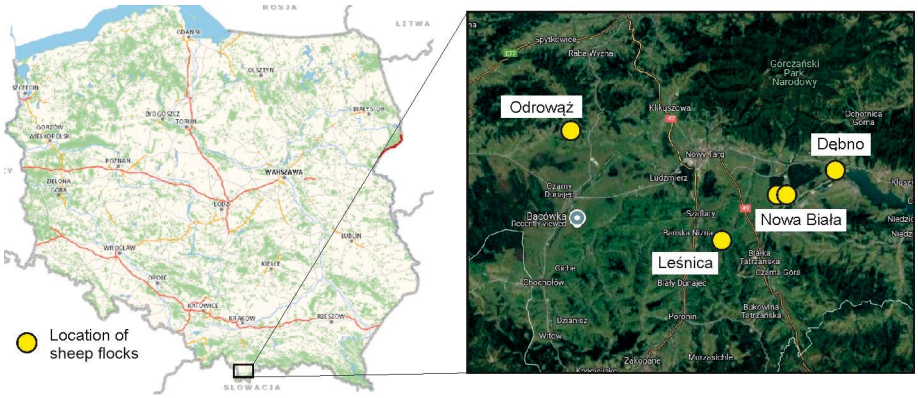

Fig. 1. Locations of the sheep herds tested for antibodies against *Brucella* sp. (ELISA and Rose Bengal Tests)

Given the above and the limited data available, this study aimed to determine the prevalence of antibodies against *Brucella* spp. in sheep from four flocks in the Małopolskie Voivodeship, where more than 1/3 of the Polish sheep population resides.

Material and methods

In 2021-2024, blood samples were collected from 241 sheep of the Polish mountain sheep breed, aged 1 to 10 years (mean age: 3.8) from four herds in Brzesko and Gorlice districts in the Małopolskie Voivodeship (Fig. 1).

Blood was collected from the jugular vein during standard veterinary procedures. The material was not specifically obtained for this study. Therefore, the procedure did not require ethics committee approval. Blood was collected into tubes with a clot activator, transported to the laboratory, and centrifuged. The obtained serum samples were stored at –20°C. Before testing, serum samples were brought to room temperature.

Serological examination

Enzyme-linked immunosorbent assay analysis (ELISA). Serum samples obtained from 241 sheep were tested with the PrioCHECK™ Brucella Ab 2.0 Strip Kit (Thermofisher Scientific, Waltham, MA, USA). The test is based on purified extract of the LPS of *Brucella* and dedicated for the detection of antibodies against *B. abortus* in serum and milk samples from cattle and *B. melitensis* in serum samples from sheep and goats. The test was performed according to the manufacturer's recommendations. The optical density (OD) was measured with an ELISA plate reader (Epoch, BioTek, USA) at 450 nm. Percent positivity (PP) of all the samples was calculated using the formula: PP = (OD450 test sample/Mean OD450 Positive Control) × 100. The result was considered reliable if the control sera met the following criteria: mean OD450 of the Negative Control (NC) < 0.2 and mean OD450 of the Positive Control (PC) ≥ 1.000. Samples with PP lower than 25% were considered negative. Samples with PP > 25% were considered positive.

Rapid slide agglutination with Rose Bengal. 241 serum samples were tested by RSA (rapid slide agglutination) with Rose Bengal (ID. VET, Grabels, France). This assay detects antibodies against *Brucella abortus*, *Brucella melitensis*, or *Brucella suis* in bovine, ovine, caprine, and porcine species. The test was performed according to the manufacturer's instructions.

Statistical analysis. The results obtained by both methods were subjected to statistical analysis. Results were analyzed using basic descriptive statistics with the use of the Wilson score to calculate the 95% confidence interval (CI) (https://www.statskingdom.com/proportionconfidence-interval-calculator.html).

We performed a statistical analysis of antibody prevalence against *Brucella* spp. in sheep using generalized linear binary models in IBM SPSS Statistics. We applied two separate models. The first model explained antibody prevalence (ELISA test results for serum samples) as a function of animal age, sex, and herd location. We used a comparison of all model variants to obtain the best-fitting model (4). We used the Akaike Information Criterion (AIC) to select the highest-ranking model (best-fitting model, i.e., the one with the lowest AIC value). The second model assessed the association between the prevalence of antibodies against *Brucella* spp. in sheep (ELISA test results on serum samples) and Rose Bengal test results. Due to the presence of only one explanatory variable, model selection was not applied.

Results and discussion

Antibodies against *Brucella* spp. were detected by ELISA in 41/241 (17%; 95% CI: 12.6-22.36) of sheep, while by RSA with Rose Bengal in only 7/241 (2.9%; 95% CI: 1.38-5.94) of sheep.

Neither sex nor age of animals influenced the prevalence of antibodies against *Brucella* spp. in sheep. Both variables were excluded during model selection. The only variable influencing antibody prevalence in sheep was herd location (Tab. 1). The Odrowąż herd had the lowest antibody prevalence and differed from all others except the Leśnica herd. The herd in Leśnica had a slightly higher antibody prevalence, but it differed significantly only from the herd Nowa Biała 1 (Fig. 2).

The prevalence of antibodies against *Brucella s*pp. in sheep, as determined by ELISA, was also associated

Tab. 1. Effects of flock location (Flock) on the occurrence of antibodies against *Brucella* sp. in sheep, sex and age of animals were excluded during model selection (model statistics: Chi-square = 24.476, p < 0.001)

Source	Beta	SE	Chi-square	p
Intercept	–2.587	0.392	43.558	< 0.001
Flock (Leśnica)	0.795	0.487	2.667	0.102
Flock (Nowa Biała 1)	1.968	0.611	10.369	0.001
Flock (Nowa Biała 2)	2.453	0.649	14.279	< 0.001
Flock (Dębno)	2.181	0.657	11.029	< 0.001
Flock (Odrowąż)	0*			

Tab. 2. Relation of Rose Bengal test results with antibodies against *Brucella* sp. in sheep (model statistics: Chi-square = 5.971, p = 0.015)

Source	Beta	SE	Chi-square	p
Intercept	–1.672	0.179	87.111	< 0.001
Rose Bengal (positive)	1.960	0.785	6.242	0.012
Rose Bengal (negative)	0*			

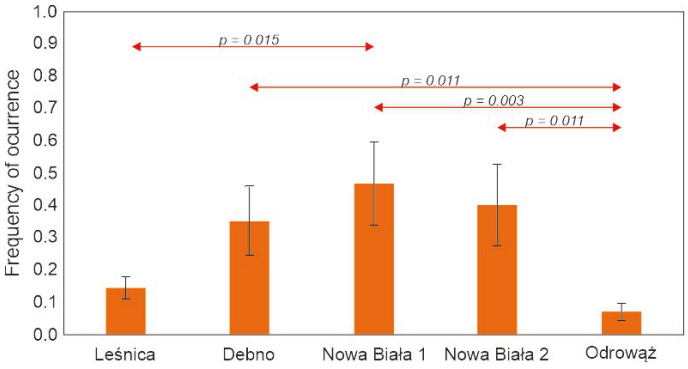

Fig. 2. Frequency of occurrence (marginal means) of antibodies against *Brucella* spp. in sheep in various sheep herds (positive vs. all tested animals in given herd) and pairwise comparison in generalized linear binary model (red arrows with p-value indicate significant differences between herds)

with the RSA with the Rose Bengal results. Positive RSA Rose Bengal results coincided more frequently with ELISA results than negative RSA Rose Bengal results (Tab. 2).

Antibodies against *Brucella* spp. in our studies have been found in 2.9% to 17.0% of examined sheep sera, depending on the test used (RSA or ELISA respectively). According to data from the General Veterinary Inspectorate, positive results also sometimes occur in annual serological monitoring of sheep, suggesting a suspicion of the disease; however, reference laboratories have not confirmed brucellosis in sheep or goats in recent years. As false-positive results are common in *Brucella* testing due to difficulties with serological diagnosis and cross-reactions with other bacterial species (2), our results should also be interpreted with caution. This is especially true given the high sensitivity and low specificity of RSA with Rose Bengal. This means that an animal deemed negative based on RSA with Rose Bengal is very likely free of infection. However, the probability that animals deemed positive are infected is low due to the low specificity of this test. Positive RSA with Rose Bengal results should be confirmed by other tests with high specificity and low sensitivity, including ELISA. It should be noted that even if individual animals tested positive for both tests (ELISA and RSA with Rose Bengal), the probability that they are infected is also low, given the low positive predictive value of infection, which depends not only on the sensitivity and specificity of the test used but also, to a large extent, on the prevalence of infection in the population. If the disease occurs very rarely in the population (prevalence close to zero), then the positive predictive value is also very low, also close to 0. This is precisely the situation that was encountered in the course of the study. Perhaps more detailed studies should be performed to identify the epidemiological situation in Małopolskie Voivodeship; however, it is generally considered a *B. melitensis* – free area.

Although antibodies against *Brucella* spp. were detected in 17% of samples by ELISA, only 2.9% showed a positive Rose Bengal test reaction. Similar results were reported by Rossetti et al. (18), who observed a common false-negative reaction to the Rose Bengal test, likely due to the examination of sera with high antibody titers. If there were strong suspicions of brucellosis based on clinical symptoms, which we did not encounter in these studies, this could be due to the Prozone effect, characterized by excessive antibody binding to the antigen, forming tiny complexes that do not agglutinate visibly, leading to false-negative results despite the high antibody titer.

Antibody detection tests remain the most cost-effective approach for screening the sheep population for *B. melitensis*-infected herds (18). In our study, we used multiple serological tests, as it is strongly recommended to use at least two different serological techniques to evaluate *Brucella* status in herds (25). Our goal was to screen the population, so we used indirect ELISA and the Rose Bengal test. However, in the future, it would be recommended to also use fluorescence-polarized antigen or complement fixation as complementary or confirmatory tests, respectively. As Poland is generally considered *B. melitensis*-free area, we would recommend performing a Brucelin skin test to exclude cross-reactions with other bacteria (like *Yersinia enterocolica* O:9, *Escherichia coli* O157 or *Salmonella urbana*) (2, 5, 13).

Controlling brucellosis in sheep is highly important not only because of the potential economic losses to farmers and occupational risks, but also because of the consumption of unprocessed dairy products (15). In Małopolskie Voivodeship, traditional sheep cheeses (oscypek, bundz) are not pasteurized and can be a source of *Brucella* infection in humans. Brucellosis in humans

is a serious disease characterized by undulating fever, weight loss, hepatomegaly, and splenomegaly (11).

Even though the epidemiological situation in Poland shows that *B. melitensis* is unlikely to occur in sheep flocks, our results should not be disregarded, and further research should be undertaken. Serological testing for brucellosis in animals should be based on multiple test results. Using a single test will not allow for accurate interpretation of the results.

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