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# SEROLOGICAL EVIDENCE OF *BORRELIA BURGDORFERI* SENSU LATO IN HORSES AND CATTLE FROM POLAND AND DIAGNOSTIC PROBLEMS OF LYME BORRELIOSIS

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**Abstract:** In the course of epizootological research on Lyme borreliosis in animals, the serological evidence of this zoonosis in horses and cattle from different voivodships of Poland was screened. We also discussed some diagnostic problems of Lyme borreliosis resulting from, in addition to other factors, genetic and geographical heterogeneity isolates *B. burgdorferi* s.l. used as antigens. Using ELISA from 395 sera of horses the total mean seroprevalence for anti-*Borrelia* IgG antibodies 25.6% was observed. In the respective years, significant differences in the mean seroprevalence were not recorded. In the voivodships, the total mean seroprevalence and mean seroprevalence for the respective years varied from 16.6–66.6%. An analysis of seroprevalence depending on the age showed a significant difference between 0–2 year-old horses compared to older horses. The total seroprevalence in the set of 98 serum samples was lower with the strain of *B. garinii* (25.5%) compared to a mixture of *B. burgdorferi* sensu stricto with *B. afzelii* (36.7%) and *B. afzelii* (42.8%). The highest correlation of findings was reached comparing the strains of *B. afzelii* (South Poland) and a mixture of *B. burgdorferi* s. s. + *B. afzelii* (East Slovakia). Lower correlation was between *B. garinii* and mixture of *B. burgdorferi* s. s. + *B. afzelii*. On the contrary, the lowest correlation of findings was observed between the Slovak strain of *B. garinii* and Polish *B. afzelii*. In a group of 26 cow sera, the mean seroprevalence for anti-*Borrelia* IgG antibodies was 26.9%. In the remaining clinical signs the seroprevalence was 28.5–66.6%. In Western blot, out of 25 examined sera of horses 15 (60.0%) were positive, out of 6 cows 5 (83.3%) were positive (2 lameness, 2 phlebitis, 1 clinically healthy). The horses and cows sera recognised the proteins: 93 (MEP)-, 83-, 75-, 66-, 55-, 43-, 45-, 41 (flagellin)-, 39-, 34-, 35 (OspB) and 25-, 28 (OspC)-kDa. These results alert veterinarians to take into account the aetiology of Lyme disease in differential diagnoses.

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## INTRODUCTION

Lyme borreliosis is the most common tick-associated disease in the Northern hemisphere. The causative agent *Borrelia burgdorferi* is transmitted by the bites of ticks and can infect human and free-living, domestic and farm animals, including horses and cattle [18, 16, 19, 13, 20, 10, 48, 45]. Parasitism by ticks infected with *Borrelia burgdorferi* is presumably an important route of infection in cattle and horses [30, 32]. The agent of this zoonosis was found in the urine of infected cattle and a urinary-oral mode of transmission has been postulated [7, 33].

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Asymptomatic infection appears to be common in cattle and horses as well as in other animal species. The predominant clinical signs reported in cattle and horses is lameness with or without joint swelling, sometimes accompanied by fever [13, 25, 26, 32]. Less frequently reported clinical signs include laminitis [33], uveitis, abortion, weight loss, and in cattle depressed milk production [8, 9, 12, 30]. With the possible exception of cattle [33], domestic animals do not commonly demonstrate an erythematous skin lesion at the site of tick bite. In cattle, clinical disease occurs as a herd problem with calf heifers most severely affected [30].

Diagnosis is particularly difficult in horses as the anatomy and athletic use of this species predisposes them to a wide variety of musculoskeletal disorders with resulting lameness [25]. The aetiology of the cattle lameness known as digital dermatitis is also very variable [15]. Presumptive diagnosis of Lyme borreliosis therefore requires elimination of other causes of lameness. Serological evidence of Lyme disease in horses and cattle was reported [3, 21, 26, 28, 50]. *Borrelia* infection rate in ticks in Poland proved their important infestation with borreliae [35, 36, 51]. This finding presumed exposure of animals to this zoonosis.

Within the research of Lyme borreliosis in the veterinary field we present the findings of *Borrelia burgdorferi* bases on the serological and immunochemical study in horses and cattle from different districts of Poland.

## MATERIAL AND METHODS

**Animals and sampling.** During 2001–2003, serum samples from 395 horses and 26 samples from cattle were examined for anti-*Borrelia* antibodies. Blood sera were collected from several geographical areas in Poland (Tab. 1, 4). The samples were collected by veterinary practitioners upon request. The following records were kept: date of sampling, county of origin, when available, clinical signs.

**Antigens.** Our own endemic strains of *Borrelia garinii* and *Borrelia burgdorferi* s. s. isolated from ticks *I. ricinus* in the agglomeration of Košice and *B. afzelii* isolated from *Ixodes ricinus* in Poland were used as antigens, prepared by the method of Tresová *et al.* [47]. Predominant of serum samples were examined by the mixture of the strains *B. burgdorferi* s.s. and *B. afzelii*. Comparative study with *B. garinii*, *B. burgdorferi* s.s. and *B. afzelii* in isolation was performed in 98 sera of horses from three voivodships (Kujawsko-Pomorskie, Łódzkie and Mazowieckie). The protein content was assessed by Bradford Protein Assay (Bio-Rad GmbH, Munich, Germany). The working dilution of the antigens was determined by box titration.

**Enzyme-linked immunosorbent assay (ELISA).** The sera were examined by the modified ELISA as described previously [40]. Horse and cattle sera with clinical signs such as lameness and swollen joints and those that had proved positive in repeated titrations, were used as positive controls. Horse sera proved negative in repeated titrations, with their absorbance value less than 0.4, served as negative controls. Sera from calves aged 1 month, which were not pastured or kept outdoors and were negative in repeated titrations, served as negative controls. These control sera were obtained from the University of Veterinary Medicine of Košice and were also confirmed by Western blotting. Cut-off was determined as the value of three standard deviations above the mean optical density (OD) for negative serum samples (horses: no. = 15; cattle: no. = 50),

**Table 1.** Seroprevalence in horses for *B. burgdorferi* in the regions studied.

| Voivodships | 2001 | | 2002 | | Total | |
|---|---|---|---|---|---|---|
| | No. examined | No. positive (%) | No. examined | No. positive (%) | No. examined | No. positive (%) |
| Kujawsko-Pomorskie | 38 | 11 (28.9) | 8 | 2 (25.0) | 46 | 13 (28.3) |
| Łódzkie | 37 | 7 (18.9) | 19 | 6 ( 31.6) | 56 | 13 (23.2) |
| Lubelskie | 7 | 3 (42.8) | — | — | 7 | 3 (42.8) |
| Mazowieckie | 127 | 20 (15.7) | 59 | 19 (32.2) | 186 | 40 (21.5) |
| Małopolskie | 3 | 1 (33.3) | — | — | 3 | 1 (33.3) |
| Opolskie | 6 | 2 (33.3) | — | — | 6 | 2 (33.3) |
| Podlaskie | 11 | 5 (45.1) | 14 | 2 (14.3) | 25 | 7 (28.0) |
| Podkarpackie | 3 | 2 (66.6) | — | — | 3 | 2 (66.6) |
| Pomorskie | 3 | 2 (66.6) | 6 | 2 (33.3) | 9 | 4 (44.4) |
| Świętokrzyskie | 20 | 8 (40.0) | 10 | 1 (10.0) | 30 | 9 (30.0) |
| Warmińsko-Mazurskie | 11 | 4 (36.4) | 2 | 1 (50.0) | 13 | 5 (38.5) |
| Wielkopolskie | 6 | 1 (16.6) | 5 | 1 (20.0) | 11 | 2 (18.2) |
| Total | 272 | 67 (24.6) | 123 | 34 (27.6) | 395 | 101 (25.6) |

which originated from areas where occurrence of ticks is rare. Anti-horse and anti-bovine peroxidase conjugates (Sigma) were used and its working dilution was carried out by box titrations.

**Reproducibility of the ELISA.** A panel of sera with the absorbance values in the ranges > 1.2, 0.6–0.9 and < 0.4 were each assayed several times. Statistical evaluation was carried out by the $\chi^2$ test and the Pearson's coefficient of correlation ($r^2$). [34].

**Western blotting.** Sonicated antigen *B. burgdorferi* s.s. and *B. afzelii* was dissolved in sample buffer (containing β-mercaptoethanol as reducing agent), boiled for 5 min and subjected to SDS-PAGE (12.5% polyacrylamide gel) using the system of Laemmli [23]. Low-range-molecular-mass standards (BioRad) were used in gel.

Electrophoresis was carried out at a constant current of 25 mA for 1 hour. Proteins separated on gel were then transferred to a nitrocellulose membrane using the systém of Towbin *et al.* [44]. The nitrocellulose membrane was cut into 3 mm wide strips. One part of each membrane was stained with amidoblack dye, to assess the efficiency of transfer, the other was used to react with cows sera. The strips were blocked for 1 hour at room temperature with 5% non fat milk in BBS buffer, pH 8.2 (10 mM $H_3BO_3$, 25 mM $NA_2B_4O_7$ and 75 mM NaCl). The membranes were incubated for 2 h with horses' and cows' sera diluted in 3% non-fat milk-BBS. After three washes in 3% milk-BBS for 5 min, the strips were treated for 1 h with anti-bovine, resp.anti-horse IgG peroxidase conjugate (Sigma) diluted in 3% milk-BBS. After two additional washes for 15 min, the strips were developed by the addition of a freshly prepared solution of 0.075% (w/v) 4-chloro-1-naphtol (Sigma), 0.06% hydrogen peroxide and 25% methanol in Tris buffer (pH 6.8). The reaction was stopped by washing the strips with distilled water [37].

## RESULTS

In the set of sera from 395 horses the total mean seroprevalence for anti-*Borrelia* IgG antibodies 25.6% was observed. In the respective years, significant differences in the mean seroprevalence were not recorded (24.6 and 27.6%, respectively). In the voivodships, the total mean seroprevalence and mean seroprevalence per respective years varied, ranging from 16.6–66.6% (Tab. 1). An analysis of seroprevalence made with respect to age showed that there was significant difference between 0–2 year-old horses compared to older horses (Tab. 2) ($\chi^2$ test, $p < 0.05$). The total seroprevalence in the set of 98 serum samples was lower with strain *B. garinii* (25.5%) in comparing with a mixture of *B. burgdorferi* s. s. with *B. afzelii* (36.7%) and *B. afzelii* (42.8%). This trend was also observed in all voivodships except the Wielkopolskie province (Tab. 3). Consistency of positive and negative findings between used strains *B. burgdorferi* s. s. ranged from 80.6–91.8%. The highest correlation of findings was reached comparing the strains of *B. afzelii* (South Poland) and a mixture of *B. burgdorferi* s. s. + *B. afzelii* (East Slovakia) ($r^2$=0.74, df=94, t=3.83, p<0.001). Lower correlation was between *B. garinii* and a mixture of *B. burgdorferi* s. s. + *B. afzelii* ($r^2$=0.44, df=95, t=6.86, p<0.001). On the other hand, the lowest correlation of findings was observed between the Slovak strain of *B. garinii* and Polish *B. afzelii* ($r^2$=0.31, df=94, t=6.83, p<0.001) (Fig. 1).

**Table 2.** Anti-*Borrelia* antibodies in horses according to age.

| Age | No examined | No positive | Seroprevalence |
|---|---|---|---|
| 0–2* | 86 | 14 | 16.3 |
| 3–5 | 16 | 6 | 37.5 |
| 6–8 | 38 | 13 | 34.2 |
| 9–12 | 164 | 44 | 26.8 |
| 12 > | 91 | 28 | 30.8 |

* significantly lower compared to other groups ($p < 0.05$, test $\chi^2$)

**Table 3.** Anti-*Borrelia* antibodies in horses using different isolates *B. burgdorferi* sensu lato as antigens (N – number examined).

| Voivodships | N | No. positive (%) | | |
|---|---|---|---|---|
| | | *B. garinii* | *B. b.* s.s. + *B. afzelii* | *B. afzelii* |
| Kujawsko-Pomorskie | 12 | 6 (50.0) | 7 (58.3) | 7 (58.3) |
| Łódzkie | 15 | 5 (33.3) | 6 (40.0) | 7 (46.6) |
| Lubelskie | 6 | 2 (33.3) | 3 (50.0) | 3 (50.0) |
| Mazowieckie | 38 | 5 (13.1) | 7 (18.4) | 9 (23.7) |
| Opolskie | 3 | 1 (33.3) | 2 (66.6) | 2 (66.6) |
| Podlaskie | 5 | 1(20.0) | 3 (60.0) | 4 (80.0) |
| Świętokrzyskie | 11 | 3 (27.3) | 5 (45.4) | 6 (54.5) |
| Warmińsko-Mazurskie | 6 | 1(16.6) | 2 (33.3) | 3 (50.0) |
| Wielkopolskie | 2 | 1 (50.0) | 1(50.0) | 1 (50.05 |
| Total | 98 | 25 (25.5) | 36 (36.7) | 42 (42.8) |

**Table 4.** Anti-*Borrelia* antibodies in cattle associated with clinical sings from region Podkarpackie.

| Clinical signs | No. examined | No. positive | Seroprevalence (%) |
|---|---|---|---|
| Lameness | 3 | 2 | 66.6 |
| Weakness | 2 | 0 | 0 |
| Mastitis, phlebitis | 7 | 2 | 28.5 |
| Clinicaly healthy | 14 | 3 | 21.4 |
| Total | 26 | 7 | 26.9 |

**Table 5.** Reactivity of horses and cows sera with strain I r 112 *Borrelia garinii* using ELISA and Western blotting.

| Horses sera | ELISA Titres | Western blotting kDa |
|---|---|---|
| 1 | 1 : 1600 | 28, 34, 41, 66, 98 |
| 2 | 1 : 3200 | negative |
| 3 | 1 : 400 | 25, 34, 41, 55 |
| 4 | 1 : 3200 | negative |
| 5 | 1 : 1600 | negative |
| 6 | 1 : 1600 | negative |
| 7 | 1 : 800 | negative |
| 8 | 1 : 1600 | 25, 35, 41, 43, 45, 83, 93 |
| 9 | 1 : 1600 | 31, 34, 35, 41, 45, 55, 66, 93 |
| 10 | 1 : 1600 | 28, 31, 39, 43 |
| 11 | 1 : 3200 | 35, 66, 75, 98 |
| 12 | 1 : 1600 | 25, 35, 41, 45, 75, 98 |
| 13 | 1 : 800 | 28, 43,. 75, 93, 95 |
| 14 | 1 : 3200 | 34, 83, 93, 94 |
| 15 | 1 : 1600 | negative |
| 16 | 1 : 1600 | 43, 75, 83, 93, 94 |
| 17 | 1 : 3200 | negative |
| 18 | 1 : 1600 | 34, 43, 67, 83, 93 |
| 19 | 1 : 800 | negative |
| 20 | 1 : 1600 | 41, 43, 71, 75, 93 |
| 21 | 1 : 800 | negative |
| 22 | 1 : 1600 | 25, 41, 45, 67, 75, 93, 98 |
| 23 | 1 : 1600 | 34, 45, 66, 83 |
| 24 | 1 : 800 | negative |
| 25 | 1: 1600 | 31, 34, 68, 93 |
| Cow sera | | |
| 1 | 1 : 1600 | 67, 68, 75, 95 |
| 2 | 1 : 1600 | 25, 41, 43, 45, 49, 66, 93 |
| 3 | 1 : 400 | 25, 45, 49, 67, 93 |
| 4 | 1 : 1600 | 41, 43, 45, 75, 83 |
| 5 | 1 : 400 | 35, 41, 43, 45, 51, 66, 93 |
| 6 | 1 : 800 | negative |

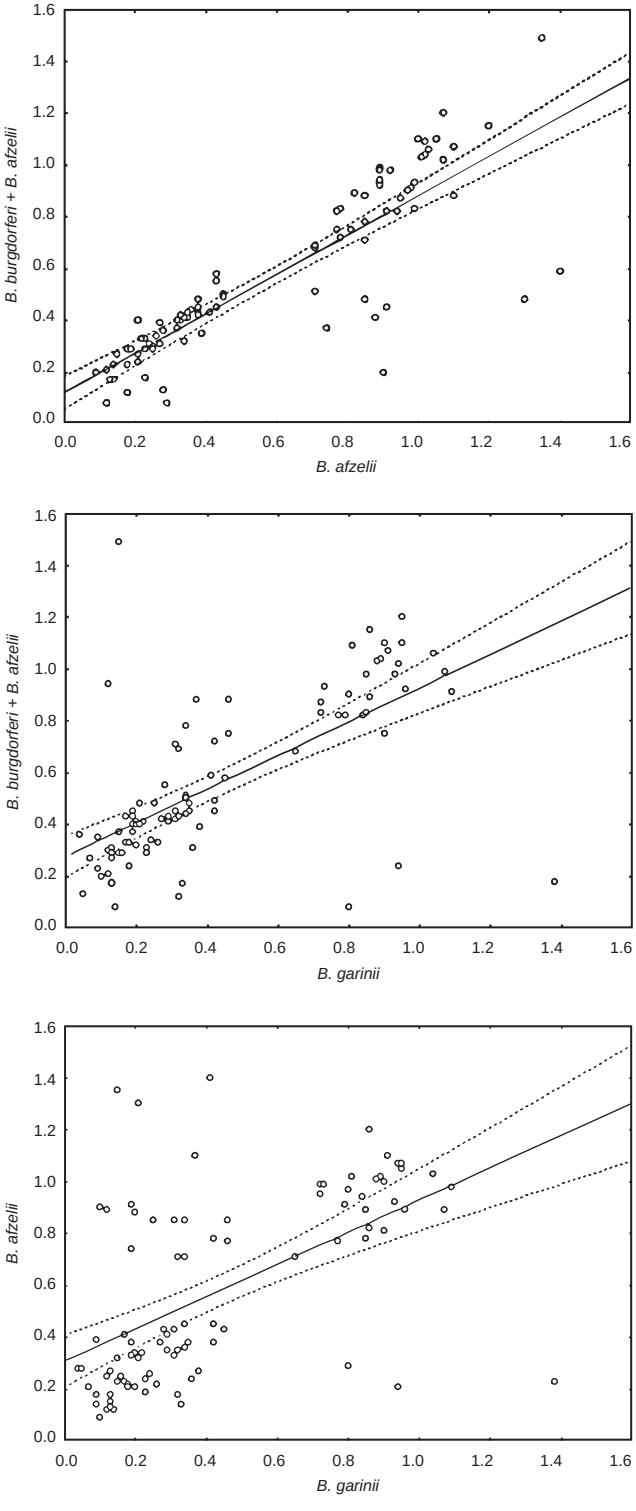


**Figure 1.** Correlation of anti-*Borrelia* IgG antibodies by ELISA in horses with different whole-cell antigens.

In a group of sera from 26 cows, the mean seroprevalence for anti-*Borrelia* IgG antibodies of 26.9% was found. In the remaining clinical signs the seroprevalence was 28.5–66.6%, but the number of animals examined was low (3–7 cows) (Tab. 4).

Western blotting (Wb) was performed using 25 sera from horses and 6 sera from cows positive in the ELISA. Out of 25 horses, 15 (60.0%) sera were positive, out of 6 cows – 5 (83.3%) were positive (2 lameness, 2 phlebitis, 1 clinically healthy) (Tab. 5, Fig. 2, 3). The IgG reactivity with at least

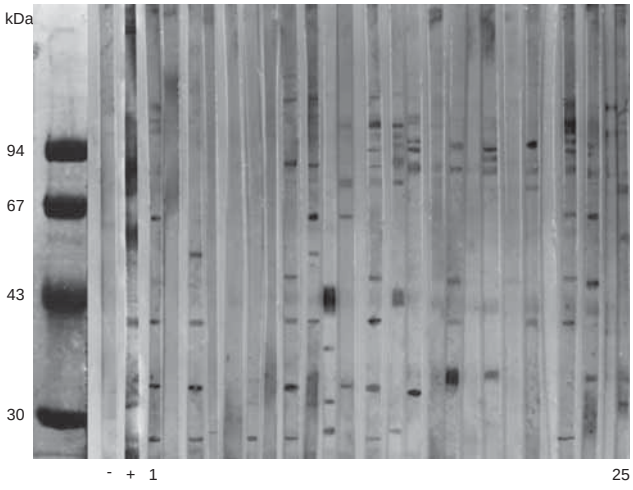


**Figure 2.** Immunoblot of 25 sera of horses and low-molecular-mass standards.

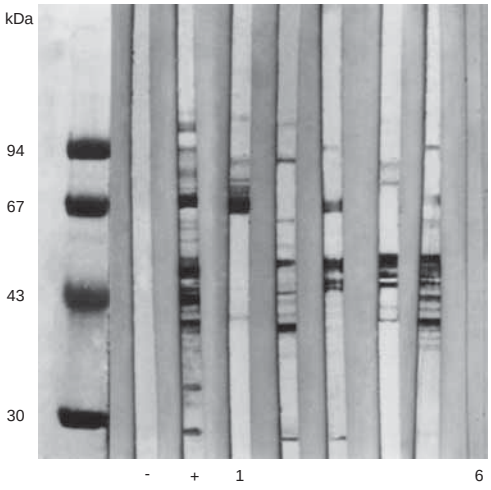


**Figure 3.** Immunoblot of 6 sera of cattle and low-molecular-mass standards.

4 borrelia-specific proteins (18, 21, 24, 28, 31, 34, 39, 41, 45, 55, 60, 66, 75 and 93 kDa) was evaluated according to Norman *et al.* [33]. The horses' and cows' sera recognised proteins: 93 (MEP)-, 66-, 75-, 55-, 45-, 41 (flagellin)-, 31-, 34-, 39 (OspB) and 25, 28 (OspC)-kDa.

## DISCUSSION

The results on seroprevalence of Lyme borreliosis in horses showed that out of 395 serum samples collected from 12 Polish voivodships, 25.6% was positive with seroprevalence differing with individual years and regions. Cows associated with clinical signs which originated from the Podkarpackie Voivodship showed seropositivity in 26.9%.

A seroepidemiological survey carried out in the USA has proved that in the north-eastern part of the country 14–25% of horses are seropositive [26, 28], in the western USA – 6–35% or more [27]. In Japan, the seroprevalence in horses was only 2.6–4.6% [43]. Horses in Texas have no record of seropositivity [13]. In the Czech Republic, 7.8% positive horses were found [46] and in Easten Slovakia it was 48.7% [40].

Although *Borrelia burgdorferi* infection in cattle is probably widespread in endemic regions, less is known regarding Lyme borreliosis in this species than in dogs [1, 6, 22] or horses [27, 28, 30]. Only a few reports deal with serological evidence of this zoonosis in cattle. For example, Borko [4] found out that the infection rate in cattle in Slovenia differed with both the areas and the infection rate in ticks, varying from 21.1–39.5% at the pasture, and from 36.5% to 64.0% after being at pasture. Wells *et al.* [52] recorded 38% seropositivity in cattle in spring and 50% in summer in Minnesota and Wisconsin herds. Stefančíková *et al.* [41] found the mean seropositivity of 23.3%, in Slovakia in particular regions the seropositivity ranged from 6–34.3%.

The differences in *Borrelia* prevalence are determined by the following ecological factors: the animal species and their individual susceptibility, size of infective doses, primal infection or re-infection, focal occurrence of ticks, local and seasonal variability of *Borrelia* infected ticks within the area from which the animals originated. Apart from these, the list may include the use of different methods (IFA, ELISA, WB) or their various modifications, which is reflected in the different interpretations of results in terms of determining the reaction positivity. Furthermore, a different number of examined serum samples may also play its role. In our case, in Kujawsko-Pomorskie, Łódzkie, Mazowieckie, Podlaskie, Świętokrzyskie Voivodships, the appropriate number of sera was examined. In the remaining voivodships the number was low, lacking the objective reflection of seropositivity, but pointing out the distribution of agents and their possible contact with horses from the studied regions. The same small number of cattle sera was examined; however, our seroprevalence results refer to a potential occurrence of this zoonosis in Poland. This is also supported by a significant infestation of ticks with borreliae in this regions of the country [31, 36].

The heterogeneity of *Borrelia burgdorferi* sensu lato is one of the intricate problems in serological diagnosis of Lyme borreliosis. This is reflected in the antigenic diversity of isolates used as antigens for serological examinations, and consequently, in the different sensitivity of the method [2]. Most of the previous studies [5, 29, 39, 42] have proved that antigens prepared from local *Borrelia* isolates give a higher sensitivity of reaction, reflecting a higher seroprevalence. Comparison of 3 isolates of *B. burgdorferi* sensu lato using ELISA in a group of 98 sera of horses from Poland has also confirmed this dependency.

Considering the age, we observed a higher seroprevalence in older horses compared to the younger ones. Within the study of the circulation of antibodies to *Borrelia burgdorferi* in sera of clinically healthy horses from East Slovakia, there was no significant difference between age groups of this animals [40]. We found a significant difference only between dogs in the categories 1–3 years and under 1 year of age [38], and a higher seroprevalence in older cows

compared to heifers [41]. Our previous studies showed that breed, age and sex do not significantly influence to the seroprevalence of this zoonosis. The decisive role, however is, played by the time of the exposure of animals to the environment infested by *Borrelia*-contamined ticks.

In our group of horses, almost 25% of seropositive cases had high titres of antibodies, with no clinical signs in cows with lameness and one clinically healthy cow. This may be indicative of Lyme borreliosis. The results of Western blotting supported this assumption. Wells *et al.* [52] also observed high value for antibodies against *B. burgdorferi* associated with lameness in dairy cattle. On the other hand, cows with confirmed infections with agent Borreliae, in which the organism has been demonstrated in fluids and tissues, may produce only low titres of antibodies [7]. Only scarce data are available on the duration of spirochaetemia and persistence of antibodies in horses and cattle [4, 27, 48]. The diagnosis of this zoonosis based on clinical signs, elimination of other causes of lameness, serology and response to therapy, is particularly difficult in horses and cattle [12, 30]. The aetiology of lameness is multifactorial, being associated with environmental factors that cause trauma and subsequently allow specific microorganisms to infect the wound. Other factors include the climate and season, housing systems, herd size and management [15]. Infectious arthritis has been linked to a variety of microorganisms [49]. *B. burgdorferi*, the infectious agent associated with Lyme disease, has been isolated from lame cattle in North America suffering from arthritis and concurrent systemic disease [7, 9]. Attempts to isolate *B. burgdorferi* from the tissues of experimentally infected cattle were unsuccessful [4, 48]. Dairy cows with clinical Lyme borreliosis may be more likely to shed spirochetes in the urine than asymptomatically infected cows [11].

It should be considered that sonicated antigens contain more than 100 proteins, some of which are equivalent to antigens of more than 60 different bacterial species, and some of them may cross-react with borreliae [17, 24]. *Treponema* spp., which bear similar antigenic characteristics to *B. burgdorferi* [3], have been identified as possible pathogens in cases of digital dermatitis [49]. In our group of horses and cows positive in Wb, the following bands of 34, 39 kDa, 41 and 55 kDa were also detected [41]. The same bands have also been described by Demirkan *et al.* [14], which were significant cross-reacting epitopes shared by *B. burgdorferi* and *Treponema* spp. in cattle with digital dermatitis. Our previous studies showed that anti-Borrelia positive sera gave very low positivity against Leptospira serovars [41, 42].

In spite of the problems of diagnosis of Lyme borreliosis, in discovering the *B. burgdorferi* circulation in animal populations from Poland, veterinarians must pay attention to this disease in their clinical practice and include it in differential diagnostics. Besides, the agent – animal contact, points to the possible occurrence in humans which contributes a problem for the protection of human health.

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