

Seroprevalence of *Leptospira* spp. and *Borrelia burgdorferi* sensu lato in Italian horses

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Ebani VV, Bertelloni F, Pinzauti P, Cerri D. Seroprevalence of *Leptospira* spp. and *Borrelia burgdorferi* sensu lato in Italian horses. Ann Agric Environ Med. 2012; 19(2): 237-240.

Abstract

The aim of the study was to determine the seroprevalence of *Leptospira* spp. and *Borrelia burgdorferi* sensu lato in healthy horses living in 7 provinces of central Italy. In the period 2007-2009, sera from 386 horses were tested by microagglutination test (MAT) to detect antibodies to *Leptospira* spp., employing the following serovars as antigens: Bratislava, Ballum, Canicola, Icterohaemorrhagiae, Grippotyphosa, Hardjo, Pomona, Tarassovi. 3 animals were positive for the serovars Icterohaemorrhagiae, 2 to Bratislava, and 1 to Pomona, for a total 1.5% seroprevalence. All sera were examined by immunofluorescence antibody test (IFAT) to reveal anti-*B. burgdorferi* s.l. antibodies. 94 (24.3%) horses were positive with antibody titres ranging from 1:64 to 1:1,024. The seroprevalence was significantly higher in >10 year-old horses compared to younger subjects. No significant differences in the mean seroprevalence were observed in the respective years. The total mean seroprevalence were strictly related to the environmental conditions of the areas in which the horses lived. No cross-reactions between *Leptospira* and *Borrelia* were observed. This is the first serological survey on antibodies to *B. burgdorferi* s.l. in Italian horses.

Key words

Leptospira spp., *Borrelia burgdorferi* sensu lato, horse, MAT, IFAT

INTRODUCTION

Species of the genus *Leptospira*, and *Borrelia burgdorferi* sensu lato (s.l.) are spirochetes able to infect several domestic and wild animals, including horses. They can also infect humans, causing severe illness.

Equine leptospirosis is usually associated with recurrent uveitis [1, 2, 3], abortion, stillbirth or weak neonatal foals [4, 5, 6, 7]. Cases of renal and hepatic dysfunction have also been reported [8, 9]. Horses are incidental hosts for most serovars, mainly Icterohaemorrhagiae, Canicola, Pomona, Ballum. Recent studies have suggested that horses may be a maintenance host for serovar Bratislava [6, 10, 11].

Usually horses become infected by direct transmission, via contaminated urine or placental fluids of infected horses, or indirectly from a contaminated environment in which leptospire have been shed by other animal species.

Previous serological surveys have detected different values of seroprevalence in relationship with the geographical location and the serovars assessed. In Italy, the latest data about the seroprevalence of equine leptospirosis are those from a survey carried out from 1995-2001. The study found 11.40% of seropositive horses among the 938 tested, of which 0.96% reacted to Pomona, 18.26% to Icterohaemorrhagiae, and 80.76% to Bratislava [10].

B. burgdorferi s.l. causes an arthropod-borne multisystemic disease called Lyme borreliosis which, in Europe, is usually

transmitted by the ticks *Ixodes ricinus*, which attach themselves to domestic and wild animals. Several species of *Borrelia burgdorferi* s.l. may cause clinical signs of disease in dogs, cattle, and sheep, as well as horses, but asymptomatic infections appear common in all these animals.

The predominant clinical signs observed in horses include sporadic lameness, laminitis, swollen joints, muscle tenderness, and weight loss. Other manifestations have been reported, such as hepatitis, panuveitis, depression, facial paralysis, encephalitis [12, 13, 14, 15].

Previous European studies on the seroprevalence of *B. burgdorferi* s.l. detected a prevalence of 47.8% seropositive horses in Slovakia [16], 29.0% in Denmark [17], 25.6% in Poland [15], 16.8% in Sweden [13], 16.1% in Germany [18], and 6.3% in Turkey [19]. In Italy, no data are available about the seroprevalence of *B. burgdorferi* s.l. among horses.

The aim of the presented research was to estimate the seroprevalence of *Leptospira* spp. and *Borrelia burgdorferi* s.l. among healthy horses living in suburban and rural areas of different provinces of central Italy.

MATERIAL AND METHODS

Samples. In the period from spring 2007 to autumn 2009, sera from 386 horses were collected and investigated: 65 horses were 1-4 years old, 124 5-10 years old, and 197 more than 10 years old. All the animals, which were clinically healthy and actively racing, lived in 7 central Italian provinces: Arezzo (43°28'N – 11°35'E), Firenze (43°46'N – 11°16'E), Grosseto (42°46'N – 11°7'E), Livorno (43°33'N – 10°19'E), Lucca (43°50'N – 10°29'E), Pisa (43°43'N – 10°24'E), Pistoia (43°55'N – 10°49'E).

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Received: 14 October 2011; accepted: 12 March 2012



Whole-blood samples were collected from jugular vein of each animal. Sera were separated by centrifugation and stored at -20°C until tested.

Microagglutination test. All samples were tested by microagglutination test (MAT) of Martin and Pettit to detect antibodies against *Leptospira* spp. Live cultures of the following 8 serovars were used as antigens: Bratislava (strain Riccio 2), Ballum (strain Ballico), Canicola (strain Alarik), Icterohaemorrhagiae (strain Bianchi), Grippotyphosa (strain Moskow V), Hardjo (strain Hardjoprajitno), Pomona (strain Mezzano), Tarassovi (strain Johnson). The serovars tested in this study represent a spectrum that was expected to be prevalent in Italy, according previous studies [10]. Antigens were 4-14 days cultures, containing 1-2 x 10⁸ leptospires/ml, grown in *Leptospira* Medium Base Ellinghausen-MacCullough-Johnson-Harris (EMJH – Difco, Becton, Dickinson and Company, Sparks, MD, USA) at 30°C and checked for purity, mobility and agglutination power [20]. Sera were diluted 1:50 with sterile saline solution in wells of 96 U-shaped plates. The same volume of the antigen suspension was added to each well and mixed by agitation. Plates were incubated at 30°C for 2 hours. A loopful of the suspension in each well was placed on a slide and examined for agglutination using a dark field microscope.

Sera with antibody titre 1:100 were considered positive. Two-fold serial dilutions were successively tested to determine the endpoint titre.

Immunofluorescence antibody test. The blood sera samples were tested by indirect immunofluorescence antibody test (IFAT) using substrate slides with *Borrelia burgdorferi* s.l. antigen (Fuller Laboratories Fullerton, CA, USA). The samples were diluted 1:64 in phosphate-buffered saline (PBS, pH 7.2), and incubated on wells of the slides in a humidified chamber at 37°C for 30 min. The slides were rinsed 3 times in PBST (PBS + 0.4% Tween 80 (Sigma-Aldrich, St. Louis, Missouri, USA), once in distilled water, and air-dried.

Each well of the slides was probed with a rabbit fluorescein isothiocyanate-conjugated anti-horse IgG (Sigma-Aldrich) diluted 1:32 in Evans Blue (Sigma-Aldrich) solution. The slides were incubated, then washed and dried as described above. Finally, the slides were examined with a fluorescence microscope.

Sera resulted positive at 1:64 dilution were successively 2-fold tested to determine the final antibody titre.

Statistical analysis. Statistical evaluation was carried out by the χ^2 test to analyze the results in relationship to provinces considered, years in which samples were collected, and age of horses.

RESULTS

Six (1.5%) horses scored positive to *Leptospira* spp., in particular, 3 animals to serovar Icterohaemorrhagiae with 1:100 antibody titre, 2 to Bratislava with titre 1:200, and 1 to Pomona with titre 1:200. Data relative to age of these seropositive horses and provinces in which they lived are shown in Table 1.

Among the 386 horses tested, 94 were seropositive to *B. burgdorferi* s.l. with 24.3% total mean seroprevalence. No significant differences in the mean seroprevalence were observed in the respective years. The total mean seroprevalence detected in province of Arezzo was the highest (55.0%). However, in the different provinces, the seroprevalence varied in relationship with the year considered, ranging from 0%-80% (Table 2).

The antibody titres varied between 1:64 - 1:1,024 (Table 3). The seroprevalence was significantly higher in >10 year-old horses compared to younger subjects (χ^2 test, p<0.05).

None of the *B. burgdorferi* s.l. positive horses had antibodies against *Leptospira* spp.

Table 1. Data relative to age, province, and antibody titre of *Leptospira* sp. positive horses

Horse	Age (years)	Province	MAT
1	5-10	Pisa	IH 1:100
2	5-10	Pisa	IH 1:100
3	1-4	Pisa	IH 1:100
4	> 10	Florence	BR 1:200
5	1-4	Pisa	BR 1:200
6	>10	Pisa	PO 1:200

MAT: microagglutination test
IH: serovar Icterohaemorrhagiae
BR: serovar Bratislava
PO: serovar Pomona

Table 3. *Borrelia burgdorferi* s.l. positive horses according to age and antibody titre

Age (years)	No. examined	No. positive at the given titre					Total
		1:64	1:128	1:256	1:512	1:1024	
1-4	65	5	3	3	1	0	12 (18.4%)
5-10	124	11	8	2	4	0	25 (20.1%)
>10	197	26	16	5	9	1	57 (28.9%)
Total	386	42	27	10	14	1	94 (24.3%)

Table 2. Prevalence of anti-*Borrelia burgdorferi* s.l. antibodies among horses from different central Italian provinces

Province	2007		2008		2009		Total	
	No. examined	No. positive (%)	No. examined	No. positive (%)	No. examined	No. positive (%)	No. examined	No. positive (%)
Arezzo	3	1 (33.3)	10	8 (80.0)	7	2 (28.5)	20	11 (55.0)
Florence	9	1 (11.1)	5	1 (20.0)	15	1 (6.6)	29	3 (10.3)
Grosseto	9	4 (44.4)	15	1 (6.6)	8	0	32	5 (15.6)
Livorno	46	9 (19.5)	30	6 (20.0)	18	5 (27.7)	94	20 (21.2)
Lucca	15	1 (6.6)	8	3 (37.5)	12	2 (16.6)	35	6 (17.1)
Pisa	54	15 (27.7)	48	11 (22.9)	52	18 (34.6)	154	44 (28.5)
Pistoia	7	3 (42.8)	5	0	10	2 (20.0)	22	5 (22.7)
Total	143	34 (23.7)	121	30 (24.7)	122	30 (24.5)	386	94 (24.3)



DISCUSSION

A low percentage (1.5%) of *Leptospira* spp. positive horses was detected, with 3 animals positive to Icterohaemorrhagiae, 2 to Bratislava, and 1 to Pomona. All the animals appeared clinically healthy at the time of sampling and had no history of leptospirosis-related syndrome, thus the low antibody titres detected could be the results of subclinical infections. The positive reactions are probably related to the environmental diffusion of maintenance hosts, such as rats *Rattus norvegicus* for the serovar Icterohaemorrhagiae, and hedgehogs *Erinaceus europaeus* for Bratislava. Serovar Pomona, a typical agent of infection mainly in pigs, but also in sheep, cattle and horses, may be harboured also by wild animals such as wild boars (*Sus scrofa*).

The low seroprevalence observed in the presented study is probably due to environmental features in which the horses lived under good management conditions and in areas with a low presence of stagnant water. Moreover, these results seem to be in accordance with the actual epidemiological situation of leptospirosis. In fact, in recent years, a significant decrease of seroprevalence to the serovars traditionally present in Italy was observed among domestic and wild animals (personal data not published).

Human Lyme borreliosis was first recognized in Italy in 1985 [21], and is actually endemic in several regions. Most human cases have been reported in northern Italy, but the infection has also been detected in other regions [22, 23, 24, 25]. In Tuscany, the presence of *B. burgdorferi* s.l. in ticks has been previously reported. In particular, Stefanelli *et al.* (1994) [26] found *Borrelia garinii* in *I. ricinus* in an area of coastal Tuscany, where neuroborreliosis was reported in people, whereas *Borrelia lusitaniae* and *Borrelia afzelii* were detected in *I. ricinus* ticks collected in a natural reserve in this region [27, 28].

Knowledge about the distribution of *B. burgdorferi* s.l. among the domestic animal population in Italy is deficient; therefore, we have reported for the first time the presence of *B. burgdorferi* s.l. antibodies in horses in central Italy.

The significant seroprevalence recorded (24.3%) indicates the exposure of the equine population to *B. burgdorferi* s.l. in the provinces considered. Most of the horses studied lived in areas with environmental conditions which favour diffusion of the arthropods: abundant vegetation and the presence of other animal species, particularly wild ruminants and medium-sized mammals (cotton-tail rabbit *Sylvilagus floridanus*, red squirrel *Sciurus vulgaris*, edible dormouse *Glis glis*, mice *Apodemus* spp.).

The highest mean seroprevalence was found in the province of Arezzo. This fact could be related to the particular topography and ecological characters of this province, which include narrow valleys set between slopes which, at times, are rocky and in other places covered by dense forest. The mountain area, called Foreste Casentinesi, is particularly rich in wild fauna which includes wolf (*Canis lupus*), roe deer (*Capreolus capreolus*) and fallow deer (*Dama dama*).

A high mean seroprevalence for anti-*Borrelia* IgG antibodies was also found in Pisa province where the horses live in areas close to the San Rossore natural reserve, a large protected area covered mainly by pinewoods hosting farm animals and an enormous variety of wildlife, including fallow deer, wild boar and many different species of birds.

The lowest mean seroprevalence was observed in Florence province in which all horses tested lived in suburban areas, far from wild animals.

Each of the other regions of Tuscany is characterized by areas with different ecological features, thus the percentage of *B. burgdorferi*-positive horses was related to the area in which they lived.

A higher seroprevalence was observed in older horses than in younger ones, probably due to the longer time of the exposure of the animals to the environment infested by *B. burgdorferi*-contaminated ticks.

Cross-reactions between anti-*Borrelia* and anti-*Leptospira* antibodies were not observed in the equine sera tested. Other authors have found minimal cross-reactivity between borreliae and leptospirae [29].

All horses examined did not show clinical signs, indicating the rare incidence of clinical borreliosis in the equine population. The considerable seroprevalence recorded during the presented study shows that Lyme borreliosis may affect horses; therefore, this infection should be taken in consideration in differential diagnoses, in particular in cases of lameness, and when horses live in an environment where cases of borreliosis have previously been reported and /or where risk of exposure factors are present.

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