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Short Communication

# First detection of anti-*Besnoitia* spp. specific antibodies in horses and donkeys in Italy

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ABSTRACT

Among Apicomplexa protozoa infecting equids, *Besnoitia* spp., *Toxoplasma gondii* and *Neospora* spp. represent important issues from a sanitary and zootechnical viewpoint. However, only scarce epidemiological data are available on the spread of the infections in horses and donkeys in Europe. Therefore, a serosurvey was planned to estimate the prevalence of these Sarcocystidae species in Italian equids. Serum samples from 268 horses and 18 donkeys raised in Italy were collected and serologically analyzed to detect anti-*Besnoitia* spp., anti-*T. gondii* and anti-*Neospora* spp. antibodies: an approach based on an initial screening by in-house ELISA followed by a confirmatory WB was used. Two horses (0.7%) and four donkeys (22.2%), showed antibodies anti-*Besnoitia* spp. Ten horses (3.7%) resulted positive to *T. gondii* and one of these (0.4%) was seropositive also to *Neospora* spp. This is the first detection of anti-*Besnoitia* spp. specific antibodies in Italian horses and donkeys. The study confirmed the circulation of *Besnoitia* spp. among equids in Europe. Low prevalence of *T. gondii* and *Neospora* spp. in horses raised in Italy was reported. Nevertheless, it is noteworthy to consider that consumption of horse meat could represent a source for human toxoplasmosis.

In equids, infections by Apicomplexa protozoa are of concern from a veterinary and zootechnical viewpoint: particularly, *Besnoitia* spp., *Toxoplasma gondii* and *Neospora* spp. were reported to affect both horses and donkeys. Besnoitiosis in equids, caused by *Besnoitia bennetti*, is considered an emerging disease of donkeys in the United States [1]. *Besnoitia* spp. specific antibodies were detected for the first time in Europe in equids from areas where bovine besnoitiosis is endemic in Spain [2]. The infection was never explored in Italian equids, even if outbreaks of the disease in cattle were recently diagnosed in Northern regions [3, 4]. Anti-*T. gondii* antibodies were demonstrated in both species in serological surveys worldwide, although there is not any confirmed report of clinical disease in equids [5]. On the contrary, *Neospora hughesi* is recognized as an etiological agent of the equine protozoal myeloencephalitis (EPM), an important neurological disease of horses [6]. Worldwide, a range of seroprevalence values between < 1 and 65.6% and between 0 and 85.7% was reported for *T. gondii* and *Neospora* spp. infections in equids, respectively [5, 6]. In Italy, the presence of antibodies anti-*T. gondii* (3–8%) and anti-*Neospora* spp. (2.3–28%) has been reported in equids reared in Southern Italy [7–10]. In Northern Italy data are limited to *T. gondii* infection in horses destined for human consumption (17.6%) [11], although the presence of the parasite in this area was recently reported in other domestic and wild species [12–15]. Therefore, the study aimed to contribute to the knowledge of *Besnoitia* spp., *T. gondii* and *Neospora* spp. by estimating their seroprevalence in equids from Italy.

A minimum sample size of 246 horses was determined considering a population in Lombardy and Piedmont of 78,490 animals (National Zootechnical Database, https://www.vetinfo.sanita.it/), a 20% expected prevalence, a 95% confidence interval and a 5% desired absolute precision. From April 2016 to March 2017, blood samples from 268 horses (*Equus caballus*) apparently healthy from 33 stables located in Northern Italy (Lombardy and Piedmont regions) were collected by puncturing of the jugular vein using a Vacutainer® sterile collection system and preserved refrigerated in tubes without anticoagulants during the transportation to the laboratory. Once in laboratory, sera were separated by centrifugation (2120*g*, 15 min) and then stored at −20 °C until serological analysis. Moreover, serum samples from 18 donkeys *(Equus asinus)* previously referred to the laboratory for routine

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parasitological examinations were also included in the study. Epidemiological data were collected interviewing the owners at sampling times; to avoid bias in the data collection, farms were visited by the same investigator. To detect anti-*Besnoitia* spp., anti-*T. gondii* and anti-*Neospora* spp. antibodies, all serum samples were initially screened by ELISA; subsequently, positive results were confirmed by Western Blot (WB), as recommended [2, 16, 17]. For both serological tests, antigens of *B. besnoiti* and *N. caninum* originally isolated from cattle were used, because strong cross reactions with the respective species infecting equids (*B. bennetti* and *N. hughesi*) were demonstrated [2]. An 'in-house' indirect ELISA was used to detect antibodies of *Besnoitia* spp., *T. gondii* and *Neospora* spp. in serum samples, as previously described [2, 17, 18]. A blocking solution of Phosphate Buffered Saline (PBS) containing 0.05% Tween 20 and 5% Bovine Serum Albumin was used; Protein G (recombinant peroxidase labeled, Sigma-Aldrich®, Saint Louis, USA) diluted at 1:1500 was used as conjugate. Absorbance was measured as Optical Density (OD) values at 405 nm using a microplate reader. Samples were analyzed in duplicate and the mean value of the OD was converted into a relative index per cent (RIPC) using the following formula: RIPC = (OD sample-OD negative control)/(OD positive control-OD negative control) × 100. For each pathogen, the cut-off value was calculated as the mean plus three standard deviations of RIPC values considering a panel of negative control sera ($n = 20$). For *T. gondii*, serum samples from horses previously referred to the laboratory for diagnostic purposes, were analyzed using a commercial indirect immunofluorescence antibody assay (IFAT), according to Gazzonis et al. [12], using a FITC anti-horse IgG (MegaCor Diagnostik, Horbranz, Austria) as conjugate and considering 1:20 dilution as the cut-off [11]. For *Neospora* spp. and *Besnoitia* spp., negative sera previously analyzed were included in the panels [2]. Samples having a RIPC value higher than 12.3, 18.7 and 21.5 were considered ELISA-positive results respectively for *Besnoitia* spp., *T. gondii* and *Neospora* spp. and then submitted to confirmatory WB, performed and interpreted as previously described [19–21]. A total of $4 \times 10^7$ *B. besnoiti* tachyzoites under non-reducing condition and $2 \times 10^7$ *T. gondii*- and *N. caninum* tachyzoites under reducing condition were employed for electrophoresis. Tachyzoite antigens were transferred to nitrocellulose membranes and incubated with sera from horses and donkeys at a 1:20 dilution, followed by a peroxidase-conjugated anti-horse IgG (H + L) antibody diluted at 1:1500 (INGENASA®, Madrid, Spain). For both serological tests, positive and negative control sera were included. In particular, for *T. gondii*, ovine positive and negative control sera were employed; for *Neospora* spp. and *Besnoitia* spp., both equine (horse and donkey, respectively) and bovine control sera were used [2].

The presence of anti-*Besnoitia* spp. antibodies was demonstrated by ELISA in 21 horses and in four donkeys. Antibodies against *T. gondii* and *Neospora* spp. were detected in 19 and 22 horses, respectively. Seropositivity against *Besnoitia* spp. was confirmed by WB in six equids, specifically four donkeys and two horses. WB analysis confirmed seropositivity to *T. gondii* in ten horses and to *Neospora* spp. only in one horse, contemporary infected also by *T. gondii* (Table 1, Supplementary Fig. 1–2). Seroprevalence of the investigated parasites in different cohorts of the examined equids are reported in Table 2. Individual and managerial data regarding the seropositive animals are reported in Supplementary Table 1.

The present study provided serological data on selected cystogenic coccidia in Italian equids, for which only scarce data are available in Europe. This is the first detection of anti-*Besnoitia* spp. specific antibodies in Italian horses and donkeys. Moreover, the circulation of *T. gondii* and *Neospora* spp. was confirmed in horses raised in Italy. European countries should be aware of these parasitic diseases. Surveillance should be implemented; harmonized diagnostic procedures and standardized techniques are needed in order to get comparable results and infer reliable conclusions. The diagnostic approach used in this study consisted of an initial screening by ELISA followed by a confirmatory WB: the use of a confirmatory technique is recommended due to the possibility of cross-reactions between closely related Apicomplexa, i.e. *T. gondii*, *Neospora* spp., *Besnoitia* spp. and *Sarcocystis* spp. [16]. In this study, six equids resulted positive to *Besnoitia* spp., with an overall seroprevalence of 2.1% (0.7% in horses and 22.2% in donkeys). Seropositive horses were raised in two farms in the south-western part of the study area, where outbreaks of bovine besnoitiosis were recently reported [3, 4]; besides, it is noteworthy consider that in this area cattle are more frequently maintained on extensive pasture, in contrast to the Po Valley, where animals are kept mainly in intensive farms. Concerning *Besnoitia* spp. infected donkeys, these animals came from both northern and southern areas: interestingly, in all these farms the co-presence of other species, in particular domestic ruminants, was reported. Other countries where there are cases of bovine besnoitiosis should consider the possibility that also equids could be seropositive to *Besnoitia* spp. Indeed, *Besnoitia* spp. specific antibodies were recently detected in Spanish equids [2] with a seroprevalence of 7.1% in areas where bovine besnoitiosis is endemic. Similar to the present study, also in Spain the seroprevalence of *Besnoitia* spp. infection was higher in donkeys (15.3%) than in horses (2.9%); however, a higher susceptibility of donkeys for *Besnoitia* spp. infection was not demonstrated. Considering individual data, anti-*Besnoitia* spp. antibodies were found only in animals older than 5 years. Furthermore, equids housed only outdoor and without box resulted more infected with *Besnoitia* spp., probably because these animals could be at greater risk of exposure to the bite of vector insects. Outside of Europe, *Besnoitia* spp. infections in equids were attributed to *B. bennetti* in sub-Saharan countries and in the United States besnoitiosis is considered an emerging disease of donkeys [1]. Molecular studies would be advisable to clarify which *Besnoitia* species is involved in the infection of Italian horses and donkeys. Concerning *T. gondii* infection, ten horses

**Table 1**
Seropositivity to *Besnoitia* spp., *Toxoplasma gondii* and *Neospora* spp. in ELISA and Western Blot (WB) in 268 horses and 18 donkeys examined.

| | | Overall | | Horses | | Donkeys | |
|---|---|---|---|---|---|---|---|
| | | ELISA | WB | ELISA | WB | ELISA | WB |
| *Besnoitia* spp. | Pos/Ex[a] | 25/286 | 6/286 | 21/268 | 2/268 | 4/18 | 4/18 |
| | P%[b] | 8.7 | 2.1 | 7.8 | 0.7 | 22.2 | 22.2 |
| | (95% CI)[c] | (6–12.6) | (1–4.5) | (5.2–11.7) | (0.2–2.7) | (9–45.2) | (9–45.2) |
| *Toxoplasma gondii* | Pos/Ex[a] | 19/286 | 10/286 | 19/268 | 10/268 | 0/18 | 0/18 |
| | P%[b] | 6.6 | 3.5 | 7.1 | 3.7 | 0 | 0 |
| | (95% CI)[c] | (4.3–10.1) | (1.9–6.3) | (4.6–10.8) | (2–6.7) | (0–17.6) | (0–17.6) |
| *Neospora* spp. | Pos/Ex[a] | 22/286 | 1/286 | 22/268 | 1/268 | 0/18 | 0/18 |
| | P%[b] | 7.7 | 0.3 | 8.2 | 0.4 | 0 | 0 |
| | (95% CI)[c] | (5.1–11.4) | (0.1–1.9) | (5.5–12.1) | (0.1–2.1) | (0–17.6) | (0–17.6) |

[a] Pos/Ex, Positive/Executed.
[b] P%, Prevalence %.
[c] 95% CI, 95% Confidence interval.