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Serological and molecular detection of *Theileria equi* infection in horses in Hungary

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ABSTRACT

The prevalence of *Theileria equi* infection was studied in 324 healthy horses from 27 farms in Hungary with cELISA and IFAT and the blood samples of 101 horses selected randomly were also examined by PCR. The results indicate that there are many stud farms where one or more horses are infected with *T. equi*. Among 27 farms 17 (67.9%) were found to have seropositive horses. The seroprevalence of theileriosis among the tested stud farms ranged between 0 and 100%. No marked differences were found in seropositivity between geographical areas. The overall prevalence of positive samples was 32.0% with cELISA as well as with IFAT. The results obtained with cELISA and IFAT in this study had the strongest agreement, except for 9 samples in which the two serological tests gave different results. The prevalence of infection among 101 horses was 49% with PCR. All 14 sequenced samples were found by BLAST analysis to be closest to the *T. equi* 18S rRNA gene sequences in GenBank with a similarity of ≥99%.

No significant association was found between the seropositivity and the age of horses. Horses below 5 years of age had three times higher chance to be PCR-positive, than older ones. There was no significant association between the gender and the results of diagnostic tests (cELISA: $p = 0.40$; IFAT: $p = 0.25$; PCR: $p = 0.41$). Based on the findings, the prevalence of equine theileriosis is much higher than expected and it occurs in many regions of the country unlike equine babesiosis. To the authors' knowledge, this is the first report of the serological and molecular survey of *T. equi* infection in horses in Hungary.

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1. Introduction

Equine piroplasmosis (EP) is caused by two intra-erythrocytic haemoprotozoan parasites, *Theileria equi* (previously known as *Babesia equi*) and *Babesia caballi* or even by a mixed infection (Mehlhorn and Schein, 1998). This blood-borne parasitic infection affects horses, donkeys, mules, and zebras. The occurrence of EP is mainly related to the distribution of several tick species known to be vectors of these parasites (de Waal, 1992; Friedhoff and Soule, 1996). However, equine piroplasms are also spread by the transfer of blood from infected to naïve equids iatrogenically via the reuse of blood-contaminated needles, syringes or surgical instruments, and blood or serum transfusions from untested, infected donor animals (Rothschild and Knowles, 2007). Equine piroplasmosis is a cause of great concern in the global horse industry because it is widely distributed throughout the tropical and subtropical regions of the world and is known to occur to a lesser extent in temperate regions. The OIE data show that 27 countries

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reported cases of EP in 2008 (OIE, 2009). Both species are endemic to southern Europe, including Portugal, Spain, France, Belgium, Italy, and the Balkan Peninsula (de Waal, 1992; Rothschild and Knowles, 2007). Seroprevalence of EP is highly variable in Europe, ranging from about 8 to 35 percent for *B. caballi* and from 20 to 40 percent for *T. equi* (Camacho et al., 2005; Hornok et al., 2007; Sevnic et al., 2008).

Equine piroplasms cause economic losses and impact the international transport of horses (Friedhoff et al., 1990; Rothschild and Knowles, 2007). Many parasites-free countries like the United States, Canada and Australia restrict seropositive horses from entering to participate in international events or for export because the introduction of carrier animals into areas where tick vectors are present can lead to an epizootic spread of the parasites (Friedhoff et al., 1990; Bruning, 1996).

Both protozoan species can cause acute, subacute or chronic disease in horses with variable and non-specific clinical signs. The symptoms of EP range from acute fever, lack of appetite, depression to anemia and jaundice. Sudden death can occur among horses native to endemic regions, depending on the pathogen strain, the health of the animal and treatment (de Waal and van Heerden, 2004). In the chronic phase of the disease horses have anorexia, lethargy, weight loss, anemia, poor exercise tolerance or can appear normal, but the infectious parasites persist. Parasitaemia is often absent in carriers, but can reoccur after immunosuppression. Chronic EP is clinically indistinguishable from other chronic diseases. Equine piroplasmosis cannot be diagnosed based on clinical signs alone, and *B. caballi* and *T. equi* infections cannot be distinguished clinically although differentiation between the two infections may be important for successful treatment and control (Rothschild and Knowles, 2007).

T. equi is considered the more pathogenic species because it is responsible for a greater incidence of death (Posnett et al., 1991). Infection caused by *T. equi* may be suppressed by anti-theilerial drugs but only some of them can clear infection. After recovery from theileriosis, horses may remain carriers for years or lifelong without further clinical disease and these animals can act as a source of infection to tick vectors and naïve equids (de Waal, 1992; Bruning, 1996). The persistence of the parasites may cause repeated antigenic stimulation and maintain detectable antibody levels. Although antibodies may control the level of parasitaemia, they fail to eliminate *T. equi*, allowing the development of a stable state between the parasite and the host (de Waal and van Heerden, 2004). It was suspected (Phipps and Otter, 2004) and recently confirmed (Allsopp et al., 2007; Georges et al., 2011) that transplacental transmission of *T. equi* from carrier mares to asymptomatic foals can occur. Intrauterine infections with *T. equi* may result in abortion and neonatal death (Potgieter et al., 1992). Piroplasmosis caused by *T. equi* infection in carrier mares is cited as the most common cause of equine abortion (de Waal, 1992). Observations by some researchers suggest that foals may be born as carriers yet remain apparently healthy (Allsopp et al., 2007).

In Hungary the presence of EP caused by *B. caballi* was first described by Buza et al. (1953, 1955). Half a century later a serological survey confirmed the stable endemic focus of *B. caballi* in the same area of the country where it was found first (Hornok et al., 2007). No information had been available about the other piroplasm species until 2004 when the first autochthonous case caused by *T. equi* was detected in a diseased horse by serological and molecular methods (Hevesi et al., 2006).

The objective of this study was to get information about the occurrence of *T. equi* infection in horses in Hungary. Clinically healthy horses were screened by a competitive enzyme-linked immunosorbent assay (cELISA) and an indirect fluorescent antibody test (IFAT) and the blood samples of 101 horses selected randomly were also examined by PCR.

2. Materials and methods

2.1. Blood samples

A total of 324 apparently healthy horses from 27 farms located in 24 geographically separate locals were examined (Fig. 1). Equine piroplasmosis had been detected only on one farm where *B. caballi* infection occurred. On a farm from 3 to 40 individuals were sampled taking into account the total number of horses. Data on the sampled animals (e.g. gender, age, origin) were recorded during sample collection. There were 199 (61.4%) mares, 88 (27.2%) geldings and 37 (11.4%) stallions. The age of tested horses ranged from 1 to 21 years.

Two blood samples per animal were collected from the jugular vein into sterile vacuum tubes (one without anticoagulant for serology and another containing EDTA for molecular biology). Serum and blood was stored at −20 °C until analysed.

2.2. Serology

2.2.1. Competitive enzyme-linked immunosorbent assay (cELISA)

B. equi Antibody Test Kit, cELISA from VMRD Inc. (Pullmann, WA, USA) was performed with each sample following the manufacturer's instructions. The principle of the test is that serum antibodies to *B. equi* inhibit primary monoclonal antibodies of the detection system from binding to the antigen-coated plate. The binding of primary monoclonal antibody is detected with horseradish-peroxidase-labelled secondary antibody. Optical density (OD) values were determined using Multiskan Plus microplate reader model RS-232 C (Labsystems, Helsinki, Finland). The results were expressed as a value of the percent inhibition (%I) according to the following formula: (%I): %I = 100 − [(sample O.D. × 100)/(mean negative control O.D.)]. Samples were classified as positive if the %I value was above 40% and negative if the %I value was less than 40%. The manufacturer of the kit established these cut-off values.

2.2.2. Indirect fluorescent antibody test (IFAT)

MegaScreen® FLUOTHEILERIA equi ad us. vet. in vitro diagnosticum test-kit (Megacor, GmbH, Austria) was used for the detection of anti-*Theileria equi*-IgG-antibodies in

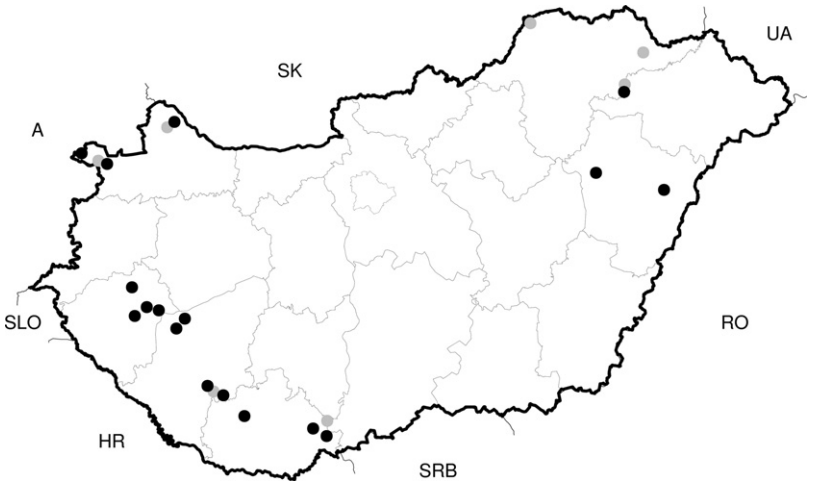

Fig. 1. Location of farms where horses were sampled. The grey dots represent the places where no animal was found as positive using either method. The black dots show the places where at least one animal was positive with at least one diagnostic test.

1:80 screening dilutions for all horse sera following the manufacturer's instructions. The stained substrate slides were read at 400× magnification using a Jenamed SH-50 (Carl Zeiss Jena, Germany) fluorescence microscope with filter system for FITC (maximum excitation wavelength 490 nm, mean emission wavelength 530 nm). Each well was compared with the visual intensity and appearance of the *T. equi* fluorescence pattern seen in the positive and negative control wells.

2.3. DNA extraction

DNA was extracted from 101 randomly selected horse blood samples with the QIAamp DNA Mini Kit (QIAgen GmbH, Hilden, Germany) following the "Blood and body fluid" protocol of the manufacturer.

2.3.1. Polymerase chain reaction

A 450 bp long section of the 18S rRNA of *Piroplasma* spp. was amplified with the primers PIRO-A1 (Muhlnickel et al., 2002) [5′-AGG GAG CCT GAG AGA CGG CTA CC-3′] and PIRO-B (Olmeda et al., 1997) [5′-TTA AAT ACG AAT GCC CCC AAC-3′]. Reaction mix contained 1× concentration of CoralLoad Buffer, 1.5 mM of $MgCl_2$, 0.2 mM for each dNTP, 25 pmol of each primer and 1 U of HotStarTaq Plus DNA Polymerase (QIAgen GmbH, Hilden, Germany) in a final volume of 25 μL. A thermal program carried out with a BIOER GenePro BIOER TC-E-BD device (Bioer, Hangzhou, PR China) was as follows: initial 5 min 95 °C was followed by a cycle 94 °C for 30 s, 60 °C for 30 s and 72 °C for 40 s repeated 35 times and finished with 72 °C for 10 min. PCR products were visualized on 1.5% agarose gel stained with ethidium–bromide. Selected positive products were sequenced in the facilities of Macrogen Inc. (Seoul, South Korea). Sequences were aligned to references with NCBI BLAST, National Institutes of Health, USA (http://www.ncbi.nlm.nih.gov). All procedures (DNA isolation, PCR and electrophoresis) were performed in separate rooms using different pipettes and racks, with separate coats and disposable gloves worn in each laboratory to prevent carry-over contamination and to avoid false-positive results. PCR mixtures were prepared in a sterile PCR box. All liquid handling procedures were performed using disposable sterile filter tips. In each PCR reaction negative control (sterile deionized water) and positive control (*Babesia canis* DNA) were included.

3. Statistical analysis

To analyse the agreement of the three diagnostic methods Cohen's kappa was used (Cohen, 1960). The association of diagnostic test results and the age of horses was analysed using logistic regression (Gelman and Hill, 2007) and Fisher's Exact test (Agresti, 2002). All statistical analyses were performed using R Development Core Team (2012).

4. Results

From the 324 serum samples that were analysed, 104 and 103 horses were found to have specific antibodies against the *T. equi* antigen with cELISA and IFAT, respectively (Table 1). The overall seroprevalence of positive samples was 32.0% (95% CI: 27.0–37.0%) with cELISA as well as with IFAT. All but 9 of the samples gave identical results in the two tests. The prevalence of *T. equi* infection in 101 horses tested with PCR was 49% (95% CI: 39–58%) (Table 1). In the comparision of IFAT and PCR or cELISA and PCR, weaker agreements (kappa = 0.83 and kappa = 0.80, respectively) were obtained. Of the positive samples obtained with PCR method, 14 were sequenced in order to verify the result.

All sequenced PCR products were found ≥99% identical to the *T. equi* 18S rRNA GenBank references (HM229408.1, HM229407.1, DQ287951.1, AY534882.1, AB515310.1) by BLAST analysis. Neither *B. caballi* nor dual infections with two protozon species were found. Eighty-four of 101 samples gave the same results with two serological and molecular methods. The serological and PCR results differed from each other in 10 samples collected on 6 farms. Five horses were found to be seropositive but PCR

Table 1
Prevalence estimation of *T. equi* infection in horse farms. Sample size is n_1 for cELISA and IFAT, n_2 for PCR.

County	n_1	cELISA			IFAT			PCR			
		Positive	Prevalence	95% CI	Positive	Prevalence	95% CI	n_2	Positive	Prevalence	95% CI
Baranya	52	15	0.29	0.18–0.42	15	0.29	0.18–0.42	19	7	0.37	0.19–0.59
Borsod-Abaúj-Zemplén	63	5	0.08	0.03–0.17	6	0.10	0.04–0.19	13	3	0.23	0.08–0.50
Győr-Moson-Sopron	77	6	0.08	0.04–0.16	5	0.06	0.03–0.14	20	4	0.20	0.08–0.41
Hajdú-Bihar	34	21	0.62	0.45–0.76	20	0.59	0.42–0.74	8	8	1.00	0.67–1.00
Somogy	41	28	0.68	0.53–0.80	28	0.68	0.53–0.80	20	14	0.70	0.48–0.85
Zala	57	29	0.71	0.55–0.82	29	0.71	0.55–0.82	21	13	0.62	0.41–0.79
Overall	324	104	0.32	0.27–0.37	103	0.32	0.27–0.37	101	49	0.49	0.39–0.58

negative and the opposite results were obtained in the other 5 animals.

Among 27 farms visited 17 (67.9%) were found to have seropositive horses, and these premises were located in six out of seven counties of Hungary. The seroprevalence of theileriosis among the tested stud farms ranged between 0 and 100%. In two farms (Bőszénfa and Kéthely) all the tested horses had theileriosis while no infection was found in 8 farms. No significant association was found between the seropositivity and the age of horses using logistic regression (cELISA: $p = 0.33$; IFAT: $p = 0.3303$). Nevertheless, when the continuous age variable was converted to two categories (under and over 5 years old), significant association between PCR results and age classes was found by Fisher's Exact test. The younger horses had three times greater odds to be PCR positive than the older horses (OR: 3.22, 95% CI: 1.33–8.04, $p = 0.005$). No significant association was between the gender and the results of diagnostic tests (cELISA: $p = 0.4028$; IFAT: $p = 0.25$; PCR: $p = 0.41$).

5. Discussion

To the authors' knowledge, this is the first report of the serological and molecular survey of *T. equi* infection in horses in Hungary. The results indicate that there are many stud farms where one or more horses are infected with *T. equi*. As regards the geographical distribution of horse theileriosis in the country infected animals were found in the western as well as in the eastern part of Hungary but without marked differences in seropositivity between the geographical areas as reported from Italy (Moretti et al., 2010), Turkey (Karatepe et al., 2009) and Greece (Kouam et al., 2010). Differences in these countries were attributable to differences in management of the horses including their nutrition and tick control (Salim et al., 2008), host activity (Kouam et al., 2010), and variation in climate among the areas (Moretti et al., 2010). The overall seroprevalence (32%) of horse theileriosis in the present study is higher than that found in other countries (Sevnic et al., 2008; Kouam et al., 2010; Grandi et al., 2011) but it is similar or lower than reported in other papers (Shkap et al., 1998; Moretti et al., 2010). The difference in the prevalence of equine theileriosis among countries may be due to differences in sensitivity of the diagnostic tests used, the occurrence and abundance of competent vectors, the management practices and effectiveness of any tick control measures. A variety of serological methods have been developed to monitor theileriosis in horses during the latent stage when microscopically undetectable parasitaemia occurs. In this study cELISA using recombinant antigens and IFAT were used. These tests were found to be more specific methods than the complement fixation test for the serodiagnosis of piroplasmosis (Bruning et al., 1997; Shkap et al., 1998; Ikadai et al., 2002; OIE, 2009). The results obtained with cELISA and IFAT in this study have the strongest agreement (kappa = 0.97) except for 9 samples the two serological tests gave the same results. This observation agrees with the results of Shkap et al. (1998) who reported that when the cELISA was compared with the IFAT, there was up to 95.7% agreement between the two assays. This showed that both tests are well suited for serodiagnosis and epidemiological studies. Almost half of the 101 horses chosen for PCR analysis were positive. Parasites from these horses can spread by way of ticks and other methods. Without reliable diagnosis *T. equi* can be transmitted to the other stud farms or abroad.

DNA of *T. equi* was detected in five seronegative horses which most likely have become recently infected, i.e., blood samples were taken before specific antibodies developed in the animals.

Based on the findings in this survey, combining *T. equi* and *B. caballi* infections the prevalence of horse theileriosis is much higher than expected and it occurs in many regions of the country, not like equine babesiosos in an endemic focus (Hornok et al., 2007). The results obtained abroad also showed that prevalence rates of *T. equi* are generally higher compared to *B. caballi* in endemic countries (Bruning, 1996; Rüegg et al., 2007).

What is/are the reason(s) that *T. equi* is more prevalent and distributed than *B. caballi* in Hungary? It is known that horses become infected with these protozoa from tick vectors. To date, several species of Ixodid ticks belonging to the genera *Boophilus*, *Hyalomma*, *Dermacentor*, and *Rhipicephalus* have been identified as vectors of both *B. caballi* and *T. equi* organisms (de Waal, 1992; APHIS, 2008). In the present study, apparent tick infestations were observed only on a few horses during the sampling time indicating that potential tick vectors are present at least in some sampled regions. One of the explanations for the difference in prevalence rates of the two species may be that the tick vector(s) of *T. equi* are different from those transmitting *B. caballi*, which would thus be virtually absent in most areas sampled by us. However, no data are currently available to support this notion. A more complete survey of different tick species on Hungarian horse farms is required to increase our knowledge of the tick vectors of EP. There

are some other explanations for the higher prevalence of equine theileriosis. It may be associated with the longer persistence of *T. equi* than *B. caballi* after infection. The higher prevalence of theileriosis may also be due to the higher parasitaemia in the infected horses which increases the risk of the infection of tick vectors. Further on, transplacental transmission of *T. equi* – not documented in case of *B. caballi* – may also contribute to this phenomenon (Allsopp et al., 2007; Georges et al., 2011). However, this route of infection should be also taken into account if abortion can occur amongst mares infected with *T. equi* (Potgieter et al., 1992; de Waal, 1992).

No significant correlation was found between the seropositivity and the age of horses when the data were analysed with logistic regression. This result is in accordance with most other studies (Shkap et al., 1998; Karatepe et al., 2009; Grandi et al., 2011; Amir et al., 2012). However, other studies found a positive correlation between increased age and positivity, which supports the claim that infection with *T. equi* is life-long (Rüegg et al., 2007; Sevnic et al., 2008; Kouam et al., 2010). When the continous variable age was converted to two categories (under and over 5 years old) in this study, significant association between PCR results and age classes was found by Fisher's Exact test. The younger horses had three times greater odds to be PCR positivity than the older horses. This finding indicates that more younger horses become infected with *T. equi* and harbour the parasites than older animals which remain seropositive but the parasites circulate in fewer of them. However, we do not believe that *T. equi* is a new, emerging problem in Hungary because younger horses are more likely to be positive. As in some previous studies, gender was not found to be a risk factor for *T. equi* infection (Karatepe et al., 2009; Grandi et al., 2011; Amir et al., 2012). Other authors did find a correlation between gender and positivity (Shkap et al., 1998; Rüegg et al., 2007; Sevnic et al., 2008; Moretti et al., 2010), although their results were often inconsistent. Shkap et al. (1998) found that stallions were significantly less affected than mares and geldings in Israel, but this was due to the fact that stallions, maintained under strict control for breeding were less exposed to tick infestation.

It is unknown how long *T. equi* has been persisting in Hungarian horses. One possibility would be that *T. equi* has been already present for a long time. It might be possible that the local tick vectors and/or host species acquired the parasites from horses that arrived in the country with armies during the Second World War. This protozoan species might have been recently introduced after importing seemingly healthy but infected horse(s) from abroad because there are not any special policies of Hungary for checking the imported horses with regard to *T. equi* infection. The parasite could spread by horses within the country because most infected horses are apparently healthy without any obvious clinical signs. Often clinical signs of *T. equi* infection in horses are too general to allow specific diagnosis and may be confused with other diseases especially in endemic areas. Therefore the correct diagnosis and treatment because no safe and effective sterilization regimen of *T. equi* exists compared to *B. caballi* (de Waal and van Heerden, 2004; Bruning, 1996).

In conclusion, the results of the first serological and molecular survey of *T. equi* infection of Hungarian horses indicate that the parasite is subclinically present in many geographical areas of the country. Although the sampled horses were asymptomatic, some of these animals act as parasite carriers for tick transmission and facilitate the spread of these agents because the parasites could be detected in their blood samples with PCR. Based on these findings a closer look on the management is needed, including tick control, where the seropositive horses are present to prevent the spread of the parasite to susceptible animals. Further studies of the vector species are also needed in the country because no information is available about those tick species which are the most common vectors of *T. equi*. Considering the fact that equine piroplasmosis caused by both parasite species is a notifiable disease for the Office International des Epizooties and may cause great economic losses, improved and long-term monitoring of the prevalence of infection is necessary.

Conflict of interest

The authors have no conflicts of interest concerning the work reported in this paper. None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

Disclosure statement

No competing financial interests exist.

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