

## SEROLOGICAL EVIDENCE FOR *BABESIA CANIS* INFECTION OF HORSES AND AN ENDEMIC FOCUS OF *B. CABALLI* IN HUNGARY

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In order to evaluate the seroconversion of horses to *Babesia caballi* and *B. canis* in Hungary, blood samples were collected from 371 animals on 23 different locations of the country. The presence of antibodies to *B. caballi* was screened with a competitive ELISA. All 29 positive samples came from one region (the Hortobágy). The prevalence of infection did not show correlation with sexes, and reached 100% in the age group of 2–5 years. *Babesia canis*-specific antibodies were demonstrated by IFAT in 6.74% of animals kept in 7 regions. The titres were low or medium level (1:40 to 1:160), indicating that the horses had previously been exposed to this piroplasm, but their infection must have been limited. The highest seropositivity rate was observed in the age group of 3–4 years, and males (stallions and geldings) were significantly more frequently infected than females. However, neither *B. caballi* nor *B. canis* could be identified in the peripheral blood samples of infected horses by PCR. Since most of the *B. caballi*-positive horses remained negative in the *B. canis* IFAT, whereas seroconversion solely to *B. canis* was detected in several regions of the country, serological cross-reaction between the two species can be discounted. This is the first serological evidence of horses being naturally infected with *B. canis*, supporting the view that piroplasms are less host specific than previously thought.

**Key words:** Horse babesiosis, *Babesia canis*, *Babesia caballi*, seroprevalence, Hungary

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*Babesia caballi* is the large piroplasm of horses, which infects red blood cells. It is transmitted by hard tick species belonging to the genera *Dermacentor*, *Rhipicephalus* and *Hyalomma* (de Waal, 1992). According to the geographical habitats of these vectors, equine babesiosis has a worldwide distribution, occurring particularly in the tropical or subtropical climate (Butler et al., 2005). On the other hand, during the past decades it has been showing a tendency to become more prevalent in the temperate zone (Friedhoff and Soule, 1996). Thus, in Europe it is endemic to France (Leblond et al., 2005), Portugal (Bashiruddin et al., 1999), Spain (Camacho et al., 2005) and Italy (Savini et al., 1997). Imported cases – sometimes suggestive of establishment and autochthonous infection – were also reported from other countries such as the UK (Joyner et al., 1981), Switzerland (International Collating Centre, 1997), Germany (Zahler and Gothe, 2000), Belgium (Mantran et al., 2004) and the Netherlands (Butler et al., 2005).

Equine babesiosis is especially relevant from the point of view of international animal trade. In certain northern countries it was introduced several times from endemic areas more to the south (Zahler and Gothe, 2000; Butler et al., 2005). In the United States, Canada, Australia and Japan the prerequisite for the import of any horses is seronegativity for *B. caballi* (Friedhoff et al., 1990). Equine piroplasmosis is also a List B disease of the Office International des Epizooties (OIE), notifiable within 72 hours. Therefore, long-term monitoring of the prevalence of infection is very important in each area where it has been present.

In Hungary *B. caballi* was first described in horses with clinical signs of acute babesiosis in the Hortobágy (Buza et al., 1953; Buza et al., 1955). However, no data on the status of the disease (whether or not it became endemic) have since been reported in this country. Therefore, to evaluate the current seroprevalence of *B. caballi* infection, blood samples were collected in various regions of the country.

Recent data based on molecular epidemiological studies suggest that piroplasms are not as host specific as previously thought (Criado et al., 2006). Since *B. canis* was also identified in a horse in Spain (Criado-Fornelio et al., 2003), and the formerly restricted endemicity of this piroplasm (Hornok et al., 2006) and the distribution of its vector (Sréter et al., 2005) were demonstrated to expand in Hungary, another aim of the present study was to screen the same samples for seroconversion to *B. canis*.

## Materials and methods

### *Animals and sampling places*

Horses for this study were chosen from areas where equine babesiosis had occurred previously (the Hortobágy), where relevant clinical signs (mainly haemoglobinuria) were noticed over the past few years by the local veterinarians,

or tick infestation of horses and canine babesiosis were reported. In this way the survey focused around Western and Northeastern Hungary (Fig. 1), including altogether 371 horses (150 stallions/geldings and 221 mares) on 23 different locations. Two blood samples per animal were collected with jugular venipuncture (one without anticoagulant for serology and another containing EDTA for molecular biology). Serum and blood were stored at  $-20^{\circ}\text{C}$  until evaluation. The local veterinarians were consulted for relevant clinical signs.

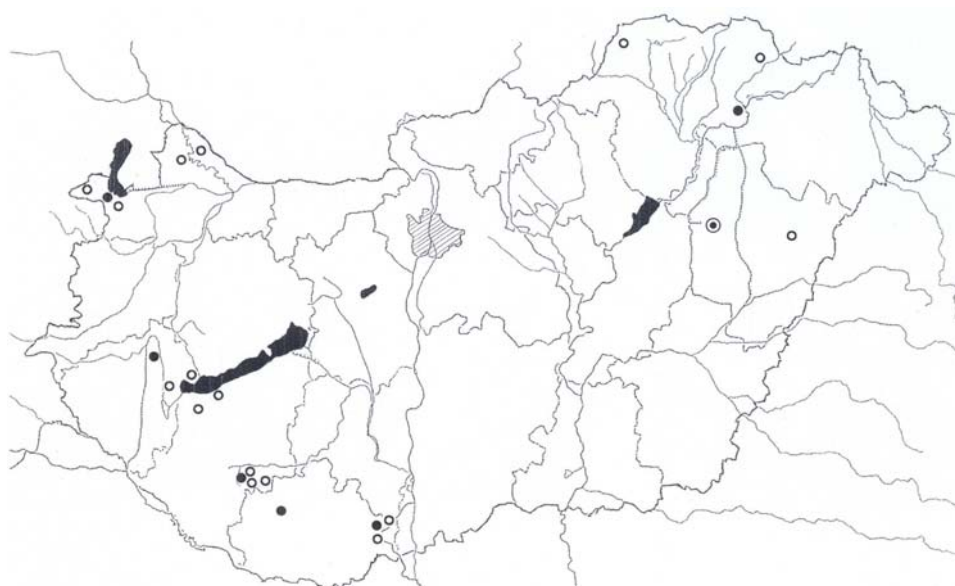


Fig. 1. Map of Hungary showing sampling sites where no seropositive animals were found (empty dots), where seroconversion to *B. canis* was detected (filled dots), and where horses were found to be infected with both *B. canis* and *B. caballi* (circled dot)

#### Serology for *B. caballi*

A competitive enzyme-linked immunosorbent assay (cELISA) was performed with each sample using the *Babesia caballi* Antibody Test Kit from VMRD Inc. (Pullman, WA, USA) following the manufacturer's instructions. This assay detects serum antibodies to a rhoptry-associated protein (RAP-1) of *B. caballi* (Kappmeyer et al., 1999). Optical density (OD) values were determined using an automatic UVmax kinetic microplate reader (Molecular Devices Corporation, Palo Alto, CA, USA), and the percentage of inhibition was calculated as follows:  $I (\%) = 100 - (\text{sample OD} \times 100) / (\text{mean OD of three negative controls})$ . Samples with an inhibition  $\geq 40\%$  were regarded as positive.

### *Serology for B. canis*

To evaluate the seroconversion to *B. canis* an indirect fluorescent antibody test (IFAT) used for canine babesiosis was adapted from the standard operating procedures (SOP S020-1) of the Institute of Parasitology and Zoology (Department of Pathobiology, University of Veterinary Medicine, Vienna, Austria). In brief, all sera were screened at dilutions of 1:20 and 1:40 in PBS. Fluorescein isothiocyanate (FITC) -labelled sheep anti-horse IgG (heavy and light chains) immunoglobulins (The Binding Site, Birmingham, UK) were used as conjugate, at a dilution of 1:30 in PBS. The conjugate dilution for horses was optimised with *B. caballi* antigen and control sera (courtesy of Dr. C. Epe, Institute of Parasitology, College of Veterinary Medicine, Hanover, Germany). Serum samples showing fluorescence at the dilution of 1:20 were further titrated using 2-fold serial dilutions. Horses were considered seropositive to *B. canis* when having titres of 1:20 or higher. This cut-off value was the same as that determined for dogs.

### *PCR to detect B. caballi*

DNA was isolated from 200 µl of EDTA blood using QIAamp DNA blood mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The PCR was performed with primers PIRO-A1 and PIRO-B as described previously (Földvári et al., 2005).

### *PCR to detect B. canis*

The method is based on the protocol of the Institute of Parasitology (Department of Pathobiology, University of Veterinary Medicine, Vienna, Austria). DNA was extracted from 200 µl EDTA blood with the High Pure PCR Template Preparation Kit (Roche) as described by the manufacturer. To evaluate the presence of *B. canis*, primers BAB C2 (5'-CCGAATTCTTTGTGAACCTTATCA-3') and BAB C1 (5'-CGGGATCCTTCACTCGCCGTTACT-3') were used with an expected product length of 871 bp. One µl of extracted DNA was added to a 20 µl reaction mixture containing 0.75 units of Promega Taq polymerase, 1.5 mM MgCl<sub>2</sub> Taq buffer, 10 pmol of each primer and 200 µM of each dNTP. Amplification was performed using a T-Gradient Cycler (Whatman-Biometra GmbH, Göttingen, Germany). An initial denaturation step at 94 °C for 5 min was followed by 37 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s and extension at 72 °C for 1 min. Final extension was done at 72 °C for 7 min. Conventional gel electrophoresis (2% agarose) was performed, followed by digital photography under UV light and analysis of the results using the GelWorks software.

### *Clinical laboratory procedures*

For haematology and biochemical analysis fresh heparinised and EDTA blood was drawn from 6 horses which were identified as seropositive to either *B. caballi* or *B. canis*, and their *Theileria equi* infection was excluded by serology and PCR (data not shown). Haematological values were determined using an Abacus haematology analyser (Diatron GmbH, Vienna, Austria), and biochemical parameters were measured by an automatic spectrophotometer (RX Daytona, Randox Laboratories Ltd., Crumlin, UK).

### *Statistical analysis*

Exact confidence intervals for the prevalence rates were calculated according to Sterne's method. Data were compared by using Fisher's exact test, and differences were considered significant when  $P \leq 0.05$ .

## **Results**

Twenty-nine samples showed positivity in the *B. caballi* cELISA: all of them originated from one region, the Hortobágy (Fig. 1). The inhibition values of samples from 4 animals (3 females and 1 male) were below 60%, those of 19 horses (7 females and 12 males) were in the interval of 60–80%, and those of 7 further ones (2 females and 5 males) exceeded 80%. Although more females had serum samples with inhibition values below 60% (3 out of 12, 25%) than males (1 out of 17, 5.9%), and more males had samples with inhibition values above 80% (5 out of 17, 29.4%) than females (2 out of 12, 16.7%), these differences were not significant ( $P = 0.278$  and  $P = 0.664$ , respectively). Similarly, the overall percentage of seropositivity had no significant association with sexes. In the group of 1-year-old foals the prevalence was 18% (7 out of 39), whereas in the age group of 2–5 years it was 100% (22 out of 22). Four older horses in the same herd had no detectable levels of antibodies to *B. caballi*.

*Babesia canis*-specific antibodies were present in 25 (6.74%; 95% confidence interval: 4.4%–9.8%) out of the 371 horses sampled, on 7 different locations (Fig. 1). Six, 8, 6 and 5 horses had IFAT titres of 1:20, 1:40, 1:80 and 1:160, respectively. There was no correlation between the age or sex of the affected animals and their level of seroconversion. More male horses, including stallions and geldings (18 out of 150, 12%), had antibodies to *B. canis* than females (7 out of 221, 3.2%), which was a significant difference ( $P = 0.001$ ). Positive samples were most frequently obtained from 3–4 years old horses, and were evenly distributed in the other age groups (Fig. 2).

Ten horses had concurrent *B. caballi* and *B. canis* infection. Out of the remaining 34 seropositive horses 19 were infected only with *B. caballi* and 15

others only with *B. canis*. Regarding these, demonstration of the former piroplasm from the blood of 9 animals (which were selected on the basis of seronegativity to *T. equi* – data not shown), and of the latter piroplasm from samples of 11 others (which had a titre of 1:80 to 1:160 in the *B. canis* IFAT) was not successful with PCR.

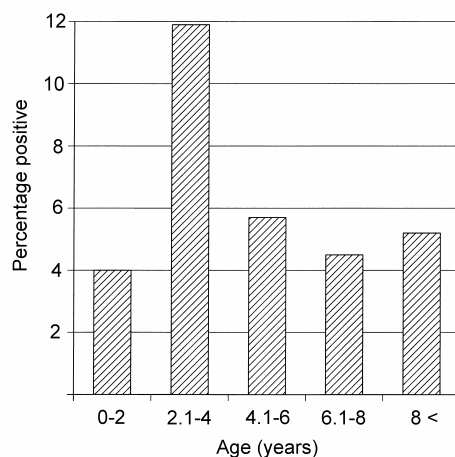


Fig. 2. Seropositivity to *B. canis* in different age groups of horses

**Table 1**

Haematological and biochemical parameters of six horses that showed seroconversion to *B. caballi* or to *B. canis* (and their *T. equi* infection was excluded)

	Normal values	Foal A	Foal B	Foal C	Horse A	Horse B	Horse C
Keeping place		H	H	H	B	B	B
Seroconversion to <i>B. caballi</i>		+	+	-	-	-	-
Seroconversion to <i>B. canis</i>		-	-	+	+	+	+
MCH	12–20 pg	11.0	10.4	11.4	15.8	16.1	15.8
MCHC	310–400 g/l	298	304	311	346	330	345
Thrombocytes	100–400 G/l	81	98	77	155	158	62
ALP	150–320 U/l	769	1114	1062	687	487	501

H: Hortobágy; B: Bükkösd; MCH: mean corpuscular haemoglobin; MCHC: mean corpuscular haemoglobin concentration; ALP: alkaline phosphatase. Only those values are shown which were below or above the normal values at least in two infected animals

Seropositive horses (to either *B. caballi* or *B. canis*) had no apparent clinical signs at the time of sampling, except for one animal having a titre of 1:40 against *B. canis*. This horse showed fever, anorexia, salivation, had bilirubinaemia (72 µmol/l, normal value < 45 µmol/l), recovered after treatment with imidocarb (Imizol), but was also negative in the PCR. Some haematological values

and biochemical parameters of six further animals are shown in Table 1. A mild reduction of mean corpuscular haemoglobin (MCH) and its concentration (MCHC) could be observed in certain horses (in two horses infected by *B. caballi* and in one horse infected by *B. canis*), and four of these animals had low thrombocyte counts. All examined horses showed prominently elevated alkaline phosphatase (ALP) values.

### Discussion

This is the first report on the prevalence of *B. caballi* infection in Hungary. Equine babesiosis was exclusively found in the same area (Hortobágy) where it had appeared more than a half century before the present survey was conducted (Buza et al., 1953), and where its status was hitherto unknown. Of the vectors of *B. caballi* *D. reticulatus* and *D. marginatus* occur in the country (Babos, 1965). Horses in the relevant herds are grazing free until approximately two years of age when they are stabled and regularly ridden. Thus, there are ample occasions (mostly as foals, but also later) to have contact with ticks, which explains that the prevalence of *B. caballi* infection was relatively low among 1-year-old foals, whereas it became 100% in the 2–5 years old animals. Antibodies to this piroplasm were not detectable with the cELISA in the four oldest horses, which does not mean that they did not have babesiosis, as formerly infected animals may clear these parasites in 2–3 years and become seronegative (Holman et al., 1993).

*Babesia caballi* was not found with PCR in the seropositive animals examined, since – except for the acute phase of the disease – this piroplasm is extremely scarce in the peripheral blood (Frerichs et al., 1969), and horses tend to enter a carrier state entailing a complete absence of parasites in the circulation (Holman et al., 1993). The establishment of a carrier state during chronic babesiosis also implies that ticks are less likely to become infected and to transmit babesiae from such horses, which might explain the stability of the endemic focus for decades. The opposite was observed in case of *T. equi* that had a more widespread (epidemic-type) distribution when compared to the restricted occurrence of *B. caballi* in the same region (Mancianti et al., 2000), partly as a consequence of higher and more persistent parasitaemia (Mehlhorn and Schein, 1998).

This is also the first report on the naturally acquired seroconversion of horses to *B. canis*. Formerly this piroplasm was demonstrated by molecular biological tools from a horse showing clinical signs (Criado-Fornelio et al., 2003). Similarly, in the present study seroconversion of horses to *B. canis* indicates previous exposure to the parasite. On the other hand, low to medium-level titres can be a consequence of a transient infection or restricted development in horses as unusual hosts. Seropositive animals were identified in different regions of the country, as the vector, *D. reticulatus*, and the causative agent have become more

prevalent in the past few years (Sréter et al., 2005; Hornok et al., 2006), suggesting an increased selective pressure for horses to become infected. However, the presence of *B. canis* could not be confirmed with PCR in peripheral blood samples of the seropositive horses examined, most likely due to the clearance of these parasites from the circulation (just like in the case of *B. caballi*). Higher incidence of seropositivity among male horses than females, and in the age group of 3–4 years, may have been influenced by more frequent (preferential) usage of certain animals for riding according to sex or age, thus increasing the chance for contact with potential vectors.

Some haematological and biochemical parameters in the present study suggest pathogenic effects that may be attributed to *B. canis*, as they are similar to those elicited by *B. caballi*. Equine babesiosis was reported to induce hyperbilirubinaemia (Allen et al., 1975a) and decrease in platelet counts (Allen et al., 1975b). The involvement of *B. canis* in such changes is further substantiated by the successful imidocarb treatment of a seropositive horse that showed clinical signs. The significant increase of ALP activity in all seropositive horses examined indicates liver damage as it was observed during *B. caballi* infection (Camacho et al., 2005), even when taking into account that foals have more elevated ALP values than adults (Price et al., 1995). To minimise the contributory role of other factors (nutritional anomaly, concomitant infections) to the above changes, horses from two locations were evaluated.

Detectable and significant serological cross-reaction between the two species can be excluded, since most of the *B. caballi*-positive horses remained negative in the *B. canis* IFAT, while seroconversion to *B. canis* was detected in several regions of the country, where horses were negative in the *B. caballi* cELISA. This means that phenotypic (antigenic) differences between these two piroplasms allow species-specific diagnosis despite existing concomitant infections. On the other hand, *B. canis* and *B. caballi* are genetically closely related (Baneth et al., 2004), and the degree of polymorphism between *B. caballi* and *B. canis* isolates is similar to that between the three subspecies of *B. canis* (Zahler et al., 1998); therefore, cross-infection of their hosts is not unlikely. The tick species most frequently found on horses and the vector of *B. caballi* in Hungary is *D. marginatus* (Babos, 1965) which can transmit *B. canis* (Pavlovic et al., 2002). *Dermacentor reticulatus*, the main vector of *B. canis*, can also be found on horses (Babos, 1965) and can transmit *B. caballi* (de Waal, 1992). This is further substantiated by concurrent *B. caballi* and *B. canis* infection in more than 30% of the seropositive horses in our study.

The present results confirm that the pathogenic role of *B. canis* in horses needs to be investigated further.



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