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Letter to the Editor

Molecular detection of *Theileria equi* and *Babesia caballi* in the bone marrow of asymptomatic horses

1. Introduction

Equine piroplasmiasis is a tick-borne intra-erythrocytic protozoal disease caused by *Babesia caballi*, *Theileria equi* (Mehlhorn and Schein, 1998) or even by a mixed infection. Although the disease is endemic to (sub) tropical regions of the world, piroplasmiasis has been reported in many countries and continents thus making this disease a cause of great concern in the global horse industry. As such, early identification of the disease and its subsequent treatment would be important in controlling the spread of this disease.

Current diagnosis of equine piroplasmiasis relies on different serological tests such as: an enzyme-linked immunosorbent assay (Hirata et al., 2002), an indirect fluorescent antibody test (Asgarali et al., 2007; Hirata et al., 2005) immunochromatographic tests (Huang et al., 2004), or alternatively on identification of the agent by microscopic examination (Bose et al., 1995) or molecular tools (Nagore et al., 2004; Nicolaiewsky et al., 2001; Rampersad et al., 2003). Many PCR methods have been described recently including single round and multiplex PCR, to allow simultaneous identification of both *B. caballi* and *T. equi* (Alhassan et al., 2005), and real-time PCR (Heim et al., 2007; Kim et al., 2008).

To date, up to 12 species of Ixodidae ticks have been identified as vectors of both *B. caballi* and *T. equi* organisms. In utero infection by *T. equi* was suspected as an alternative source of the disease, in foals born with no overt clinical signs (Phipps and Otter, 2004); transplacental transmission from carrier mares to asymptomatic foals was recently confirmed (Allsopp et al., 2007). The clinical signs of this disease are not pathognomonic and may vary considerably between horses. Acute and sub-acute cases are the most commonly observed. The horse may exhibit apathy, lethargy, fever, anaemia, icterus, weight loss and oedema of distal limbs, leading, in some cases, to death (Knowles, 1996). The presence of both parasites (*T. equi* and *B. caballi*) may also persist in clinically recovered horses for at least several years and thus acting as reservoirs for subsequent infecting ticks (Holbrook, 1969). However, little is known whether these horses suffer from a chronic subclinical form of piroplasmiasis or merely represent asymptomatic carriers of the protozoans. Unlike experimental conditions in which *B. caballi* infection can be

resolved (Schwint et al., 2009), treating such horses in naturally occurring infections with either, buparvaquone against *T. equi* (Zaugg and Lane, 1989) or, high-doses of imidocarb against *T. equi* and *B. caballi* (Butler et al., 2008) shows no consistent effectiveness. Therefore, the precise mechanism for survival and the localisation of these persistent protozoans still remain to be determined.

The aim of the present paper is to report the detection of *T. equi* and *B. caballi* by PCR analysis in the bone marrow of clinically healthy horses.

2. Materials and methods

2.1. Study design

Between January 2008 and May 2009, bone marrow was harvested from the sternebrae of 35 horses that were being used for orthopaedic clinical research purposes (stem cell therapy). The study was approved by the ethical committee of Alfort National Veterinary School (France). Prior to surgery, each horse underwent a thorough clinical examination in order to insure that no obvious clinical abnormality was present. The surgical procedure was performed according to a previously described technique (Smith et al., 2003). All bone marrow samples were kept at room temperature in EDTA and shipped within 6 h to the laboratory for a complete standardised evaluation in order to exclude the presence of contagious micro-organisms.

2.2. DNA extraction and PCR amplification

One hundred microliter samples of bone marrow were diluted in the same volume of phosphate-buffered saline (PBS) and DNA was then extracted through the use of a QIAamp DNA Blood Mini Kit, according to the manufacturer's instructions (Qiagen, Hilden, Germany). The nucleotide sequences of the primers used in this study and the three different PCR amplifications (Table 1) were performed according to previously published procedures (Alhassan et al., 2005; Battsetseg et al., 2001; Kim et al., 2008). For each batch of samples, a negative control (H₂O) and "field" positive controls (*T. equi* and *B. caballi* nucleic acid extracts) were included.

2.3. Cytological examination

A qualitative evaluation was performed on Giemsa-stained bone marrow smears, by light microscopy ($\times 100$

Table 1
Primer sequences and PCR conditions for detection of *T. equi* and *B. caballi* DNA in horses.

PCR method	Target organisms	Amplicon size (bp)	Primer and probe designation	Sequence 5'–3'	Cycling conditions
Classical (Alhassan et al., 2005)	<i>Babesia</i> spp.	867–913	Bec-UF1 Bec-UR	GTTGATCCTGCCAGTAGTCA CGGTATCTGATCGTCTTCGA	5 min/95 °C 1 min/96 °C 1 min/60.5 °C 1 min/72 °C 10 min/72 °C
	<i>Theileria equi</i>	392	Bec-UF2 Equi-R	TCGAAGACGATCAGATACCGTCC TGCCTAAACTTCCTTGGCAT	×40
Real time (Kim et al., 2008)	<i>Theileria equi</i>	81	Be18SF Be18SR Be18SP	GCGGTGTTTCGGTGATTGATA TGATAGGTCAGAACTTGAATGATACATC AAATTAGCGAATCGCATGGCTT	10 min/95 °C 20 s/95 °C 1 min/55 °C
Classical (nested) (Battsetseg et al., 2001)	<i>Babesia caballi</i>	454	BC48-F1	ACGAATTCACACAACAGCCGTGTT	2 min/98 °C 30 s/94 °C 30 s/55 °C 1 min/72 °C
			BC48-R3 BC48-F11	ACGAATTCGTAAGCGTGCCATG GGCGACGTGACTAAGACCTTAT	×35
		7 min/72 °C 2 min/98 °C 30 s/94 °C 30 s/55 °C	BC48-R31	GTTCTCAATGTCAGTAGCATCCGC	1 min/72 °C 7 min/72 °C
			×35		

Table 2
Diagnosis of piroplasmosis in bone marrow of asymptomatic horses.

Animal investigated	Sampling date	PCR performed		
		Real-time <i>T. equi</i> (Ct value)	<i>T. equi</i> /Babesia spp.	<i>B. caballi</i>
Horse 1	17/06/2008	+ (34)	+/+	–
Horse 2	10/02/2009	+ (29)	+/+	–
Horse 3	10/02/2009	+ (31)	+/+	+

(+) Positive sample; (–) negative sample.

magnification), in order to determine the presence, or not, of either *Theileria* or *Babesia*.

3. Results

The bone marrow of three clinically healthy horses tested positive for piroplasmosis (Table 2). None of the horses had previously been treated with imidocarb during, at least, the preceding twelve months. *T. equi* was detected in Horse 1 using both classical and real-time PCR procedures. Bone marrow smears from this horse, which were evaluated subsequently by light microscopy, also confirmed the presence of the parasite. Again, using both PCR methods, the bone marrow sample of Horse 2 was also found to be positive for *T. equi*. Horse 3 tested positive for both *T. equi* and *B. caballi* as demonstrated by each procedure performed. Unfortunately, blood samples from these three different horses were unavailable for analysis.

4. Discussion

To our knowledge, this paper is the first to report on the identification of both *T. equi* and *B. caballi* in the bone marrow of naturally infected and asymptomatic horses. However, this unexpected finding is in accordance with a recent study, in which the post-mortem presence of *T. equi* in bone marrow and in many other equine tissues was identified, following the acute phase of experimentally induced piroplasmosis (Alhassan et al., 2007). The observa-

tions of these latter authors suggest a vascular dissemination of the parasite to virtually all the host organs during the acute clinical phase of the disease. Detection of *Babesia* sp. by molecular and microscopic means was also reported in the bone marrow of an immunosuppressed dog that exhibited clinical and haematological signs compatible with piroplasmosis (Birkenheuer et al., 2004).

Even if the pathogenesis of *T. equi* and *B. caballi* within the equine host are slightly different, the acute phase of the disease is characterised for both parasites by their multiplication within red blood cells, which may subsequently rupture and release merozoites, thus allowing the temporal infection of other cells (Ali et al., 1996). In the chronic condition, parasites may remain present durably in asymptomatic carrier horses (Holbrook, 1969). Nonetheless, the long-term localisation of these parasites, as well as the underlying mechanisms of their persistence, still remains unknown.

Although frequently looked for, microscopic detection of the parasites in blood samples from asymptomatic carrier horses is not a constant finding (Bose et al., 1995). The sensitivity of the PCR assay is higher than that of a classical microscopic examination (Rampersad et al., 2003). Therefore, since parasites in the bone marrow were also observed in the present study at the microscopic level, the hypothesis of false positive results due to a possible lower specificity of PCR assays can be rejected. This argument is also reinforced by the findings of the PCR amplification of three different genome fragments.

Since the active duration of a red blood cell is about five months (Marcilese et al., 1966) and since parasites may persist in asymptomatic carrier horses for several years, a recurrent low-grade erythrocytic multiplication may be suspected. Conversely, since bone marrow is a precursor of red blood cells, it should not be excluded as one of the potential reservoir sites of *T. equi* and *B. caballi* in clinically healthy horses.

In conclusion, further studies including long-term investigations after experimentally induced infection, are required in order to understand the mechanisms that lead to the chronic and asymptomatic form of piroplasmiasis in horses. The possible presence and persistence of *T. equi* and *B. caballi* parasites in equine bone marrow should be thoroughly considered in order to develop a fully effective treatment for dealing with these naturally occurring infections.

Conflict of interest statement

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.

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