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Evidence of transplacental transmission of equine piroplasms *Theileria equi* and *Babesia caballi* in an Italian breed mare

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Abstract: Equine piroplasmosis (EP) is a vector borne disease caused by apicomplexans protists *Babesia caballi* (Nuttal et Strickland, 1910) and *Theileria equi* (Laveran, 1901). Carrier mares may transmit the infection transplacental resulting in neonatal piroplasmosis or abortions. This event has been described for *T. equi* by several authors over the world, but no evidence for *B. caballi* has been reported in Europe. In this study, vertical transmission for both parasites in an Italian breed mare has been confirmed using molecular and microscopic tools. Transplacental transmission is an underestimated problem mainly in endemic areas as it not only contributes to the spread and maintenance of the infection, but also produces significant economic losses.

Keywords: abortions, vertical transmission, serological assays, polymerase chain reaction

Equine piroplasmosis (EP) is a tick-borne disease caused by *Babesia caballi* (Nuttal et Strickland, 1910) and *Theileria equi* (Laveran, 1901) that affects horses, mules, donkeys and zebras. Both parasites are transmitted by ticks of the genera *Dermacentor* Koch, *Rhipicephalus* Koch and *Hyalomma* Koch (Wise et al. 2013). This disease is globally distributed and causes peracute, acute, subacute and chronic forms (WOAH 2011). Diagnosis can be performed by stained blood identification, serological tests such as complement fixation test, indirect fluorescent antibody test (IFAT), enzyme-linked immunosorbent assay (ELISA) or polymerase chain reaction (PCR) methods.

EP causes economic losses and it is a major constraint to the international movement of equines (Wise et al. 2013). Transplacental transmission of *T. equi* has been reported in horses from different continents and in Brazilian mules (Françoso et al. 2018), whereas no evidence has been provided for *B. caballi* in Europe (Sant et al. 2016). *In utero* infections may result in abortions, stillbirths, or live foals with neonatal piroplasmosis (Allsopp et al. 2007). Foals born to infected mares are naive at birth and acquire antibodies through the colostrum intake (Kumar et al. 2008). Therefore, direct methods are needed to prove vertical transmission. Here, we describe a case in which transplacental transmission of *T. equi* and *B. caballi* was demonstrated using several molecular methods.

MATERIALS AND METHODS

Anamnestic data

The mare was a five years old Italian heavy draft horse living semi-feral in Rieti Province (Latium Region). She suffered an abortion during the last trimester. Only unspecific symptoms (lethargy, mild fever) were correlated to the abortion. No specific treatment was applied. The foetus was submitted to determine the cause of the abortion. During the necropsy, main findings were: petechiae in the small intestine and in the epicardium, sero-haemorrhagic pericardial effusion, liver and renal degeneration, and congestion in both kidneys, meninges and brain. However, histological examination of haematoxylin and eosin-stained sections of brain and heart did not reveal any significant lesions.

The foetus tested negative for other infectious pathogens such as equine viral arteritis, equine herpesvirus 1 and 4, *Leptospira* spp., *Toxoplasma gondii* (Nicolle et Manceaux, 1908) and *Neospora caninum* Dubey, Carpenter, Speer, Topper et Uggla, 1988 and *Coxiella burnetti* using PCR methods, and *Salmonella* spp., and Enterobacteriaceae using bacterial culture tests. Due to the breeding system, it was difficult to follow up the case or collect more samples. It was only possible when the herd was moved back from the mountains to lower pastures.

Samples and preparation

Foetal heart clot was extracted and stored at -20°C. Organs (foetal heart, liver, spleen and lung) were stored at -80°C until pro-

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Table 1. Results of direct tests (smear and PCR protocols) used for the detection of equine piroplasmosis. Accession numbers in bold are the selected sequences submitted to GenBank®; in italics, percent identity and accessions numbers after sequence analysis using BLAST (Basic Local Alignment Search Tool); query coverage was always over 96%. NP not performed; + positive result; - negative result).

	Smear	Real time *Theileria equi*	EMA 5/6 *T. equi*	RLB		Real time *B. caballi*
				T. equi	*Babesia caballi*	
Foetal clot	+	*97% JX177673*	**KU923592**	**KU92346**		-
Foetal liver	NP	*96% JX177673*	*99% JQ782603*	*100% KC465785*		*100% MT355491*
Foetal spleen	NP	*96% JX177673*	*99% JQ782603*	*100% KC465785*		*100% MF120936*
Foetal heart	NP	*95% JX177673*	*99% JQ782603*	*99% KC465785*		-
Foetal lung	NP	*95% JX177673*	*99% JQ782603*	*99% KC465785*		-
Mare	+	*100% AB733379*	-	**KU923628**	*99% AY534883*	*100% MF120936*

cessed. Blood samples were collected from the mare jugular vein and kept in serum and EDTA tubes. Sera (from blood and foetal clot) were obtained by centrifugation at 3,580 g for 10 minutes, then stored at -20°C and thawed at 37°C immediately before testing.

Microscopical examination

Thin smears from blood were prepared and stained with Diff Quick and then observed microscopically (1,000×) to determine the presence of intracellular parasites.

Serological tests

Indirect fluorescent antibody test (IFAT) was used for the detection of specific IgG antibodies against *Theileria equi* and *Babesia caballi* infections. Tests were carried out according to the manufacturer's instructions (Fuller Laboratories, Fullerton, California, USA). Samples with a strong fluorescence at a dilution of 1 : 80 were considered positive. Two commercial competitive enzyme-linked immunosorbent assays (cELISA): *Babesia equi* Antibody test kit VMRD® and *B. caballi* Antibody test kit VMRD® were used according to manufacturer's instructions.

PCR amplification protocols and sequencing

DNA extraction from blood was conducted using QIAamp DNA Blood Mini kit (Qiagen, Hilden, Germany) following the protocol described by the manufacturers. The DNA was eluted in 200 µl of buffer AE. DNA yield was determined with a spectrophotometer (Eppendorf BioPhotometer, Eppendorf AG, Hamburg). To extract DNA from the organs, the same protocol was used adding one previous step: 100 milligrams of organ in 600 µl of 1X PBS were placed in a FastPrep tub and then homogenise in a FastPrep FP120 Cell Disrupter (Thermo Electron, Waltham, Massachusetts, USA) twice 5 min at 12,000 rpm. Supernatant was collected and use to assess DNA extraction.

Real time PCR 18S rRNA of *B. caballi* amplified a 95 bp fragment in the V4 hypervariable region of 18S rRNA gene of *B. caballi*. Primers and probe employed (F: Bc-18SF402; R: Bc18SR496; Probe: TaqMan MGB™ probe FAM-MGB, Bc-18SP) were those reported in the literature (Bhoora et al. 2010).

The real rime 18S rRNA *T. equi* amplified an 81 bp fragment in the V4 hypervariable region of the 18S rRNA gene. Primers and probe employed (F: Be18SF; R: Be18SR) TaqMan probe (VICTAMRA, Be 18SP) were those reported in the literature (Kim et al. 2008). For both Real Time PCRs, TaqMan® Universal PCR Master Mix kit (Applied Biosystems, Foster City, CA, USA) was used. Positive controls were constituted by the plasmid vectors pCRII®-TOPO TA Cloning® Invitrogen, Carlsbad, CA, USA) in which the targets of the real time PCR *T. equi* and *B. caballi* have been cloned. All real time PCRs were carried out using ABIPRISM 7900 HT Sequence Detection System (Applied Biosystems).

The target for EMA end point PCR detecting *T. equi* was a 268 bp internal fragment at the merozoite antigen 1 (EMA-1) gene coding for a major parasite surface antigen and the primers used were EMA-5/EMA-6 (Battsetseg et al. 2002). Another nested PCR protocol that amplifies the hypervariable V4 region of the 18rRNA gene of the genera *Theileria* and *Babesia* was also carried out. This protocol has products of approximately 430 bp and 390 bp for species of *Theileria* and *Babesia* respectively, and the primers used are RLB F1/ RLB R1, RLB F2/RLB R2 (Nagore et al. 2004). In order to avoid non-specific reactions, the thermal profile was optimised by changing the annealing temperature from 51°C indicated in the literature to 54°C.

AmpliTaq Gold® DNA Polymerase (Applied Biosystems, Life Technologies, Austin, TX, USA) to prepare the master mix and the GeneAmp® PCR System 9700 (Applied Biosystems, Foster City) were used in all protocols. PCR products were visualised after electrophoresis in a 1.5% Tris-Boric Acid-EDTA agarose gel, stained with GelRed 10,000X (Biotium, Hayward, CA, USA) and recovered from agarose gel using the QIAquick® PCR Purification kit (Qiagen) according to the manufacturer's instructions and sequenced using the PCR primers Bc-18SF402/Bc-18SR496, Be18SF/Be18SR, EMA-5/EMA6 and RLB F2/RLB R2 with the BigDye Terminator Cycle Sequencing Ready Reaction kit, version 3.1 (PerkinElmer, Applied Biosystems) in an automated sequencer (3500 Genetic Analyzer, Applied Biosystems).

The nucleotide sequences obtained were analysed using the Genetic Analyzer Sequencing v5.4 (Applied Biosystems, Foster City). Sequencing search and alignment were performed using nucleotid Basic Local Alignment Search Tool (BLASTn).

RESULTS

Samples from the mare and foetal organs were tested for EP aetiological agents using direct (smear and several PCRs) and indirect (ELISA and IFAT) methods. The examination of thin blood smears detected parasites within the erythrocytes in both animals. Foetal samples (clot, heart, liver, spleen and lung) tested positive for *Theilera equi* by all PCR protocols (real time, end point and RLB). PCR products were sequenced confirming the results. RLB and EMA sequences were submitted and registered in GenBank. For *Babesia caballi*, clot, heart and lung samples resulted negative using real time Bc18S, while liver and spleen tested positive. Real time-PCR products (liver and spleen) could be sequenced showing 100% homology with *B. caballi* registered sequences. Foetal serum tested negative by both serological methods.

Table 2. Serological results.

	IFAT *Babesia caballi*	IFAT *Theileria equi*	ELISA *B. caballi*	ELISA *T. equi*
Foetal serum	-	-	-	-
Mare	+	+	+	+

+ positive result; - negative result

Mare blood samples tested positive for both piroplasms by real time protocols Bc18S, Be18S and using nested PCR (RLB); results were confirmed by sequencing. RLB sequence was submitted and registered in GenBank. However, EMA *T. equi* protocol tested negative. Serum from the mare was positive for *B. caballi* and *T. equi* both by IFAT and ELISA methods. The results of direct methods (smear and PCR), and sequences are described in Table 1, while serological results are shown in Table 2.

DISCUSSION

Placental transmission could be influenced by individual horse genetics, or strain (Wise et al. 2013). Vertical transmissions in *Theilera equi* infections have been observed by several authors in horses from different countries such as South Africa, Trinidad, India (Allsop et al. 2007, Georges et al. 2011, Chhabra et al. 2012) and mules in Brazil (Françoso et al. 2018). However, *Babesia caballi* transplacental transmission has been reported before only in Trinidad by Sant et al. (2016). So, as far as the authors know, this could be the first report of transplacental transmission of *B. caballi* in Europe and it was only possible using real time PCR protocols.

Being field samples and in particular breeding conditions, it was difficult to follow up the case and perform more samplings. Abortion occurred in the last pregnancy trimester and the prognosis was good. Despite not having followed any treatment, the mare recovered perfectly and practitioners informed that the following season the mare got pregnant, no abnormalities during the pregnancy and no complications in the delivery were reported.

Regarding the results, mare was infected with *B*. *caballi* and *T. equi*. The detection of both parasites was performed by direct methods (blood smears and PCR). The negative result using EMA PCR could be due to differences in PCR sensitivity. The presence of antibodies was confirmed for both parasites by IFAT and ELISA tests.Vertical passage in foetus was confirmed; parasites were detected using PCR and stain smears. The foetus resulted serologically negative by all techniques as expected; the lack of antibodies in the infected foetuses and their acquisition with the colostrum have been described by Kumar et al. (2008).

Babesia caballi detection can sometimes be difficult. It may be due to the low parasitaemia and the use of long amplicons (over 400 bp), remaining under the detection limit as observed also by Sant et al. (2016). Real time amplicons are much shorter, which can also overcome the problem of DNA degradation. Another important factor for successful diagnosis were the tissues; positive results were found in blood and organs (liver, spleen) that have physiological specific characteristics (e.g., haemocatheresis) concentrating more blood cells, so more parasites could be found.

RLB PCR is a very useful tool as it catches different parasites and is helpful for genotyping but when several variants coexist the most predominant strain sequence is detected masking the presence of the minority ones. Despite the different product length (430 bp *Theileria* and 390 bp *Babesia*), it may occur that the two products bands do not separate clearly in the agarose gel and become indistinguishable as both seemed grouped in a thick band. Contamination with mare blood was unlikely due to the fact that maternal circulation is always independent from the foetal one; therefore, blood from the mother and foetus never mixes and also no passage of antibodies is possible. In horses, the type of placenta is epitheliochorial (Grosser's classification), which is the most superficial kind, and there is no significant invasion of the uterine lining with no destruction or invasion of the maternal tissues (Climent and Bascuas 1989). This is also supported by the serological results observed in the foetus samples which resulted negative.

It is complicated to verify if the abortion was caused by piroplasms, fever, comorbidities, or other infectious or non-infectious causes. However, several tests were carried out to diagnose different infectious agents and all resulted negative. Abortions caused by equine piroplasms are usually an underestimated problem, and frequently when infectious origin is suspected, investigations focus on the presence of different agents such as equine herpesvirus type 1, equine arteritis virus, *Salmonella* spp., *Leptospira* spp., *Toxoplasma gondii* or *Neospora caninum*, while EP protists are rarely included in differential diagnoses. Even if piroplasmosis abortion could seem an infrequent event (Tirosh-Levy et al. 2020), abortions due to *T. equi* can reach 11% in endemic areas (Lewis et al. 1999). In areas with high prevalence of EP, the use of imidocarb as a preventive treatment to avoid abortions or neonatal piroplamosis has been considered, although more studies are needed on safety and effectiveness during pregnancy to treat foetuses (Lewis et al. 1999).

It is obvious that EP economic losses in equine husbandry related to reproductive issues must be considered as well as the effect of vertical transmission in the maintenance of the parasitic infections.

This study is the first evidence of transplacental transmission of both piroplasms *B. caballi* and *T. equi* in Europe. Vertical transmission of piroplasms is an underestimated problem mainly in endemic areas, causing abortion and neonatal mortality which generates important economic losses. Appropriate preventive measures should be advised to avoid equine infection as well as adequate treatment protocols to manage infected mares.

Serological tests should be accompanied by direct diagnostic tools, the most sensible, as evidenced in this study, were real-time PCR protocols. It is necessary that equine sector practitioners are aware about new available diagnostic tools and the proper use of specific tubes indicated for each assay as a requirement to obtain accurate results. Further investigation about transplacental transmission mechanisms in endemic areas, the role to maintain infections

and new strategies of treatment for carriers are needed to overcome these issues.

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Author contribution. All authors made significant contributions to this article in particular, LBP performed all direct and indirect detection tests, as well as DNA extraction and purification and sequences alignment; she conceptualised the study and wrote the original draft. AM provided critical review and meaningful insights to the original manuscript, MZ carried out all sequencing experiments and data cleaning and AC was responsible for the methodology design and supervision of the study.

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