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Original article

Evaluation of *Theileria equi* vertical transmission rate and routes in a cohort of asymptomatic mares and their foals

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ABSTRACT

Equine piroplasmosis is a tick-borne disease mainly caused by *Theileria equi* and *Babesia caballi*. The objectives of this study were to analyse the frequency and routes of vertical transmission of these blood parasites from 179 asymptomatic mares to their foals. Foals were sampled within 72 h post-partum. The seroprevalences determined by Indirect Immunofluorescent Antibody Test (IFAT) and based on a subset sample of 107 couples, were 59.8% and 42.1% for *T. equi* and *B. caballi*, respectively in the mare population, and 54.2% and 40.2% in the foal population. A species-specific nested PCR was performed on all blood samples (358) and on available samples of placenta (24), umbilicus (6) and colostrum (18). For mares, 30.2% (54/179) and 2.2% (4/179) were PCR-positive for *T. equi* and *B. caballi*, respectively. Vertical transmission was not observed in the case of *B. caballi*, and four foals were born *T. equi* PCR-positive, giving a transmission rate of 7.4% (4/54). The blood smear evaluation showed viable *T. equi* parasites for the four foals without clinical signs of neonatal equine piroplasmosis, but one foal had acute renal failure. *Theileria equi* DNA was detected in umbilical cords, placenta and/or colostrum from PCR-positive mares, without correlation with the carrier status of the foal. One foal was born carrier but *T. equi* DNA had not been detected in the placenta. The 18S rRNA genotype E of *T. equi* was characterized in the four foals, foetal parts of the placenta and colostrum. The routes of transmission and particularly the possibility of colostral passage warrant further investigation.

1. Introduction

Equine piroplasmosis is a tick-borne disease caused by the hemoprotozoan parasites, *Theileria equi* and *Babesia caballi*. The recently described *T. haneyi* is also infective for equids (Knowles et al., 2018). Tropical and subtropical regions are endemic, and disease has been reported in Asia, Africa, southern Europe, south and central America and some parts of the USA (Onyiche et al., 2019).

The disease is primarily transmitted by ticks belonging to the genera *Dermacentor, Rhipicephalus, Hyalomma, Amblyomma* and *Haemaphysalis* (De Waal, 1992; Scoles & Ueti, 2015). Different clinical presentations have been described, ranging from asymptomatic carriers to chronic, acute and peracute disease (Wise et al., 2013). Peracute disease has been described in cases of neonatal equine piroplasmosis (Phipps & Otter, 2004; Georges et al., 2011; Chhabra et al., 2012).

Vertical transmission of piroplasms from the mare to her foal has been reported in several countries, but data is currently limited (Phipps and Otter 2004; Allsopp et al., 2007; Georges et al., 2011; Sant et al., 2016; Françoso et al., 2018; Bartolomé del Pino et al., 2023). Vertical transmission of piroplasmosis agents has also been previously reported for other species of *Babesia* and *Theileria* in other animal species like cattle (Baek et al., 2003; Swilks et al., 2017; Agrawal et al., 2020; Henker et al., 2021; Andrade et al., 2024), sheep (Zakian et al., 2014), dogs (Fukumoto et al., 2005; Simões et al., 2011; Mierzejewska et al., 2014), voles (*Microtus* spp.) (Tołkacz et al., 2017), white-footed mice (*Peromyscus leucopus*) (Tufts and Diuk-Wasser, 2018), and humans (Joseph

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et al., 2012; Krause & Vannier, 2012). Transplacental transmission has been reported to cause abortions, stillbirths, neonatal diseases, or birth of carrier foals (Allsopp et al., 2007). The work by Allsopp et al. (2007) suggests that transmission of *T. equi* occurs during histiotrophic nutrition of the foetus (day 40 to day 150 of gestation), which allows infected red blood cells of the mare to pass the placenta into the foetal circulation. It is also thought that this transmission occurs before the development of the foetal immune system (prior to day 240 of gestation), which could explain the birth of healthy carrier foals (Allsopp et al., 2007; Sant et al., 2016). However, these foals are potentially a source of infection for other animals. In contrast, several cases of neonatal piroplasmosis have been described (Georges et al., 2011; Chhabra et al., 2012; Levi et al., 2018) and it has been speculated that the outcome of vertical transmission will be influenced by the number of parasites crossing the placenta, the strain's virulence or a lack of immune response from the foal or the mare (Allsopp et al., 2007; Levi et al., 2018). In cases of abortion and neonatal piroplasmosis, high parasitaemia levels, of up to 63 %, have been described (De Waal & Van Heerden, 2004; Georges et al., 2011; Chhabra et al., 2012). It also has been speculated that the innate immune response together with maternal colostral antibodies control parasitaemia levels during early months of life (Allsopp et al.,2007; Kumar et al., 2008).

Equine piroplasmosis is an endemic disease in France with high levels of prevalence reported in some areas, especially in the southern regions (Guidi et al., 2015; Rocafort-Ferrer et al., 2022; Nadal et al., 2022; Jouglin et al., 2024). The genotype E of *T. equi* is predominant in the French horse population (98 % of 201 genotyped isolates) and the genotype A is the sole genotype described for *B. caballi* (Jouglin et al., 2024). A classfication and nomenclature system has been outlined by Tirosh-Levy et al. (2020a). The high prevalence may have economic and sanitary consequences since export to certain countries, such as the USA and Australia, might be restricted. The same consequences apply to foals born infected, and especially in cases of abortions, the economic losses can be major. It might therefore be important for breeders to know the prevalence of vertical transmission and the possible consequences, and to take preventive measures, like treating the infected broodmares before breeding. Little information is currently available about the safety of treating pregnant mares with imidocarb diproprionate. It has been demonstrated that the concentrations in the blood of foetus and dam are similar, confirming placental crossing and that it may show fetotoxicity (Lewis et al., 1999).

The objectives of our study were to estimate the frequency of vertical transmission of piroplasmosis from asymptomatic PCR-positive broodmares to their neonatal foals in an endemic region, to evaluate transmission routes, to genotype the isolates in the PCR-positive foals, foetal parts of the placenta and colostrum, and to determine the health status of carrier foals in the immediate postpartum period.

2. Materials and methods

2.1. Selection of mares and foals

Veterinarians and breeding farms known and/or suspected to have a relative high prevalence of piroplasmosis were contacted and were included when agreed to participate. The farms were mainly localized in the region Auvergne-Rhône-Alpes, but others were localized in Occitanie and Normandy.

Mares were included when they were confirmed to be pregnant and in the last trimester of gestation, spending more than 6 months per year on pasture and when the parturition was planned to take place in a box or paddock without access to grass. Foals of included mares were included themselves if they were less than 72 h of age and were born in a box or paddock without access to grass, in order to avoid early contact with ticks. Foals were excluded if they were born in the pasture or were in contact with pasture before the time of sampling.

This research was approved by the VetAgro Sup Research Ethics Committee, Approval number 2211.

An informed consent was signed and a questionnaire on environment and health was completed by the owners before sampling.

The following questions were included in the questionnaire of the mares: 1. location; 2. is the mare currently showing any clinical abnormalities, compatible with equine piroplasmosis? (yes/no); 3. if yes, which ones? (text); 4. has the mare previously been diagnosed with equine piroplasmosis? (yes/no); 5. if yes, which diagnostic test has been used (serology/PCR/blood smear evaluation); 6. which parasite has been detected? (*T. equi, B. caballi*, both); 7. does the mare have a history of previous resorptions (yes/no), abortions (yes/no) and or stillbirths (yes/no)?; 8. if yes, which diagnostic tests have been performed on the aborted foetus/stillbirth? (text).

The following questions were included in the questionnaire of the foals: 1. how was the parturition? (normal/dystocia); 2. how was the foal during the postpartum period (72 h after birth time)? (normal/showed clinical abnormalities); 3. if clinical abnormalities, which ones? (text); 4. were placental abnormalities observed? (yes/no); 5. if yes, which ones? (text).

2.2. Sampling

The veterinarian responsible of the breeding farm was informed about the protocol and sampling procedure. Sampling was performed between the months February and July of the years 2022 and 2023.

Ten millilitres of blood were collected aseptically from the jugular vein of mares during the last trimester of gestation or around birth and 5 ml from the foals after birth during a routine perinatal examination into heparinized and pro-coagulation tubes. The heparinized tubes were stored at 8 °C and transported to the laboratory in ambient temperatures (UMR BIOEPAR, Nantes) for PCR analysis and blood smear evaluation. The smears were Diff-Quick stained (Labor + Technik, Berlin, Germany) and read a posteriori to control for the presence of viable parasites in the blood of PCR-positive foals. The pro-coagulation tubes were centrifuged and serum was stored at – 20 °C until performance of the indirect immunofluorescent antibody test in the on-campus laboratory (LAV, VetAgro Sup, Marcy l'Étoile).

When possible, samples of umbilical cord, placenta and colostrum were also collected, stored at −20 °C and transported in cooled temperature to the laboratory for analysis (UMR BIOEPAR, Nantes). They were analysed once the mare and foal PCR status was determined. In the case of PCR-negative mares, two fragments of about 25 mg of umbilical cords and placentas were sterilely collected at different places of each organ, and one of these was used for DNA extraction and PCR testing. In the case of PCR-positive mares for *T. equi*, six fragments were collected. A first set of DNA extraction and PCR was performed on **three** fragments. If negative results were obtained, the second set of fragments was extracted and analysed. For the colostrum, up to seven PCR replicates were performed in the case of PCR-positive mares for *T. equi.* For the *B. caballi* positive mares, no colostrum or placenta samples were available.

2.3. Laboratory analysis

2.3.1. Serological testing - Indirect immunofluorescent antibody test (IFAT)

Presence and quantification of IgG antibodies against *T. equi* and *B. caballi* in sera was determined using indirect fluorescent antibody test (IFAT). Sera were tested using twofold dilutions from 1:80 up to 1:1280 in phosphate buffered saline (PBS 1X). Twenty microlitres of diluted serum were placed on commercial slides that contained fixed horse erythrocytes infected with *B. caballi* or *T. equi* (MegaFLUO® *Babesia caballi* or MegaFLUO *Theileria equi*, Megacor, GmbH, Hörbranz, Austria). The slides were incubated in a humid chamber at 37 °C for 30 min, successively rinsed and soaked in phosphate buffered saline (1X) for 10 min. Twenty microlitres of FITC anti-horse IgG conjugate (Megacor, GmbH, Hörbranz, Austria) were applied to each well and then

incubation and rinse–soak steps were repeated. Finally, the slides were dried and mounted with commercial mounting medium (Megacor). The slides were observed under an epi-fluorescent microscope (OLYMPUS CX43 – x400). A positive reaction appeared as peripheral fluorescent green inclusion bodies within the infected erythrocytes and compared to that of the internal positive and negative controls. Antibodies titre is given by the highest dilution showing a positive reaction.

2.3.2. DNA extraction

Genomic DNA was extracted from 200 µL of pelleted red blood cells diluted 1:1 in PBS1X, or from 200 µL of colostrum, using the DNA Nucleospin blood extraction kit following the manufacturer's instructions (Macherey-Nagel, Hoerdt, France). DNA was also extracted from several placenta or umbilical cord fragments of 25 mg each using the DNA Nucleospin tissue extraction kit, after an overnight tissue lysis at 56 °C. DNA was stored at −20 °C until further use.

2.3.3. Nested PCR

A first PCR was performed using CRYPTOF and CRYPTOR primers to amplify the 18S rRNA gene of piroplasms (Malandrin et al., 2010). Briefly, reactions were carried out in 30 µL reaction mixtures containing 1 X buffer, 2 mM $MgCl_2$, 0.2 mM of each dNTP (Promega, Madison, USA), 1-unit GoTaq G2 Flexi DNA Polymerase (Promega), 0.5 µM of each primer and 7 µL of DNA template. PCR cycling conditions were as followed: 5 min at 95 °C, 40 cycles at 95 °C for 30 s, 30 s at 63 °C, 1 min 40 s at 72 °C, and a final extension at 72 °C for 5 min. Two nested PCR were then conducted separately with species specific primers as described previously (Jouglin et al., 2024). In brief, primers specific for *B. caballi* (BabF4 and BabR4, expected amplicons length of 856 bp) and *T. equi* (TeqF3 and TeqR5, expected amplicons length of 1470 bp) were used. For this second PCR reaction, the first amplicons were diluted to 1/40 and 10 µL of this dilution was added to a 20 µL reaction mixture containing the same components as the first PCR. Cycling conditions were the same as the primary reaction except the annealing temperature was 54 °C in both specific second amplifications.

PCR amplicons were visualized in 1 % agarose gel with 1X SYBR Safe DNA Gel stain (Invitrogen, Carlsbad, USA). Amplified fragments from PCR-positive foals were purified with ExoSAP-IT reagent following manufacturer's recommendations (Affymetrix, Santa Clara, USA) and were subsequently sequenced bi-directionally using the same primers (Eurofins Genomics, Germany). Sequences were then assembled using the Geneious Prime 2022.2.1 software (https://www.geneious.com) and an online BLAST was performed for sequence homology searches (http://blast.ncbi.nlm.nih.gov). Sequence alignments and similarity analysis with reference sequences of each genotype was performed using Clustal Omega software (https://www.ebi.ac.uk) to compare hypervariable regions. Homologies higher than 99 % were obtained between sequences of the same genotypes, and lower than 98 % with sequences of different genotypes (Jouglin et al., 2024).

2.4. Data analysis

A power calculation was performed to estimate the minimum sample size needed to reach a sensitivity of 95 % (α=0.05) and a confidence interval precision of four. Results showed that 175 *T. equi* positive mares and their foals were needed for an expected transmission rate of 8 %.

Nominal data was reported as absolute numbers and percentages of horses in which the analysis yielded a positive result and 95 % confidence intervals (CI) were calculated when appropriate. Maps were made with Microsoft Excel.

The questionnaires were analysed, answers for open-end questions were collected and categorised. Categorical variables were reported as absolute numbers and percentages.

3. Results

3.1. Description of the sampled population and answers to the questionnaire

A total of 179 mares and their foals were included in the study, 71 during the foaling season of 2022 and 108 during the foaling season of 2023. They were localized in 54 different communes mainly in the regions Auvergne-Rhônes-Alpes, Occitanie and Normandy (Fig. 1a). Questionnaires were available for 114 mares and 99 foals. No clinical signs of acute piroplasmosis were reported in mares or foals. A previous diagnosis of equine piroplasmosis was reported in five mares (5/114). Of these five mares, one was diagnosed with both *B. caballi* and *T. equi,* but the diagnostic test used was unknown. For the remaining four mares with a history of equine piroplasmosis (4/114), the owner did not recall whether *T. equi* and/or *B. caballi* was diagnosed and which diagnostic method was used. For 108 mares (108/114), no previous diagnosis of equine piroplasmosis was reported and for one mare (1/114), the owner could not answer. Four mares had a history of previous abortions, but no diagnostic test was performed to determine the origin of the pregnancy loss.

3.2. Indirect immunofluorescent antibody test (IFAT)

Serology was performed for 69 mares and their foals of the 2022 foaling season and for 38 pairs of mares and their foals of the 2023 foaling season. For two pairs of the 2022 foaling season, no pro-coagulation tubes were collected. Reason for the partial analysis of mares and foals from 2023 was a worldwide unavailability in commercial slides needed to perform the IFAT at the time. Antibodies were detected in a total of 69/107 mares, among them, 64/107 mares (59.8 %) were seropositive for *T. equi* and 45/107 (42.1 %) seropositive for *B. caballi*. In 40 (37.4 %) of these seropositive mares, antibodies against both parasites were detected. Antibodies were found in a total of 67/107 foals, among them, 58/107 foals (54.2 %) were seropositive for *T. equi* and 43/107 (40.2 %) seropositive for *B. caballi*. In 34 (31.8 %) of these seropositive foals, antibodies against both parasites were detected. Four of the 57 seropositive foals for *T. equi* were born from seronegative mares. The titres in these foals were low, 1:80. Similarly, five of the 42 foals seropositive for *B. caballi* were born from seronegative mares, titres were also low, 1:80, and 1:160 in one foal. Ten foals born from *T. equi* seropositive mares and seven foals from *B. caballi* seropositive mares were seronegative. For these mares, the titre for *T. equi* was at the limit of seropositivity (1:80) except in two mares (1:160). The titre for *B. caballi* was also at the limit of seropositivity (1:80) in four cases, 1:160 in one mare and relatively high at 1:320 and 1:640 in two other mares. Proportions of seropositive mares and foals and their respective 95 % confidence interval are presented in Table 1 and seroprevalence in the different counties is presented in Fig. 1b.

3.3. Prevalence of PCR-positive mares and transmission rate

In total, a positive PCR result was detected in 56/179 mares. *Theileria equi* was detected in the blood of 54/179 (30.2 %) of the mares and *B. caballi* in 4/179 (2.2 %) of them. Co-infection with *T. equi* and *B. caballi* was identified in 2/179 of the mares (1.1 %) The percentage of mares positive in the different counties is presented in Fig. 1c.

For 33 of these *T. equi* PCR-positive mares, the IFAT was performed, 32 mares were seropositive for *T. equi* and one mare was seronegative despite being PCR-positive. For two *B. caballi* PCR-positive mares, the IFAT was performed and both were seropositive. On the other hand, from the 64 *T. equi* seropositive mares, 51.6 % were positive by nested PCR and from the 44 *B caballi* seropositive mares, only 2 (4.5 %) were positive by nested PCR.

In the foal population, 4/179 foals (2.2 %) were PCR-positive for *T. equi* and 0/179 (0 %) were PCR-positive for *B. caballi*. When

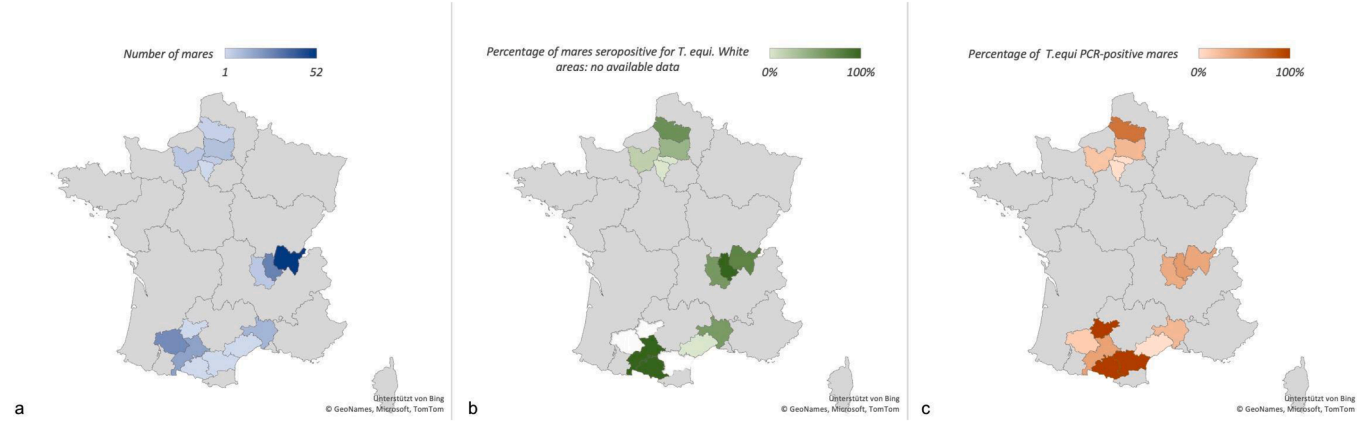

Fig. 1. Geographical distribution of the mare population. a - Distribution of mares over the different counties. b - Percentage of seropositive mares for *T. equi* in the different counties. Counties marked in white indicate the absence of serology results, while no mares were included in the study from counties shaded grey. c - Percentage of *T. equi* PCR-positive mares in the different counties, as determined by nested PCR.

Table 1
Proportion of piroplasms PCR-positive and antibody-positive mares and foals determined by nested PCR and IFAT analysis, respectively, for each piroplasm with the respective confidence intervals of 95 %.

			Sampled mares			Sampled foals		Transmission rate	
	Total number of positive results		Numbers of positive (%)	95 % CI	Total number of positive results	Numbers of positive (%)	95 % CI	Proportion	95 % CI
Nested PCR ($n = 179$)	56	*T. equi*	54 (30.2 %)	[23.5 %; 36.9 %]	4	4 (2.2 %)	[0.1 %; 4.3 %]	7.4 %	[0.4 %; 14.4 %]
		B. caballi	4 (2.2 %)	[0.1 %; 4.3 %]		0		0	
		Both	2 (1.1 %)	[0 %; 2.7 %]		0			
IFAT ($n = 107$)	69	*T. equi*	64 (59.8 %)	[50.5 %; 69.1 %]	67	58 (54.2 %)	[44.8 %; 63.7 %]		
		B. caballi	45 (42.1 %)	[32.7 %; 51.4 %]		43 (40.2 %)	[30.9 %; 49.5 %]		
		Both	40 (37.4 %)	[28.2 %; 46.6 %]		34 (31.8 %)	[22.9 %; 40.6 %]		

considering only the foals born from *T. equi* PCR-positive mares, a vertical transmission rate of 7.4 % (4/54) for *T. equi* in foals could be evaluated considering both sampling years.

None of the foals born to PCR-negative mares were PCR-positive. The blood smear of the four PCR-positive foals was positive and the presence of parasites in their red blood cells was confirmed. IFAT results were available for four PCR-positive foals and were positive in three of them.

Proportions of PCR-positive mares and foals and their respective 95 % confidence interval are presented in Table 1.

3.4. Evaluation of T. equi transmission route

Samples (48) of umbilical cord (6), placenta (foetal membranes) (24) and colostrum (18) were collected from 29 different mares, five of them were asymptomatic *T. equi* PCR-positive mares (Table 2). Piroplasms' DNA could not be detected in any of the samples collected from the 24 PCR-negative mares (piroplasmosis free), representing a total of 39 tested samples. *B. caballi* was never detected in the 48 different samples.

Umbilical samples were available for a total of six mares, of which two were *T. equi* PCR positive mares. *Theileria equi* could be detected in both umbilical samples from the PCR-positive mares. A total of 24 placenta samples were available. Four samples came from *T. equi* PCR-positive mares and *T. equi* could be detected in two of these four samples. Multiple fragments of the placenta and umbilicus samples had to be tested to demonstrate the presence of *T. equi* in the respective samples. Colostrum samples were available for 18 mares, two of them were *T. equi* PCR-positive mares. One colostrum sample was found to be positive for *T. equi* DNA, while the colostrum from the second PCR-positive mare was negative despite seven attempts to amplify piroplasm DNA (Table 2).

Different situations were highlighted for the detection of *T. equi* in the five PCR-positive mares, foetal parts of the placenta or colostrum, and their foals (Table 2). Foals apparently free of piroplasms could be born from PCR-positive mares with umbilical cord, placenta and/or colostrum carrying *T. equi*. The carrier foal was born from a mare with a placenta in which *T. equi* could not be detected in 12 different placental

Table 2
Individual results of *T. equi* detection in the placenta, umbilical cord or colostrum and in the blood of foals born from *T. equi* PCR-positive mares and IFAT status of the foals.

T. equi PCR positive mare reference	PCR Umbilical cord	PCR Placenta	PCR Colostrum	PCR *T. equi* foal status	*T. equi* foal IFAT status
J22–149	+ (2/6)	+ (2/4)	na	–	+ (1:640)
J23–116	+ (2/3)	na	+ (1/1)	–	nt
J23–178	na*	- (0/12)	na	+	–
J23–206	na	+ (1/3)	na	–	nt
J23–238	na	- (0/6)	- (0/7)	–	nt

*na: non available samples. nt: not tested. The numbers into brackets indicate the number of positive fragments/number of tested fragments of umbilical cord or placenta, or the number of positive PCR/number of PCR replicates in the case of colostrum.

fragments tested. Umbilicus or colostrum samples were not available from this mare.

3.5. Genotyping of T. equi in foals, foetal parts of the placenta and colostrum

Theileria equi identification and 18S rRNA genotyping was performed by sequencing all PCR amplification products from the four foals as well as from each positive organ or colostrum. Sequences of 1327 bp to 1381 bp were obtained and were highly similar (99.3 to 100 % nucleotide identity). Comparisons (Blastn analysis) with sequences previously described as genotype E from France (Jouglin et al., 2024; GenBank accession number PQ044750, query cover 100 %) or from Mongolia (GenBank accession number LC781893, query cover 100 %) highlighted homologies of 100 %, confirming their belonging to this widespread genotype in Eurasia. Finally, partial 18S rRNA sequences of *T. equi* from foals and from different organs or colostrum were deposited in GenBank (accession numbers PP905479 to PP905482 for the **four foals** blood parasites, and PP905476 to PP905478 for the fetal organs and colostrum).

3.6. Follow up of foals

Questionnaires were available for 99/179 foals. For one of the *T. equi* positive foals the questionnaire was available and for the remaining three foals, oral information was provided by the responsible veterinarian. Of the 102 foals for which information was available, thirteen showed clinical abnormalities after birth**.** The reported pathologies included dysmaturity (6), neonatal encephalopathy (4), meconium retention (3), bladder rupture (1) and acute renal failure (1). The foaling was reported to be normal in all foals, except for one foal that was born dead after dystocia. No placental abnormalities were detected for any of the foals. Among the four *T. equi* positive foals, no clinical signs compatible with neonatal piroplasmosis was observed in three and one was born through caesarean section and had acute renal failure. This foal died after hospital discharge. Among the three others, two foals were reported to be healthy six months after sampling. No information was available on the health status of the third one.

4. Discussion

The results of our study demonstrate the possibility of vertical transmission of *T. equi* in a European equine piroplasmosis endemic region, from asymptomatic mares to their foals. Despite the large sample studied (179 couples' mare/foal), we were however unable to confirm vertical transmission of *B. caballi*, probably due to the low prevalence of *B. caballi* PCR-positive mares (2.2 %).

It is important to first acknowledge some limitations and biases. The 95 % confidence interval determined for the *T. equi* PCR-positive foals was relatively large, 95 % CI [0.4 %; 14.4 %], due to the lower number of positive mares included in our study (54) than needed (175) for a precision of the confidence interval of four. Mares were selected based on the information of a relative high prevalence of piroplasmosis on the breeding farm selected by the responsible veterinarian. This could have biased our sample selection because we observed that on some breeding farms the prevalence of piroplasmosis was actually 0 %. Also, information provided by owners was inconsistent and it is possible that a more objective selection of mares, with selecting mares being previously diagnosed with either *T. equi* and/or *B. caballi,* could have increased the number of PCR-positive mares and refined our results. It is possible that the rate of transmission was influenced by parasitaemia levels in mares. It has previously been speculated that the number of parasites crossing the placenta would influence the outcome (Allsopp et al., 2007) and the same might be true for the possibility of transmission. One might expect that in cases of chronic infections in mares, the parasitaemia levels were low. Blood smear examinations is a useful diagnostic tool to detect acute cases. However, this method is known to have a low sensitivity and it is usually not possible to detect parasites when the parasitaemia is too low (Mendoza et al., 2024).

We chose a nested PCR method as it is generally accepted to be more sensitive than qPCR, even if a sensitivity threshold remains (Lv et al., 2022; Mendoza et al., 2024). Moreover, it has the advantage to allow genotyping from the PCR products. Heparin was used as anticoagulant despite possible inhibitory effects on PCR amplifications. We previously demonstrated that piroplasms remain viable for up to two weeks when this anticoagulant was used, ensuring good DNA quality preservation and allowing eventual *in vitro* culture initiation (Malandrin et al., 2004). The use of nested PCR allows dilution of eventual residues of heparin between the two PCRs.

The IFAT showed that overall, 59.8 % of mares were seropositive for *T. equi* and 42.1 % for *B. caballi.* A higher seroprevalence than prevalence by nested PCR (30.2 % and 2.2 %**,** respectively) was a result we expected. For *B. caballi*, spontaneous clearance of this parasite has been described in horses with persistence of circulating antibodies for longer periods (Friedhoff et al., 1990; de Waal 1992). Persistence of circulating antibodies against *T. equi* after clearance has also been described (Ueti et al., 2012) but spontaneous clearance of *T. equi* has not been observed. We cannot exclude that in some seropositive cases, especially cases with high titres, the nested PCR resulted in a false-negative result. Even if it is a sensitive technique, low parasitaemia levels may always result in false-negative PCR. In contrast, we observed that some nested PCR negative mares, had relatively low antibody titres, which could have been false-positive results.

The overall seroprevalence in foals was higher than the prevalence determined by nested PCR, 54.2 % and 40.2 % for *T. equi* and *B. caballi***,** respectively. This is a finding we expected since it has been reported that foals born from seropositive mares will become seropositive after ingestion and absorption of colostrum containing antibodies against *T. equi* and *B. caballi* and this maternal immunity is thought to be transitory (Kumar et al., 2008; Sant et al., 2016; Tirosh-Levy et al., 2020b). Four of these 57 seropositive foals for *T. equi* were born from a seronegative mare and five of the seropositive foals for *B. caballi* were born from a seronegative mare. In all cases relatively low titres were determined and we suggest that these results could be false-positive due to serological cross-reactions. Fifteen foals were born seronegative even if the mare was seropositive for either *T. equi* or *B. caballi.* Some of these mares had low antibody titres and false positive results in these cases cannot be excluded. Another explanation could be that the antibody level in the mother was reduced during gestation and/or that the transfer of antibodies by ingestion of colostrum was insufficient to lead to a seropositive result in the foal (Sant et al., 2016). The latter might explain why foals born from mares with high antibody titres were seronegative. Since we had to rely on other veterinarians for the sampling, we cannot exclude that these foals might have been sampled before adequate colostrum intake. The possibility of false negative results in the foals could not be excluded.

In this population, a 30.2 % prevalence of *T. equi* positive mares was determined by nested PCR, with an observed transmission rate of 7.4 % for *T. equi*. Few studies described transmission rates. In a population of 111 positive mare-foal pairs, Sant et al. (2016,2019) reported a *T. equi* transmission rate of 14.8 % in Trinidad. In these studies, *B. caballi* transmission rate could not be evaluated due to the low prevalence of PCR-positive mares (3.6 %), and probably to the high level of foetal losses attributed to *B. caballi* infections. In their study, on 18 mules in Brazil, a similar *T. equi* transmission rate of 15.4 % was found (Françoso et al., 2018). In some studies, extremely high *T. equi* transmission rates were published: 100 % in the experimental study by Allsopp et al., in 2007, where 12 mares were included, of which six delivered at term and six underwent an induced abortion. Another example is the 50 % transmission rate in the case study of Phipps and Otter (2004) where an infected mare imported from Portugal (endemic) to UK (sporadic) gave birth to two infected and two uninfected foals over eight years.

Comparing these papers with our study is difficult because of the different geographic regions, case selection and diagnostic tests used. For example, the nested PCR for detection of *T. equi* used in our study has been described to have an increased sensitivity and the diagnosis of subclinical infections may be more reliable (Nicolaiewsky et al., 2001; Rampersad et al., 2003).

Little is known about the vertical transmission mechanisms of blood parasites, and only few reports exist. The mechanism of transplacental transmission of blood parasites such as *T. equi* and *B. caballi* is still a matter of debate as there is no blood exchange between the mare and the foetus, that are separated by an epitheliochorial placenta, consisting of six layers (Pozor, 2016). After birth, only the foetal membranes are expelled and the maternal endometrial tissue is preserved (Morresey, 2011; Pozor, 2016). However, abortions are reported in the literature, with either parasites detected in the foetus' blood or organs, with sometimes very high parasitaemia, demonstrating that *in utero* transmission of both parasite species is indeed possible, even if the mechanisms of this transmission remain obscure. The described abortions occur rather late, between the seventh and tenth months of pregnancy (Allsopp et al., 2007; Sudan et al., 2015; Sant et al., 2016; De Sousa et al., 2017; Oliveira et al., 2019; Dirks et al., 2021). Allsopp et al. (2007) suggested in their study that vertical transmission of *T. equi* could take place during histiotrophic nutrition of the foetus. This takes place from day 40 to day 150 of gestation. During this period, infected red blood cells from the mother can cross the placenta as a source of iron and infect the foetus. To date, no other study has been performed to confirm this hypothesis. In contrast, it has previously been suggested that transmission could be due to abnormal placentation like placental damage or reverse erythroblastosis (Du Plessis & Basson, 1966; Erbsloh, 1975). In our study, no placental abnormalities were reported by the veterinarians for any of the *T. equi* positive foal. We had to rely upon the information provided since none of the placenta was examined by one of the authors and it is possible that minor changes could have been missed. Allsopp et al. (2007) suggested that transmission takes place before development of the foetal immune system (prior to day 240 of gestation) so that parasites would be recognized by the neonate themselves, leading to apparently healthy carrier foals. It is also speculated that ingested maternal antibodies together with the innate immune response may act to control parasitaemia levels during the foal's early life. This hypothesis does not explain why in some cases vertical transmission is responsible for abortions, stillbirths or peracute disease in neonatal foals. Peracute disease in foals has been reported to cause weakness, icterus, decreased suckling, recumbency and death (Georges et al., 2011; Chhabra et al., 2012; Sant et al., 2016; Levi et al., 2018). In these cases, high levels of parasitaemia have been described (De Waal & Van Heerden, 2004; Georges et al., 2011; Chhabra et al., 2012) and it has been speculated that the outcome of transmission will be influenced by the number of parasites crossing the placenta, the strain's virulence phenotype or a lack of immune response from the foal or the mare (Allsopp et al., 2007; Levi et al., 2018).

We also hypothesize that differences in genotypes might explain the different outcomes of vertical transmission reported in foals. No piroplasmosis complication was noticed for three of the carrier foals, the fourth one suffered from acute renal failure. In this case the cause of the renal failure was unknown. The only description of a genotype E vertical transmission was in Austria, in a case of acute piroplasmosis occurring at the fifth month of pregnancy, followed by an abortion at the eighth month (Dirks et al., 2021). However, the authors declared that *T. equi* was not the likely cause of abortion, as other complications occurred during the pregnancy. Five genotypes of *T. equi* have been described worldwide based on 18S rRNA sequencing and named A to E (reviewed in Tirosh-Levy et al., 2020a). Based on a recent study performed on a cohort of 566 asymptomatic horses, the genotype E is predominant and represents 98 % of the isolates originating from different geographical areas in France. This is therefore not surprising that the foals from our study were infected with parasites of this genotype. In many cases of abortions reported in the literature, the foetus carried a genotype A of *T. equi* (Oliveira et al., 2019; Bartolomé del Pino et al., 2023) or when not determined, occurred in countries (India, Brazil) where the genotype A is predominant (Sudan et al., 2015; De Sousa et al., 2017; Tirosh-Levy et al., 2020a). As for the abortions, in seven cases of neonatal peracute piroplasmosis occurring few hours to few days and up to three weeks after birth, the genotype A of *T. equi* was determined for four of them (Levi et al., 2018; Sant et al., 2019), or suspected in the three other cases as it is the predominant genotype in the India (Chhabra et al., 2012) and Trinidad (Georges et al., 2011). One intriguing description of abortion was published recently (Bartolomé del Pino et al., 2023). When we performed the BLAST analysis of the sequences deposited in GenBank, we discovered that the mare carried the genotype E of *T. equi* while the foetal organs were infected by the genotype A. One possible explanation is that the mare carried both genotypes, with a higher proportion of genotype E parasites that were therefore preferentially detected, but with genotype A parasites that could express a higher ability to be transmitted to the foetus through the placenta. All these published cases, combined with the fact that the four foals infected with genotype E from our study were asymptomatic, except for one foal with renal failure of unknown origin, could point towards a differential virulence between genotypes A and E.

Transplacental transmission of several blood pathogens such as *Anaplasma marginale, A. ovis* and *A. phagocytophilum, Babesia bovis, B. bigemina, B. gibsoni* and *B. microti* has been demonstrated in various animals (Grau et al., 2013; Reppert et al., 2013; Costa et al., 2016; Tufts & Diuk-Wasser, 2018), **and** the study on *B. gibsoni* highlighted the absence of transmission via the colostrum (Fukumoto et al., 2005).

Our study describes for the first time the detection of *T. equi* DNA in the colostrum of an infected mare, raising a novel possibility of vertical transmission route. Many different pathogens are transmitted *via* the colostrum of different mammalian hosts, mainly viruses (Preziuso et al., 2004; Grego et al., 2008; Martínez-Guinó et al., 2009; Mayo et al., 2010; Desgraupes et al., 2021; Yuan et al., 2022), but also bacteria (Stabel, 1998). For the blood parasites, to our knowledge, only the transmission of *Trypanosoma evansi* through the colostrum has been demonstrated (Campigotto et al., 2015) and *Theileria orientalis* DNA was also detected in cattle colostrum (Hammer et al., 2016). *Theileria equi* could not be detected in the milk of 11 *T. equi* carrier jennies (Perrucci et al., 2021). However, the cell composition of milk and colostrum differs. Equine colostrum immune cells are comprised mainly of maternal T-lymphocytes (about 93 %) and B-cells (about 5 %) (Perkins et al., 2014) and as the host cell range of *T. equi* includes monocyte/macrophages, as well as T lymphocytes and B lymphocytes (Ramsay et al., 2013), mare's colostrum represents a suitable environment for the maintenance or even the growth of *T. equi*.

In our study, the foal for which the colostrum and umbilicus were PCR-positive for *T. equi* was negative for PCR in the blood at the time of sampling. It is not yet known what the mechanism of passage of the parasite or its DNA in the colostrum could be. Possible explanations could be an ability of the parasite or its DNA to cross the intact mammary barrier, breaches in the mammary barrier or even a small wound on the teats.

In one of the PCR-positive foals in the blood, *T. equi* was not detected in the available placental sample, and unfortunately the colostrum and umbilicus were not available for this foal. Conversely, for another foal, the detection of *T. equi* DNA in the placenta did not correlate with a PCR-positive status of the foal. Based on our results, the analysis of several placental or umbilicus fragments is necessary to improve the reliability of *T. equi* detection, as the parasite may not be homogeneously distributed in these organs.

In any case, the interpretation of our results must be done with caution because a positive PCR does not mean the presence of viable parasite in the sample. It must also be taken into account that false-negative results can be observed if the parasitaemia is too low. Our results therefore do not allow us to conclude whether the vertical

transmission route was via the placenta or whether ingestion via colostrum could play a role in transmission. Further studies should be undertaken to look for the presence of live and infectious *T. equi* parasites in colostrum, umbilicus and/or placenta.

5. Conclusion

This study confirms the previously reported low (7.4 %) vertical transmission rate of *T. equi*, and that the transmission of *T. equi* genotype E results in asymptomatic carrier foals, except for one foal with acute renal failure of unknown origin. We were unable to confirm vertical transmission *B. caballi*. This study is the first to describe the identification of *T. equi* by nested PCR in equine placenta, umbilicus and more importantly in colostrum samples. The exact mechanism of vertical transmission remains unknown and differences in strain's virulence may influence the outcome. Cautious interpretation of the results is warranted and larger studies with more statistical power are needed. The results of this study may have important implications for the monitoring of breeding mares and the understanding of vertical transmission of equine piroplasmosis in Europe, especially in countries where the *T. equi* genotype E is present.

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Ethics statement

This research was approved by the VetAgro Sup Research Ethics Committee. Approval number: 2211.

CRediT authorship contribution statement

Lisa-Marie Hermans: Writing – review & editing, Writing – original draft, Project administration, Methodology, Funding acquisition, Data curation, Conceptualization. **Claire Bonsergent:** Writing – review & editing, Project administration, Investigation. **Anne Josson:** Writing – review & editing, Resources. **Gloria Rocafort-Ferrer:** Writing – review & editing, Resources. **Marine Le Guyader:** Investigation. **Sophie Angelloz-Pessey:** Investigation. **Agnès Leblond:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Funding acquisition, Conceptualization. **Laurence Malandrin:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

None.

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Data availability

Data will be made available on request.

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