Equine piroplasmosis in different geographical areas in France: prevalence heterogeneity of asymptomatic carriers and low genetic diversity of Theileria equi and Babesia caballi

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Original article

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ABSTRACT

Equine piroplasmosis is a worldwide tick-borne disease caused by the parasites *Theileria equi* sensu lato and *Babesia caballi*, with significant economic and sanitary consequences. These two parasites are genetically variable, with a potential impact on diagnostic accuracy.

Our study aimed to evaluate the frequency of asymptomatic carriers of these parasites in France and describe the circulating genotypes. We developed a species-specific nested PCR protocol targeting the 18S small sub-unit (SSU) rRNA gene and used it on blood samples collected from 566 asymptomatic horses across four National Veterinary Schools.

The carrier frequency varied from 18.7 % around Paris (central-north) to 56.1 % around Lyon (southeast), with an overall prevalence of 38.3 %. *Theileria equi* carriers were ten times more frequent (91.7 %; 209/228 isolates) compared to *B. caballi* carriers (8.3 %; 19/228 isolates). Notably, *T. equi* carrier frequency was significantly lower in the northern region (Ile de France) compared to the southeastern regions. A positive relationship was observed between the frequencies of asymptomatic carriers and the frequency of previous acute piroplasmosis reported from the owner across all four geographic areas. Neither horse gender nor age showed a significant effect on the frequency of asymptomatic carriers. In some areas, a substantial proportion of horses (22.2 % to 37.5 %) carried *T. equi* before the age of three years, indicating high infection pressure.

Genotyping of 201 *T. equi* isolates revealed a predominance of genotype E (98 %) and few isolates belonging to genotype A (2 %). Notably, two of the four *T. equi* genotype A isolates were detected in horses originating from Spain. All 19 *B. caballi* isolates belonged to the genotype A.

The discussion section explores the link between these results, the tick distribution and abundance, and the frequency of detection of *T. equi* and *B. caballi* in febrile cases attributed to piroplasmosis.

1. Introduction

Equine piroplasmosis, a tick-borne disease of equids (horses, donkeys, mules, and zebras), is caused by two protozoan parasites: *Theileria equi* (formerly *Babesia equi*) and *Babesia caballi* (de Waal, 1992; Mehlhorn and Schein, 1998). *Theileria equi* is a cluster of genetically well-defined genotypes, within which the species *Theileria haneyi* has been delineated (Bhoora et al., 2020; Knowles et al., 2018). Over 30 tick species are implicated as possible vectors of equine piroplasmosis (listed in Scoles and Ueti, 2015). However, only species of the genera *Dermacentor, Rhipicephalus*, and *Hyalomma* have been confirmed as competent vectors (de Waal, 1992). Equine piroplasmosis has a wide geographical

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distribution, with its incidence depending on the distribution of these parasites' vectors (Onyiche et al., 2019; Tirosh-Levy et al., 2020). Endemic in tropical, subtropical, and some temperate regions, the disease is responsible for significant economic losses to the equine industry due to the treatments and their side effects, reduced animal performance and the negative impact on international trade in horses (Rothschild, 2013).

A few countries, including the United States, Canada, Australia, New Zealand, and Japan, are effectively free of equine piroplasmosis. Consequently, testing for equine piroplasmosis in horses is mandatory, whether for participation in international events or for export to these countries (Knowles, 1996; WOAH, 2023).

Equine piroplasmosis presents with a wide range of clinical signs, from subclinical (apathy, lack of appetite, poor exercise tolerance) to severe (fever, anemia, jaundice, hemoglobinuria, petechial hemorrhages) (Onyiche et al., 2019; Tirosh-Levy et al., 2020; Wise et al., 2013). These symptoms are variable in severity and presentation, they are non-specific, and do not allow differentiation between *B. caballi* or *T. equi* infections. The disease may also be asymptomatic, with infected animals recovering from acute or primary *T. equi* infection remaining carriers for life, even with appropriate treatment. In contrast, horses infected with *B. caballi* may remain carriers for up to four years or be cleared after treatment (Tirosh-Levy et al., 2020).

Clinical signs are therefore unreliable for definitive diagnosis, and carrier status with low parasitemia is challenging for accurate diagnosis. While blood smears can diagnose acute cases, identifying carriers requires more sensitive techniques (Mendoza et al., 2024). Serological tests like the now-abandoned complement fixation test (CFT) have been developed. However, these methods, including the reliable but time-consuming indirect fluorescent antibody test (IFAT) and the simpler enzyme-linked immunosorbent assay (ELISA), have limitations in sensitivity and specificity due to parasite genetic variability (Mendoza et al., 2024; Onyiche et al., 2019; Wise et al., 2013). The World Organization for Animal Health (WOAH) recommends using IFAT or competitive ELISA (cELISA) for serological diagnosis alongside molecular tests based on parasite DNA amplification (simple, nested, multiplex, Reverse-Line-Blotting [RLB], or real-time PCR) (WOAH, 2023). This combined approach offers the most accurate diagnosis for both acute cases and carrier animals.

These tools have also been used to investigate the prevalence of equine piroplasmosis, which is endemic in many parts of Asia, Arabia, South and Central America, Africa, and Europe. Studies have reported widely variable prevalence, reaching up to 88 % (reviewed in Onyiche et al., 2020). In Europe, a decreasing south-north gradient of equine piroplasmosis seroprevalence appears to exist, with Italy (Bartolomé Del Pino et al., 2016; Moretti et al., 2010; Piantedosi et al., 2014; Sgorbini et al., 2015), Spain (Camacho et al., 2005; Montes Cortés et al., 2017), Portugal (Ribeiro et al., 2013), and Greece (Kouam et al., 2010) being the most affected. In Ireland (Coultous et al., 2020), the United Kingdom (Coultous et al., 2019), the Netherlands (Butler et al., 2012), and Switzerland (Sigg et al., 2010), the seroprevalence of equine piroplasmosis remains below 5 % and a few autochthonous cases are reported in the Netherlands (Butler et al., 2012) and Austria (Dirks et al., 2021) (reviewed in Nadal et al., 2021). This north-south trend has also been observed across France, with higher serological prevalence in the south (Guidi et al., 2015) compared to the north (Le Metayer, 2007; Nadal et al., 2022; Soulé et al., 1990). The spatial analyses were carried out using the CFT technique, on fairly old data (20 years), and without any knowledge of the clinical status of the animals. A locally high prevalence of equine piroplasmosis in France has been identified through molecular detection of parasites in specific regions in the south, including Camargue, the city of Marseille, and Corsica (Dahmana et al., 2019; Rocafort-Ferrer et al., 2022) - or in geographically unspecified regions (Fritz, 2010).

Molecular tools offer several advantages: they enable sensitive, specific, and reliable detection of pathogens even at low parasite levels (parasitemia) common in carrier horses. Additionally, these tools allow sequencing for studying the genetic variability of isolates, improving our understanding of equine piroplasmosis epidemiology. The genetic diversity of *T. equi* (5 clades referred to as A to E) and *B. caballi* (3 clades A, B1 and B2) has been highlighted using the 18S SSU rRNA gene sequences (reviewed in Tirosh-Levy, 2020). A fourth clade (B3) within *B. caballi* has recently been identified, along with a subdivision of clade A into five subclades, designated A1 to A5 (Rar et al., 2024). Importantly, this diversity can influence the accuracy of serological detection due to the lack of cross-reactions between the antigens used in these tests (Bhoora et al., 2010; Mahmoud et al., 2016; Rapoport et al., 2014).

The existing data on equine piroplasmosis prevalence in France suffers from several limitations: reliance on large but outdated data (over 20 years old), collected from a horse population with unspecified status (symptomatic vs. asymptomatic, age, gender, travel history or origin of the horses), and analyzed with detection tools with low sensitivity/specificity (CFT) that are no longer used (Le Metayer, 2007; Nadal et al., 2022), or on geographically limited horse populations (Dahmana et al., 2019; Rocafort-Ferrer et al., 2022). Our study aimed to address these gaps. We employed molecular tools to investigate the carriage of *T. equi* and *B. caballi* in asymptomatic horses across four large geographically distinct regions of France. We focused on asymptomatic horses because they represent a significant parasite reservoir, particularly for *T. equi*. Additionally, we assessed the genetic diversity of these two pathogens, as this heterogeneity can impact diagnostic results.

2. Material and methods

2.1. Animals and sampled areas

Blood samples were collected from equids presented at four French Veterinary Schools in Maisons-Alfort (ENVA, north-central of France), Nantes (Oniris, west), Lyon (VetAgro Sup, southeast), and Toulouse (ENVT, southwest). The horses recruited were presented at veterinary schools for various reasons (surgery, reproduction, lameness, etc.). The sole inclusion criterium for horses in this study was outdoor or mixed (mainly outdoor pasture and indoor) type of housing. The only exclusion criterium was the presence of symptoms of acute equine piroplasmosis at the time of blood sampling. Each school was considered as a focal point with horses located in their surroundings. For clarity in tables and figures, the veterinary schools will be referred to by the main city names: Nantes (Oniris), Paris (ENVA), Toulouse (ENVT) and Lyon (VetAgro Sup).

The study was presented to and approved by the Oniris Ethics Committee for Veterinary Clinical and Epidemiological Research (CERVO-2019–1-V). After explaining the purpose of the study, written consent was obtained from each horse owner for their animal's participation. During sample collection, owners completed questionnaires providing information about their horses' characteristics, including horse residence at sampling time, breed, gender, age, previous geographic places of residence, and previous history of acute piroplasmosis (diagnostic method used and identified species).

Blood was collected by jugular venipuncture into sterile heparinized tubes. Heparin was chosen as the anticoagulant because it preserves parasite viability for up to 9 days at room temperature, ensuring good DNA quality for analysis (Malandrin et al., 2004). The blood samples and questionnaires were sent to the BIOEPAR research unit in Nantes for analysis.

As this study was part of the national citizen science program PiroGoTick, the results were made anonymous and accessible to participants on the project website (https://www.pirogotick.fr). Each participant received an information note explaining the project and how to retrieve the results using their unique reference on the PiroGoTick website.

2.2. Generation of geographical location maps of horses

For each of the four veterinary schools (Paris, Toulouse, Lyon, and Nantes), we generated geographical location maps of the horses included in this study. These maps were constructed using data from the French National Geographic Institute (IGN) (including GPS location of communes and a map of mainland France in Lambert-93 format) and information on horse origin, movement and current place of residence collected through questionnaires at admission time. All data were then summarized into maps using QGIS software (v3.36).

2.3. Molecular detection of blood piroplasms

Genomic DNA was extracted from 200 µL of pelleted red blood cells diluted 1:1 in PBS1X using the DNA Nucleospin blood extraction kit following the manufacturer's instructions (Macherey-Nagel, Dueren, Germany). The extracted DNA was then stored at −20 °C for later use.

To detect the presence of piroplasms, we first amplified a region of the 18S rRNA gene, a common genetic marker for these parasites, using a standard PCR technique and the primers CRYPTOF and CRYPTOR (Malandrin et al., 2010). Reactions were carried out in 30 µL reaction mixtures containing 1 X buffer, 2 mM $MgCl_2$, 0.33 mM of each dNTP (Eurobio, Les Ulis, France), 0.15 µL (1 unit) GoTaq G2 Flexi DNA Polymerase (Promega, USA), 1 µM of each primer and 7 µL of DNA template. PCR cycling comprised 5 min at 95 °C, 40 cycles at 95 °C for 30 s, 60 s at 58 °C, 40 s at 72 °C, and a final extension at 72 °C for 5 min. Nested PCRs were then performed to specifically and separately amplify *B. caballi* and *T. equi*, with primers specific for *B. caballi* (BabF4 5′-TTG TAATTGGAATGATGGC-3′and BabR4 5′-TCCCTACAACTTYTCGRTGT-3′, expected amplicons length of 856 bp) and *T. equi* (TeqF3 5′-TCAGT TGCGTTTATTAGAC-3′ and TeqR5 5′-CACAAAACTTCCCTAGACG-3′, expected amplicons length of 1470 bp). We used 10 µL of 1/100 diluted amplicons in a 30 µL reaction mixture containing the same components as the first PCR. Cycling conditions were the same as for the primary reaction, with the exception of the annealing temperature, which was 54 °C in both cases of second specific amplification. The primers specific to *B. caballi* (BabF4 and BabR4) and the primers specific to *T. equi* (TeqF3 and TeqR5) were designed for the present study based on alignments of NCBI sequences selected to represent the different genetic clades.

Amplified fragments were purified with ExoSAP-IT reagent following the manufacturer's instructions (Affymetrix, USA). Mono or bi-directional Sanger sequencing was then performed by Eurofins Genomics, Germany, using the original PCR primers. The resulting sequences were assembled using Geneious R6 software (https://www.geneious.com). To identify sequence similarities, an online BLAST search was conducted (https://blast.ncbi.nlm.nih.gov). For further analysis, ClustalW software (https://www.ebi.ac.uk) was used to perform sequence similarity analysis. Finally, representative sequences were deposited in GenBank (accession numbers PQ044745 to PQ044786 for *T. equi*; accession numbers PQ044745 to PQ044795 for *B. caballi*).

2.4. Statistical analysis

Data analysis was conducted in R (v4.3.3) (R Core Team, 2023). The normality of all continuous variables was tested using the Shapiro-Wilk normality test. Continuous variables were expressed as mean, median and range. Differences in the proportional distribution of categorical variables among groups were evaluated using Fisher's exact tests (for two-by-two tables) or Chi-squared tests (for larger tables). Differences in the distribution of continuous variables between groups were assessed by non-parametric tests due to their non-normal distribution, using Wilcoxon rank sum tests (for two groups) or Kruskal-Wallis rank sum tests (for more groups). For multiple pairwise comparisons of categorical or continuous variables, a Bonferroni correction was applied: the significance level α was set to 0.05/n, with n the number of comparisons.

2.5. Phylogenetic analysis

To construct maximum likelihood phylogenetic trees, the advanced PhyML+SMS/OneClick workflow available on the NGPhylogeny.fr website was employed (https://ngphylogeny.fr). In brief, this pipeline performs several steps: first, it aligns sequences in FASTA format using MAFFT software (Katoh and Standley, 2013). Then, it cleans the alignments using BMGE software (Criscuolo and Gribaldo, 2010). Finally, it constructs phylogenetic trees using PhyML software (Guindon et al., 2010). This process includes automatic selection of the most suitable evolutionary model using SMS software (Lefort et al., 2017) and generates the final tree in Newick format (Junier and Zdobnov 2010). The parameters used are the default ones except for the PhyML+SMS analysis where the following choices were made: use of the BIC (Bayesian Information Criterion) for the model selection criterion, the NNI (Nearest Neighbor Interchange) for the tree topology and a boostrapping (FTB+TBE) of 1000 replicates for inference (Lemoine et al., 2018).

Phylogenetic analyses of the sequences were additionally performed using Bayesian inference using MAFFT software (Katoh and Standley, 2013), followed by cleaning of these alignments using BMGE software (Criscuolo and Gribaldo, 2010). We then used the BEAST2 software (v2.7.6) (Bouckaert et al., 2019) with the bModelTest plugin (v1.3.3) (Bouckaert and Drummond, 2017) to build the trees.

The generated trees were then formatted using TreeViewer v2.2.0 (Bianchini and Sánchez-Baracaldo, 2024).

3. Results

3.1. Description of sampled equids' population

From November 2019 to January 2023, blood samples from 566 horses were collected and analyzed, all asymptomatic for piroplasmosis. The place of residence of each horse is shown in Fig. 1 to highlight the geographical distribution of the equine population studied.

The study in Nantes ran from 2019 to 2023 and enrolled 169 horses from 11 counties (Table 1). At the other veterinary schools, blood was collected between 2020 and 2022 from 107 equids located in 22 different counties around Paris, 135 equids from 20 counties around Toulouse and 155 equids from 16 counties around Lyon (Table 1). In total, equids from 62 different counties were sampled (Fig. 1). Horses from seven counties were referred to different veterinary schools, either Nantes/Paris or Lyon/Toulouse, depending on geographic proximity and the owner's preference. A highly variable number of equids were sampled in the 62 counties, from 1 to 100. A detailed breakdown of the equine population studied by sampling year and department is provided in Supplementary Tables 1 and 2, respectively.

Incomplete questionnaires from some horse owners resulted in missing data for about 2 % of the population. The sex distribution of the horses sampled across the four veterinary schools differed significantly ($p = 0.011$, Chi-squared test). Overall, the study population consisted of a similar proportion of females ($n = 250$) and geldings ($n = 249$), and approximately 10 % of stallions ($n = 55$) (Table 1). Notably, the ratio of geldings to females was balanced in Nantes and Toulouse, but skewed towards females in Lyon and geldings in Paris.

The age distribution of the horses differed significantly between veterinary schools ($p = 0.043$, Kruskal-Wallis rank sum test). The mean and median age of the horse population studied were 11.5 and 11 years respectively, and ages ranged from 1 to 31 years. Ranking veterinary schools by increasing mean age order, horses in Nantes were younger (mean 10.4 years, range 1–31 years), followed by Paris (mean 11.8 years, range 2–26 years), Toulouse (mean 11.9 years, range 1–31 years), and Lyon (mean 12 years, range 1–27 years). A violin diagram illustrating the age distribution across the veterinary schools is provided in Supplementary Fig. 1.

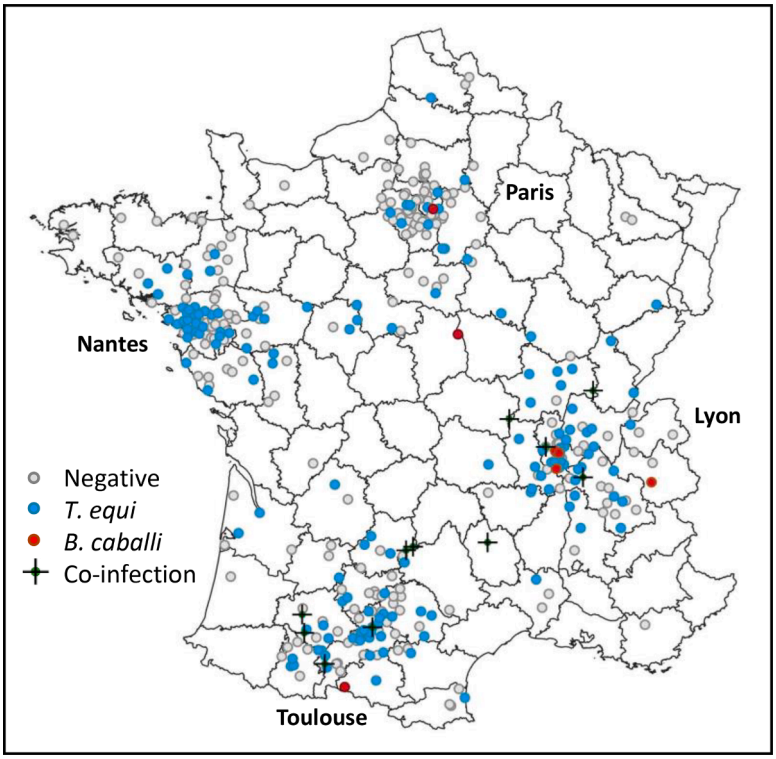

Fig. 1. Geographical location and carrier status of the horse population sampled in the four veterinary schools in France between 2019 and 2023 (107 horses around Paris, 169 horses around Nantes, 135 horses around Toulouse, 155 horses around Lyon). The french counties borders are indicated.

Table 1
Description of the sampled horse population (sampled area, year of sampling and gender).

	Number of tested equids per year					Sex				
Veterinary School	2019	2020	2021	2022	2023	Female	Gelding	Stallion	ND	Total
Paris	0	39	53	15	0	40	61	6	0	107
Nantes	71	62	32	0	4	73	74	22	0	169
Toulouse	0	5	52	78	0	57	64	12	2	135
Lyon	0	18	87	50	0	80	50	15	10	155
Total	71	124	224	143	4	250	249	55	12	566

3.2. *Frequency of asymptomatic piroplasmosis carriers according to the geographical location*

Our study revealed an overall carrier frequency of 38.3 % (217/566 horses) for equine piroplasmosis, with *T. equi* as the predominant parasite (91.7 % of the isolates; 209/228 isolates), and *B. caballi* much less frequent (8.3 % of the isolates; 19/228 isolates) (Table 2). A small proportion of horses (1.9 %; 11 horses) was co-infected with both parasites. Over half (57.8 %; 11/19 horses) of *B. caballi* carriers were co-infected with *T. equi*. Single infections with *B. caballi* were less common (1.4 %; 8 horses).

The frequency of equine piroplasmosis carriers varied significantly between the four horse populations sampled ($p = 4.64e\text{-}09$, Chi-squared test): 18.7 % around Paris (20/107 horses), 32.0 % around Nantes (54/169 horses), 41.5 % around Toulouse (56/135 horses), and 56.1 % around Lyon (87/155 horses) (Table 2). *Theileria equi* carrier frequencies were significantly lower around Paris compared to the other three populations and significantly higher in Lyon compared to the two northern populations (Nantes and Paris) (Table 3). The frequencies of equine piroplasmosis in Nantes and Toulouse were not significantly different, neither were those between Toulouse and Lyon.

Most *B. caballi* carriers were identified in the south of France, around

Table 2
Sampled population and prevalence of asymptomatic carriers of piroplasmosis, *Theileria equi* and *Babesia caballi* in equids sampled in the 4 veterinary schools between 2019 and 2023.

			Carrier frequency % [95 % CI] (number) of			
Veterinary School	Period	Number of sampled equids	Piroplasmosis carriers	*Theileria equi* carriers	*Babesia caballi* carriers	Co-infected carriers
Paris	2020–2022	107	18.7 [12.1–27.6] (20)	16.8 [10.5–25.6] (18)	1.9 [0.3–7.2] (2)	0 [0.0–4.3] (0)
Nantes	2019–2023	169	32.0 [25.1–39.6] (54)	32.0 [25.1–39.6] (54)	0 [0.0–2.8] (0)	0 [0.0–2.8] (0)
Toulouse	2020–2022	135	41.5 [33.2–50.3] (56)	40.7 [32.5–49.5] (55)	5.2 [2.3–10.8] (7)	4.4 [1.8–9.8] (6)
Lyon	2020–2022	155	56.1 [47.9–64.0] (87)	52.9 [44.8–60.9] (82)	6.5 [3.3–11.9] (10)	3.2 [1.2–7.8] (5)
Total	**2019–2023**	**566**	**38.3 [34.3–42.5] (217)**	**36.9 [33.0–41.1] (209)**	**3.4 [2.1–5.3] (19)**	**1.9 [1.0–3.6] (11)**

Table 3
Comparison of piroplasmosis carrier frequencies between the 4 sampled populations. P-values of pairwise comparisons are indicated, using Fisher's exact test. The significance threshold was set at p-value<0.0083 (0.05/6) after the Bonferroni correction and the p-values for significant differences are highlighted in bold.

	Nantes	Toulouse	Lyon
Paris			
Piroplasmosis carriers (20/107 - 18.7 %)	0.018	**1.6e-04**	**1.1e-09**
Theileria equi carriers (18/207 - 16.8 %)	**0.005**	**6.8e-05**	**1.8e-09**
Babesia caballi carriers (2/207 - 1.9 %)	0.149	0.306	0.130
Nantes			
Piroplasmosis carriers (54/169 - 32.0 %)		0.094	**1.2e-05**
Theileria equi carriers (54/169 - 32.0 %)		0.119	**1.9e-04**
Babesia caballi carriers (0/169 - 0 %)		**0.003**	**5.4e-04**
Toulouse			
Piroplasmosis carriers (56/135 - 41.5 %)			0.014
Theileria equi carriers (55/135 - 40.7 %)			0.045
Babesia caballi carriers (7/135 - 5.2 %)			0.803
Lyon			
Piroplasmosis carriers (87/155 - 56.1 %)			
Theileria equi carriers (82/155 - 52.9 %)			
Babesia caballi carriers (10/155 - 6.5 %)			

Toulouse and Lyon (89.5 %; 17 horses), with none found in Nantes and two (10.5 %) identified around Paris. Despite the low overall frequency of *B. caballi* carriers, equids in the areas around Toulouse and Lyon had a significantly higher frequency compared to Nantes, but not compared to Paris. There was no difference between *B. caballi* frequencies in Lyon and Toulouse (Table 3).

More detailed geographical representations of the horse location and carrier frequency by French region are shown in Figs. 1 and 2, respectively. Data for Normandy, Grand-Est, Provence-Alpes-Côte d'Azur, and Corsica were excluded due to limited samples (<10 horses) (see Supplementary Table 2 for details). The frequency of equine piroplasmosis carriers was significantly higher in Burgundy-Franche-Comté (82.4 %) compared to the five western or northern regions, but not compared to the southern or neighboring regions (Supplementary Table 3). The Ile-de-France region had a significantly lower carrier frequency compared to the southeastern regions: Burgundy-Franche-Comté, Auvergne-Rhône-Alpes, and Occitanie.

3.3. Comparison of frequencies of asymptomatic and symptomatic horses in the sampled population

Questionnaires asked horse owners about any previous episode of acute equine piroplasmosis in their horses, including the diagnostic methods used and the parasite species identified. Information about the occurrence of a previous acute piroplasmosis was obtained from 523 questionnaires (104 in Paris, 161 in Nantes, 129 in Toulouse, and 129 in Lyon). Owners were more certain of the past clinical signs of piroplasmosis than of the specific methods or parasite species involved. The reported frequencies of previous acute piroplasmosis cases were lower but positively related to the carrier frequencies observed in this study (Fig. 3). Most owners were not aware of the identity of the parasite involved, but a few of them remembered, with 21 declared cases of *T. equi* and 17 cases of *B. caballi* as the presumed causative agent.

3.4. Influence of horse movements or origin on piroplasmosis carrier status

The aim of this section was not to analyze horse movements in detail, but to highlight how they can influence the estimated frequency of carrier status, particularly in regions of low endemicity, therefore we chose Paris as our case study. Among the 20 carrier horses around Paris, nine (45 %) were born or lived in regions where equine piroplasmosis is highly endemic: five originated from French areas (Jura, Yonne, Haute-Garonne, Corsica) and four from southern countries (Italy, Spain). In contrast, only 13 of the 87 non-carrier horses (15 %) originated from these same regions or countries. Thus, originating from a region or country with high piroplasmosis frequency was associated with a statistically significant higher risk of infection ($p = 0.005$, Fisher's exact test). This highlights how horse movement can potentially inflate carrier frequency estimates in areas with low endemicity, such as the Paris region.

3.5. Influence of age and gender on the piroplasmosis carrier status

Due to differences in carrier frequencies observed between regions and between parasite species, we performed separate statistical analyses for carrier frequency according to age groups for each region.

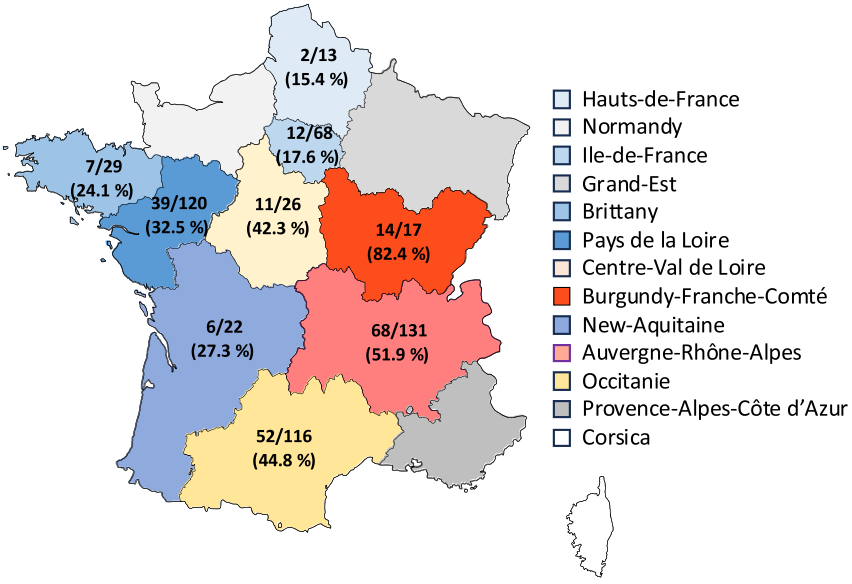

Fig. 2. Frequency of equine piroplasmosis carriers in the regions of France. The numbers of positive and tested horses are indicated in each region as well as the deduced frequency. Data are not indicated in the regions Normandy, Grand-Est, PCA and Corsica due to the too low number or absence of sampled equids (respectively 8, 2, 1 and 0, shades of grey). The regions where the frequency of carriers is lower than the mean prevalence in France are indicated with shades of blue. The regions where the frequency of carriers is higher than the mean prevalence in France are indicated with increasing shades of yellow/orange/red.

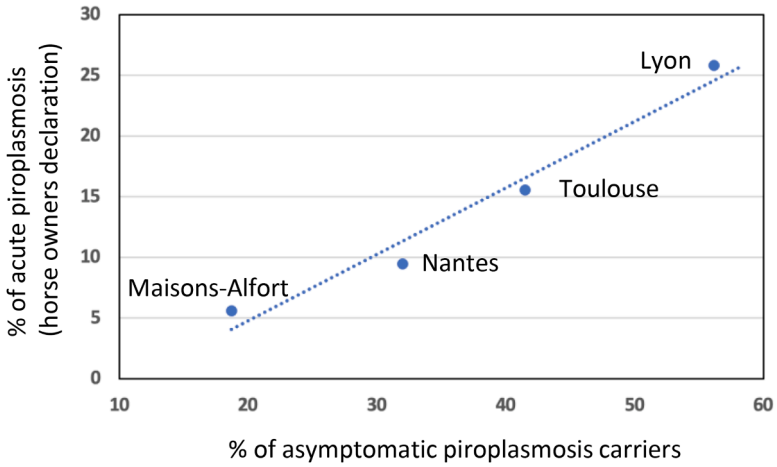

Fig. 3. Relationship between the frequency of carriers of equine piroplasmosis and the percentage of horses with reported acute piroplasmosis in the past, in each of the 4 equine populations analyzed. Acute piroplasmosis occurence is based on owners declarations in the questionnaires: 104 around Paris, 161 around Nantes, 129 around Toulouse and 129 around Lyon.

Additionally, analyses were performed separately for *T. equi* and *B. caballi* carriers due to their distinct infection dynamics. *Theileria equi* persists for life, potentially leading to a higher carrier prevalence with age. Conversely, *B. caballi* infections can be cleared, with a possible decrease in carrier frequency among older horses due to acquired immunity (Tirosh-Levy et al., 2020).

The frequencies of *T. equi* and *B. caballi* carriers by age group for each collection site are detailed in Table 4a and b respectively. We analyzed carrier frequency in specific age groups (e.g. ≤5 years, 6–10 years, etc.). A separate category was designated for young horses (less than three years of age, defined as ≤2 years), highlighted in grey in the Tables. This category was also included in the broader ≤5 years age group. This analysis revealed that horses get infected at a young age with up to 44 % of horses (16/36) already carrying *T. equi* around Lyon before the age of six years. More than one horse in three of the horses were already infected as foals around Lyon, compared to about one horse in four around Nantes and Toulouse (Table 4a). Applying a Bonferroni correction to adjust the significance level ($p < 0.001$), no significant effect of age on the frequencies of *T. equi* carriers was detected in pairwise comparisons of the five different age groups within each four sampled populations (data not shown). The observed gradient in *T. equi* carrier frequency across the four regions (Paris < Nantes < Toulouse < Lyon) remained consistent for all age groups.

For *B. caballi*, the highest frequency of carriers was found for foals in Toulouse and Lyon, and was significantly higher compared to the frequency in the remaining horse population (>3 years) in Lyon (4/16 compared to 6/136; $p = 0.012$, Fisher's exact test) (Table 4b). In Lyon, *B. caballi* infected foals represented 40 % (4/10) of the *B. caballi* infected horses. In Toulouse, 43 % (3/7) of the *B. caballi* infected horses were less than six years of age. As with *T. equi*, there was no significant effect of age on the frequencies of *B. caballi* carriers in pairwise comparisons of the five different age groups.

The population of sampled stallions was significantly younger than that of females ($p = 1.131e\text{-}13$, Wilcoxon rank sum test) or geldings ($p = 1.631e\text{-}14$, Wilcoxon rank sum test) in general, and also in each sampled area (data not shown) (Table 5). Despite this age difference, piroplasmosis carrier frequencies were not significantly different between stallions (36.4 %; 20/55) and geldings (35.7 %; 89/249) ($p = 1$, Fisher's

Table 4
Frequencies of piroplasmosis carriers (*Theileria equi* 4a and *Babesia caballi* 4b) by age classes in the 4 sampled horse populations. Frequencies of carriers in the population of foals (below three years of age) is indicated as a separate category (in grey), and is then included in the below 6 years of age category. This separation was performed to highlight the piroplasmosis frequency in foals.

a- Frequencies (%) and numbers of *Theileria equi* carriers by age classes in the 4 sampled horse populations.

	Paris	Nantes	Toulouse	Lyon
<= 2 Y	0.0 (0/1)	27.8 (5/18)	22.2 (2/9)	37.5 (6/16)
<= 5 Y	11.1 (1/9)	20.0 (10/50)	28.1 (9/32)	44.4 (16/36)
6 to 10 Y	16.2 (6/37)	33.3 (16/32)	44.8 (17/38)	55.2 (16/29)
11 to 15 Y	18.9 (7/30)	31.0 (9/29)	40.8 (11/27)	51.2 (22/43)
16 to 20 Y	23.5 (4/17)	43.5 (10/23)	40.0 (8/20)	53.6 (15/28)
>20 Y		44.4 (8/18)	42.9 (9/21)	56.3 (9/16)

b- Frequencies (%) and numbers of *Babesia caballi* carriers by age classes in the 4 sampled horse populations.

	Paris	Toulouse	Lyon
<= 2 Y	0.0 (0/1)	11.1 (1/9)	25.0 (4/16)
<= 5 Y	0.0 (0/9)	9.4 (3/32)	13.9 (5/36)
6 to 10 Y	5.4 (2/37)	5.3 (2/38)	3.5 (1/29)
11 to 15 Y	0.0 (0/30)	0.0 (0/27)	2.3 (1/43)
16 to 20 Y	0.0 (0/17)	10.0 (2/20)	10.7 (3/28)
>20 Y		0.0 (0/21)	0.0 (0/16)

exact test) or females (40.4 %; 101/250) ($p = 0.65$, Fisher's exact test), nor between females and geldings ($p = 0.31$, Fisher's exact test). This analysis was performed for each sampled area separately with the same conclusions (data not shown).

3.6. Genetic diversity of *T. equi* and *B. caballi* and phylogenetic analysis

In total, 201/209 *T. equi* positive samples produced 18S rRNA sequences identified as *T. equi*. These sequences were from horses in the areas around Paris (18), Nantes (52), Toulouse (53) and Lyon (78). Sequence lengths ranged between 230 and 1412 bp. Sequence identities were obtained via a blast analysis with reference sequences selected to represent geographically distant locations (AY534882 from Spain and KF559357 from China for the genotype E, KJ573370 from Brazil, AY150062 from Spain and KX227625 from Israel for the genotype A). Isolates and sequences description is detailed in the Supplementary Tables 4 and 5. A majority of genotype E (98 %; 197/201 sequences) and a few sequences of genotype A (2 %; 4/201 sequences) were characterized.

Within clade E, the sequence identities were higher than 99 % identity to each other or with geographically distant genotype E isolates, while identities were lower than 98.5 % with genotype A reference sequences. The genotype E isolates originated from equids living in 43 different counties in France. Of the 197 genotype E sequences analyzed, 136 (69 %) were identical to the KF559357 sequence from China, one of the most closely related sequences from a geographically distant region. The remaining 61 sequences exhibited high levels of similarity, with 50 sharing greater than 99.5 % identity to KF559357 and 11 sharing between 98.97 % and 99.46 % identity. Most of these variations were due to single or double nucleotide polymorphisms (34 and 10 sequences respectively), often located at the sequence ends or within single-read sequences, suggesting potential sequencing errors. Only one of these variants exhibited 100 % identity to a GenBank sequence (MZ326991). A detailed analysis of mismatches and insertions/deletions is provided in Supplementary Table 4.

The four sequences belonging to *T. equi* genotype A demonstrated identities higher than 99 % to each other and to geographically distant reference sequences. Identities with genotype E sequences were below 97 %. The genotype A isolates originated from equids living in 3 different counties in France. Two of these horses originated from Spain. These four sequences formed two pairs of identical sequences, with 99.2 % identity between the pairs. Two sequences (PQ044783 and PQ044785) were 100 % identical to *T. equi* sequences from the Americas (USA, Brazil, Cuba) and Israel. Interestingly, the horses carrying these sequences originated from Spain. The remaining two sequences (PQ044784 and PQ044786) were unique, with the closest matches (99.93 % identity, with a single mismatch) found in sequences from Mongolia (LC781916).

All 19 sequences identified as *B. caballi* belonged to the genotype A, and they shared identities higher than 99 % to each other and to sequences of *B. caballi* genotype A from geographically distant countries (Supplementary Table 6). Most of the sequences belong to sub-clade A1 described by Rar et al. (2024), with sequences from Western Siberian horses as well as from horses from different regions of the world. The exception is isolate CVT21–43, which is identical to a sequence from *Dermacentor silvarum* (OR789565) in the same region and closely related (99.87 %) to horse isolates (OR794991) of sub-clade A3, whose members were all from Western Siberian, as described by the same group.

Representative sequences of *T. equi* genotype E (38 sequences from different counties with accession numbers PQ044745 to PQ044782), *T. equi* genotype A (four sequences with accession numbers PQ044745 to PQ044786), and *B. caballi* (nine sequences from different counties with accession numbers PQ044745 to PQ044795) were deposited in GenBank. Details on sequence lengths, identities and geographic locations are given in the Supplementary Tables 4 to 6.

For each *T. equi* genotype and for *B. caballi* evidenced in this study, representative sequences were selected to confirm their phylogenetic positioning, with either the Maximum likelihood method (Figs. 4 and 5), or with the Bayesian inference method (Supplementary Fig. 2 and 3). Positioning in the different genotype clades was identical for each sequence whatever the phylogenetic method used.

4. Discussion

The aim of this study was to investigate the prevalence of asymptomatic carriers of equine piroplasmosis in France, analyze the relative proportion of each causative agent (*T. equi* and *B. caballi*), and to genetically characterize these parasite species. One of the inclusion criteria for horses in this study was outdoor or mixed (mainly outdoor pasture and indoor) type of housing, in order to focus on animals in contact with ticks, some species of them being competent vectors of equine piroplasmosis. Blood samples were taken from horses in four areas around each national veterinary school in France.

In the four regions studied, the frequency of *T. equi* carriers was much higher (16.8–52.9 %) than that of *B. caballi* (0–6.5 %), and *B. caballi* was not detected in asymptomatic horses from the north-

Table 5
Piroplasmosis carrier frequencies (noted freq.), mean and range (into brackets) ages of the horse population in the 4 sampled areas according to the gender and infectious status (NI: not infected, I: infected).

	Piroplasmosis carrier frequency (%) and mean age (min-max) of each category							
	Studied population (554)		Female (250)		Gelding (249)		Stallion (55)	
Sampled area	Carrier freq. (Number)	Age total *Age NI* **Age I**	Carrier freq. (Number)	Age total *Age NI* **Age I**	Carrier freq. (Number)	Age total *Age NI* **Age I**	Carrier freq. (Number)	Age total *Age NI* **Age I**
Paris	**18.7 %** (20/107)	11.8 (2–26) *11.9 (2–26)* **11.6 (5–18)**	**17.5 %** (7/40)	11.2 (4–23) *11.2 (4–23)* **11.1 (5–16)**	**21.3 %** (13/61)	12.5 (2–26) *12.6 (2–26)* **11.9 (7–18)**	**0 %** (0/6)	9.2 (3–19) *9.2 (3–19)* **none**
Nantes	**32.0 %** (54/169)	10.4 (1–31) *9.6 (1–31)* **12.1 (1–24)**	**26.0 %** (19/73)	10.4 (1–26) *9.8 (1–26)* **12.1 (2–24)**	**36.5 %** (27/74)	11.5 (2–31) *10.4 (2–31)* **13.5 (2–23)**	**36.4 %** (8/22)	6.0 (1–24) *5.7 (1–24)* **6.6 (1–16)**
Toulouse	**41.4 %** (55/133)	11.9 (1–31) *11.6 (1–28)* **12.4 (1–31)**	**45.6 %** (26/57)	13.3 (1–31) *13.5 (1–28)* **13.1 (1–31)**	**37.5 %** (24/64)	12.3 (1–25) *11.6 (1–25)* **13.5 (6–25)**	**41.7 %** (5/12)	3.3 (1–10) *3.3 (1–10)* **3.2 (1–6)**
Lyon	**56.1 %** (81/145)	12.0 (1–27) *11.4 (1–23)* **12.6 (1–27)**	**61.3 %** (49/80)	13.4 (2–27) *13.2 (2–23)* **13.5 (4–27)**	**50.0 %** (25/50)	12.4 (2–25) *11.5 (2–23)* **13.5 (2–25)**	**46.7 %** (7/15)	3.6 (1–14) *3.4 (1–7)* **3.9 (1–14)**
Total population	**37.9 %** (210/554)	11.5 (1–31) *11.0 (1–28)* **12.3 (1–31)**	**40.4 %** (101/250)	12.2 (1–31) *11.6 (1–28)* **13.0 (1–31)**	**35.7 %** (89/249)	12.1 (1–31) *11.5 (1–26)* **13.3 (2–25)**	**36.4 %** (20/55)	5.1 (1–24) *5.3 (1–24)* **4.7 (1–16)**

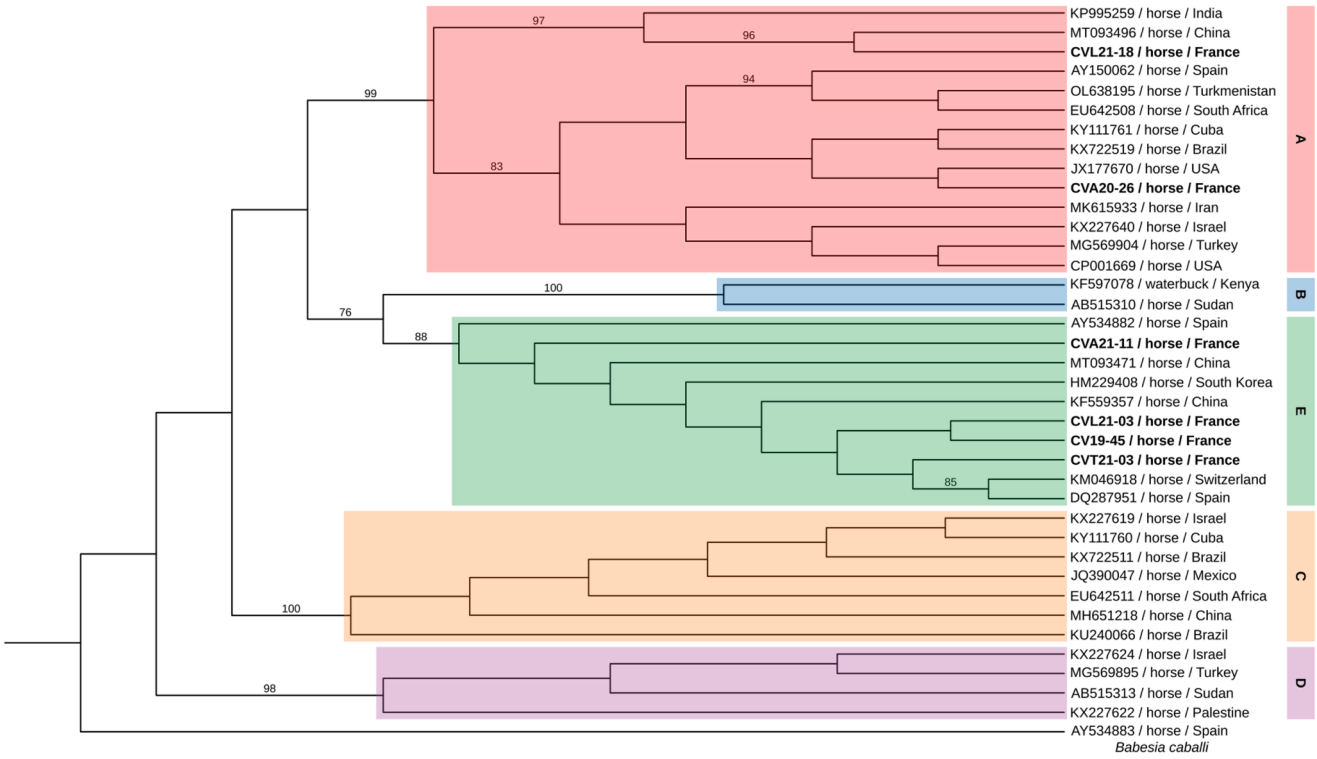

Fig. 4. A maximum likelihood tree of *Theileria equi* isolates based on sequences derived from the amplified 18S rRNA hypervariable regions. The tree includes 38 sequences with 1295 analyzed positions. The Tamura-Nei model has been used with 1000 repetitions. Significant bootstrap values (>70) are indicated on the branches. *Theileria equi* sequences reported in the litterature are denoted by their GenBank accession number, host and country of origin. Representative sequences obtained in the present study are indicated in bold. Previously described genotypes (A-E) as described in Tirosh-Levy et al. in 2020 are highlighted.

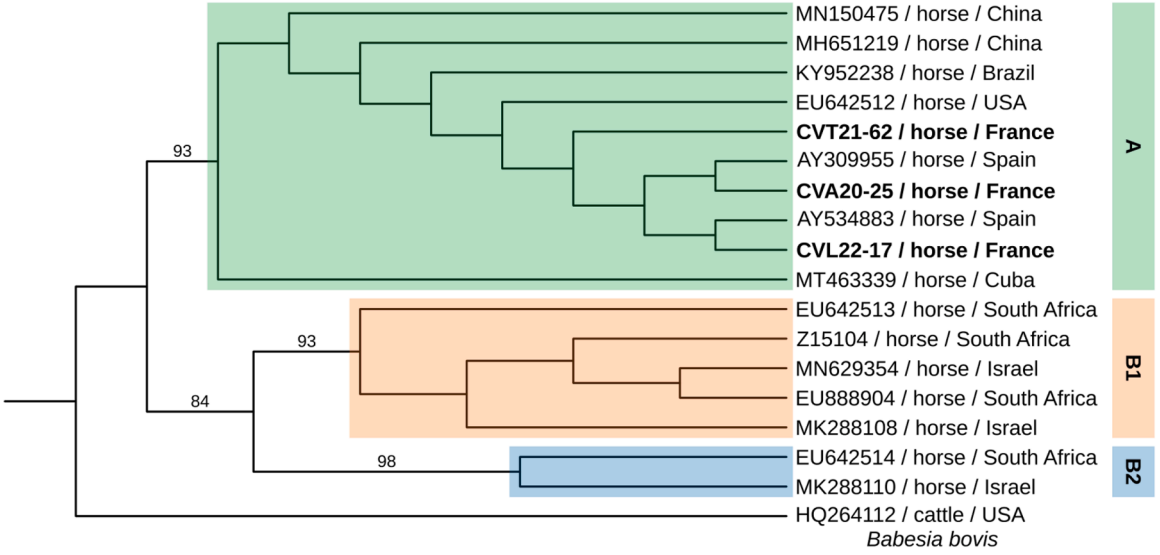

Fig. 5. A maximum likelihood tree of *Babesia caballi* isolates based on sequences derived from the amplified 18S rRNA hypervariable regions. The tree includes 18 sequences with 860 analyzed positions. The Tamura-Nei model has been used with 1000 repetitions. Significant bootstrap values (>70) are indicated on the branches. *Babesia caballi* sequences reported in the litterature are denoted by their GenBank accession number, host and country of origin. Representative sequences obtained in the present study are indicated in bold. Previously described genotypes (A, B1 and B2) as described in Tirosh-Levy et al. in 2020 are highlighted.

western part of France (Nantes). This pattern aligns with two other studies involving asymptomatic horses in southern France (Dahmana et al., 2019; Rocafort-Ferrer et al., 2022), and most other European countries where equine piroplasmosis is endemic (Montes Cortés et al., 2017; Nadal et al., 2022). This frequency difference occurs despite the presence of vector ticks common to both piroplasm species in these countries. It probably reflects the fact that infected animals that survive a first infection completely eliminate *B. caballi* one to four years after infection or after appropriate treatment, whereas *T. equi* remains as a lifelong infection (de Waal, 1992).

We observed variations in the frequencies of equine piroplasmosis across different regions in France. The highest carrier frequencies were noted in the two southern areas studied, particularly around Lyon (56.1 %). An intermediate frequency was recorded around Nantes (32.0 %), in

the north-western part of France, and a lower frequency was found around Paris (18.7 %), in the central-northern part of France. A significantly higher prevalence was measured in the Burgundy-Franche-Comté (82.4 %), and high prevalence was also found in Auvergne-Rhône-Alpes (51.9 %) and Occitanie (44.8 %). This apparent gradient in the prevalence of piroplasmosis, increasing from the north-west to the south-east, has also been documented using serological data based on the Complement Fixation Test (CFT) at the scale of French departments (Le Metayer, 2007), and later re-analyzed at the regional level (Nadal et al., 2022), using data from a dataset of 16,127 horse sera.

According to these analyses, the regions of Burgundy-Franche-Comté, Auvergne-Rhône-Alpes and Occitanie also exhibited the highest percentages of seropositivity. However, the seroprevalence measured 20 years ago in these regions was low (maximum 30 %), compared to the prevalence of carriers determined in the present study. These differences could be explained by an increase in the prevalence of equine piroplasmosis, variations in the sensitivity and specificity of the detection method used, or changes in climate. In the southernmost Mediterranean part of France, equine piroplasmosis agents were detected in the blood of 75 % of horses from stables where unexplained fever or weight loss had been identified by local veterinarians (Rocafort-Ferrer et al., 2022). In the same area, using the same kind of method (real-time PCR), lower prevalence was measured (29.4 %) in horses from equestrian centers, where access to pasture and therefore to vector ticks may have been more restricted (Dahmana et al., 2019). Differences in the prevalence of equine piroplasmosis between horses with similar types of housing (pasture in the present study) but in different geographical locations could be related to different climatic conditions. These conditions would influence not only the presence of different vector tick species, but also their abundance and activity period length (García-Bocanegra et al., 2013; Grandi et al., 2011; Kouam et al., 2010). Climatic factors such as temperature, humidity or rainfall influence tick habitats, and most likely the transmission dynamics of equine piroplasmosis. In this study, the four sampling areas are subject to different climates (Joly et al., 2010). Nantes experiences an oceanic climate, while the climate around Paris is described as a degraded oceanic climate, the area around Lyon as a mixture of continental and mountain climates with a Mediterranean influence, and Toulouse as a south-western basin climate. It remains to be determined whether these different climates impact tick populations and their abundance, and whether this can be linked to the prevalence of equine piroplasmosis. In France, four tick species could be involved in the transmission of equine piroplasmosis agents: *Dermacentor reticulatus, D. marginatus, Hyalomma marginatum*, and *Rhipicephalus bursa*, and they have been found to be infesting horses in France (Chastagner et al., 2013; Grech-Angelini et al., 2016; Rocafort-Ferrer et al., 2022; Vial et al., 2016). *Hyalomma marginatum* and *R. bursa* are known to prefer Mediterranean climates (Santos-Silva and Vatanserver, 2017; Vatanserver, 2017), and could therefore be more abundant in the southern part of France. However, data on their prevalence in horses in France is unknown.

A frequency of 38.3 % of equine piroplasmosis carriers was assessed in the overall sampled population. The 4 geographic areas analyzed represent approximately one-third of the French equine population, with the Pays de la Loire and Auvergne-Rhône-Alpes being two of the four regions with the most abundant equine populations (IFCE, 2023). Our study lacks data on the French region with the largest equine population, Normandy, where prevalence is likely to be low because this breeding region is located in the northern part of France and a seroprevalence of <5 % was determined using the CFT method (Nadal et al., 2022). In conclusion, we found one in three equids carrying piroplasmosis in France, ranging from one in five in the northwest to one in two in the south-east of France.

Based on this molecular analysis of piroplasmosis carriers and questionnaire responses, we found a positive relationship between the frequency of carriers and the frequency of previous episode of acute piroplasmosis, reported in horses in the four regions sampled. This relationship supports the difference in circulation of the etiological agents of equine piroplasmosis between the four regions sampled. Answers provided through the questionnaires highlight the fact that owners are often not fully aware of the infectious status of their horses. Many factors could explain the difference between the frequency of carriage and the frequency of declared acute piroplasmosis such as the acquisition by the owners of already infected equine or silent infections without clinical symptoms. According to the questionnaires, diagnostics during acute piroplasmosis revealed *B. caballi* (17 cases) almost as often as *T. equi* (21 cases, including serological diagnostic) as the potential causal agent. In the diagnosis of acute piroplasmosis with fever, *T. equi* can be mistaken as the causal agent of fever because of its lifelong persistence. When diagnosed during a fever, a *T. equi* carrier will be detected by serological and molecular means (this study), even if it is not the causal agent of the symptoms.

The French network for the surveillance of equine diseases (RESPE) is an online veterinary and laboratory-based reporting and information system designed to detect equine syndromes and non-notifiable diseases early in France (Lupo et al., 2023). A network of voluntary veterinarians, known as Sentinel Veterinarians, report clinical cases of isolated fever syndrome in a standardized manner and submit biological samples for screening for *Anaplasma phagocytophilum, B. caballi, T. equi*, Equine Infectious Anemia, and West Nile Fever (Lupo et al., 2022). On the samples collected and analyzed from 2013 to 2022 in France, *T. equi* was diagnosed in 34.6 % (2381 samples tested) and *B. caballi* in 13.9 % (2646 samples tested) of samples (Péju, 2023). The frequency of detection of *T. equi* was similar whether the sample consisted of asymptomatic or symptomatic horses (36.9 % in our study and 34.6 % in the RESPE analysis, respectively). However, the frequency of *B. caballi* differed, with 3.4 % in asymptomatic horses of our study compared to 13.9 % in horses with a fever in the RESPE analysis. This comparison suggests that the most symptomatic piroplasmosis cases likely stem from *B. caballi* infection, while in at least part of the RESPE study population, the detection of *T. equi* may result from *T. equi* carriers with *T. equi* not being the causal agent of the symptoms. This is further supported by the observation that equids with *T. equi* detection presented with milder clinical signs (mild fevers with body temperature below 39 °C, weight loss, reduced performance, no anemia) compared to the severe signs (severe fevers, anemia, weakness, anorexia, jaundice) associated with *B. caballi* infection (Péju, 2023). A quantitative analysis of the molecular detection would be particularly valuable in evaluating parasitemia levels in asymptomatic versus symptomatic horses.

The role of age as a risk factor for *T. equi* infection remains debatable, with studies both supporting or contradicting this association (refer to the discussion and references in Guidi et al., 2015 and Montes Cortès et al., 2017). These discrepancies may be linked to the endemicity level in each study area, to the age of the population sampled and to the methods used in each study. In highly endemic region, foals might acquire infections early through tick exposure or even by vertical transmission (Allsopp et al., 2007; Hermans et al., 2023; 2024), leading to high prevalence already in foals (approximately one in four foals are infected with *T. equi* around Nantes, Toulouse or Lyon, and with *B. caballi* in Lyon). Conversely, in regions with low endemicity, age might be a more influential factor in infection risk, as older animals accumulate greater tick exposure over time. However, no significant age-related differences in *T. equi* carrier frequency were observed across the four study areas. To reconcile this discrepancy, three potential explanations are considered. Firstly, while unlikely given existing literature, the possibility of horses naturally clearing *T. equi* infections cannot be entirely excluded. This would result in a stable carrier frequency within a region, regardless of age, as the population would consist of horses cleared from *T. equi* as well as newly infected horses. Secondly, fluctuations in parasitemia, potentially decreasing with age, might impact detection rates, notably as methods have inherent limitations in terms of blood volume analyzed. There are no detailed studies regarding the effect of age on parasitemia. A third possibility is that local

environmental factors influence infection risk. Regional climate can impact the suitability of habitats for competent tick vectors, while local factors such as pasture type and vegetation further shape tick populations. Given the limited movements of horses, individuals are consistently exposed to the same local tick burden. Consequently, horses in locally low-risk environments may remain uninfected, whatever the time they are exposed and the risk related to the climatic region. Therefore the *T. equi* carrier frequency in the older age classes (over 16 years of age) would represent the average risk of a horse to get infected in a given climatic region, this value being then variable according to local environmental conditions. This suggests that within a specific environment, most horses should either be rapidly infected (at risk environment, meaning favorable to ticks) or remain uninfected (no risk environment), unless individual variations in tick attraction or individual infection susceptibility/resistance exist among horses sharing the same pastures. As for age, the role of gender as a potential risk factor for infection also remains unclear, with conflicting results reported in the literature (Guidi et al., 2015; Onyiche et al., 2020). Our study did not identify any significant effect of gender on infection rates. A comprehensive understanding of the dynamics of *T. equi* and *B. caballi* infections, including transmission risk, host susceptibility, and parasite persistence, is still lacking. To address these knowledge gaps, long-term studies following horses from a young age are essential.

In this study, we sequenced partial 18S rRNA genes from most detected equine piroplasmosis agents, using the genotype references A to E for *T. equi* and A, B1 and B2 for *B. caballi* as described in the review by Tirosh-Levy et al. (2020). This analysis identified a predominant presence of the *T. equi* genotype E in France (98 % of isolates, 197 sequences) across all studied regions. Only a few isolates (4) belonged to genotype A and were found scattered across different areas (near Lyon, Toulouse, and Paris). Data on *T. equi* sequences found in France is scarce. We often had to determine genotypes by performing bioinformatic analysis on GenBank sequence references, either deposited directly or referenced as containing identical sequences (Bernard et al., 2024; Dahmana et al., 2019; Fritz, 2010; Grech-Angelini et al., 2020; Joly--Kukla et al., 2024). Studies have shown genotype E to be present in all positive horse samples from the northern part of France (Fritz, 2010) and from Marseille on the Mediterranean coast (Dahmana et al., 2019, *Theileria* sp. Europa var1). In Corsica, using standard conventional and real-time PCR methods, Dahmana et al. (2019) identified genotype A (46.1 %, *T. equi* var2 and var3), genotype E (35.9 %, *Theileria* sp. Europa var1), and genotype D (15.4 %, *Theileria* sp. Africa var 4) in asymptomatic horses. In the south of France and in Corsica, using high-throughput BioMark microfluidic amplification, only genotype A was found in *H. marginatum* and *R. bursa* ticks collected from horses (Bernard et al., 2024; Grech-Angelini et al., 2020; Joly-Kukla et al., 2024).

The genotype E is widespread in Eurasia (reviewed by Tirosh-Levy et al., 2020). Limited data from eastern and northern French neighboring countries highlight the presence of genotype E only in Ireland, Switzerland, Austria, Hungary and Czech Republic (Belkova et al., 2021; Coultous et al., 2020; Dirks et al., 2021; Farkas et al., 2013; Hornok et al., 2020; Liu et al., 2016). In contrast, southern European countries like Spain, Portugal, Italy, Croatia, Romania, and Greece typically show a mix of genotypes, including genotype E (Camino et al., 2020; Chisu et al., 2023; Coultous et al., 2022; Criado et al., 2006; Criado-Fornelio et al., 2004; Fuehrer et al., 2020; Gallusova et al., 2014; Toma et al., 2017; Veronesi et al., 2014). This distribution of genotypes is likely linked to the greater diversity of tick vectors present in southern Europe compared to northern regions.

In Spain, genotype E is well-represented, particularly in the north (Camino et al., 2020; Gimenez et al., 2009). Studies there have also suggested an association between genotype E and asymptomatic horses, while genotype A appears more frequent in symptomatic cases (Criado et al., 2006; Criado-Fornelio et al., 2004; Nagore et al., 2004). As our study focused on asymptomatic horses in order to evaluate the equine piroplasmosis reservoir, it should be complemented by a similar study on symptomatic horses in order to assess any potential difference this might introduce into our genetic diversity findings. Two of the four horses carrying the *T. equi* A genotype originated in Spain. They likely acquired this variant there and remained carriers.

All 19 *B. caballi* sequences obtained in this study belonged to genotype A, which is the most prevalent genotype worldwide (Tirosh-Levy et al., 2020). However, due to the low number of *B. caballi* carriers in our study, other genotypes may still exist in France. Genotypes B1 and B2 have been described in *H. marginatum* and *Rhipicephalus annulatus* ticks in Italy (Toma et al., 2017), and B1 has been found in horses in Spain (Camino et al., 2020; Criado et al., 2006; Criado-Fornelio et al., 2004; Nagore et al., 2004). To gain a more comprehensive understanding of *B. caballi* genetic diversity in France, we would need to increase sampling on symptomatic horses as well as ticks.

5. Conclusion

Equine piroplasmosis is endemic in France with an overall carrier frequency of 38.3 %, mostly due to *T. equi*, with a frequency of *T. equi* carriers higher in the southern parts of the country, and a low frequency of *B. caballi* carriers. No significant effect of horse age or gender on these frequencies could be demonstrated. The genetic diversity of isolates in France is rather low, with a great majority of *T. equi* genotype E and *B. caballi* genotype A. These results show that breeders and veterinary practitioners in endemic countries should pay more attention to systematic screening and prevention of piroplasmosis in horses because the economic losses associated with chronic *T. equi* carriage are likely to be significant. For example, screening tests should be more systematically offered at the time of transactions.

Further studies are still needed to assess the economic consequences of chronic *T. equi* carriage in horses and researchers should propose methods for long-term monitoring of infected horse populations. Finally, in the long term, it will be important to be able to propose measures to prevent infection, such as the use of acaricidal products. Local studies are still necessary to understand more precisely the respective role of factors linked to the horse (tick attractivity, immunity), the parasites (virulence, genotype), the vectors (transmission efficiency, vectorial capacity) and the environment (climate, favorable local conditions).

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work, LM used Gemini in order to improve the English language. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

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CRediT authorship contribution statement

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Declaration of competing interest

The authors declare that they have no competing interests.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ttbdis.2024.102434.

Data availability

The datasets used and/or analyzed during the current study are available from L.M. on reasonable request.

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