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Original Article

Great diversity of *Piroplasmida* in *Equidae* in Africa and Europe, including potential new species

Handi Dahmana[a], Nadia Amanzougaghene[a], Bernard Davoust[a,b], Thomas Normand[b], Olivier Carette[b], Jean-Paul Demoncheaux[b], Baptiste Mulot[c], Bernard Fabrizy[d], Pierre Scandola[b], Makhlouf Chik[a], Florence Fenollar[e], Oleg Mediannikov[a,*]

[a] IRD, AP-HM, MEPHI, IHU-Méditerranée Infection, Aix Marseille Univ, Marseille, France
[b] Working Group on Animal Epidemiology, French Forces Medical Service, Toulon, France
[c] ZoologicalResarch Center, Saint-Aignan-sur-Cher, ZooParc of Beauval, France
[d] Clinique Vétérinaire CyrnevetLupino, Bastia, France
[e] IRD, AP-HM, SSA, VITROME, IHU-Méditerranée Infection, Aix Marseille Univ, Marseille, France

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ABSTRACT

Piroplasms are *Apicomplexa* tick-borne parasites distributed worldwide. They are responsible for piroplasmosis (theileriosis and babesiosis) in Vertebrata and are therefore of medical and economic importance.

Herein, we developed a new real time PCR assay targeting the 5.8S rRNA gene and three standard PCR assays, targeting 18S rRNA, 28S rRNA, and *cox1* genes, for the detection of piroplasmids. These assays were first optimized and screened for specificity and sensitivity. Then, they were used to study a total of 548 blood samples and 97 ticks collected from *Equidae* in four sub-Saharan countries (Senegal, Democratic Republic of the Congo, Chad, and Djibouti) and France (Marseille and Corsica).

DNA of piroplasms was detected in 162 of 548 (29.5%) blood samples and in 9 of 97 (9.3%) ticks. The highest prevalence in blood samples was observed in Chad in 2016 with 72.9% positivity rate. Sequencing allowed the identification of four species of piroplasms, including two potentials new species. *Theileria equi* was mainly found. The highest prevalence was observed in Senegal (14 positive out of 23, 60.87%). *Babesia caballi* was detected in one horse in Senegal. Two new potential *Theileria* species were detected: *Theileria* sp. "*Africa*", observed in all areas excepted in Marseille and *Theileria* sp. "*Europa*", observed in Marseille and Corsica.

In conclusion, sensitive and specific PCR assays were developed for epidemiological studies of *Piroplasmida*. The circulation of multiple species of piroplasms, including two potentials new species, observed among Equidae from sub-Saharan Africa and France.

1. Introduction

Apicomplexa protists are stealth invaders, they can escape the immune response in host cells while using them as a source of nutrients (Striepen et al., 2007). Almost all apicomplexan are parasites, including multiple pathogenic species, both for humans and animals like malaria, toxoplasmosis, cryptosporidiosis, and piroplasmosis.

The parasites belonging to the apicomplexan order *Piroplasmida* include three genera namely: *Babesia*, *Theileria*, and *Cytauxzoon* (Schreeg et al., 2016). Equine piroplasmosis is an infectious tick-borne disease caused by the hemoprotozoan parasites *Theileria equi* and *Babesia caballi* (Wise et al., 2013). These piroplasms affect equid species, including horses, donkeys, mules and zebras(Wise et al., 2013). *T. equi* was initially named *Piroplasma equi* by Laveran in 1901, but after the discovery of schizogony in horse lymphocytes, which is known for *Theileria* but not for *Babesia*, this parasite was reclassified by Mehlhorn and Schein as *T. equi* (Uilenberg, 2006). In 1912, *B. caballi*, formerly named *Piroplasma caballi*, was first identified as another parasite infecting equids and different from *T. equi* and was assigned to the genus *Babesia* (Nuttall and Strickland, 1912).

Equine piroplasmosis caused by *B. caballi* and *T. equi* is endemic in tropical and subtropical zones; the latter is most prevalent (Friedhoff and Soulé, 1996). Southern Europe and Africa are highly endemic (Wise et al., 2014). Moreover, a high genetic diversity of these parasites has been observed in Tunisia(Ros-García et al., 2013) and Jordan (Qablan et al., 2013).

* Corresponding author at: MEPHI, IRD, APHM, IHU-Méditerranée Infection, 19-21 Boulevard Jean Moulin, 13385 Marseille Cedex 05, France.
E-mail address: olegusss1@gmail.com (O. Mediannikov).

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Equine piroplasmosis has serious impacts on horses' health. Agricultural production is strongly affected with high cost of control rules and impact on the carriage of merchandises and international commerce(International Office of Epizootics. Biological Standards Commission, 2012). In the United States, with a population of about 9.2 million horses, the direct economic impact of the equine industry is about 39 billion dollars per year. This industry also supports approximately 1.4 million full-time jobs (The American Horse Council, 2005). It took 25 years and $23 million for Southern California to eradicate equine piroplasmosis (USDA, 2010).

For a long time, *T. equi* and *B. caballi* were considered specific to their hosts. However, both parasites were recently identified in clinically healthy dromedaries by PCR, in Jordan (Qablan et al., 2012). Both were also detected in a dog in Croatia in 2009 (Beck et al., 2009). In France, in 2010, Fritz et al. analyzed 166 dogs; 31 were infected by *T. equi* and one by *B. caballi* (Fritz, 2010). Finally, *Babesia canis*, *Babesia rossi* and *Babesia capreoli* were recently reported to infect also horses (Fritz, 2010; Zanet et al., 2017).

Piroplasmosis is a typical zoonotic vector-borne infection (Schnittger et al., 2012). However, *T. equi* and *B. caballi* are not considered to infect humans (Maslin et al., 2004). Ticks and iatrogenic blood transfers are efficient modes of transmission in equids (Ueti et al., 2005). More than 21 tick species, mainly hard ticks, are associated with the transmission of these parasites. Co-infections are frequently reported in *Equidae*, often associated with co-infestation by tick species of the genera *Dermacentor*, *Hyalomma* and *Rhipicephalus*, (Tamzali, 2013). *Babesia* and *Theileria* exhibit different ecological relationships with their vectors and hosts. The reservoirs of *Babesia* include chronically infected animals and ticks (Yabsley and Shock, 2013). Infected ticks are able to transovarial and transstadial transmission of *B. caballi* from female ticks to its offspring. In contrast, *Theileria* are only transmitted transtadially (Ueti et al., 2005). Besides, *Equidae* are the primary reservoirs of *Theileria*.

Clinical signs of equine piroplasmosis are similar for both parasites (Tamzali, 2013).They occur after transmission within 10–30 days for *B. caballi* and 12–19 days for *T. equi*(de Waal, 1992). In endemic areas, most of infected horses are asymptomatic carriers with low level of parasitaemia. In case of concurrent disease or stress, they may develop clinical equine piroplasmosis (Allsopp et al., 2007). Acute forms of equine piroplasmosis can include fever (over 40 °C), sweating, congested mucous membranes, limb and supraorbital edema, icterus, anorexia, tachypnoea, tachycardia, anemia, and occasionally petechiae or ecchymoses (Tamzali, 2013). Death may occur in severe cases.

In this study, our aim was to develop sensitive and specific molecular tools able to potentially detect all piroplasm species and to analyze their phylogeny, in order to perform an epidemiological study on *Equidae* from sub-Saharan Africa and France.

2. Materials and methods

2.1. Study area and samples collection

In total, 548 blood samples and 97 hard ticks were collected from *Equidae* (horses and donkeys). Blood samples were collected in four countries from sub-Saharan Africa (Chad, Senegal, Djibouti, and Democratic Republic of the Congo [DR Congo] and from France (Marseille and Corsica) (Fig. 1). Hard ticks have only been collected in Chad. All equines were apparently healthy at the time of sampling (Table S1).

In Chad, we had two periods of sampling of saddle horses belonging to the Chadian National Guard (GNT). Ninety-six male horses (from 4 to 15-year-old) were sampled in 2012 and 60 in 2016 (59 male and one female). The infestation of horses by ticks is noticeable during wet periods especially, with 97 ticks collected in March 2016. In Senegal, an epidemiological survey, conducted from 2011 to 2014, allowed the inclusion of 126 horses and 54 donkeys from north-west villages. In Djibouti, in the Decan Reserve, 5 horses and 11 Somali wild donkeys (*Equus asinus somalicus*) were sampled in March 2010. In DR Congo, a screening survey performed in August 2012 at the Equestrian Club in Kinshasa covered 48 sport and leisure horses (34 males and 14 females from 5 to 33 year-old). In the city of Marseille (France), a study was conducted in February 2015 on 51 horses (30 males and 21 females from 7 to 35-year-old). Finally, in Corsica (French island in the Mediterranean Sea), 98 horses (59 males and 39 females from 1 to 29-year-old) were sampled in September 2014, in 13 equestrian centers.

The blood sampling was done aseptically using an *ad-hoc* device (BD Vacutainer® system; Becton Dickinson, Franklin Lakes, New Jersey, USA) and a single use needle (20G – 0.9 × 40 mm) at the jugular vein. Vacuum tubes with EDTA K3 were used to collect the blood. Then, specimens were stored and transported at +4 °C. On arrival at the laboratory, specimens were frozen at −80 °C.

The 97 ticks were collected manually from Chadian horses in 2016 and stored in 70% ethanol until they were identified under a binocular microscope. We used the available taxonomic keys and morphometric table to classify the ticks by family, genus and species (Walker et al., 2003). Each tick was washed three times in distilled water and stored at −20 °C until DNA extraction.

2.2. DNA extraction

EZ1 DNA Kits (Qiagen, Courtaboeuf, France) have been used for the DNA extraction. The DNA was extracted from 200 µl of blood and from half of a tick. DNA extracts were then stored at −20 °C until PCR analysis.

2.3. Piroplasmida specific-PCR tools design

In order to detect piroplasms DNA, we designed primers and probe targeting the most conserved regions of encoding ribosomal RNA genes (28S, 18S, and 5.8S), as well as primers targeting mitochondrial gene (*cox1*). House-keeping genes that are typically constitutive genes, required to maintain basal cell function and that are reliable to study piroplasms phylogeny (Schreeg et al., 2016).

First, a real time PCR assay for the screening of all piroplasms targeting a conserved region of the 5.8S gene was developed. Then, for species identification, we designed three conventional PCR assays targeting 969-bp,750-bp, and 480–720-bp sequences from the 18S, 28S, and *cox1* genes, respectively. In order to detect an eventual co-infection by the two equine piroplasmosis agents, PCR assays for specific amplification of *B. caballi* were developed targeting a 2930-bp sequence of the 28S gene.

All primers and probe for real time PCR and conventional PCR assays were designed using free web Primer3 software, version 4.0 (http://frodo.wi.mit.edu/primer3/); their sequences were listed in Table 1. The specificity and sensitivity of all PCR assays were tested *in silico* using primer-BLAST (NCBI, USA) and were validated using a panel of DNA extracts from several species of piroplasms, arthropods and bacteria, as well as from human, donkey, horse, cattle, mouse and dogs (Table S2).

2.4. Piroplasmid DNA detection, PCR amplification and phylogenetic analysis

The analytical sensitivity of the 5.8S real time PCR was assessed using 10-fold dilution from horse infected blood, which harbored 1.64 E +5 parasites per ml (100 ul of blood extracted and eluted in 50 ul using EZ1 DNA Kits (Qiagen, Courtaboeuf, France).

The initial screening was performed using the 5.8S-based real time PCR assay. Then, the identification of *Piroplasmida* species in real time PCR-positive samples was based on the amplification of a 969-bp fragment of 18S rRNA gene. Finally, the 28S rRNA and *cox1* genes were amplified from selected samples representing different genetic variants

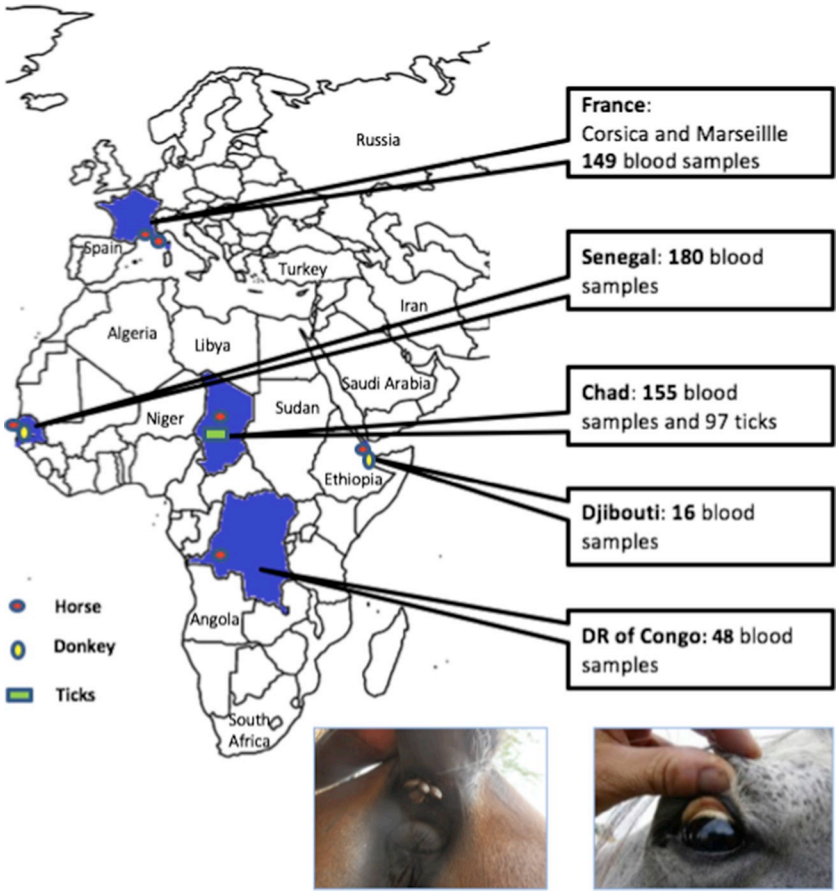

Fig. 1. Map of countries where samples were collected in sub-Saharan Africa and Europe.

based on the 18S rRNA sequence analysis.

Reaction mix for the 5.8S real-time PCR contained 5 µl of the DNA template, 10 µl of Eurogentec Takyon™ Mix (Eurogentec, Liège, Belgium), 0.5 µl (20 µM) of each reverse and forward primers, 0.5 µl (5 µM) of the FAM-labeled probe (Table 1) and 3.5 µl of distilled water DNAse and RNAse free, for a final volume of 20 µl. The real time PCR amplification was carried out in a CFX96 Real-Time system (Bio-Rad Laboratories, Foster City, CA, USA) using the following thermal profile: Incubation at 50 °C for 2 min for UDG action (eliminating PCR amplicons contaminant), then activation step at 95 °C for 3 min, followed by 40 cycles of denaturation at 95 °C for 15 s and annealing-extension at 60 °C for 30 s.

Table 1
Oligonucleotide sequences of primers and probe used for qPCRs and conventional PCRs in this study.

Targets	Targeted gene	Name	Primers (5′–3′) and probe	Annealing temperature
Piroplasmida	5.8S	5.8S-F5	AYYKTYAGCGRTGGATGTC	60 °C
		5.8S-R	TCGCAGRAGTCTKCAAGTC	
		5.8S-S	FAM-TTYGCTGCGTCCTTCATCGTTGT-MGB	
	18S	piro18S-F1	GCGAATGGCTCATTAIAACA	58 °C
		piro18S-F4	CACATCTAAGGAAGGCAGCA	
		piro18S-F3	GTAGGGTATTGGCCTACCG[a]	
		piro18S-R3	AGGACTACGACGGTATCTGA[a]	
	28S	28S-F2	CACCCCYGKCCGTACDGAT	58 °C
		28S-R2	CCTCAYTGAGYTYGCCTTRGGAC	
B. caballi	28S	28Scab-F4	GCAGAAAAGAAAATAACCATG	62 °C
		28Scab-R3	GCGTTCAGTCATTATCCAACGG	
		28Scab-F6	GCTTTAGCACGGTTGCTAGGA[a]	
		28Scab-F7	GGCATAGCAGTCCGGYYTTCGG[a]	
		28Scab-F8	TGTCCCTATCTGCCATCTAG[a]	
		28Scab-R6	TCGCCCCTATACCCGGATTTG[a]	
		28Scab-R7	CCGAARRCCGGACTGCTATGCC[a]	
	cox1	piro-F	AGGAAGTGGWACWGGITGGA	57 °C
		piro-R	GATGISCCCAIACIARACAWCC	
Theileria sp. "Europe"	*cox1*	383-F3	GAAGTGGWACHGGDTGGAC	57 °C
		900-R1	CCHGADGTATACATATGRTG	
Theileria sp. "Africa" & *Theileria equi*	*cox1*	COI-F	GTGAYGTTGTTTTTCCAAG	57 °C
		COI-R	CCWGTTGTACCTCCAAYDAC	

[a] Primers used for sequencing only.

Conventional PCR amplifications were performed in a Peltier PTC-200 model thermal cycler (MJ Research Inc., Watertown, MA, USA). PCR reactions contained 5 µl of the DNA template, 25 µl of Amplitaq-Gold STAR™ Mix (Eurogentec, Liège, Belgium), 10 µM (1 µl) of each primer, and 18 µl of distilled water DNAse and RNAse free.

Conditions for conventional PCR were one incubation step at 95 °C for 15 min, 40 cycles of 1 min at 95 °C, 30 s annealing at a different hybridization temperature for each PCR assay and 1 min at 72 °C followed by a final extension for 5 min at 72 °C (Table 1). Negative and positive controls were included in each molecular assay. The success of amplification was confirmed by electrophoresis on a 1.5% agarose gel. The purification of PCR products was performed using NucleoFast 96 PCR plates (Macherey-Nagel, Hoerdt, France) according to the manufacturer's instructions.

Co-infections were confirmed by amplifying different species from the same DNA after cloning using PGEM easy vector system II A1380 (Promega, Charbonnières-les-Bains, France). The amplicons were sequenced using the Big Dye Terminator Cycle Sequencing Kit (Perkin Elmer Applied Biosystems, Foster City, CA, USA) with an ABI automated sequencer (Applied Biosystems). The obtained sequences were assembled and edited using ChromasPro software (ChromasPro 1.7, Technelysium Pty Ltd., Tewantin, Australia). Then, sequences were compared with those available in the GenBank database by NCBI BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Our sequences obtained from positive samples in addition to sequences of validated piroplasms species already available in Genbank were aligned using Bioedit software version 7.0.5.3 (ClustalW multiple alignment) (Tom Hall, 2011). For taxonomic analyses, the Maximum-likelihood phylogenetic trees constructed using MEGA software version 7.0.21(Kumar et al., 2016)with 100 bootstrap replications and species position confirmed using Topali software version V2.5 (2.5.13.04.03) (Milne et al., 2009). More specific information found in front of figures.

2.5. Statistical analysis

The Epi Info version 7 program (http://www.cdc.gov/epiinfo/index.html) was used to compare prevalence. A difference was statistically significant when *p*-values were $< .05$.

3. Results

3.1. Sensitivity and specificity of PCR assays

The 5.8S real time PCR, as well as the standard 18S RNA-based PCR assay, allowed the amplification of the following piroplasmids: *T. equi, B. caballi, Theileria ovis, Theileria annulata, Theileria buffeli, Babesia canis*, and *Babesia vogeli,* all are already identified and sequenced in our laboratory (Table S2). All 18S amplicons from controls were sequenced. The obtained sequences allowed an accurate identification of all controls. None of the DNA extracts from negative controls were amplified.

The real time PCR is able to detect the presence of parasites with an efficiency of 121.5%, slope: 2.895, Y.int: 41.44 and an almost perfect coefficient higher than 0.98. The limit of detection was fixed at 1.64E +2 parasites/ml in blood which means 0.82–1 copy per reaction (Supp. Table S5)To insure the reproducibility of the results, samples from the DRCongo ($n = 48$) were tested simultaneously by 5.8S real time PCR and 18S PCR tools. Both gave the same result (21 positive on 48 samples). None of them missed positive samples; 5.8S real time PCR tool was then used for screening in all our samples.

3.2. Results of the PCR tools on the samples

In total, 548 blood samples of *Equidae* were tested for the presence of piroplasms' DNA using the real time PCR assay. 29.56% were found positive, including 54 samples from France and 108 from sub-Saharan Africa. The overall results on blood samples are presented in Table 2.

Table 2
Overall prevalence of piroplasms in blood and tick samples.

	Blood	Ticks
Country	Number of positive/Number of tested samples (%)	
France (Marseille)	15/51 (29.4)	NA
France (Corsica)	39/98 (39.8)	NA
Senegal	23/180 (12.8)	NA
DR Congo	21/48 (43.75)	NA
Chad (2012)	20/96 (20.8)	NA
Chad (2016)	43/59 (72.9)	9/97 (9.27)
Djibouti	1/16 (6.25)	NA
Total	162/548 (29.6)	9/97 (9.27)

NA: Not available.

Table 3
Prevalence of piroplamosis agents according to the country, animals and their sex.

	Horses	Donkeys	X^2 test
	Number of positive/Number of tested samples (%)		
Senegal	22/127 (17.32%)	1/53 (1.87%)	$p \leq .02$
DR Congo	21/48 (43.75%)	0	Only horse
Marseille (France)	15/51 (29.41%)	0	Only horse
Corsica (France)	39/98 (39.8%)	0	Only horse
Chad in 2012	20/96 (20.83%)	0	Only horse
Chad in 2016	43/59 (72.88%)	0	Only horse
Djibouti	0/5 (0%)	1/11 (9.1%)	$p \leq .90$

	Male	Female	X^2 test
	Number of positive/Number of tested samples (%)		
Senegal	13/127 (10.57%)	10/53 (18.87%)	$p \leq .20$
DR Congo	13/34 (38.24%)	8/14 (57.14%)	$p \leq .5$
Marseille (France)	8/33 (24.24%)	7/18 (38.89%)	$p \leq .5$
Corsica (France)	18/59 (30.5%)	21/39 (53.85%)	$p \leq .20$
Chad in 2012	20/96 (20.83%)	0	Only male
Chad in 2016	43/59 (72.88%)	0	Only male
Djibouti	1/16 (6.25%)	0	Only male

In France, real time PCR screening detected 15/51 (29.4%) positive horses in Marseille, and 39/98 (39.8%) positive horses from Corsica. There is no statistically significant difference in the prevalence of piroplasm between these two studied sites (Table 3).

In sub-Saharan Africa, we found 23/180 (12.8%) positive *Equidae* from Senegal and 21/48 (43.75%) positive horses from DR Congo. Only one horse of 16 (6.25%) *Equidae* from Djibouti was tested positive. All 11 wild Somali donkeys were found negative. In Chad, the prevalence was significantly higher in 2016 (43/59, 72.9%) than in 2012 (20/96, 20.8%), $p < .0001$(Table S3).

Considering the species of *Equidae* we studied, the prevalence of piroplasm was significantly higher in horses (**160/484**, 33.06%) than in donkeys (**2/64**, 3.12%), $p \leq .0001$. The infection was also observed more often in females (**46/124**, 37.09%) than in males (**116/424**, 27.36%), $p < .20$. More detailed data are given in Table 3.

Of the 97 ticks collected in Chad (2016), only 9 (8 female and one male ticks) were positive (9.27%). Interestingly, all positives ticks were *Rhipicephalus decoloratus*, which were collected from two horses (coded MGM04 and MGM10) that were also found positive for piroplasms.

3.3. Piroplasms species identification

To identify piroplasms species, all positive samples were subjected to standard PCR (targeting 18S rRNA gene) coupled with sequencing. Sequencing results showed the existence of a large genetic diversity

Table 4
Piroplasms genetic variants infecting equines found in studied areas.

Genetic variants	Areas						
	Senegal	DR Congo	Chad	Djibouti	Corsica	Marseille	Total
	Number of positive in this variant/Total of positives (%)						
Theileria equi (var2)	13/23 (56.5)	3/21 (14.28)	5/63 (7.9)	0/1 (0)	11/39 (28.2)	0/15 (0)	32/162 (19.8)
Theileria equi (var3)	1/23 (4.3)	0/21 (0)	7/63 (11.1)	0/1 (0)	7/39 (17.9)	0/15 (0)	15/162 (9.3)
Theileria sp. "*Africa*" (var4)	1/23 (4.3)	0/21 (0)	14/63 (22.2)	0/1 (0)	6/39 (15.3)	0/15 (0)	21/162 (12.9)
Theileria sp. "*Africa*" (var5)	3/23 (1.3)	17/21 (80.9)	5/63 (7.9)	1/1 (100)	0/39 (0)	0/15 (0)	26/162 (16)
Theileria sp. "*Africa*" (var6)	4/23 (17.4)	1/21 (4.76)	22/63 (34.9)	0/1 (0)	0/39 (0)	0/15 (0)	27/162 (16.6)
Theileria sp. "*Africa*" (var7)	0/23 (0)	0/21 (0)	10/63 (15.8)	0/1 (0)	0/39 (0)	0/15 (0)	10/162 (6.2)
Theileria sp. "*Europa*" (var1)	0/23 (0)	0/21 (0)	0/63 (0)	0/1 (0)	14/39 (35.9)	15/15 (100)	29/162 (17.9)
Theileria sp. "*Europa*" (var1.2)	0/23 (0)	0/21 (0)	0/63 (0)	0/1 (0)	1/39 (2.6)	0/15 (0)	1/162 (0.6)
Babesia caballi	1/23 (4.3)	0/21 (0)	0/63 (0)	0/1 (0)	0/39 (0)	0/15 (0)	1/162 (0.6)
Total	23	21	63	1	39	15	**162**

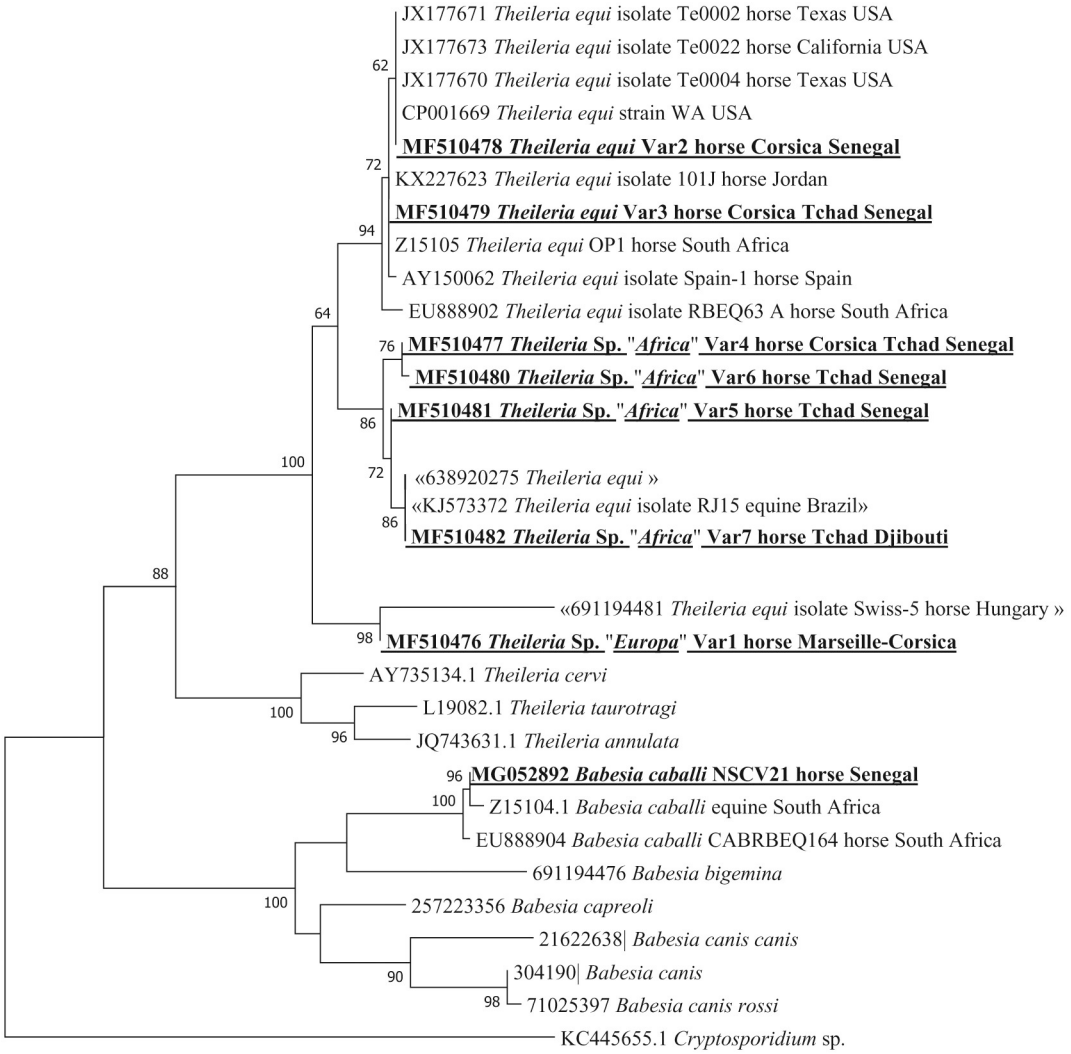

Fig. 2. Maximum-likelihood phylogenetic tree of piroplasms, including our new species based on partial 858-bp 18S gene.

(Table 4) allowing us to identify four piroplasms species: *T. equi*, *B. caballi* and two potential new species; provisionally named here *Theileria* sp. "*Africa*" and *Theileria* sp. "*Europa*". The highest prevalence was observed for *Theileria* sp. "*Africa*" (51.85%, 84/162) followed by *T. equi* (28.39%, 46/162), *Theileria* sp. "*Europa*" (19.14%, 31/162), and *B. caballi* (0.62%, 1/162). Only one horse from Senegal was positive for *B. caballi*.

We selected some samples corresponding to each 18S genetic variant to sequence using 28S and *cox1* genes (Table 1). All sequences were submitted to the Genbank and their accession numbers were obtained (Table S3). A horse from Senegal was co-infected by *T. equi* and *B. caballi* which is confirmed by amplifying the two species from the same DNA after cloning.

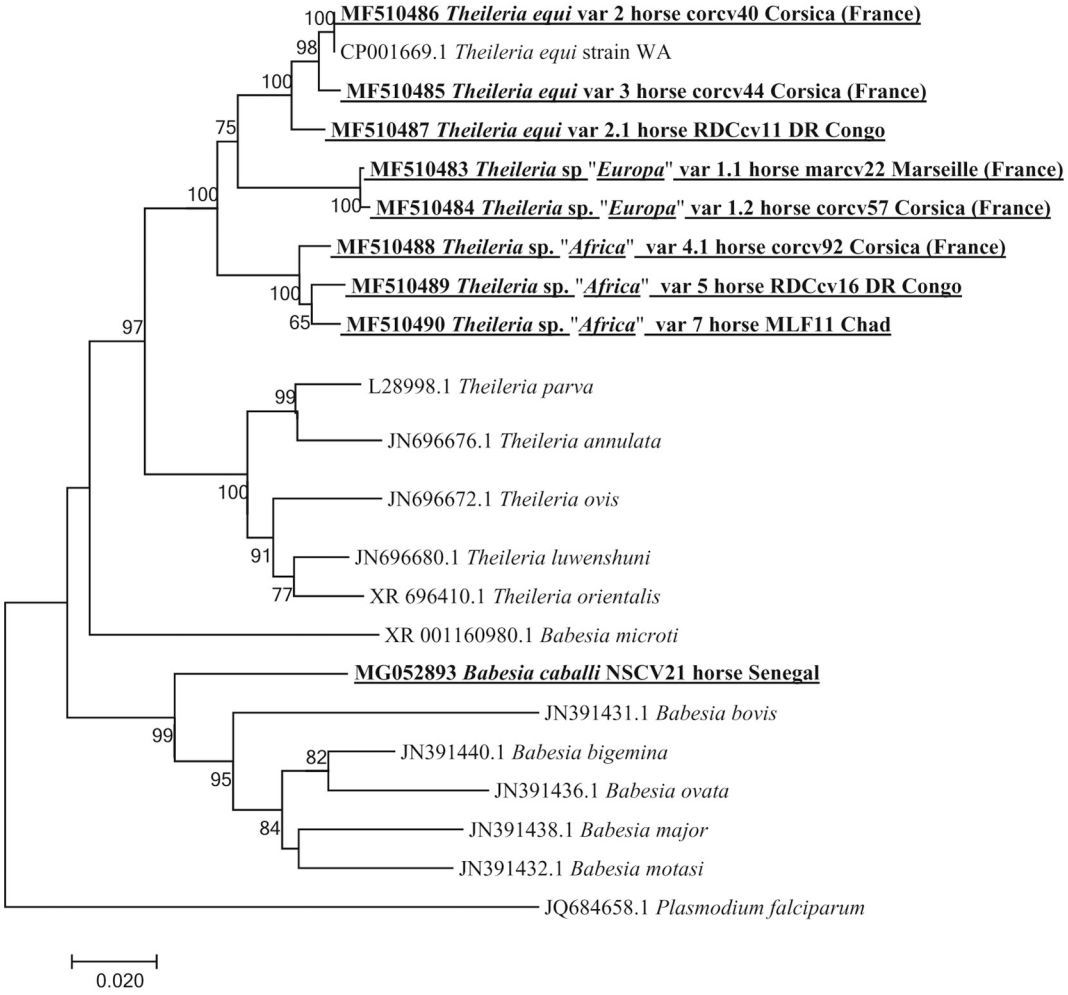

Fig. 3. Maximum-likelihood phylogenetic tree of piroplasms, including our new species based on partial 754-bp 28S gene.

3.4. Phylogenetical analysis

The phylogenetic trees constructed on the base of 18S, 28S and *cox1* genes allowed the position of the identified species to be visualized (Figs. 2, 3, and 4, respectively). Both *Theileria* sp. "*Africa*" and *Theileria* sp. "*Europa*" formed separate clades with samples of different geographical origins grouped together with a good bootstrap support (Figs. 2, 3, and 4).

In a second time, we compared sequences of three genes of *Theileria* spp. identified in the present study with *T. equi* sequences of WA strain and USDA strains available in the Genbank (Table S4). We discovered that the differences among *T. equi* and the two potential new species varied between 4 and 14%.

4. Discussion

To the best of our knowledge, all published PCR-based assays are able to amplify either only one species or a group of piroplasms (Qurollo et al., 2017; Salem et al., 1999). Herein, we have developed a real time PCR assay targeting the 5.8S gene able to detect well-known equine pathogens (*T. equi* and *B. caballi*), well-known mammal pathogens as well as potential new species. The 5.8S gene is a conservative gene easy to amplify by PCR using one pair of universal primers (Gou et al., 2012). For the design of our 5.8S real time PCR assay, highly divergent regions flanked by highly conserved regions among piroplasms have been targeted from 32 aligned sequences of *Theileria* spp. and *Babesia* spp. The designed real time PCR assay exhibited a good sensitivity. As expected *in silico*, the tool allowed an accurate *in vitro* detection of all known piroplasms, as well as those not yet described. Besides, none of the DNA extracts from the control group were amplified with this molecular tool confirming its specificity. The *cox1* gene is one of the most used markers for population genetics and phylogeographic studies (Derycke et al., 2010). The 18S and 28S genes, generally considered to be highly conserved, are actually composed of a mixture of conserved and divergent regions (Gou et al., 2012). For the design of the 28S and *cox1* PCR assays, 35 and 34 sequences were respectively aligned. The molecular phylogeny of *Babesia* and *Theileria* is usually based on 18S sequences (Gou et al., 2012). For the design of our 18S PCR assay, the combination of primers F1 and R4 showed high specificity.

Overall, we analyzed 548 blood specimens from apparently healthy *Equidae* sampling in four sub-Saharan African countries (DR Congo, Djibouti, Chad, and Senegal) and France (Marseille and Corsica). The overall prevalence of piroplasms detected in blood samples was 29.6% (162/548). Only one case of *B. caballi* was diagnosed in Senegal, in a horse. All the other samples were positive for *Theleiria* spp. Thus, the overall prevalence of *Theleiria* spp. was28.4%. Besides, two new potential *Theleiria* species were detected, *Theileria* sp. "*Europa*" and *Theileria* sp. "*Africa*". *Theileria* sp. "*Europa*" exhibited a prevalence of 5.7% (31/548). *Theileria* sp."*Africa*" was more commonly observed with a prevalence of 15.3% (84/548). This higher prevalence observed in Chadian horses in 2016 compared to 2012 can be explained by the inclusion of another sampling site such as Amguifel (Table S1). Previous studies reported PCR-negative samples while indirect fluorescent

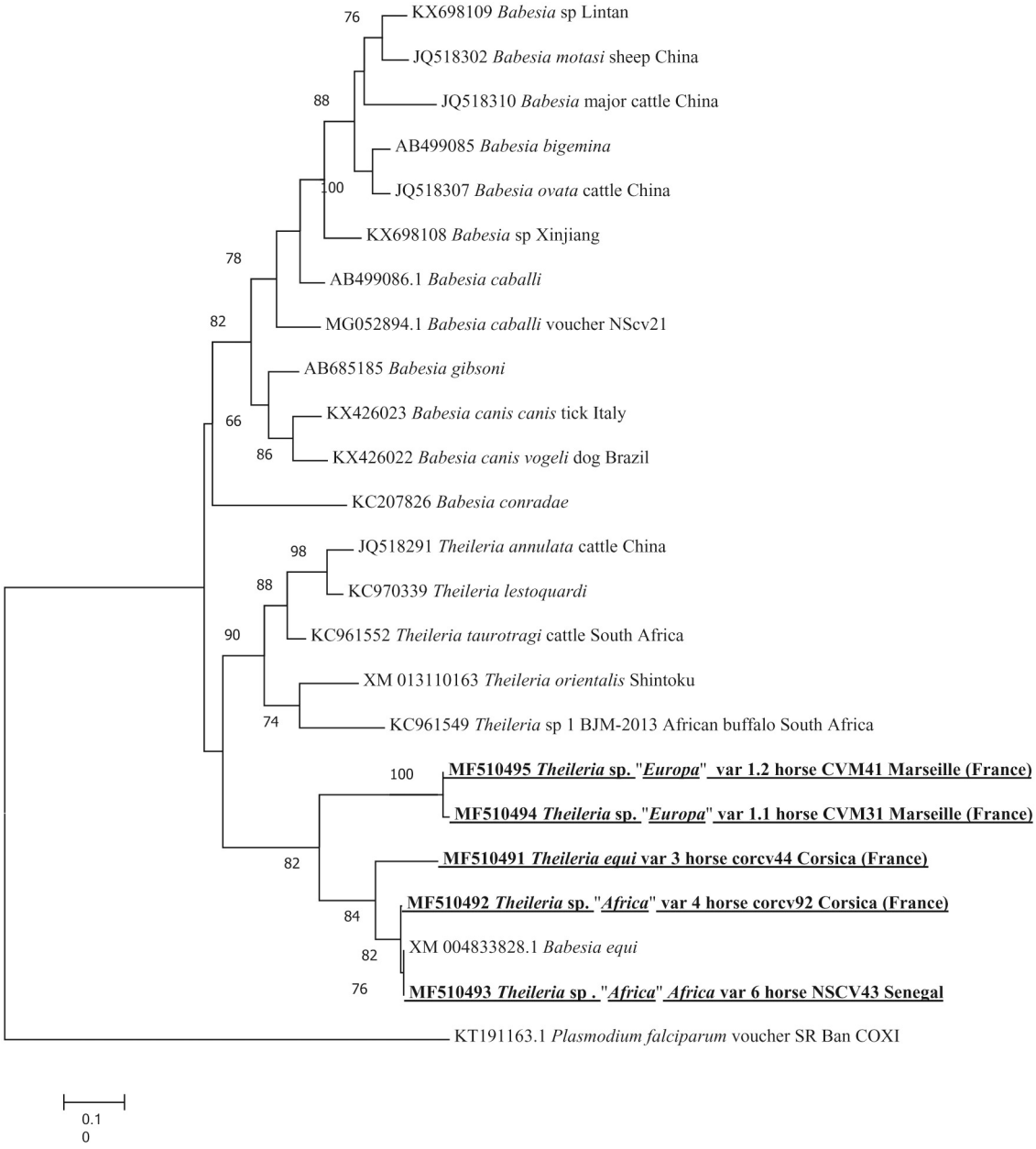

Fig. 4. Maximum-likelihood phylogenetic tree of piroplasms, including species identified in the present study based on partial 480-bp *cox1* gene.

Table 5
Prevalence of piroplasms in few European and African countries.

Country	Year	Techniques	Samples	*T. equi*	*B. caballi*	References
South Africa	2010	Nested PCR	41 horses	80%	72%	Bhoora et al. (2010)
Sudan	2013	Conventional PCR (18S)	499 *Equidae*	35.95%	0%	Salim et al. (2013)
Egypt	2016	Nested PCR	88 horses	36.4%	19.3%	Mahmoud et al. (2016)
			51 donkeys	43.1%	15.7%	
Kenya	2015	Nested PCR (18S)	71 donkeys	72%	0%	Hawkins et al. (2015)
			16 zebras	100%	0%	
Nigeria	2014	Giemsa blood smear	240 horses	40%	2.5%	Turaki et al. (2014)
Tunisia	2013	Reverse Line Blot hybridization	104 horses	11.53	1.92	Ros-García et al. (2013)
Italy	2016	Real time PCR	673 horses	27.49%	4%	Bartolomé Del Pino et al. (2016)
Italy	2017	Semi-nested PCR	135 horses	13.33%	0%	Zanet et al. (2017)
Turkey	2017	Multiplex PCR	125 horses	8.8%	0%	Guven et al. (2017)
Portugal	2013	ELISA	162 horses	17.9%	11.1	Ribeiro et al. (2013)
France	2010	Conventional PCR (18S)	166 dogs	19%	0.6%	Fritz (2010)
	2010	Conventional PCR (18S)	111 horses	80%	1.2%	Fritz (2010)
Spain	2017	Indirect Fluorescent Antibody Test	3100 horses	44%	21%	Montes Cortés et al., (2017)

antibody (IFA) tests were positive. One of the hypotheses was the existence of variants that have not yet been identified and therefore cannot be detected by the available PCR tools (Bhoora et al., 2010). Indeed, we have identified here different genotypes that can easily be omitted by conventional PCR tools (Table 4).

Ticks from the genera *Dermacentor*, *Hyalomma* and *Rhipicephalus* are natural vectors of *Babesia* and *Theileria* (Tamzali, 2013). The 97 ticks studied from Chad belonged to the two following genera: *Hyalomma* (4.1%, 4/97) and *Rhipicephalus* (95.9%, 93/97). Nine ticks (9.3%, 9/97) identified as *Rh. decoloratus* were found positive for *Theileria* sp. "*Africa*". Besides, these ticks were found on two horses which were also positive for *Theileria* sp. "*Africa*". More studies on these ticks must confirm or disprove their capacity to transmit piroplasms (Table 5).

Our study does not claim to be representative because of the reduced number of *Equidae* tested. Furthermore, our sampling was carried out in an opportunistic manner but it gives a picture on the epidemiological situation in the studied areas. The horses tested are certainly protected by a pre-immunization while latent infection persists. Tick infestations are regularly observed in areas where our study has been conducted and sporadic clinical cases of piroplasmosis are reported by veterinarians. It is therefore important to limit the infestation by ticks with external deworming measures to control the diseases they transmit. Asymptomatic infection is very persistent (this is known for *T. equi*) and tick infestation (especially nymphs) may pass unnoticed.

The sole case of *B. caballi* was identified in a horse from Senegal, which was co-infected with *T. equi.* We should notify that the sampling time plays a critical role in the detection of circulating parasites. Indeed, infections by *B. caballi* are self-limiting. Horses are generally able to eliminate the infection within 1–3 years naturally or after a sterilizing treatment (Friedhoff and Soulé, 1996).

T. equi and *B. caballi* are the parasites most frequently involved in equine piroplasmosis. However, recent studies have reported the existence of other piroplasms species in horses. Indeed, *B. canis* has recently been reported in horses in France and Italy and *B. capreoli* in Italy (Fritz, 2010; Zanet et al., 2017). The analysis of three different genes of the two-potential new *Theileria* genotypes clearly showed a distant position between these species and the others. These data strongly support the fact that they differ from other recognized species, including *T. equi* (Table S4). New species are isolated from equines and described and validated using phylogenetic analyses based on both 18S rDNA sequences and 223 nuclear-encoded protein-coding genes, extensive genome-wide differences and with predicted protein divergence with *T. equi* (Knowles et al., 2018). Finally, the pathogenicity of these potentially new species remains unknown.

5. Conclusion

Overall, sensitive and specific PCR assays have been developed to potentially identify all piroplasm species and to study their phylogeny. Four horse-infecting piroplasms, including two potentially new species, were identified in *Equidae* from sub-Saharan Africa and France. However, few epidemiological investigations on equine piroplasmosis are conducted. Our study provides a better understanding of the situation. Further studies, covering larger geographical areas and larger number of samples, are nevertheless required to improve exploration of the health status of *Equidae.* These studies will assess more specifically the actual prevalence of equine piroplasmosis and its impacts on equine populations. Moreover, the establishment of high rigorous prophylaxis plans, allowing both surveillance and control of equine piroplasmosis should be proposed. The involvement of public authorities in the detection of these diseases remains essential to contain them, or at least to reduce their spread.

Declaration of competing interests

None.

Ethical statement

1) This material has not been published in whole or in part elsewhere;
2) The manuscript is not currently being considered for publication in another journal;
3) All authors have been personally and actively involved in substantive work leading to the manuscript, and will hold themselves jointly and individually responsible for its content.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vprsr.2019.100332.

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