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Highlights

- New PCR assays were developed to potentially identify all piroplasms.
- Epidemiological study was conducted on *Equidae* from sub-Saharan Africa and France.
- Four species of piroplasms infecting horse, including two potentially new ones, were detected.
- The newly identified species were characterized by amplifying three genes.

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.

Key words: Piroplasmosis, *Equidae*, PCR assays, Sub-Saharan Africa, 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* (Megan E. 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).

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(World Organization for Animal Health. 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 to 30 days for *B. caballi* and 12 to 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) (Figure 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 to15-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 33year-old). In the city of Marseille (France), a study was conducted in February 2015on 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 – 0,9X40 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 (Megan E 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 2,930-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 (100ul of blood extracted and eluted in 50ul 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 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 3.5µl 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 two minutes for UDG action (eliminating PCR amplicons contaminant), then activation step at 95°C for three minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds and annealing-extension at 60°C for 30 seconds.

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 minutes, 40 cycles of one minute at 95°C, 30 seconds annealing at a different hybridization temperature

for each PCR assay and one minute at 72°C followed by a final extension for five minutes 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 <0.05.

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**.

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<0.0001(**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≤0.0001. The infection was also observed more often in females (**46/124**, 37,09%) than in males (**116/424**, 27.36%), p<0.20. 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)** 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.

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 (Figures 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 (Figures 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 to 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 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.

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 one to three 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.

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518 **Figure legends.**

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

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

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

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

Fig 1

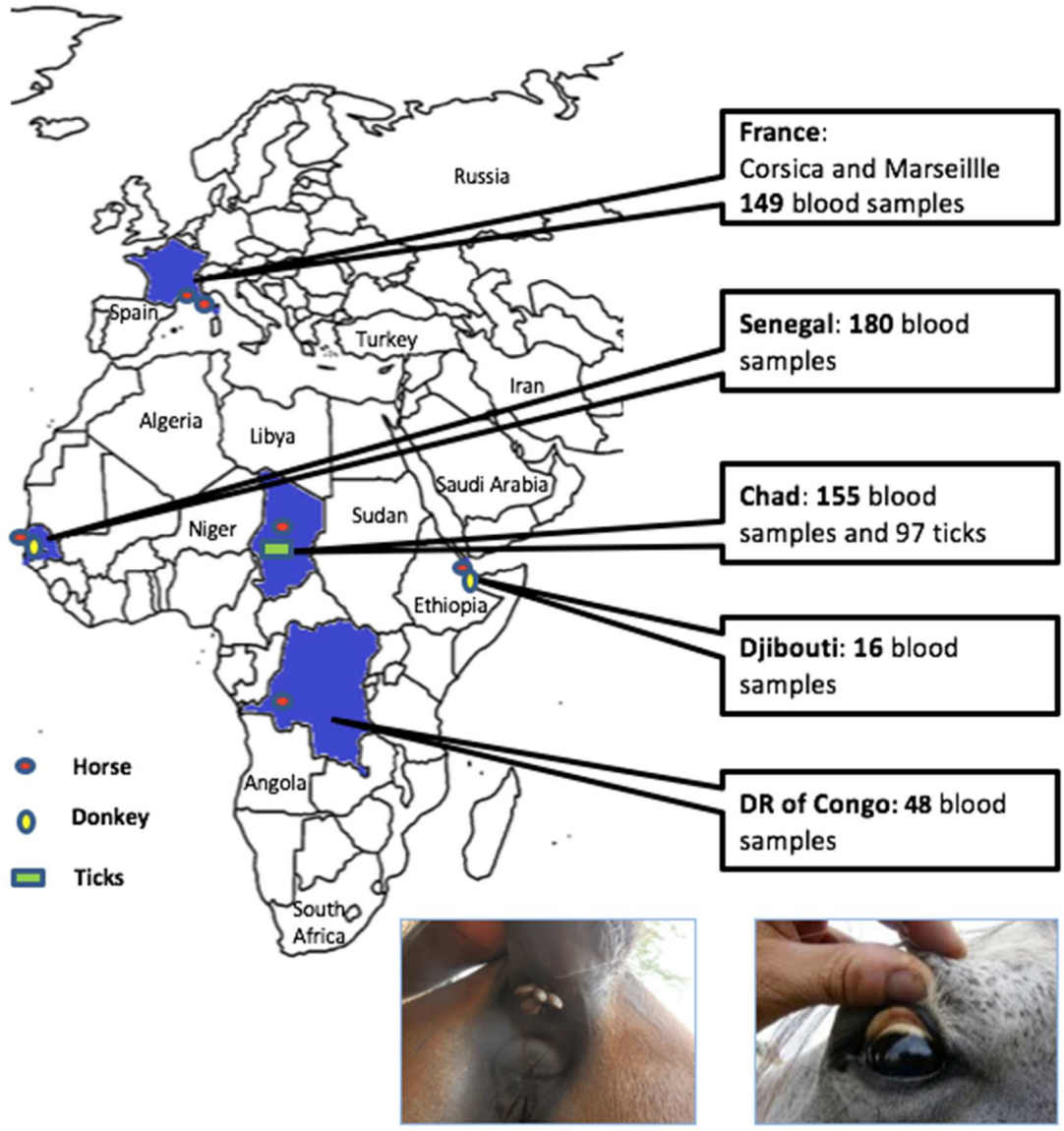
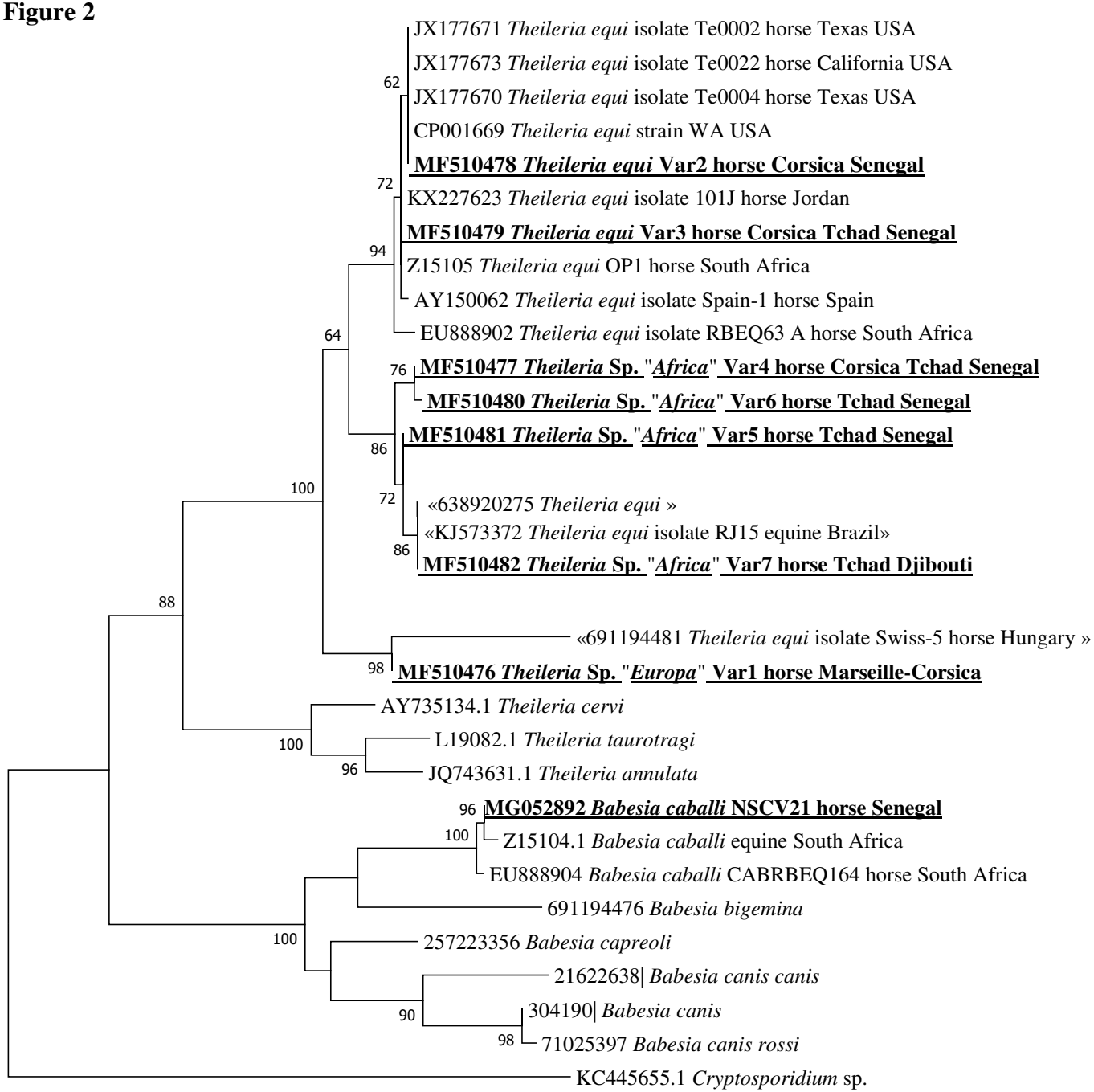

Russia
Spain
Turkey
Iran
Algeria
Libya
Saudi Arabia
Niger
Sudan
Ethiopia
Angola
South Africa
Horse
Donkey
Ticks
France:
Corsica and Marseillle
149 blood samples
Senegal: 180 blood samples
Chad: 155 blood samples and 97 ticks
Djibouti: 16 blood samples
DR of Congo: 48 blood samples

Figure 2


JX177671 Theileria equi isolate Te0002 horse Texas USA
JX177673 Theileria equi isolate Te0022 horse California USA
JX177670 Theileria equi isolate Te0004 horse Texas USA
CP001669 Theileria equi strain WA USA
MF510478 Theileria equi Var2 horse Corsica Senegal
KX227623 Theileria equi isolate 101J horse Jordan
MF510479 Theileria equi Var3 horse Corsica Tchad Senegal
Z15105 Theileria equi OP1 horse South Africa
AY150062 Theileria equi isolate Spain-1 horse Spain
EU888902 Theileria equi isolate RBEQ63 A horse South Africa
MF510477 Theileria Sp. "Africa" Var4 horse Corsica Tchad Senegal
MF510480 Theileria Sp. "Africa" Var6 horse Tchad Senegal
MF510481 Theileria Sp. "Africa" Var5 horse Tchad Senegal
«638920275 Theileria equi »
«KJ573372 Theileria equi isolate RJ15 equine Brazil»
MF510482 Theileria Sp. "Africa" Var7 horse Tchad Djibouti
«691194481 Theileria equi isolate Swiss-5 horse Hungary »
MF510476 Theileria Sp. "Europa" Var1 horse Marseille-Corsica
AY735134.1 Theileria cervi
L19082.1 Theileria taurotragi
JQ743631.1 Theileria annulata
MG052892 Babesia caballi NSCV21 horse Senegal
Z15104.1 Babesia caballi equine South Africa
EU888904 Babesia caballi CABRBEQ164 horse South Africa
691194476 Babesia bigemina
257223356 Babesia capreoli
21622638| Babesia canis canis
304190| Babesia canis
71025397 Babesia canis rossi
KC445655.1 Cryptosporidium sp.
62
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72
86
100
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100
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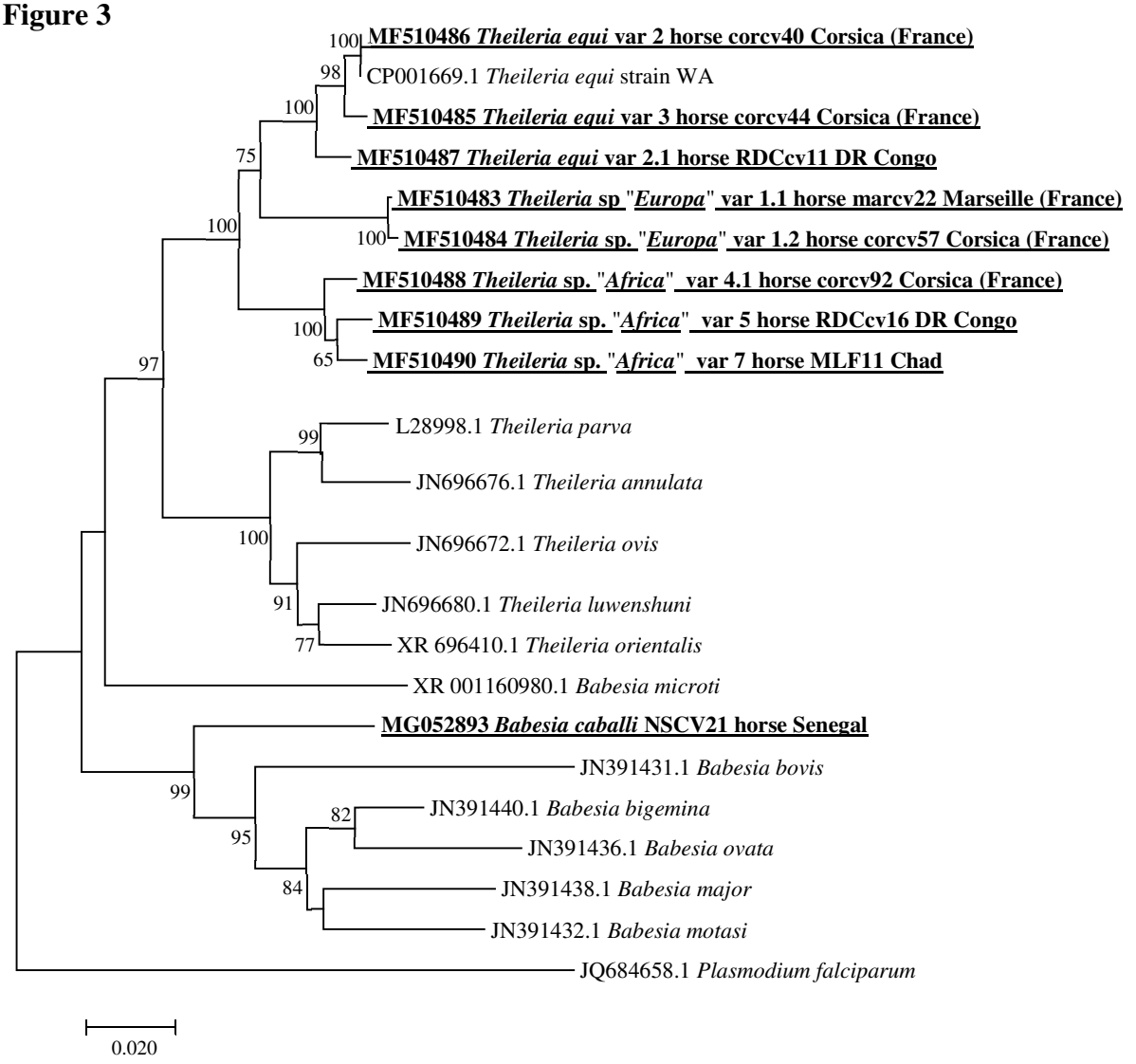
100
100
90
98

Figure 3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98 CP001669.1 Theileria equi strain WA
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75 MF510487 Theileria equi var 2.1 horse RDCcv11 DR Congo
MF510483 Theileria sp "Europa" var 1.1 horse marcv22 Marseille (France)
100 100 MF510484 Theileria sp. "Europa" var 1.2 horse corcv57 Corsica (France)
MF510488 Theileria sp. "Africa" var 4.1 horse corcv92 Corsica (France)
100 MF510489 Theileria sp. "Africa" var 5 horse RDCcv16 DR Congo
97 65 MF510490 Theileria sp. "Africa" var 7 horse MLF11 Chad
99 L28998.1 Theileria parva
JN696676.1 Theileria annulata
100 JN696672.1 Theileria ovis
91 JN696680.1 Theileria luwenshuni
77 XR 696410.1 Theileria orientalis
XR 001160980.1 Babesia microti
MG052893 Babesia caballi NSCV21 horse Senegal
JN391431.1 Babesia bovis
99 82 JN391440.1 Babesia bigemina
95 JN391436.1 Babesia ovata
84 JN391438.1 Babesia major
JN391432.1 Babesia motasi
JQ684658.1 Plasmodium falciparum
0.020

Fig 04

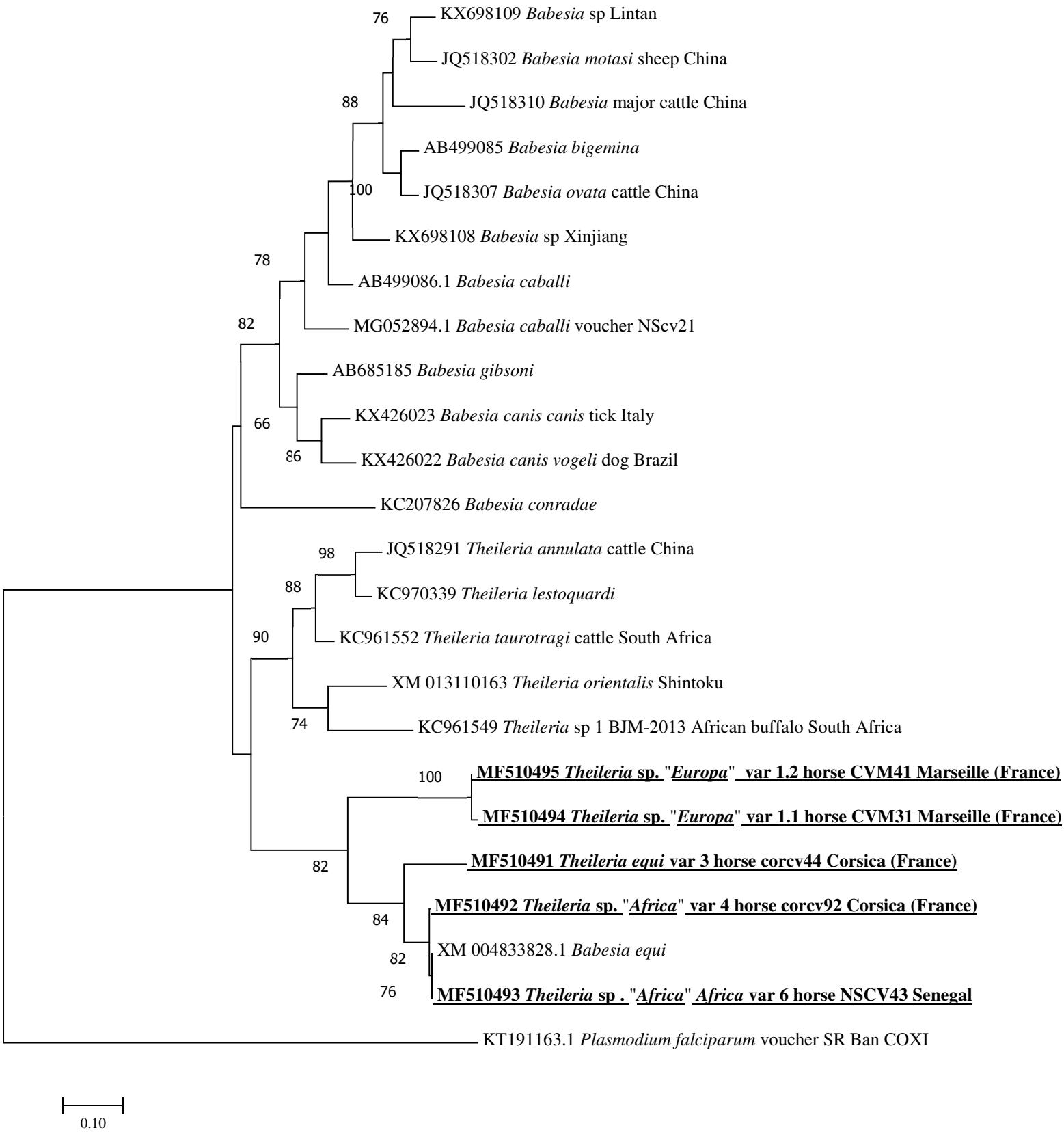
76
KX698109 Babesia sp Lintan
JQ518302 Babesia motasi sheep China
88
JQ518310 Babesia major cattle China
AB499085 Babesia bigemina
100
JQ518307 Babesia ovata cattle China
KX698108 Babesia sp Xinjiang
78
AB499086.1 Babesia caballi
82
MG052894.1 Babesia caballi voucher NScv21
AB685185 Babesia gibsoni
66
KX426023 Babesia canis canis tick Italy
86
KX426022 Babesia canis vogeli dog Brazil
KC207826 Babesia conradae
98
JQ518291 Theileria annulata cattle China
88
KC970339 Theileria lestoquardi
90
KC961552 Theileria taurotragi cattle South Africa
XM 013110163 Theileria orientalis Shintoku
74
KC961549 Theileria sp 1 BJM-2013 African buffalo South Africa
100
MF510495 Theileria sp. "Europa" var 1.2 horse CVM41 Marseille (France)
MF510494 Theileria sp. "Europa" var 1.1 horse CVM31 Marseille (France)
82
MF510491 Theileria equi var 3 horse corcv44 Corsica (France)
MF510492 Theileria sp. "Africa" var 4 horse corcv92 Corsica (France)
84
XM 004833828.1 Babesia equi
82
76
MF510493 Theileria sp . "Africa" Africa var 6 horse NSCV43 Senegal
KT191163.1 Plasmodium falciparum voucher SR Ban COXI
0.10

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*	
		piro18S-R3	AGGACTACGACGGTATCTGA*	
	28S	28S-F2	CACCCCYGKCCGTACDGAT	58°C
		28S-R2	CCTCAYTGAGYTYGCCTTRGGAC	
B. caballi	28S	28Scab-F4	GCAGAAAAGAAAATAACCATG	62°C
		28Scab-R3	GCGTTCAGTCATTATCCAACGG	
		28Scab-F6	GCTTTAGCACGGTTGCTAGGA*	
		28Scab-F7	GGCATAGCAGTCCGGYYTTCGG*	
		28Scab-F8	TGTCCCTATCTGCCATCTAG*	
		28Scab-R6	TCGCCCCTATACCCGGATTTG*	
		28Scab-R7	CCGAARRCCGGACTGCTATGCC*	
	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	

*Primers used for sequencing only

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**	**X² test**
	Number of positive / Number of tested samples (%)		
Senegal	22/127 (17.32%)	1/53 (1.87%)	P ≤ 0,02
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 ≤ 0,90
	Male	**Female**	**X² test**
	Number of positive / Number of tested samples (%)		
Senegal	13/127 (10.57%)	10/53 (18.87%)	p ≤ 0.20
DR Congo	13/34 (38.24%)	8/14 (57.14%)	p ≤ 0.5
Marseille (France)	8/33 (24.24%)	7/18 (38.89%)	p ≤ 0.5
Corsica (France)	18/59 (30.5%)	21/39 (53.85%)	p ≤ 0.20
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

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 (%)						
	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**

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	3,100 horses	44%	21%	(Guadalupe et al., 2017)