Molecular epidemiology and genotypic diversity of equine piroplasma parasites

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馬ピロプラズマの分子疫学調査と遺伝子型の多様性

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Contents

Abbreviations

Abbreviations

18S rRNA	-	Ribosomal RNA of 18S sub-unit
BLAST	-	Basic Local Alignment Search Tool
bp	-	Base pair
cELISA	-	Competitive Enzyme-Linked Immunosorbent Assay
CI	-	Confidence interval
DDW	-	Double distilled water
DNA	-	Deoxyribonucleic acid
dNTP	-	Deoxyribose nucleoside triphosphates
E. coli	-	*Escherichia coli*
EDTA	-	Ethylenediaminetetraacetic acid
ELISA	-	Enzyme-Linked Immunosorbent Assay
ema-1	-	Equi Merozoite Antigen-1 gene
EMA-1	-	Equi Merozoite Antigen-1
EP	-	Equine piroplasmosis
Hb	-	Haemoglobin
HCT	-	Haematocrit

LAMP	-	Loop-mediated isothermal amplification
MAFFT	-	Multiple Alignment using Fast Fourier Transform
MatGAT	-	Matrix Global Alignment Tool software
MCHC	-	Mean corpuscular haemoglobin concentration
MCV	-	Mean corpuscular volume
MEGA	-	Molecular Evolutionary Genetics Analysis software
No.	-	Number
PCR	-	Polymerase Chain Reaction
rap-1	-	Rhoptry Associated Protein-1 gene
RAP-1		Rhoptry Associated Protein-1
RBCs	-	Red blood cells
sp.	-	Species
UV	-	Ultraviolet
WOAH	-	World Organization for Animal Health

Unit abbreviations

^{o}C	-	degree Celsius
hr	-	hour
μ g	-	microgram
μ l	-	microliter
μ m	-	micrometer
μ M	-	micromolar
ml	-	milliliter
mM	-	millimolar
min	-	minute
%	-	percentage
sec	-	second
U	-	units

General Introduction

1. Equine piroplasmosis

Equine piroplasmosis (EP) is an infectious disease caused by two haemoprotozoan parasites, including *Theileria equi* and *Babesia caballi* (Fig. 1) in various equine species, such as horses, donkeys, mules, and zebras (Wise et al., 2014; Laus et al., 2015; Machado et al., 2012; Qablan et al., 2013; Lampen et al., 2009; Zweygarth et al., 2002). Although equines are the primary hosts for *T. equi* and *B. caballi* infections (Wise et al., 2014), the DNAs of these parasites have been detected in various non-equine hosts, such as camels (Qablan et al., 2012; Salman et al., 2022), dogs (Beck et al., 2009; Fritz, 2010; Rosa et al., 2014; de Sousa et al., 2018; Inácio et al., 2019), goats, cattle (Zhang et al., 2015), waterbuck (Githaka et al., 2014), and sheep (Zhang et al., 2015; Azmi et al., 2019). This raises concerns about the host specificity of these parasites, although the epidemiological implications of these non-equine hosts remain unknown (Wise et al., 2014; Tirosh-Levy et al., 2020a).

Both *T. equi* and *B. caballi* are transmitted primarily by tick species of the genera *Boophilus*, *Hyalomma*, *Dermacentor*, and *Rhipicephalus* to the equine hosts (Brüning, 1996; Ueti et al., 2003; Hajdušek et al., 2013; Tahir et al., 2020). In addition, they can also be transmitted iatrogenically through contaminated needles or blood transfusions (Short et al., 2012), and by transplacental transmission which sometimes results in abortion (Allsopp et al., 2007; Chhabra et al., 2012; Wise et al., 2014).

In the equine hosts, *T. equi* and *B. caballi* undergo an asexual reproduction in red blood cells (RBCs); leading to haemolytic anaemia and other clinical signs (Rothschild,

2013; Wise et al., 2013; Onyiche et al., 2019). However, most of the infected animals remain asymptomatic in endemic countries (Rothschild et al., 2013; Wise et al., 2014, Scoles and Ueti, 2015). Early diagnosis and specific chemotherapy are vital for the recovery of infected animals with acute EP. The clinical EP can be diagnosed, based on clinical presentation and demonstration of parasites within RBCs. Morphological differentiation of *T. equi* and *B. caballi*, however, requires expertise (Bruning, 1996; Wise et al., 2013; Rothschild, 2013; Onyiche et al., 2019). Alternatively, species-specific PCR assays can be conveniently used for this purpose (Alhassan et al., 2007; Mans et al., 2015; Wise et al., 2013; Rothschild, 2013). Diagnosis should be followed by prompt treatment with an effective anti-protozoal agent to ensure early recovery from the infection. However, the recovered animals and those with asymptomatic infections remain long-term carriers.

The carrier animals chronically infected with *T. equi* and *B. caballi* play a critical role in the epidemiology of EP, as they can be the source of infection for further transmission (Wise et al., 2014; Tirosh-Levy et al., 2020b). For this reason, countries that import horses, especially those considered free from the infectious disease, impose strict regulations. In particular, they require horses to be tested with diagnostic tests recommended by the World Organisation for Animal Health (WOAH) to rule out the infections prior to importation (WOAH, 2021). However, the genotypic diversity of *T. equi* and *B. caballi* can sometimes leads to false-negative test results, potentially leading to the introduction of these parasite species into non-endemic areas (Awinda et al., 2013; Mahmoud et al., 2016; Knowles et al., 2018). Therefore, a comprehensive understanding of the epidemiology and genotypic diversity of *T. equi* and *B. caballi* is essential for the global management of EP.

2. Lifecycles of *T. equi* and *B. caballi*

The lifecycles of *B. caballi* and *T. equi* involve tick vectors and equine hosts (Fig. 2). In equine hosts, the lifecycles of these parasite species begin with an injection of sporozoites by the infected ticks during their blood feeding (Mehlhorn and Shein, 1984; Wise et al., 2014; Scoles and Ueti, 2015). The sporozoites of *B. caballi* directly invade the host's RBCs, where they transform into trophozoites, and then undergo an asexual reproduction, known as merogony, to form merozoites. After maturation, the merozoites egress from the infected RBCs, invade uninfected RBCs, and continue to proliferate by the merogony (Mehlhorn and Shein, 1984; Wise et al., 2014; Scoles and Ueti, 2015). On the other hand, the sporozoites of *T. equi* first infect the host's lymphocytes, where they form multinucleated schizonts (Ramsay et al., 2013). The schizonts subsequently burst and release merozoites, which invade the host RBCs and then proliferate by merogony (Mehlhorn and Shein, 1984; Wise et al., 2014; Scoles and Ueti, 2015). Some of the *T. equi* and *B. caballi* merozoites transform into gametocytes, ingestion of which initiates the lifecycle of these parasite species in tick vectors (Müller and Hemphill 2013; Wise et al., 2014. In the tick midgut, gametocytes mature into gametes and undergo a sexual reproduction, forming zygotes. The zygotes become kinetes, which reach various organs, such as the salivary glands and the ovaries, through the haemolymph. The kinetes in the salivary glands produce sporoblasts and then sporozoites, which are injected into the blood of equine hosts during the blood feeding of next tick stage. The persistence of *T. equi* and *B. caballi* from one tick stage to the next is known as transstadial persistence (Wise et al., 2014; Scoles and Ueti, 2015). In addition, *B. caballi* kinetes in the ovaries are passed to the tick eggs by a transovarial transmission and generate sporozoites in the larval stage (Mehlhorn and Shein, 1984; Müller and Hemphill 2013; Wise et al., 2014; Scoles and Ueti,

2015). During the blood feeding of infected larvae, *B. caballi* is transmitted to the equine hosts.

3. Clinical equine piroplasmosis

The clinical presentation of *T. equi* and *B. caballi* infections in equines is often variable, depending on the individual equine species (Tamzali, 2013; Wise et al., 2013; Tirosh-Levy et al., 2020b). Among the equine species, horses are more susceptible to clinical EP (Tamzali, 2013). The prepatent periods for *B. caballi* and *T. equi* infections typically are between 10 to 30 days and 12 to 19 days, respectively (Chhabra et al., 2012; Saunders, 2012). During the acute phase, horses infected with *B. caballi* and *T. equi* may display similar clinical signs, although *B. caballi* infection results in milder disease, as compared to that caused by *T. equi* (de Waal, 1992; Wise et al., 2013). The main clinical sign of EP is haemolytic anaemia, which results from the destruction of RBCs due to the asexual reproduction of parasites (Zobba et al., 2008; Rothschild et al., 2013; Wise et al., 2013). Other clinical signs include fever, icterus, haemoglobinuria, and sometimes death (Tamzali, 2013; Wise et al., 2013). Hindlimb paralysis and gastroenteritis associated with *B. caballi* infection may sometimes occur and the animal may suffer relapses (Al-Obaidi et al., 2016). The animals recovered from clinical EP become persistent carriers for *T. equi* and *B. caballi* infections (de Waal, 1992; Saunders, 2012).

4. The role of carrier stage in the epidemiology of EP

Equines that are carriers for *T. equi* and *B. caballi* infections are key to the epidemiology of EP, as they act as a source of infection for the transmission by ticks and

other modes (Friedhoff et al., 1990; Ueti et al., 2005; Saunders, 2012; Wise et al., 2014). Under an endemic setting, a vast majority of equine are unapparent carriers, with low levels of parasitaemia (Wise et al., 2013). These carrier equines can potentially disseminate the parasites in non-endemic areas where the competent tick vectors are present (Rothschild, 2013). To prevent the above scenario, international movement of equine is strictly regulated (Friedhoff et al., 1990; Ueti et al., 2005). The occurrence of EP and the stringent regulations related to the movement of horses result in serious economic losses to the global horse industry (Wise et al., 2013).

5. Control measures for EP

Control strategies for EP largely depends on diagnosis, chemotherapy, tick control, and restrictions on animal movement (Brüning, 1996; Tirosh-Levy et al., 2020b). Early diagnosis should be followed by prompt treatment with effective anti-protozoan drugs for the recovery of animals with clinical EP. For the prevention of EP, only tick control is available in endemic countries, while strict regulations are in place in EP-free countries to prevent the introduction of causative parasites.

5.1. Diagnosis

Diagnosis of EP usually involves laboratory tests, as the clinical signs are non-specific. The laboratory diagnosis of EP can be achieved through direct or indirect methods. Direct methods encompass the use of microscopy and molecular techniques. Microscopy involves the examination of Giemsa-stained blood smears to identify the parasites within infected RBCs (Barcia, 2007; Rothschild, 2013). Differential identification of *T. equi* and *B. caballi* can be achieved by examining their morphological features; *T. equi* is usually

small and polymorphic and has a characteristic 'Maltese-cross' shape, while *B. caballi* is large and found as single or paired pyriforms (Fig. 1). However, this method has limited sensitivity, and as a result, may not be suitable for detecting asymptomatic carriers with low levels of parasitaemia (Zobba et al., 2008; Solano-Gallego et al., 2016; Tirosh-Levy et al., 2020c). Alternatively, molecular-based diagnostic tests are now widely used, as they are highly sensitive and specific in detecting the *T. equi* and *B. caballi* from samples with low parasitaemia levels. Several molecular assays, such as polymerase chain reaction (PCR), nested PCR, real-time PCR, and loop-mediated isothermal amplification (LAMP) assays have been developed to detect *T. equi* and *B. caballi* DNAs in the infected animals (Alhassan et al., 2007; Lobanov et al., 2018; Montes Cortés et al., 2019; Torres et al., 2021; Lv et al., 2022). These molecular diagnostic assays, however, may not detect the infections in circulating blood, as the parasites may be sequestered in the internal organs of carrier animals (Pitel et al., 2010; Ribeiro et al., 2013). Therefore, serological tests are preferred over agent identification methods to determine whether an animal is free from the infections.

The commonly used serological tests include indirect enzyme-linked immunosorbent assay (ELISA), competitive ELISA (cELISA), and indirect immunofluorescence antibody test (IFAT) (Donnelly et al., 1982; Weiland et al., 1984; Knowles et al., 1991; Bose et al., 1995; Bruning et al., 1997; Kappmeyer et al., 1999) for detecting specific antibodies against *T. equi* and *B. caballi.* The WOAH recommends the use of cELISA and IFAT as regulatory tests mainly in the international horse trade (WOAH, 2021). Among these two tests, cELISAs are widely used due to its availability as kits, fixed cut-off value that makes the interpretation of test results easy, and suitability for large-scale studies in identifying the carrier animals (Knowles et al., 1991; Kappmeyer et al., 1999; WOAH, 2021).

5.2. Treatment

At present, only a few anti-protozoan drugs, such as diminazene aceturate and imidocarb dipropionate, are available for the treatment of EP (Kuttler & Johnson, 1986; Wise et al., 2014; Onyiche et al., 2019; Tirosh-Levy et al., 2020c). The objective of treatment for EP differs between the endemic and non-endemic countries (Wise et al., 2014; Tirosh-Levy et al., 2020c). In endemic countries, the treatment aims to resolve the clinical signs during acute infection by reducing the parasitaemia levels (Wise et al., 2014; Tirosh-Levy et al., 2020c). The current treatment options have proven to be effective in managing the clinical EP, as they can reduce the disease burden and mortality rates among the affected animals. In non-endemic countries, the objective of treatment is to completely clear the parasites from the infected animals in order to prevent further transmission. Previous studies found that repeated treatment with imidocarb dipropionate via intramuscular injection at a dose of 4.0 mg/kg of body weight four times at 72-h intervals, may completely clear the *T. equi* and *B. caballi* infections (Schwint et al., 2009; Grause et al., 2013). However, recent studies reported inconsistent results and found that this treatment strategy is ineffective in completely eradicating the parasites in a subset of the infected horses (Friedhoff et al., 1990; Bruning, 1996; Butler et al., 2008)

5.3. Tick control

Tick control is crucial to prevent the EP, as both *T. equi* and *B. caballi* are primarily transmitted by tick vectors (Scoles and Ueti, 2015). The most widely used tick control method is the application of acaricides belonging to various classes, such as organophosphates, pyrethroids, and amidines (Coles et al., 2014; De Meneghi et al., 2016). However, the emergence of acaricide-resistant ticks as a result of improper application

frequently renders the tick control ineffective (Obaid et al., 2022). Furthermore, acaricides are expensive and environmentally harmful (George et al., 2004; Coles and Dryden, 2014; Obaid et al., 2022). Therefore, in addition to educating the farmers on the proper use of acaricides, the development of an integrated, efficient, cost-effective, and eco-friendly tick control strategy is essential for EP control (Bishop et al., 2023).

5.4. Restriction on transnational movement of infected horses

The equine industry heavily relies on the import and export of horses across different regions for various purposes, such as breeding, racing, and recreational activities (Lansade et al., 2004). However, international movement of horses always carries risks of infectious diseases, especially being introduced into non-endemic regions. The EP is one of the infectious diseases that impede the global movement of horses. The importing countries take precautionary measures to prevent the introduction of *T. equi* and *B. caballi*. These measures include horses being tested for the parasite infections during the period of pre-export quarantine in exporting countries, as well as during the quarantine period in importing countries (Timoney, 2007; Sergeant et al., 2016). In addition to being costly, these regulations may sometimes not be effective in reducing infection risks due to the porous nature of borders in some countries (Medley et al., 2021).

6. Importance of epidemiological surveillance

The EP has a global distribution, while only a few countries, such as Japan, Canada, Iceland, Greenland, New Zealand, and Australia, confirmed to be free from the infectious disease (Fig. 3) (Tirosh-Levy et al., 2020c). On the other hand, there are several other countries, where the status of EP is still unknown, as epidemiological surveys of *T. equi*

and *B. caballi* infections are not conducted. This situation may have serious implications in managing EP, not only in countries with unknown epidemiological status, but also globally. The horses with EP in the countries with unknown EP status, may not receive appropriate treatments, leading to severe economic losses. On the other hand, under this situation, EP-free countries are also at a risk, since the infected horses from regions with uncertain epidemiological status may be unintentionally imported. Therefore, epidemiological surveillance of *T. equi* and *B. caballi* is important for managing the EP. These surveillance programs must be followed by studies to investigate the genotypic diversity of *T. equi* and *B. caballi*, which have potential implications for managing EP (Manna et al., 2018; Sears et al., 2020; Tirosh-Levy et al., 2021).

7. Implications of genotypic diversity in managing EP

Both *T. equi* and *B. caballi* are genetically diverse, consisting of several genotypes (Tirosh-Levy et al., 2020c). These genotypes have potential implications for the control and prevention of EP. Specifically, the genotypic diversity may lead to false-negative test results, that may influence the clinical outcome of infections, and affect the therapeutic efficacy of anti-protozoan drugs.

7.1. False-negative test results

The genotypic diversity of *T. equi* and *B. caballi* frequently leads to false-negative test results in cELISAs (Bhoora et al., 2010; Rapoport et al., 2014; Mahmoud et al., 2016). Based on ribosomal RNA of 18S sub-unit (18S rRNA) sequences, *T. equi* can be divided into five genotypes, including genotypes A-E (Tirosh-Levy et al., 2020c). The current cELISA for detecting *T. equi* antibodies was developed, based on the equi merozoite

antigen – 1 (EMA-1) of genotype A (Fig. 4) (Knowles et al., 1991). However, a recent study found that *Theileria haneyi*, belonging to genotype C, lacks the gene encoding the EMA-1 (Knowles et al., 2018). Consequently, cELISA is not suitable for detecting *T. equi* antibodies in the animals infected with genotype C (Bhoora et al., 2010a). Similarly, false-negative results are also common with the *B. caballi* cELISA (Bhoora et al., 2010b; Rapoport et al., 2014). Based on the rhoptry associated protein – 1 gene (*rap-1*) sequences, *B. caballi* can be classified into three genotypes, consisting of genotypes A1, A2, and B (Fig. 5) (Tirosh-Levy et al., 2020c). The cELISA for the serodiagnosis of *B. caballi* was developed, based on the RAP-1 antigen of genotype B (Kappmeyer et al., 1999). Recent studies found that the *rap-1* is poorly conserved among the *B. caballi* genotypes and that the cELISA was unable to detect the antibodies in the animals infected with genotypes A1 and A2 (Bhoora et al., 2010b; Rapoport et al., 2014; Mahmoud et al., 2016). The false-negative test results obtained with cELISAs may result in the introduction of *T. equi* and *B. caballi* in EP-free countries, leading to serious animal health and economic consequences. Therefore, the diagnostic tests for EP should be selected in light of the genotypic diversity of *T. equi* and *B. caballi* in the endemic countries, especially during the transnational movement of horses.

7.2. Genotype-dependent virulence of *T. equi*

The findings from previous investigations suggest an association between the *T. equi* genotypes and the clinical outcome of infection (Rothschild, 2013; Wise et al., 2013; Tirosh-Levy et al., 2020c). The majority of horses with clinical disease were found to be infected with *T. equi* genotype A in several studies that had investigated EP (Tirosh-Levy et al., 2020c). Genotype A is the most prevalent, as compared to other *T. equi* genotypes,

and was detectable in different countries across several continents (Hall et al., 2013; Manna et al., 2018; Tirosh-Levy et al., 2020b). As a result, horses throughout the world are at risk for clinical EP. The potential genotype-dependent virulence of *T. equi* highlights that a comprehensive understanding of the genotypes is vital for the successful management of EP in the endemic countries.

7.3. Implication of genotypes in drug-induced clearance of *T. equi*

Complete clearance of *T. equi* and *B. caballi* from infected horses is important, especially in EP-free countries, to prevent the disease spread. Previous studies demonstrated that imidocarb dipropionate, when administered intramuscularly at 4.0 mg per kg of body weight four times at 72-hour intervals, may clear *B. caballi* and *T. equi* from chronically infected carrier horses (Ueti et al., 2012; Grause et al., 2013). However, recent studies found that achieving complete clearance of these two parasite species, especially *T. equi*, is sometimes challenging (Frerichs et al., 1973; Schwint et al.,2009). Importantly, the efficacy of imidocarb dipropionate may be genotype-dependent. For example, imidocarb dipropionate clears *T. equi* in horses singly infected with genotype A, but not in animals infected with genotype C, which is now known as *T. haneyi* (Sear et al., 2020). Similarly, imidocarb dipropionate was unable to clear *T. equi* in animals co-infected with genotypes A and *T. haneyi* (Sear et al., 2020). Therefore, a thorough understanding of *T. equi* genotypes is important for predicting the therapeutic efficacy of anti-protozoan drugs.

8. Objectives of the present study

The lack of epidemiological data regarding *T. equi* and *B. caballi* infections is a hindrance in controlling the disease in countries with unknown EP. I hypothesized that *T. equi* and *B. caballi* are endemic in countries where the EP status is unknown, but the competent tick vectors exist. Therefore, the first objective of my study was to survey the equines from countries where competent tick vectors are found, but the EP status is unknown. To realize this objective, I selected two countries, including Sri Lanka and Paraguay located in Asia and South America, respectively, to conduct epidemiological surveys of *T. equi* and *B. caballi* infections. Both countries possess favourable climates for tick activities and are endemic for tick species capable of transmitting *T. equi* and *B. caballi* (Payne and Osorio, 1990; Gunasekara et al., 2019; Sivakumar et al., 2012; Ogrzewalska et al., 2014; Zhyldyz et al., 2019; Zayas et al., 2021). Previous studies have reported many cases of other tick-borne diseases in livestock in Sri Lanka and Paraguay (Sivakumar et al., 2012; Inácio et al., 2019; Zhyldyz et al., 2019). In addition, countries neighbouring to Sri Lanka and Paraguay have reported the occurrence of EP among their equine populations (Holman et al., 1998; Ferreira et al., 2016; Peckle et al., 2018; Gabriela et al., 2019; Kumar et al., 2020; Sebastian et al., 2021). These backgrounds strongly suggest that EP might be endemic in these two countries. My specific objectives were to survey donkeys in Sri Lanka and horses in Paraguay for *T. equi* and *B. caballi* infections (Chapter 1 & 2).

The second objective of my studies was to determine the genotypic diversity of *T. equi* and *B. caballi* in Sri Lanka and Paraguay. The current approach to investigate the genotypic diversity involves the use of either PCR-sequencing techniques or real-time PCR assays (Coultous et al., 2020; Tirosh-Levy et al., 2020c). The PCR-sequencing is the

most widely used method for genotyping, but it is costly and may not be effective in detecting minor genotypes in mixed-genotype infections since it only provides one consensus sequence (Ruettger et al., 2011; Gharsallah et al., 2012; Zhao et al., 2022). Similarly, real-time PCR assays, which require sophisticated equipment and expertise, have not been validated for a global use. On the other hand, conventional PCR assays, which can overcome the limitations of PCR-sequencing and real-time PCR assays, have not been developed for detecting the parasite genotypes. Therefore, an additional objective of my study was to develop conventional genotype-specific PCR assays tailored to identify the *T. equi* genotypes (Chapter 3).

The goal of my studies was to demonstrate the importance of surveying equines in countries with unknown EP status for *T. equi* and *B. caballi* infection, and to determine their genotypic diversity. Such investigations can provide a framework for conducting similar research in other countries where EP status is unknown.

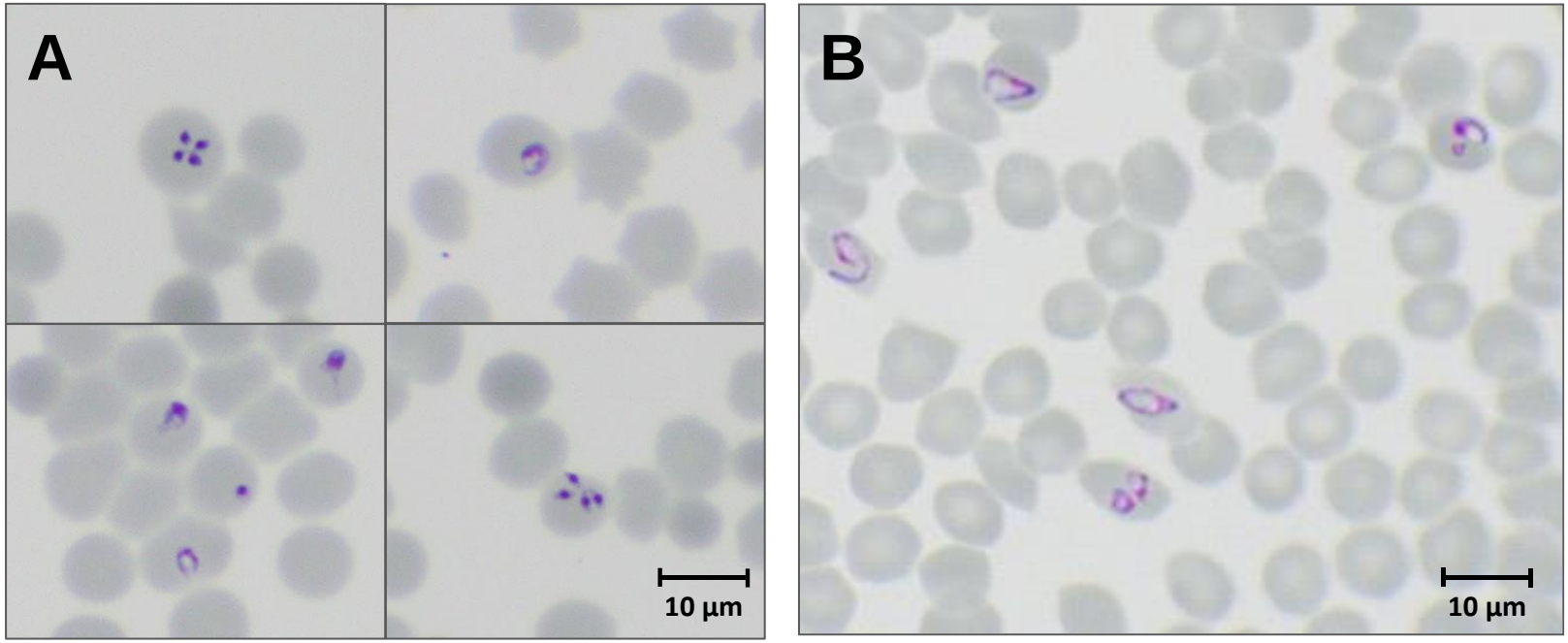

Fig. 1. Micrographs of *Theileria equi* (A) and *Babesia caballi* (B).

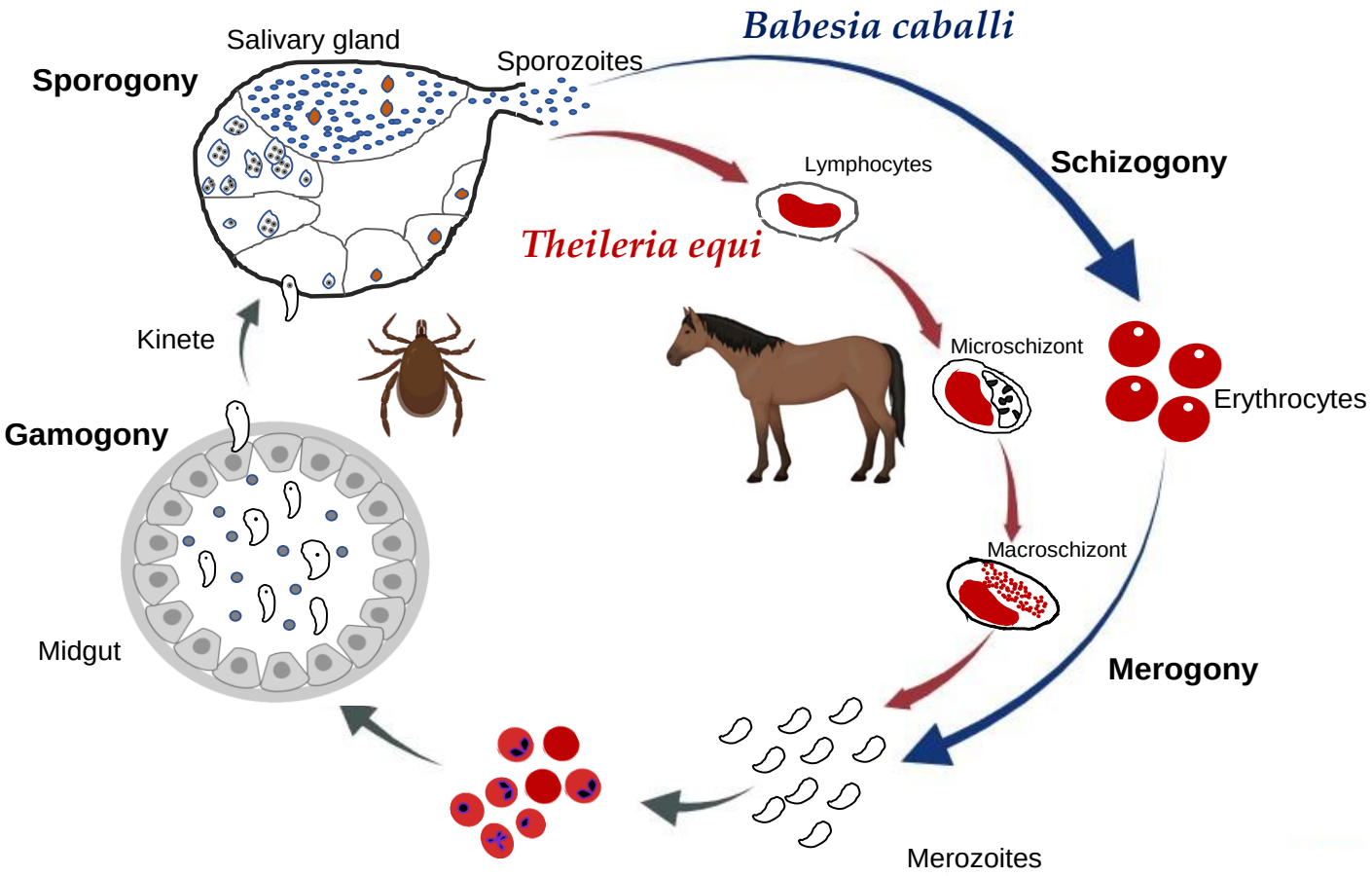

Fig. 2. Lifecycles of *Theileria equi* and *Babesia caballi*.

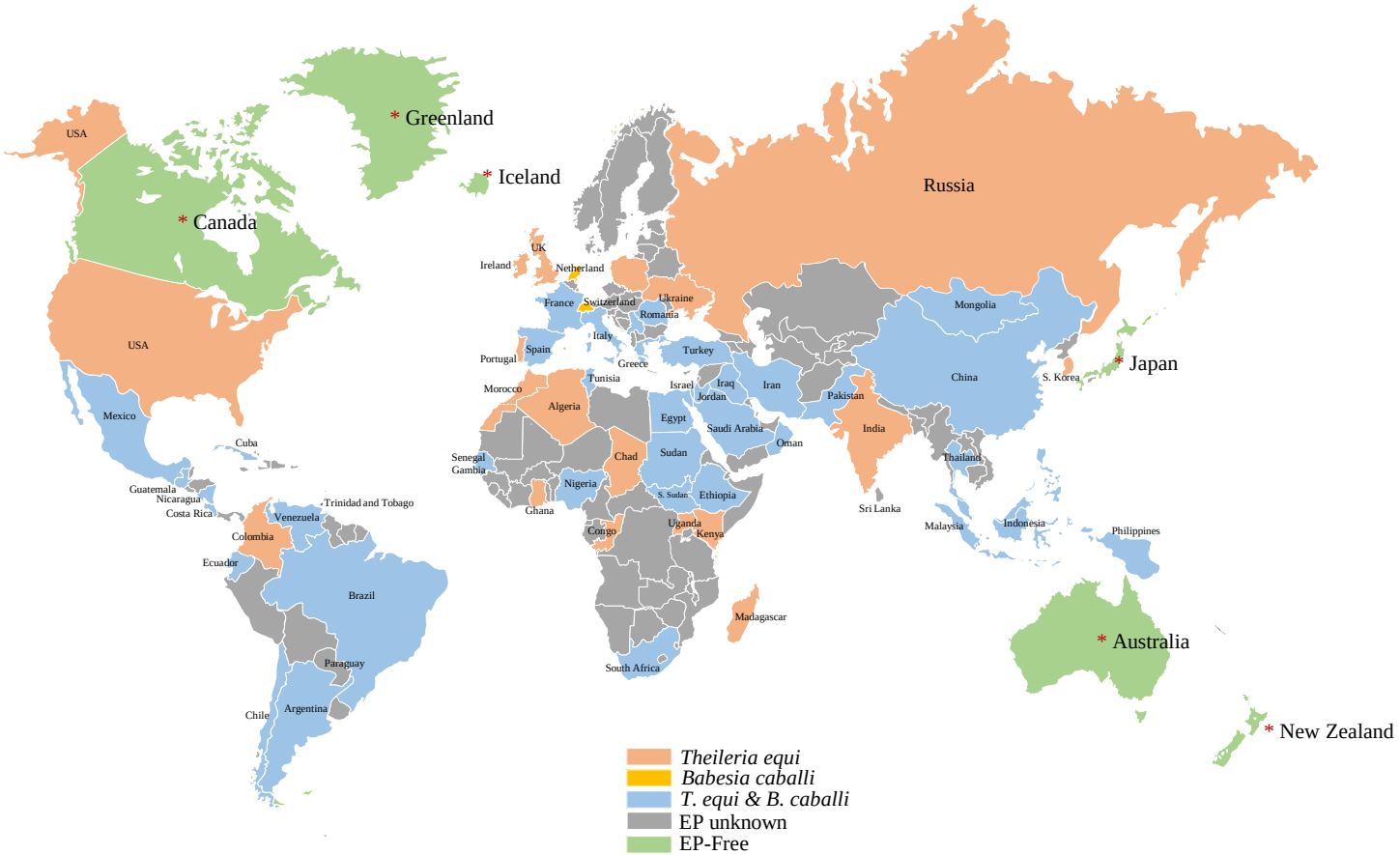

Fig. 3. Global distributions of *T. equi* and *B. caballi* (Tirosh-Levy et al., 2020c).

EP-free countries are shown in green and countries with uncertain epidemiological status are shown in grey.

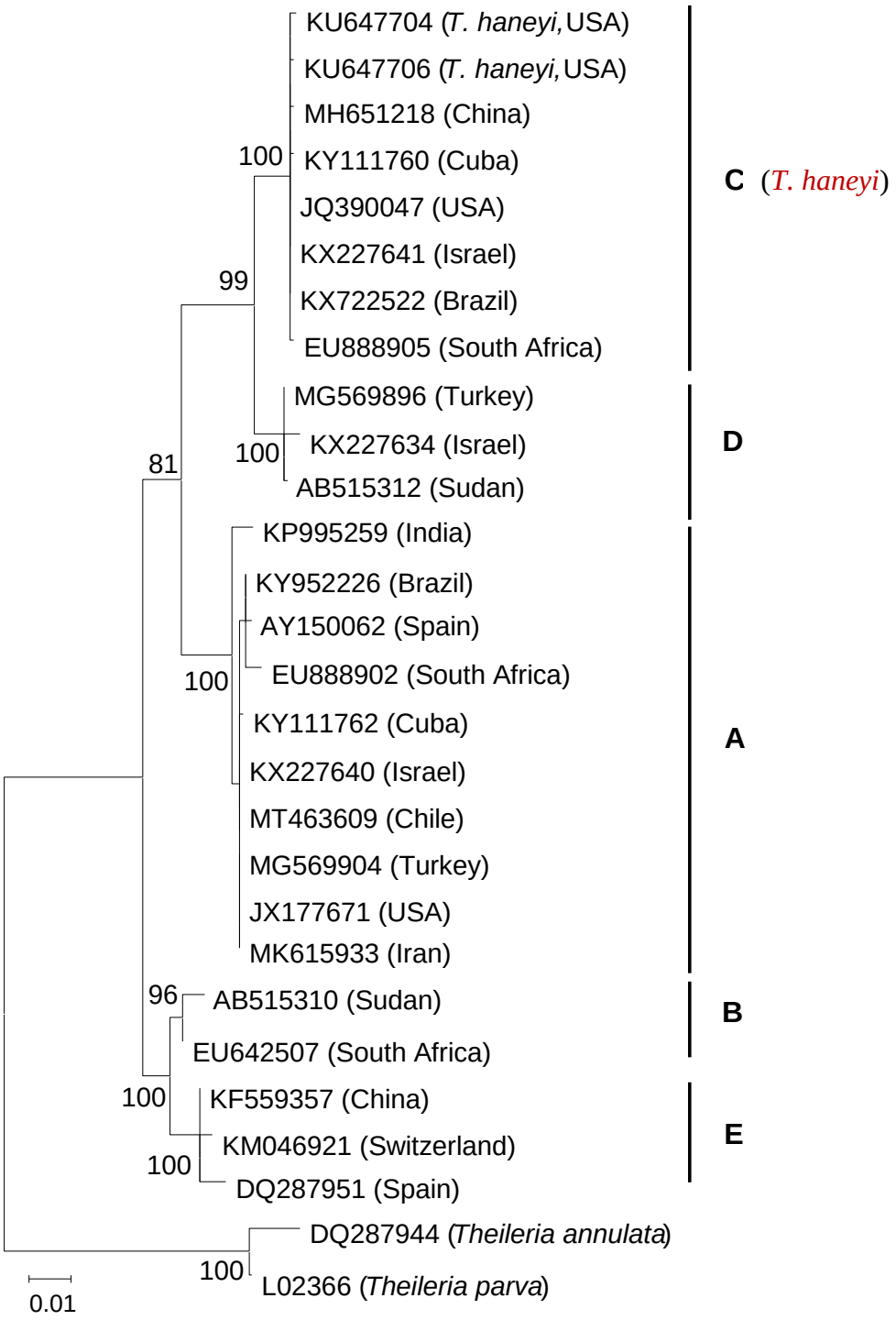

Fig. 4. Phylogenetic analysis of *Theileria equi* 18S rRNA. Based on the sequences, *T. equi* can be divided into five genotypes, including A-E.

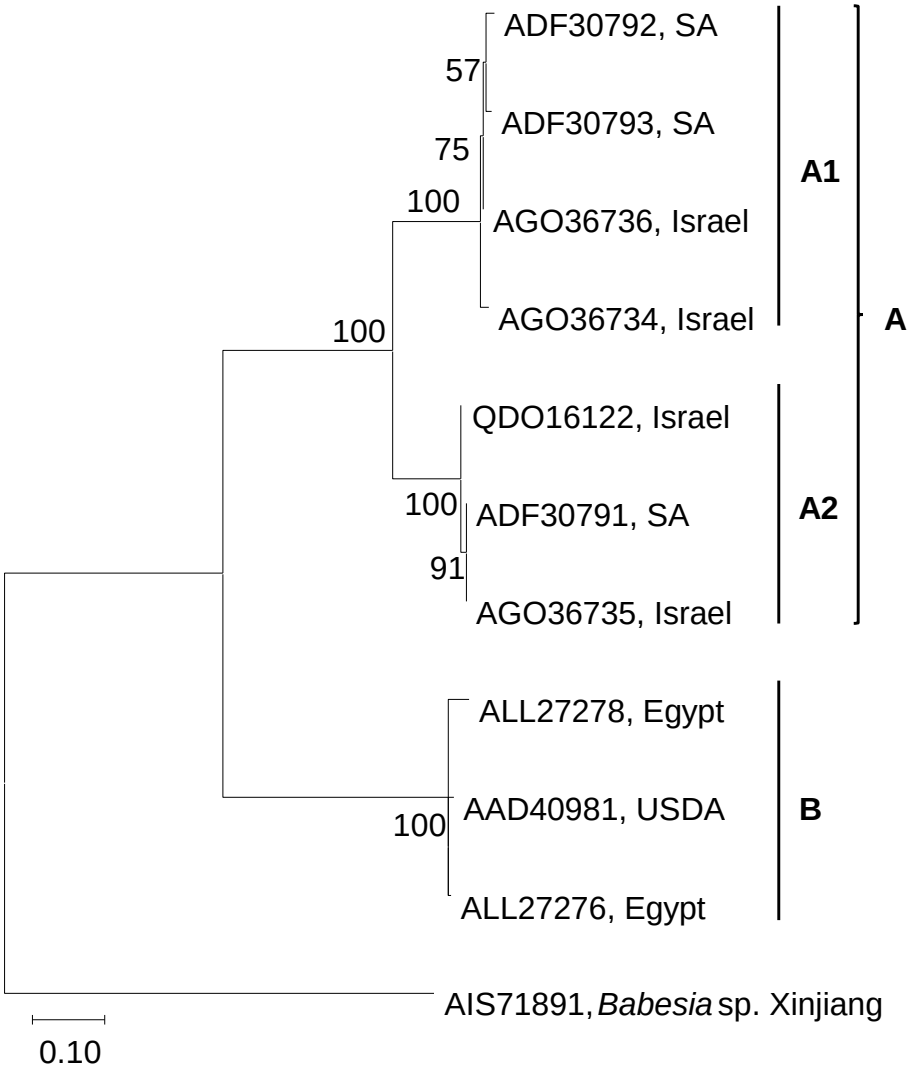

Fig. 5. Phylogenetic analysis of *Babesia caballi* RAP-1 amino acid sequences. Based on the sequences, *B. caballi* can be divided into three genotypes, including A1, A2, and B.

Chapter 1

First detection of *Theileria equi* in free-roaming donkeys (*Equus africanus asinus*) in Sri Lanka

1-1. Introduction

Equine piroplasmosis (EP) is an infectious disease caused by the intra-erythrocytic protozoan parasites, *Theileria equi* and *Babesia caballi,* in equids, including horses, donkeys, mules, and zebras (Tamzali, 2013; Tirosh-Levy et al., 2020b). These parasite species have also been reported in other animals, such as camels (Qablan et al., 2012) and dogs (Fritz, 2010; de Sousa et al., 2018), raising concerns about their host specificity. However, the epidemiological significance of these non-equine hosts is unclear. In addition to morbidity and mortality, restricted international animal trade due to EP often results in severe economic impacts on the equine industry (Wise et al., 2014; Tirosh-Levy et al., 2020b).

EP has a global distribution and is endemic in tropical, sub-tropical, and some temperate regions where competent tick vectors are present (De Waal 1990; Scoles and Ueti, 2015). Both *T. equi* and *B. caballi* are transmitted primarily by Ixodid tick species, belonging to the genera *Amblyomma*, *Dermacentor*, *Hyalomma*, and *Rhipicephalus* (Scoles and Ueti, 2015). Iatrogenic and transplacental transmissions have also been reported (Allsopp et al., 2007; Chhabra et al., 2012; Wise et al., 2014). Although a large proportion of the infected animals remains asymptomatic, clinical disease characterized by fever, haemolytic anaemia, icterus, haemoglobinuria, anorexia, weakness, weight loss,

poor exercise tolerance, and sometimes death, is common among naïve horses in the endemic countries (Tamzali, 2013; Wise et al., 2013). The animals recovered from the clinical disease and the asymptomatically infected ones may remain as carriers for a long period (De Waal 1990; Rothschild, 2013; Wise et al., 2013). In the absence of reinfection, *B. caballi*-infected animals usually clear the parasites in four years, while *T. equi* persists for life (Rothschild, 2013; Tamzali, 2013; Wise et al., 2014). These carrier animals play a key role in the disease epidemiology, because they may be a source of infection for the tick vectors (Wise et al., 2014). Therefore, the detection of carrier animals is vital for assessing the risk of EP and designing effective control measures.

In common with other protozoan parasites, *T. equi* and *B. caballi* consist of genetically diverse populations (Tirosh-Levy et al., 2020b). Five *T. equi* genotypes (A, B, C, D and E) were identified based on their ribosomal RNA of 18S sub-unit (18S rRNA) sequences and three *B. caballi* genotypes (A1, A2 and B) based on their *rap-1* sequences. (Bhoora et al., 2009; Qablan et al., 2013; Liu et al., 2016; Bishop et al., 2020; Tirosh-Levy et al., 2020c). The performance of diagnostic tests is often compromised by these genetic diversities. For instance, two competitive enzyme-linked immunosorbent assays (cELISAs) for *T. equi* and *B. caballi* infections were developed based on the equine merozoite antigen 1 (EMA-1) (Bhoora et al., 2010) and rhoptry-associated protein 1 (RAP-1) (Bhoora et al., 2010a), respectively. However, the *T. equi*-cELISA lacks the ability to detect all the *T. equi* genotypes (Bhoora et al., 2010), because *ema-1* is not found in the genotype C (Knowles et al., 2018). Similarly, false negatives are common with the *B. caballi* cELISA due to the high genotypic diversity of *rap-1* (Bhoora et al., 2010a; Rapoport et al., 2014). Genotypic diversity may also be associated with differences in virulence, as previous studies have found that clinical cases of *T. equi* are

more often associated with genotype A than those of the other genotypes (Manna et al., 2018; Tirosh-Levy et al., 2021). Moreover, drug treatment outcomes might also depend on the genotypic diversity, because previous studies have shown that repeated treatment with imidocarb dipropionate cleared the Texas strain that belongs to genotype A, but not *Theileria haneyi*, which is a newly described species that belongs to genotype C (Ueti et al., 2012; Sears et al., 2020). Therefore, identification of *T. equi* and *B. caballi* genotypes is very important, because of their diagnostic, clinical, and therapeutic significances.

Sri Lanka, an island nation in the Indian Ocean, is home to a limited number of horses and donkeys. The donkeys in this country are thought to have been brought from north-east Africa by Dutch and Arabian traders several centuries ago, and were mainly used for transporting goods (Santiapillai et al., 1999; Liyanage, 2014). During decade of civil unrest in Sri Lanka (Ganguly, 2018), the donkeys in Mannar district were abandoned, and they have been roaming freely ever since (Liyanage, 2014). In common with other tropical countries, tick infestation and infection with tick-borne pathogens are very common in Sri Lankan livestock populations (Sivakumar et al., 2012; Zhyldyz et al., 2019). Therefore, the feral donkeys are also at a high risk for infection by tick-borne pathogens including *T. equi* and *B. caballi*, but such infections have not yet been investigated. In the present study, I investigated the presence and genotypic diversity of *T. equi* and *B. caballi* in the free-roaming donkeys in Mannar and Kilinochchi districts of Sri Lanka.

1-2. Materials and Methods

Blood sampling, smear preparation, and DNA extraction

In 2019, my collaborators collected blood samples randomly from 111 apparently healthy free-roaming donkeys from two locations, namely Mannar (n = 100) and Kilinochchi (n = 11) districts in the northern province of Sri Lanka (Fig. 6). Approximately 2 ml of blood were collected from the jugular vein of each animal into a sterile ethylenediaminetetraacetic acid (EDTA) vacutainer tube. Thin blood smears were prepared from a drop of the blood, and then air-dried (Houwen, 2002). Additionally, a QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) was used to extract DNAs from the collected blood samples, and then stored at -30 °C until use.

The Animal Care and Use Committee of Obihiro University of Agriculture and Veterinary Medicine, Japan, approved all the animal procedures (approval number 21-4).

Microscopic examination for *T. equi* and *B. caballi* infections

Thin blood smears were prepared, fixed with absolute methanol, and then stained with 10% Giemsa solution (Barcia, 2007). I observed the stained smears under a light microscope, CX33 (Olympus, Tokyo, Japan) with a 100 X objective lens and emersion oil for the presence of *B. caballi* and *T. equi* parasites.

PCR screening for *T. equi* and *B. caballi* infections

I used a previously described PCR assays based on 18S rRNA sequences to screen the donkey DNA samples to detect *T. equi* and *B. caballi* (Bashiruddin et al., 1999; Alhassan et al., 2005). Briefly, 1 µl of DNA sample was added to 9 µl of reaction mixture

that contained 1× PCR buffer (Applied Biosystems, Branchburg, NJ, USA), 200 µM of each dNTP (Applied Biosystems, Vilnius, Lithuania), 0.5 µM of each forward and reverse primer (Table 1), 0.1 µl of 5U/µl Taq DNA polymerase (Applied Biosystems), and 5.9 µl of double distilled water (DDW). The reaction mixture was then subjected to pre-denaturation at 95 ºC for 5 min, followed by 40 cycles that included a denaturation step at 95 ºC for 30 sec, an annealing step at 51 ºC (*T. equi*) or 55ºC (*B. caballi*) for 30 sec, and an extension step at 72 ºC for 1 min. After the final elongation at 72 ºC for 7 min, the PCR products were resolved in a 1.5% agarose gel, stained with ethidium bromide, and then visualized under UV illumination. DNA samples that had been extracted from *in vitro* cultures of *T. equi* and *B. caballi* (Holman et al., 1994; Zweygarth et al., 1995; Avarzed et al., 1997) were used as positive controls, while reaction mixture without any template DNA was used as a negative control. Samples that produced bands of expected sizes (Table 1) were considered to be positive.

Cloning, sequencing, and phylogenetic analysis of *T. equi* 18S rRNA sequences

A long fragment of 18S rRNA (~1,600 bp) was amplified from *T. equi*-positive donkey DNA samples, using previously reported forward (Nbab_1F) and reverse (TB-rev) primers (Matjila et al., 2008; Oosthuizen et al., 2008) (Table 1). The PCR reaction mixture and cycling conditions used, were described previously (Sivakumar et al., 2019). The PCR amplicons were gel-extracted using a QIAamp DNA Blood Mini Kit (Qiagen), and then cloned into a PCR 2.1-TOPO plasmid vector (Invitrogen, Carlsbad, CA, USA). For each amplicon, two colonies were sequenced using an ABI PRISM 3100 genetic analyzer (Applied Biosystems, Tokyo, Japan). The resulting sequences were analysed using the basic local alignment search tool (BLAST) searches

(https://blast.ncbi.nlm.nih.gov/Blast.cgi) to confirm their origin and to determine the identity scores that they shared with the corresponding sequences registered in GenBank. The identity scores shared among the newly generated sequences were determined using the matrix global alignment tool (MatGAT) software (Campanella et al., 2003).

The Sri Lankan sequences, together with *T. equi* 18S rRNA sequences representing the five genotypes (A–E) derived from Sudan, Israel, Turkey, South Africa, Brazil, USA, Cuba, China, India, Spain, Chile, Iran, and Switzerland, were aligned using an online version of multiple alignment using fast fourier transform (MAFFT) (https://mafft.cbrc.jp/alignment/server/) (Katoh et al., 2002). The resulting alignment was analysed using the molecular evolutionary genetics analysis (MEGA) X (Kumar et al., 2018) to predict the best-fitting substitution model. Subsequently, a maximum likelihood phylogeny was constructed, based on the General Time Reversible substitution using Invariant plus Gamma-distributed sites (GTR+I+G) model (Felsenstein, 1981) with 1,000 bootstrap replicates to estimate reliability.

1-3. Results

In the present study, the presence and genotypic diversity of *T. equi* and *B. caballi* were investigated against free-roaming donkeys in Sri Lanka. The results showed that 64 (57.7%) and 95 (85.6%) of 111 surveyed animals were positive for *T. equi* by microscopy and PCR, respectively. The blood smears were examined microscopically for the presence of *B. caballi* and *T. equi.* No large paired pyriforms, which are indicative of *B. caballi*, were detected in any of the 111 samples. However, various forms of *T. equi*, such as Maltese-cross, ring, and irregular pyriforms, were observed (Fig. 7).

To analyze the genotypic diversity of *T. equi* infecting in the donkeys in Sri Lanka, I cloned a long fragment of 18S rRNA amplified from 33 randomly selected PCR-positive DNA samples, and then sequenced two clones per sample. Sequences of both clones from 24 samples were identical to each other, while the different sequences of both clones were obtained from the remaining nine samples. Therefore, a total of 42 18S rRNA sequences were registered with GenBank (Accession No. LC649067 – LC649098 and LC670594 – LC670603), and then used for the phylogenetic analysis. The Sri Lankan sequences shared 97.8–100% identity scores with each other, and also shared 95 – 100% identity scores with other sequences registered in GenBank. I found that, based on the phylogenetic analysis, two genotypes, namely C and D, were detected (Fig. 8). Genotype D was the most prevalent; 36 sequences from 28 donkeys occurred in the relevant clade, while the remaining six sequences from five donkeys occurred in a clade representing genotype C.

1-4. Discussion

Equine piroplasmosis has a worldwide distribution with a significant negative economic impact on the global equine industry, mainly due to restriction on the movement of infected horses (Wise et al., 2013). Only a few countries, including Canada, Australia, New Zealand, and Japan, are free from *T. equi* (Wise et al., 2014; Rothschild, 2003; Tirosh-Levy et al., 2020c), and many other countries have never been investigated including Sri Lanka. This is the first study to report on the *T. equi* infection in Sri Lankan.

Although microscopy technique is commonly used for parasite detection, it lacks sensitivity (Zobba et al., 2008; Tirosh-Levy et al., 2020b), and is unsuitable for detecting carrier animals with very low parasitaemia. Conversely, PCR assays have now

superseded the microscopy in epidemiological surveys, because of their high specificity and sensitivity (Tirosh-Levy et al., 2020b). Therefore, conventional PCR assays based on 18S rRNA sequences were subsequently used for detecting *T. equi* and *B. caballi* DNAs in the samples (Bashiruddin et al., 1999; Alhassan et al., 2005). These PCR assays have been widely used in epidemiological surveys to detect carrier animals. My findings showed that 95 (85.6%) samples were PCR-positive for *T. equi*, whereas none of the samples were positive for *B. caballi*.

The detection of *T. equi* in the surveyed donkeys was not surprising, because this parasite species has been reported in several equids other than horses, such as both domestic and wild donkeys and zebras. Similar to my findings, high infection rates of *T. equi* have been observed in donkeys (72.0%) in Kenya and Israel (89.0%), and zebras in Israel (62.0%), Kenya (100%), and South Africa (80.3%) (Bhoora et al., 2010b; Hawkins et al., 2015; Tarav et al., 2017; Tirosh-Levy et al., 2020a). The pronounced persistence of *T. equi* in the infected animals, probably for the remainder of their lives, could be the reason for the high rate of positivity (Rothschild, 2013). Compared with *T. equi*, *B. caballi* has a limited geographical distribution, and the prevalence of *B. caballi* was reported to be lower than *T. equi* globally (Tirosh-Levy et al., 2020c). The absence of *B. caballi* in the surveyed donkeys may suggest that this parasite species is probably not endemic to Sri Lanka. However, a large-scale epidemiological survey is essential to confirm this assumption, due to the small sample size used in the present study.

Although the infectious rate of *T. equi* was high in the surveyed donkeys, severe clinical disease during the acute phase of EP is uncommon in this animal species (Laus et al., 2015). However, a previous study found that *T. equi*-infected donkeys had significantly lower values for RBC counts, haematocrit, and haemoglobin concentration,

as compared with those of uninfected donkeys (Laus et al., 2015). Therefore, EP might be of health significance in donkeys, particularly as the donkey population in Sri Lanka is shrinking and in a danger of extinction probably due to either poor management practices or infectious diseases (Santiapillai et al., 1999). Preservation efforts should also focus on management of various infectious diseases, including EP in the donkeys.

A previous study found that several tick species, belonging to the genera *Amblyomma*, *Dermacentor*, *Hyalomma*, and *Rhipicephalus*, infest livestock and other animals including cattle, goats, pigs, dogs, and buffalo in Sri Lanka. However, tick species infesting in equines and prevalent in the study area are unknown (Liyanaarachchi et al., 2015). Therefore, studies to identify tick species infesting donkeys in the study area are a priority. The *T. equi*-infected donkeys can act as carriers from which the parasite can be tick-transmitted to horses, where the infection may result in severe disease (Kumar et al., 2009). However, this possibility is unlikely in Sri Lanka, because only a small number of horses are maintained in geographically distant areas. The horses are mainly found in the central region of the country with no close contact with the donkeys maintained in the northern part.

Phylogenetic analysis detected genotype C, which has a wide distribution, but had not been detected previously in donkeys (Tirosh-Levy et al., 2020c). Therefore, the present study is the first to report the genotype C in donkeys. Recently, a strain belonging to genotype C was characterized as a new *Theileria* species, known as *T. haneyi*, which lacks *ema-1* (Knowles et al., 2018). Therefore, EMA-1-based diagnostic assays, such as ELISA and cELISA (Knowles et al., 2018) might not be suitable for serological survey of *T. equi* infection in Sri Lanka. Moreover, the presence of genotype C is worrisome, because drug-induced clearance of *T. haneyi* is extremely difficult (Sears et al., 2020).

Genotype D was found in Africa, the Mediterranean region, and the Middle East (Tirosh-Levy et al., 2020b). This genotype has been commonly detected in several African countries, including Sudan (Salim et al., 2009), South Africa (Bhoora et al., 2020), Nigeria (Mshelia et al., 2020), and Gambia (Coultous et al., 2020). These observations, together with the origin of Sri Lankan donkeys, and the prevalence of genotype D may suggest that *T. equi* had been introduced from Africa into Sri Lanka.

International movement of horses is highly restricted, based on the epidemiological status of EP in the countries involved (WOAH, 2021). In Sri Lanka, however, the endemicity of EP was unknown, leading to several challenges, such as inability of the horse management agencies to import into or export from Sri Lanka. Therefore, the detection of *T. equi* in Sri Lanka is important in terms of global epidemiology of EP and international trade of horse.

1-5. Summary

Sri Lanka is a tropical country, where infections of various tick-borne parasites are common among livestock animals. However, infections of *T. equi* and *B. caballi* remain unstudied in Sri Lanka. Therefore, in the present study, I conducted an epidemiological survey to investigate the presence of *T. equi* and *B. caballi* in apparently healthy free-roaming donkeys. Blood samples were taken from 111 donkeys in Mannar and Kilinochchi districts, and blood DNA samples were screened for *T. equi* and *B. caballi* infections using species-specific PCR assays. I found that 64 (57.7%) and 95 (85.6%) of donkeys were positive for *T. equi* by microscopy and PCR, respectively, while all samples were negative for *B. caballi*. The present study reports the presence of *T. equi* and two of its genotypes, C and D. This is the first report of *T. equi* in Sri Lanka and of

genotype C in donkeys, and highlight the importance of monitoring the shrinking donkey population in Sri Lanka due to *T. equi*-induced infection.

Table 1. List of PCR primers used in the present study.

Target Gene	**Primers**	**Sequence (5`- 3`)**	**Product (bp)**	**Reference**
T. equi 18S rRNA	Bec-UF2	TCGAAGACGATCAGATACCGTCG	435	Alhassan et al., 2005
	Equi-R	TGCCTTAAACTTCCTTGCGAT		
B. caballi 18S rRNA	BCAF	TTCGCTTCGCTTTTTGTTTTTACT	659	Bashiruddin et al., 1999
	BCAR	GTCCCTCTAAGAAGCAAACCCAA		
T. equi 18S rRNA (Sequencing)	Nbab_1F	AAGCCATGCATGTCTAAGTATAAGCTTTT	~ 1,600	Oosthuizen et al., 2008
	TB Rev	GAATAATTCACCGGATCACTCG		Matjila et al., 2008

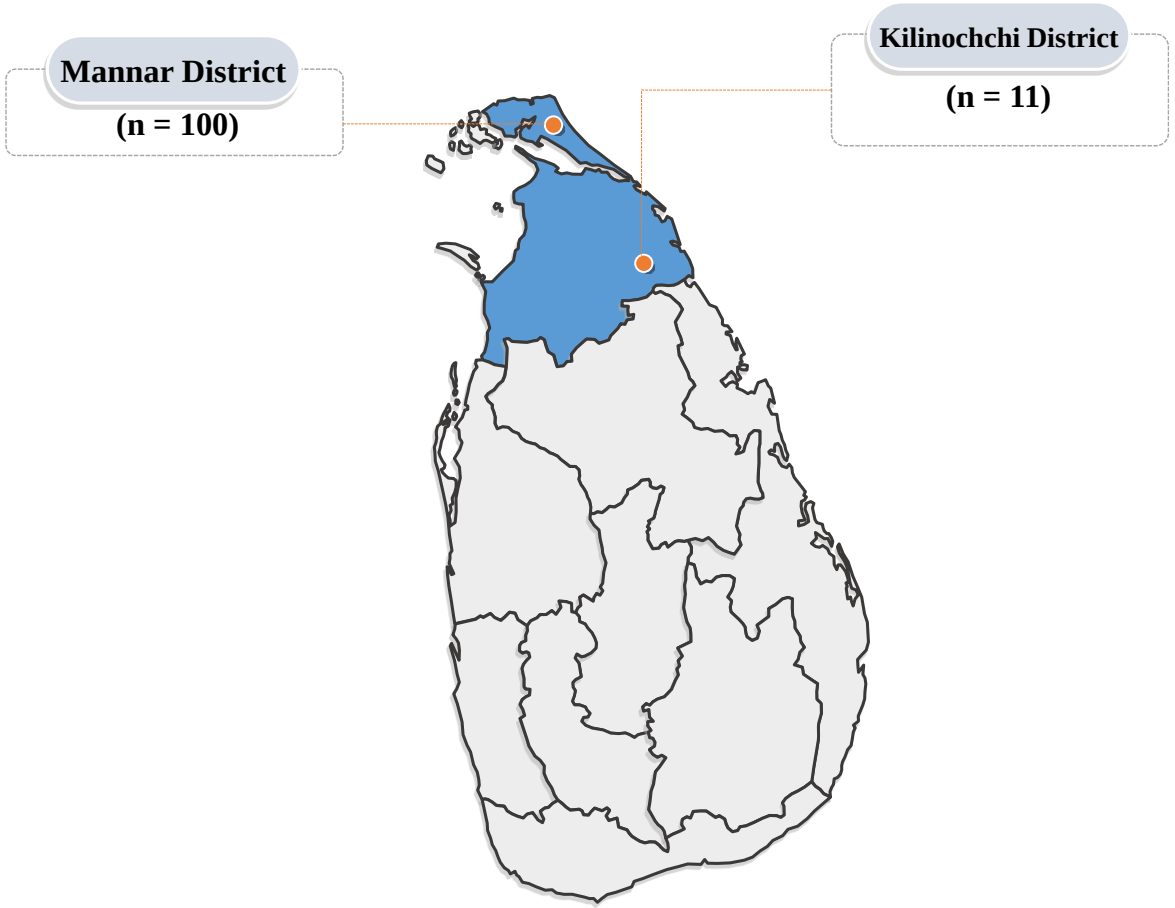

Fig. 6. Map showing the sampling districts in Sri Lanka. The donkeys were sampled from in Mannar and Kilinochchi districts.

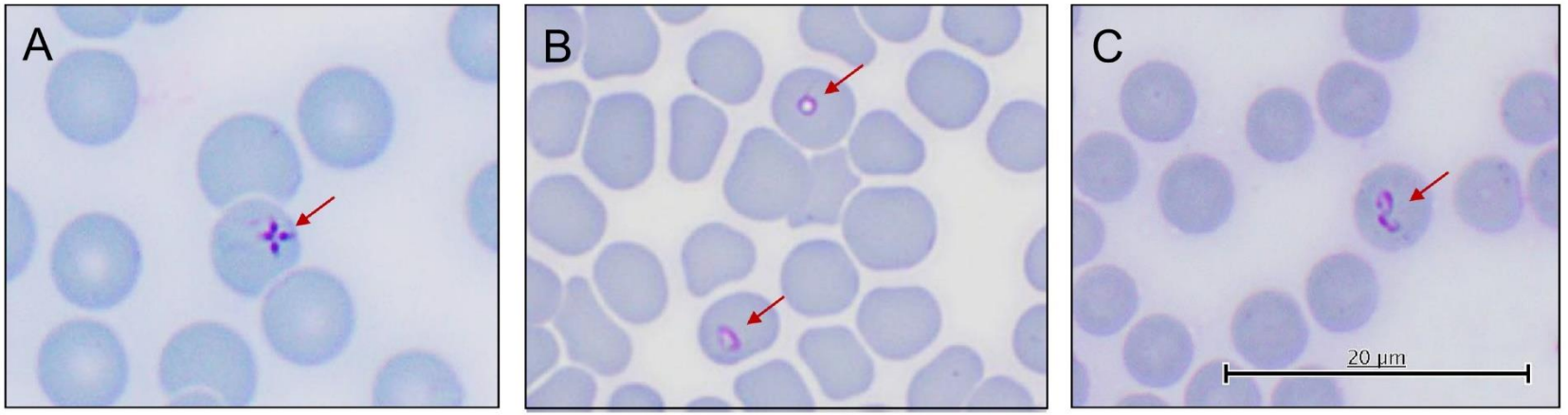

Fig. 7. Morphology of *Theileria equi* detected in free-roaming donkeys in Sri Lanka.

Panel **A**. Maltese-cross form, **B**. ring form, and **C**. irregular pyriform.

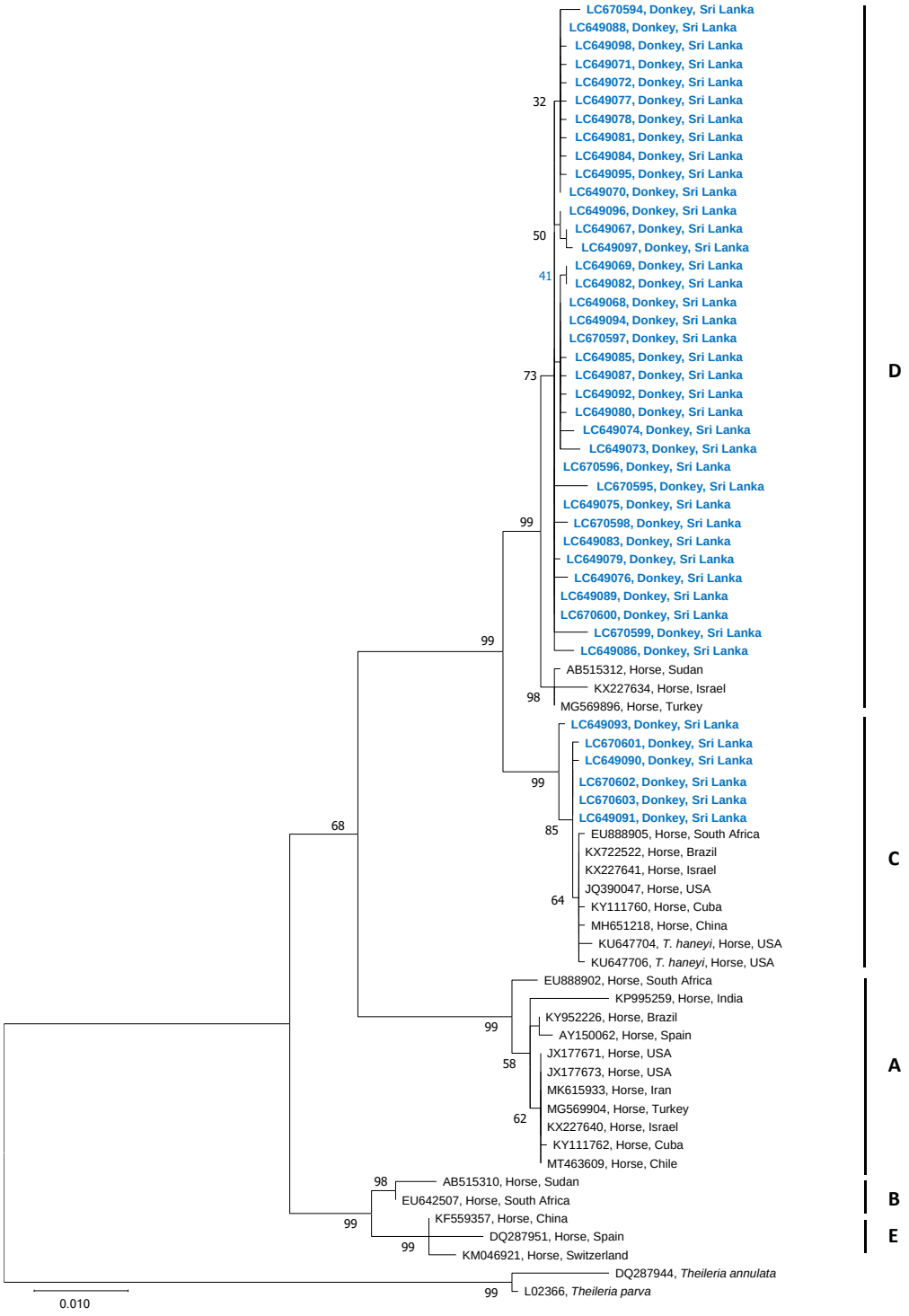

Fig. 8. Phylogenetic analysis of *Theileria equi* 18S rRNA sequences. The sequences isolated from the donkeys in the present study, together with those retrieved from GenBank, were used to construct a maximum likelihood phylogeny. The sequences determined in the present study are indicated in blue font. The Sri Lankan sequences occurred in clades C and D.

Chapter 2

PCR detection of *Theileria equi* and *Babesia caballi* in apparently healthy horses in Paraguay

2-1. Introduction

Equine piroplasmosis (EP) is a tick-borne disease caused by two intra-erythrocytic protozoan parasites, *Theileria equi* and *Babesia caballi*, in several equid species, including horses, donkeys, mules, and zebras (Friedhoff et al., 1990). This disease has a global distribution, but is highly endemic in tropical, subtropical, and some temperate countries, where most competent tick vectors exist (De Waal, 1990; Scoles and Ueti, 2015). Both *T. equi* and *B. caballi* are primarily transmitted to equids by tick species of the genera *Amblyomma*, *Dermacentor*, *Hyalomma*, and *Rhipicephalus* (Scoles et al., 2011; Scoles and Ueti, 2015). In addition, the parasites can be transmitted transplacentally and iatrogenically via blood transfusion, contaminated needles and syringes, and surgical instruments (De Waal, 2004; Ueti et al., 2008, Wise et al., 2014).

Infections with *T. equi* and *B. caballi* can cause mild subclinical to severe acute disease. Fever, anaemia, icterus, haemoglobinuria, anorexia, weakness, weight loss, poor exercise tolerance, and sometimes death characterise the acute form of EP (Zobba et al., 2008; Rothschild, 2013; Tamzali, 2013; Wise et al., 2013), and animals that recovered from acute EP and those with subclinical infections become chronic carriers (Zobba et al., 2008, Wise et al., 2014). Equines infected with *B. caballi* are able to naturally clear the

parasites within 4 years in the absence of re-infections, while those infected with *T. equi* remain carriers for the remainder of their lives. These carriers aid in the spread of EP by acting as a source of infection for tick vectors (Zobba et al., 2008, Rothschild, 2013; Tamzali, 2013; Wise et al., 2014). Therefore, the detection of carriers is crucial for devising management strategies, such as those that aim to prevent the introduction of EP into non-endemic areas/countries through the movement of infected animals that appear healthy (Wise et al., 2014; Tirosh-Levy et al., 2020c).

Paraguay is a tropical, landlocked country that shares borders with Argentina, Brazil, and Bolivia (Worldfact book, 2022). The country's primary industry is agriculture, and livestock production accounts for approximately 11% of the gross domestic product of Paraguay (World Bank, 2020). As of 2018, there were about 200,000 horses in this nation, making them a significant component of total livestock production (Zaya et al., 2021). Additionally, horses play a part in a variety of industries, including ranching, sports, and recreational activities (Lansade et al., 2004), which is the basis for their considerable economic importance (Giulotto, 2001). In Paraguay, extensive management systems are generally used to maintain livestock, including horses, which puts these animals at a high risk of tick infestations and tick-borne diseases (Payne and Osorio, 1990).

Although EP has been reported among horses bred in neighbouring countries, such as Brazil and Argentina (Holman et al., 1998; Ferreira et al., 2016; Peckle et al., 2018; Gabriela et al., 2019; Sebastian et al., 2021), infectious status remains uninvestigated in Paraguayan horses. A previous study, however, detected *T. equi* DNA in dogs in this country (Inácio et al., 2019). This observation, together with the presence of tick species of the genera *Amblyomma* and *Rhipicephalus*, which are known competent vectors of *T. equi* and *B. caballi* (Nava et al., 2007; Dixit et al., 2010), strongly suggest that EP might be

common among horses in Paraguay. In the present study, therefore, I investigated the *T. equi* and *B. caballi* infectious status of horses bred in Paraguay.

2-2. Materials and Methods

Blood sampling and DNA extraction

Between 2019 and 2020, my collaborators in Paraguay collected blood samples from 545 apparently healthy horses randomly selected from 16 of the 17 departments of Paraguay, excluding Alto Paraguay (Fig. 9). Approximately 2 ml of blood was collected from the jugular vein of each animal into a sterile ethylenediaminetetraacetic acid (EDTA)-containing vacutainer tube. Subsequently, DNA samples were extracted from 200 µl of the blood using DNAzol reagent (Themo Fisher Scientific, Waltham, MA, USA), following the manufacturer's instructions. The precipitated DNA was suspended in 50 µl of double distilled water (DDW), and stored at −30°C until use. These DNA samples were previously screened for animal trypanosoma genes (Suganuma et al., 2021).

The Animal Care and Use Committee of Obihiro University of Agriculture and Veterinary Medicine, Japan, approved all animal procedures (approval number 21-4).

Haematological analysis

All the 545 blood samples were evaluated using an automatic blood analyser HumaCount 80^{TS} (HUMA Gesellschaft für Biochemica und Diagnostica mbH, Wiesbaden, Germany). The haematological parameters, including the red blood cell (RBC) count, haemoglobin (Hb) concentration, haematocrit value (HCT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), and mean corpuscular haemoglobin concentration (MCHC), were determined.

PCR screening for *T. equi* and *B. caballi* infections

Using the parasite-specific PCR assays, I screened all the DNA samples for *T. equi* and *B. caballi* infections, as described below. A PCR assay with a set of previously described primers; forward primer, Bec-UF2 (5′-TCGAAGACGATCAGATACCGTCG-3′), and reverse primer, Equi-R (5′-TGCCTTAAACTTCCTTGCGAT-3′) (Alhassan et al., 2005), which amplify a 435-bp fragment of ribosomal RNA of 18S sub-unit (18S rRNA), were used for the detection of *T. equi*. Another PCR assay with forward primer, BC48_F (5′-CCAACCGCTGACCCTTC-3′), and reverse primer, BC48_R (5′-CTTCAGCTTCATGTACCACTTCTT-3′), which amplify a 544-bp fragment of rhoptry-associated protein-1 gene (*rap-1*), were used to detect *B. caballi*. Briefly, 1 µl of DNA sample was added into a 9-µl reaction mixture that contained 1× PCR buffer (Applied Biosystems, Branchburg, NJ, USA), 200 µM of each dNTP (Applied Biosystems), 0.5 µM of each forward and reverse primer, 0.1 µl of 5 U/µl Taq DNA polymerase (Applied Biosystems), and 5.9 µl of DDW. The reaction mixture was then subjected to pre-denaturation at 95°C for 5 min, followed by 40 cycles that included a denaturation step at 95°C for 30 sec, an annealing step at 51°C (*T. equi*) or 55°C (*B. caballi*) for 30 sec, and an extension step at 72°C for 1 min. After a final elongation at 72°C for 7 min, the PCR products were resolved on a 1.5% agarose gel, stained with a MIDORI Green Xtra (NIPPON Genetics, Düren, Germany), and then visualised under UV illumination. DNA samples extracted from *in vitro* cultures of *T. equi* and *B. caballi* (Zweygarth et al., 1995; Avarzed et al., 1997) were used as positive controls, while a reaction mixture without any DNA template served as a negative control. Samples that produced amplicons of the expected size were considered positive in the respective PCR assays.

Cloning and sequencing analysis of PCR products

The PCR amplicons were gel-extracted, purified using a QIAquick Gel extraction kit (Qiagen, Hilden, Germany), and then cloned into a PCR 2.1-TOPO plasmid vector (Invitrogen, Carlsbad, CA, USA). The inserted gene fragments were sequenced using the ABI PRISM 3130xl genetic analyser (Applied Biosystems, Tokyo, Japan). The generated sequences were then analysed, using a Basic Local Alignment Search Tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to confirm their origins.

Phylogenetic analysis

To investigate the genotypic diversities, I constructed two phylogenetic trees using the *T. equi* 18S rRNA and *B. caballi rap-1* sequences determined in the present study, as well as the GenBank sequences representing the five *T. equi* genotypes (A-E) and three *B. caballi* genotypes (A1, A2, and B). In brief, the sequences were aligned using an online version of multiple alignment using fast fourier transform (MAFFT) (https://mafft.cbrc.jp/alignment/server/) (Katoh et al., 2002), and the best-fitting substitution models were predicted using the molecular evolutionary genetics analysis (MEGA XI) software (Kumar et al., 2018). The Tamura-Nei (Tamura and Nei, 1993) and Kimura 2 - parameter (Kimura, 1980) substitution models were then used to construct two maximum likelihood phylogenetic trees for *T. equi* and *B. caballi*, respectively.

Statistical analysis

The positive rates were analysed using OpenEpi online software (https://www.openepi.com/Proportion/Proportion.htm) to calculate the 95% confidence intervals (CI) based on the Wilson score interval (Wilson, 1927). The *P* values were

calculated using an 'N-1' chi-squared test, an online software (https://www.medcalc.org/calc/comparison_of_proportions.php) (Campbell, 2007; Richardson, 2011) to determine whether differences in positive rates between groups were statistically significant. Differences were considered statistically significant, when *P* values were < 0.05. Standard deviations (SD) for the mean values of haematological parameters were calculated using Microsoft Excel.

2-3. Results

The PCR results indicated that horses in Paraguay were infected with *T. equi* and *B. caballi. Theileria equi* was the most common parasite species, infecting 178 (32.7%) of 545 horses, while *B. caballi* was detected in only 8 (1.5%) horses (Table 2). As two animals were found to be co-infected with *T. equi* and *B. caballi*, a total of 184 (33.8%) horses were infected with at least one of the surveyed parasite species. *Theileria equi* was detected in 15 of the 16 surveyed departments, with Canindeyú being the exception, whereas *B. caballi* was detected in only 6 departments, including Alto Paraná, Caazapá, Central, Ñeembucú, Presidente Hayes, and San Pedro (Figs. 9, 10, 11, 12, 13; Table 2). The two horses that had been co-infected with *T. equi* and *B. caballi* were each found in Alto Paraná and San Pedro departments (Table 2). Ten and two amplicons randomly selected from the *T. equi* and *B. caballi* PCR assays, respectively, were sequenced to validate our results. The newly determined sequences of *T. equi* (GenBank accession numbers LC721039–LC721048) and *B. caballi* (LC721049 and LC721050) shared 99.5%–100% and 98.8%–99.7% identity scores, respectively, with the sequences of *T. equi* (MN611348, MT463613, and MG052902) and *B. caballi* (AB017700) previously registered in GenBank (Figs. 11 and 13). In the *T. equi* phylogenetic tree, seven of the 10

Paraguayan sequences occurred in the genotype C clade, while the remaining three occurred in the genotype A clade (Fig. 14). The two *B. caballi rap-1* sequences from Paraguayan horses, on the other hand, were found in the genotype A clade (Fig. 15).

Since *B. caballi* was detected in only eight animals, we focused on only *T. equi* infections in further analyses. On a per department basis, the *T. equi*-positive rates ranged from 13.3% to 51.1% (Table 2). Of the 545 horses sampled, information on breed, sex, and age was available for 454, 514, and 482 animals, respectively. Based on this information, we analysed the *T. equi*-positive rates in relation to breed, sex, and age-group. In the present study, 10 horse breeds belonging to the indigenous breed (Criolla), exotic breeds (Brasilero de Hipismo, Corona Cartel, Thoroughbred, Dogo Aleman, Holando, Quarter Horses, and Silla Argentina), and a crossbreed (Mestiza), were sampled (Table 3). *Theileria equi* was detected in five breeds: the indigenous breed Criolla; the exotic breeds Brasilero de Hipismo, Thoroughbred, and Quarter horse; and the crossbreed Mestiza (Table 4). My analyses showed that the positive rates of *T. equi* did not differ among the indigenous breed (33.3%), exotic breeds (31.5%), and crossbreed (30.9%) ($P > 0.05$). Similarly, the *T. equi*-positive rates did not differ between males (31.8 %) and females (32.9%), or between ≤3 years old (35.9%) and >3 years old (29.0%) age groups (*P* values 0.7774 and 0.1078, respectively). Blood samples collected from all 545 horses were subjected to haematological analyses. The mean Hb, RBC, HCT, MCV, MCH, and MCHC values for the infected and non-infected horses are shown in Table 5. I observed no significant differences in the mean values of the haematological parameters in the *T. equi*- or *B. caballi*-infected horses, as compared with those in the non-infected horses. Two co-infected animals, however, had Hb (10.3 g/dL and 9.2 g/dL) and HCT (30% and 27%) values that were below the lower end of the normal range (11.4 g/dL and 31.0%,

respectively). Moreover, the mean values of Hb and HCT in co-infected animals (9.8 g/dL and 28.5%, respectively) were below those in the non-infected animals (11.7 g/dL and 34.5%, respectively) (Table 5). The mean HCT value, in particular, was significantly lower (P = 0.0444) in co-infected horses than that of non-infected animals.

2-4. Discussion

To the best of our knowledge, this is the first study to report on infections of *T. equi* and *B. caballi*, the causative agents of EP, in Paraguayan horses. In particular, *T. equi* was more common in infected horses than *B. caballi*. Similar findings were obtained with other studies conducted in several other countries, including Brazil and Venezuela in South America (Heim et al., 2007; Machado et al., 2012; Rosales et al., 2013; Peckle et al., 2018). The lower infectious rate of *B. caballi* is probably due to their low parasitaemia and the natural clearance of the parasites in the host animals, whereas the life-long persistence of *T. equi* may result in higher infectious rates (Rapoport et al., 2014; Tirosh-Levy et al., 2020b).

In the present study, the infectious rate of *B. caballi* was 1.5%, as compared to 17.2% in a related study in Brazil (Peckle et al., 2022). The reasons for this discrepancy may include the differences in the abundance and seasonal activities of competent tick vectors and the sensitivities of used PCR assays. On the other hand, seroprevalence of *B. caballi* is usually higher in endemic countries than the molecular prevalence (Tirosh-Levy et al., 2020b). For example, a previous investigation found that 70.6% of the surveyed horses in Venezuela were seropositive to *B. caballi* (Mujica et al., 2011). This could be attributed to the fact that *B. caballi*-infected horses may clear the parasites with time, leading to negative results in PCR assays, while the animals may still be seropositive.

Therefore, additional studies to investigate the seroprevalence are essential to determine the extent of *B. caballi* exposure in horses in Paraguay.

In contrast, *T. equi* usually persists throughout the lifetime of infected horses (Rothschild, 2013; Wise et al., 2014). As a result, a large proportion of horses in a population tend to be positive for *T. equi* at any given time (Zobba et al., 2008, Rothschild, 2013; Tamzali, 2013; Wise et al., 2014). Our study also found that *T. equi* has a wide distribution in Paraguay, as this species was detected in 15 of the 16 surveyed departments. The negative results obtained for the horses bred in Canindeyú Department do not guarantee that horses in this area are free from the infection, because only six animals were surveyed. Although the positive rates of *T. equi* infection varied among the surveyed departments, a fair comparison of the positive rates based on department was not possible, because of the small sample sizes in each location.

The positive rates of *T. equi* infection did not differ between the horse breeds, males and females, or the ≤3 years old and >3 years old age groups. In Paraguay, livestock, including horses, are mostly maintained under extensive management systems (Milán and González, 2022). As a result, horses in this country might be exposed to many tick vectors to a similar degree, regardless of their breed, sex, or age (Rapoport et al., 2014).

I also found no significant differences in haematological parameters of non-infected animals and those with single infections (*T. equi* or *B. caballi*). By contrast, the two horses co-infected with *T. equi* and *B. caballi* had Hb and HCT values below the normal ranges. A previous study found that co-infection with *Babesia* and *Theileria* may potentiate anaemia development in cattle (Sivakumar et al., 2012). Similarly, there is a possibility that co-infection with *T. equi* and *B. caballi* increases the likelihood of anaemia in horses. Therefore, additional investigations with a larger number of co-infected animals and horses with experimental infections are essential to confirm our assumption.

The genotypic diversity of *T. equi* and *B. caballi* has implications for the control of EP, because their genotypes may influence diagnostic test results (Bhoora et al., 2010; Rapoport et al., 2014), clinical outcome (Manna et al., 2018; Tirosh-Levy et al., 2020b), and therapeutic efficacy of drugs in terms of parasite clearance (Ueti et al., 2012; Sears et al., 2020). I found that the horses in Paraguay were infected with *T. equi* genotypes A and C, as well as with *B. caballi* genotype B. However, the presence of other genotypes cannot be entirely ruled out, because the current study only examined a few short 18S rRNA sequences. Therefore, to identify all genotypes of *T. equi* and *B. caballi* infecting horses in Paraguay, additional studies using a large number of long 18S rRNA fragments are essential (Schnittger et al., 2022).

The detection of *T. equi* and *B. caballi* in Paraguayan horses is an important finding with domestic and global implications. Although the majority of horses infected with *T. equi* and *B. caballi* were asymptomatic in the present study, severe forms of EP have been frequently reported in many endemic countries (Rothschild, 2013; Tamzali, 2013; Wise et al., 2014; Tirosh-Levy et al., 2020b). Therefore, horses in Paraguay should be monitored for clinical EP, and EP should be one of the differential diagnoses, when horses with anaemic signs are presented to equine clinics. Because of the clinical and economic significance and persistence of EP infections, guidelines from the World Organization for Animal Health (WOAH) strictly regulate the transnational movement of horses, particularly from endemic countries to disease-free countries (WOAH, 2021). This report on the parasite species may offer a guidance to industry players interested in safe equine trading involving Paraguay. Therefore, Paraguayan veterinary authorities should also design appropriate control strategies to minimise the impact of EP to increase economic benefits in the international equine trade.

2-5. Summary

EP has a global distribution and often leads to a significant socioeconomic impact on the equine industry. Infected animals remain as carriers and become a source of infection for tick vectors, thereby posing an immense challenge in the disease management. Paraguay is a tropical country where various tick-borne diseases are common among livestock; however, the status of EP remains unknown. Because the tick vectors capable of transmitting *T. equi* and *B. caballi* are endemic in Paraguay, I hypothesised that Paraguayan horses are infected with these parasite species. To test my hypothesis, I used blood DNA samples from a total of 545 apparently healthy horses in 16 of the 17 departments of Paraguay, and analysed them with specific PCR assays to detect *T. equi* and *B. caballi*. I found 178 (32.7%) and 8 (1.5%) of the horses were infected with *T. equi* and *B. caballi*, respectively. In analysing the risk factors, I observed that the positive rates of *T. equi* infection did not differ between horse breeds, gender, or age groups. Haematological parameters were also similar between non-infected and single-infected animals. However, two horses (0.4%) coinfected with *T. equi* and *B. caballi*, had their Hb and HCT values lower than the normal ranges. The phylogenetic analyses detected two genotypes A and C of *T. equi*, and B of *B. caballi*. This is the first report of EP infection in the horses in this country. My findings suggest that EP should be added to differential diagnoses, when anaemic horses are presented to equine clinics in Paraguay.

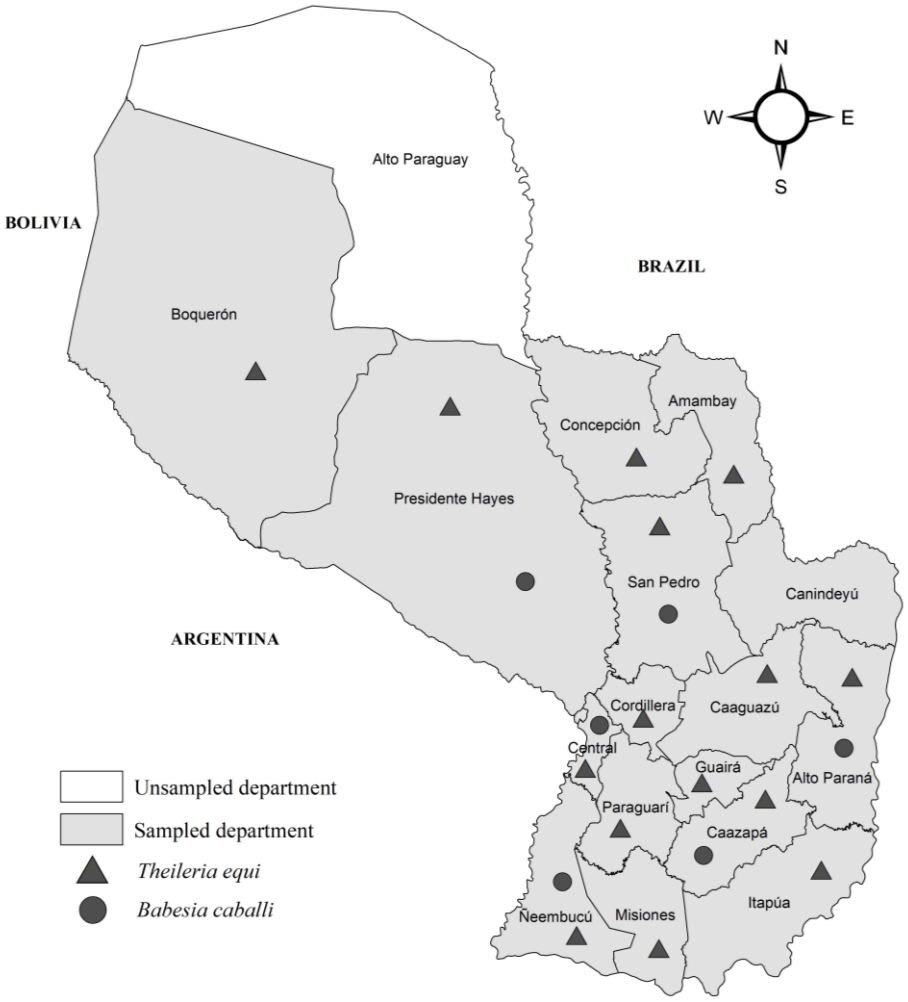

Fig. 9. Sampling locations in Paraguay. Horses were sampled in 16 of the 17 departments in Paraguay. *Theileria equi* was detected in 15 of the 16 departments surveyed, with the exception being Canindeyú, whereas *Babesia caballi* was detected in four departments, including Caazapá, Central, Ñeembucú, and Presidente Hayes.

Table 2. PCR detection of *Theileria equi* and *Babesia caballi* in 545 horses from the 16 departments in Paraguay.

Departments	**No. sample**	***T. equi***		***B. caballi***		**Co-infection**	
		No. positive	**% (CI)[a]**	**No. positive**	**% (CI)**	**No. positive**	**% (CI)**
Alto Paraná	14	6	42.9 (21.4-67.4)	1	7.1 (1.3-31.5)	1	16.7 (1.3-31.5)
Amambay	6	2	33.3 (9.7 -70)	0	0.0 (0.0-39.0)	0	0.0 (0.0-39.0)
Boquerón	15	2	13.3 (3.74-37.9)	0	0.0 (0.0-20.4)	0	0.0 (0.0-20.4)
Caaguazú	16	5	31.3 (14.2-37.9)	0	0.0 (0.0-19.4)	0	0.0 (0.0-19.4)
Caazapá	45	23	51.1 (37.0-65.0)	1	2.2 (0.4-11.6)	0	0.0 (0.0-7.9)
Canindeyú	6	0	0 (0.0-39.0)	0	0.0 (0.0-39.0)	0	0.0 (0.0-0.0)
Central	151	46	30.5 (23.7-38.2)	3	2.0 (0.7-5.7)	0	0.0 (0.0-2.5)
Concepción	15	3	20.0 (7.0-45.2)	0	0.0 (0.0-20.4)	0	0.0 (0.0-20.4)
Cordillera	30	8	26.7 (14.2-44.5)	0	0.0 (0.0-11.4)	0	0.0 (0.0-11.4)
Guairá	40	7	17.5 (8.7-32.0)	0	0.0 (0.0-8.8)	0	0.0 (0.0-8.8)
Itapúa	17	5	29.4 (13.3-53.1)	0	0.0 (0.0-18.4)	0	0.0 (0.0-18.4)
Misiones	22	9	40.9 (23.3-61.3)	0	0.0 (0.0-14.9)	0	0.0 (0.0-14.9)
Ñeembucú	64	29	45.3 (33.7-57.4)	1	1.6 (0.3-8.3)	0	0.0 (0.0-5.7)
Paraguarí	37	12	32.4 (19.6-48.5)	0	0.0 (0.0-9.4)	0	0.0 (0.0-9.4)
Presidente Hayes	27	10	37.0 (21.5-55.8)	1	3.7 (0.7-18.3)	0	0.0 (0.0-12.5)
San Pedro	40	11	27.5 (16.1-42.8)	1	2.5 (0.4-12.9)	1	2.5 (0.4-12.9)
Total	**545**	**178**	**32.7 (28.9-36.7)**	**8**	**1.5 (0.7-2.8)**	**2**	**0.4 (0.1-1.3)**

[a] CI, 95% confidence interval

Table 3. Positive rates of *Theileria equi* infection in horses in relation to breeds, sex, and age.

Factors	No. of animals	No. positive (%)	*P* value
Breeds			
Indigenous	18	6 (33.3)	
Exotic breed	368	116 (31.5)	0.8727[a], 0.8462[b], 0.9221[c]
Crossbreed	68	21 (30.9)	
Sex			
Male	274	87 (31.8)	0.7774
Female	240	79 (32.9)	
Age			
≤3-year-old	223	80 (35.9)	0.1078
>3-year-old	259	75 (29.0)	

[a] *P* value between indigenous breed and exotic breed

[b] *P* value between indigenous breed and crossbreed

[c] *P* value between exotic breed and crossbreed.

Table 4: PCR detection of *Theileria equi* and *Babesia caballi* in different horse breeds sampled in Paraguay

Breeds		**No. samples**	***T. equi* (n = 137)**		***B. caballi* (n = 6)**		**Co-infection (n = 2)**	
			No. positive	**% CI [a]**	**No. positive**	**% CI**	**No. positive**	**% CI**
Indigenous	Criolla	18	6	33.3 (16.3-56.3)	0	0.0 (0.0-17.6)	0	0.0 (0.0-17.6)
Exotic breed	Brasilero de Hipismo	2	1	50 (9.5-90.6)	0	0.0 (0.0-65.8)	0	0.0 (0.0-65.8)
	Corona Cartel	1	0	0.0 (0.0-79.3)	0	0.0 (0.0-79.3)	0	0.0 (0.0-79.3)
	Thorough bred	7	2	28.6 (8.2-64.1)	0	0.0 (0.0-35.4)	0	0.0 (0.0-35.4)
	Dogo Aleman	1	0	0.0 (0.0-79.3)	0	0.0 (0.0-79.3)	0	0.0 (0.0-79.3)
	Holando	1	0	0.0 (0.0-79.3)	0	0.0 (0.0-79.3)	0	0.0 (0.0-79.3)
	Quarter Horses	355	107	30.1 (25.6-35.1)	6	1.7 (0.8-3.6)	2	0.6 (0.2-2.0)
	Silla Argentina	1	0	0.0 (0.0-79.3)	0	0.0 (0.0-79.3)	0	0.0 (0.0-79.3)
Crossbreed	Mestiza	68	21	30.9 (21.2-42.6)	2	2.9 (0.8-10.1)	0	2.9 (0.8-10.1)

[a] CI, 95% confidence interval

Table 5. Mean values of haematological parameters in non-infected, *Theileria equi* -infected, *Babesia caballi*-infected, and co-infected animals

Parameters [a]	**Non-infected (n = 367)**	***T. equi*-infected (n = 176)**		***B. caballi*-infected (n = 6)**		**Co-infected (n = 2)**		**Normal Range**
		Mean ± SD	*P* value	Mean ± SD	*P* value	Mean ± SD	*P* value	
Hb (g/dL)	11.7±1.5	11.5±1.5	0.1465	11.3±1.4	0.5171	9.8±0.8[b]	0.0746	11.4–17.3
HCT (%)	34.5±4.2	34.1±4.6	0.3145	33.3±4.2	0.4880	28.5±2.1[b]	0.0444 [c]	31.0–50.0
RBC (x10^6/µL)	8.2±1.0	8.0±1.1	0.0352	7.8±1.0	0.3317	7±0.5	0.0911	6.2–10.2
MCV (fL)	44.1±2.4	44.2±2.2	0.6409	43.1±1.6	0.3102	41.5±0.7	0.1269	42.0-53.0
MCH (pg)	15.3±6.8	15.0±5.6	0.6114	14.5±0.6	0.7737	14±0.1	0.7873	14.0-18.0
MCHC (g/dL)	33.9±0.7	33.9±0.6	1.0000	33.9±0.2	1.0000	34.2±0.1	0.4806	32.8-36.4

[a] Mean ± SD of the haemoglobin (Hb), hematocrit (HCT), red blood cells (RBC) count, mean capsular volume (MCV), mean capsular haemoglobin (MCH), and mean capsular haemoglobin concentration (MCHC) values were calculated for non-infected, *T. equi*-infected, *B. caballi*-infected, and co-infected animals.

[b] Mean Hb and HCT values were significantly lower in co-infected animals compared to those in non-infected animals.

[c] Mean HCT values were statistically significant ($P = 0.0444$) in co-infected animals compared to the non-infected animals.

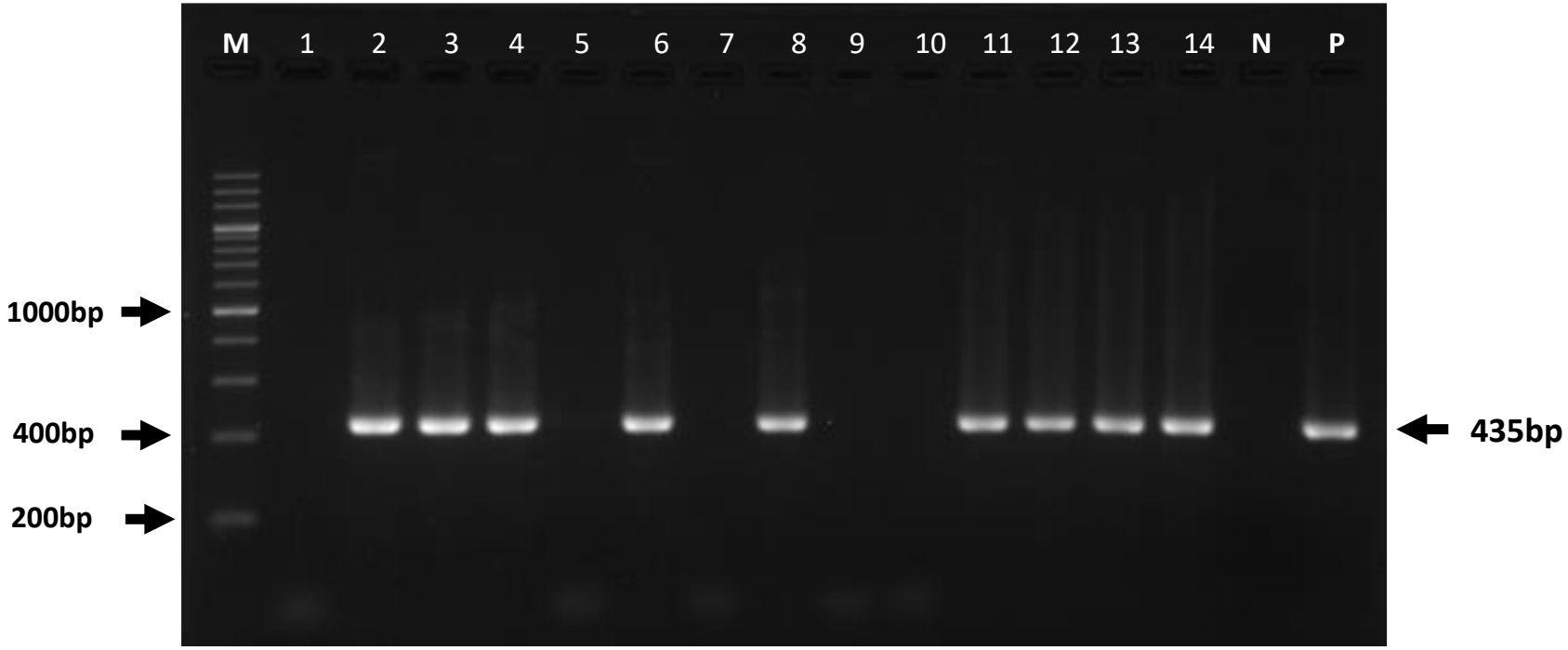

Fig. 10. PCR detection of *T. equi* from blood DNA samples from Paraguayan horses. Samples 1–14 were analysed by a *T. equi*-specific 18S RNA PCR assay. M, 200-bp DNA marker ladder, N, negative control (uninfected horse DNA), and P, positive control of *T. equi* DNA.

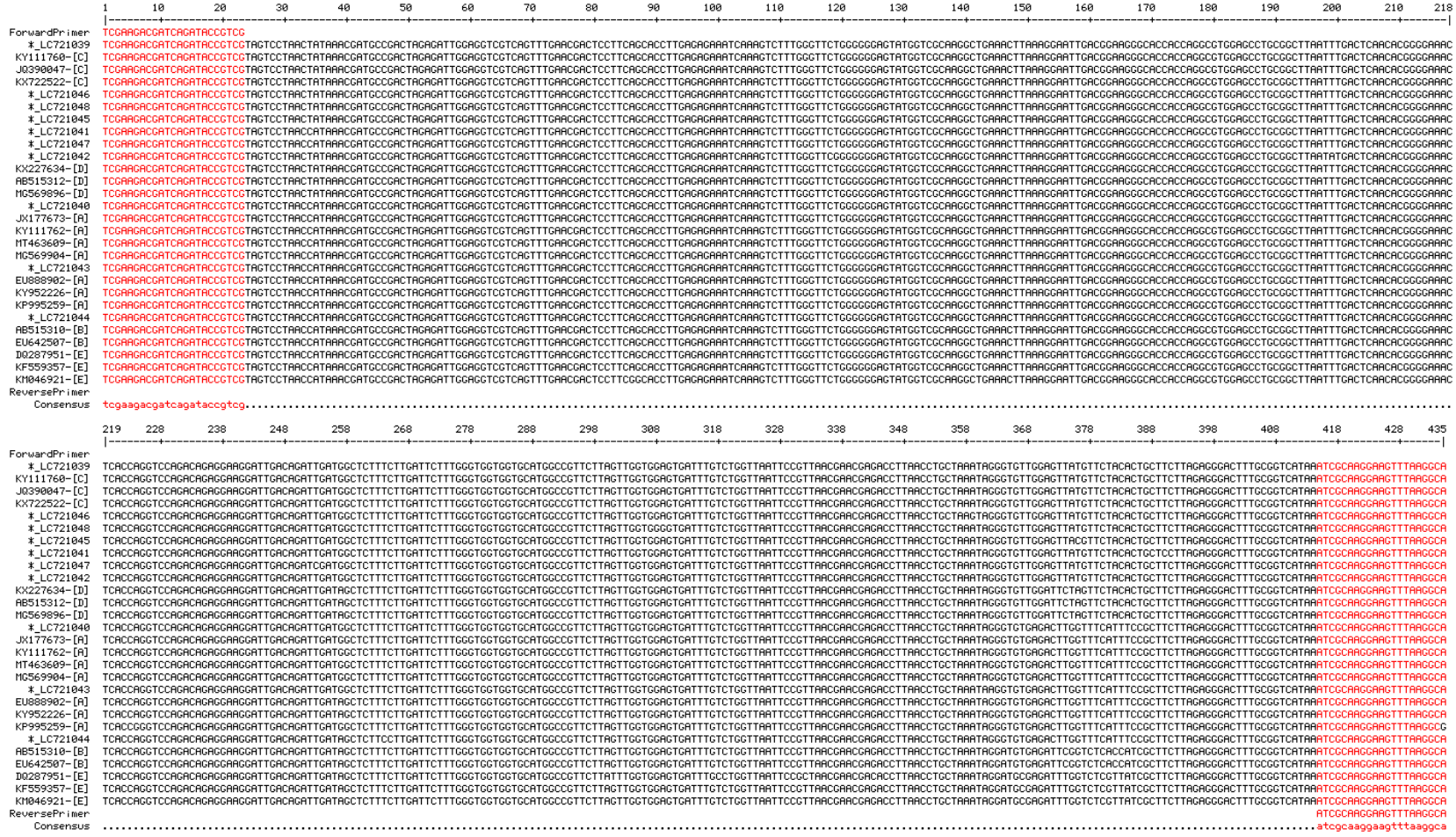

Fig. 11. The Multiple alignment of the *Theileria equi* 18S rRNA sequences. The *T. equi* 18S rRNA sequences representing each genotype (A, B, C, D, E) from the GenBank were aligned together with the Paraguayan sequences marked with asterisk (*) and the primer sequences highlighted in red.

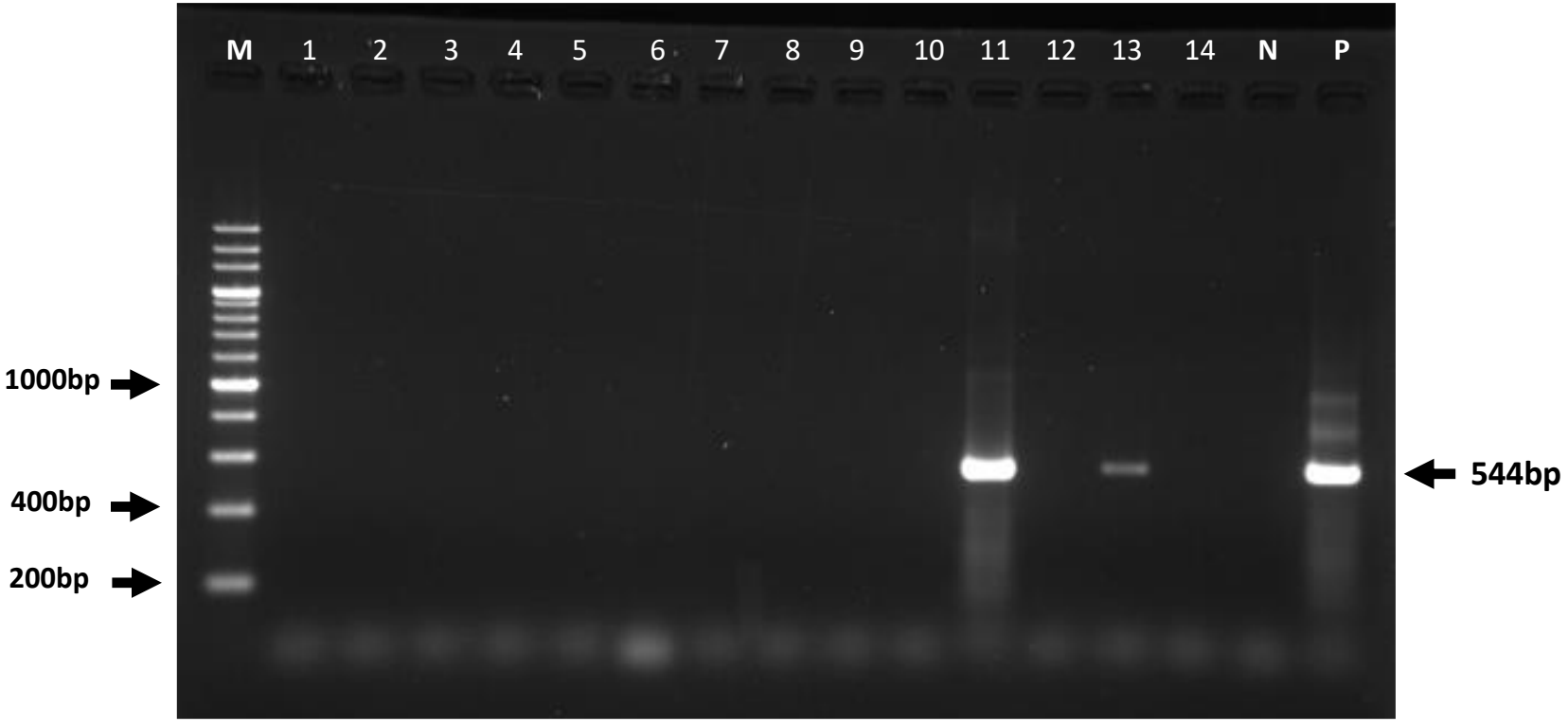

Fig. 12. PCR detection of *B. caballi* from blood DNA samples from Paraguayan horses. Samples 1–14 were analysed by a *B. caballi*-specific RAP-1 PCR assay. M, 200-bp DNA marker ladder, N, negative control (uninfected horse DNA), and P, positive control of *B. caballi* DNA.

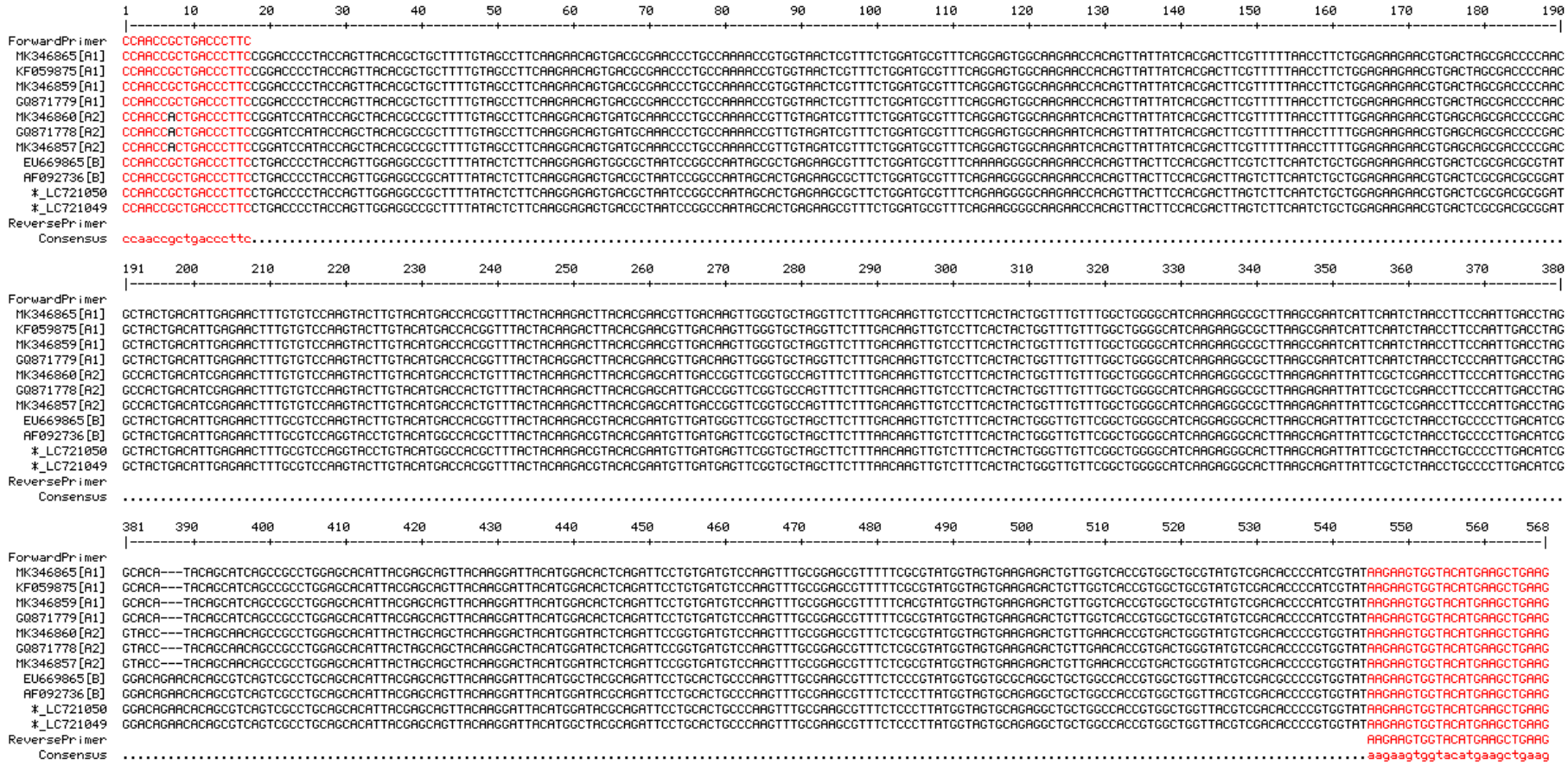

Fig. 13. The Multiple alignment of the *Babesia caballi* RAP-1 sequences. The *B. caballi* RAP-1 sequences representing each genotype (A1, A2, B) from the GenBank were aligned together with the Paraguayan sequences marked with asterisk (*) and the primer sequences highlighted in red

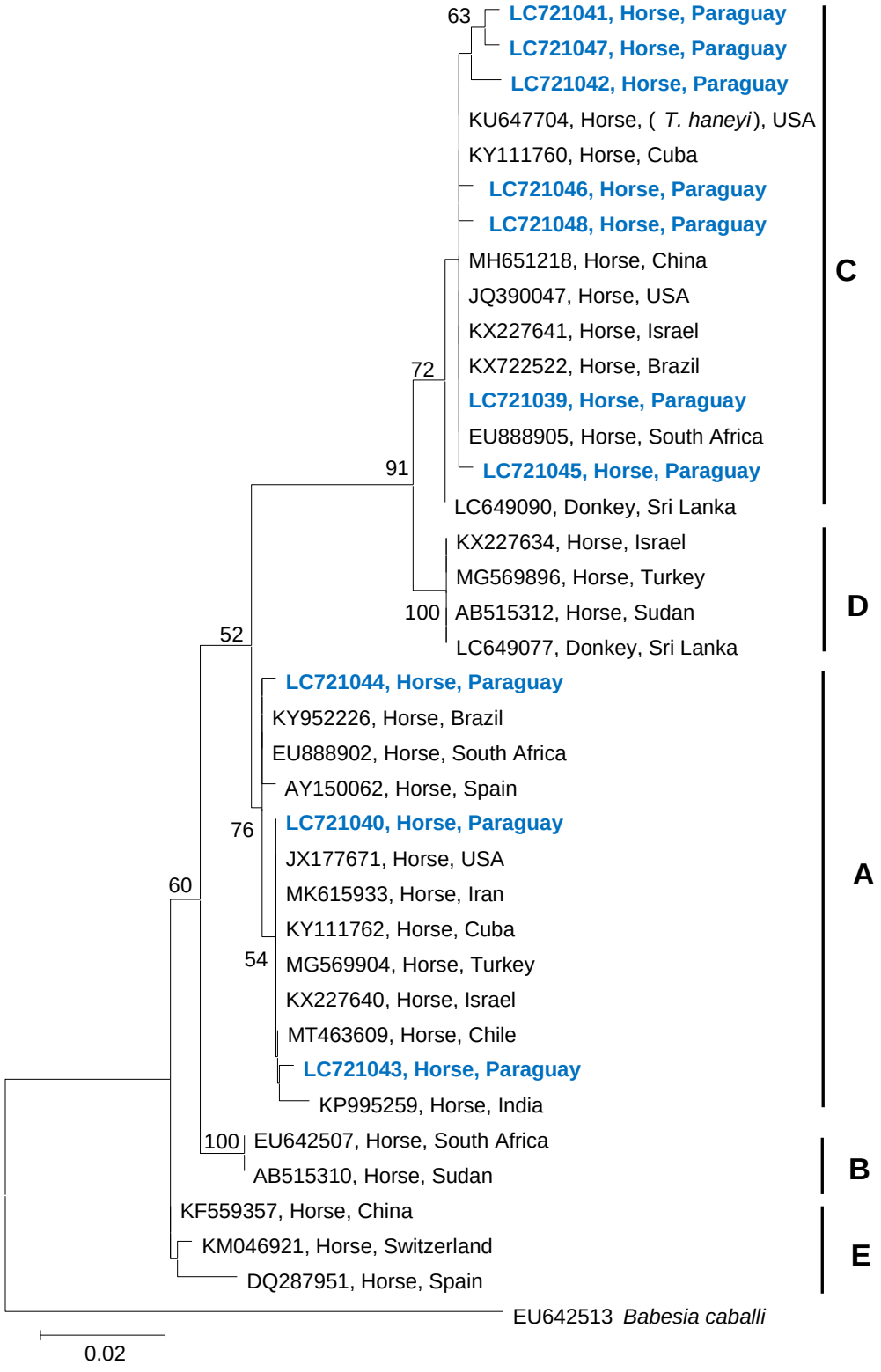

Fig. 14. Phylogenetic analysis of *Theileria equi* 18S rRNA sequences. The *T. equi* 18S rRNA sequences determined in the present study and those retrieved from the GenBank were used to construct a maximum likelihood phylogenetic tree. The Paraguayan *T. equi* sequences (highlighted in blue) occurred in clades A and C.

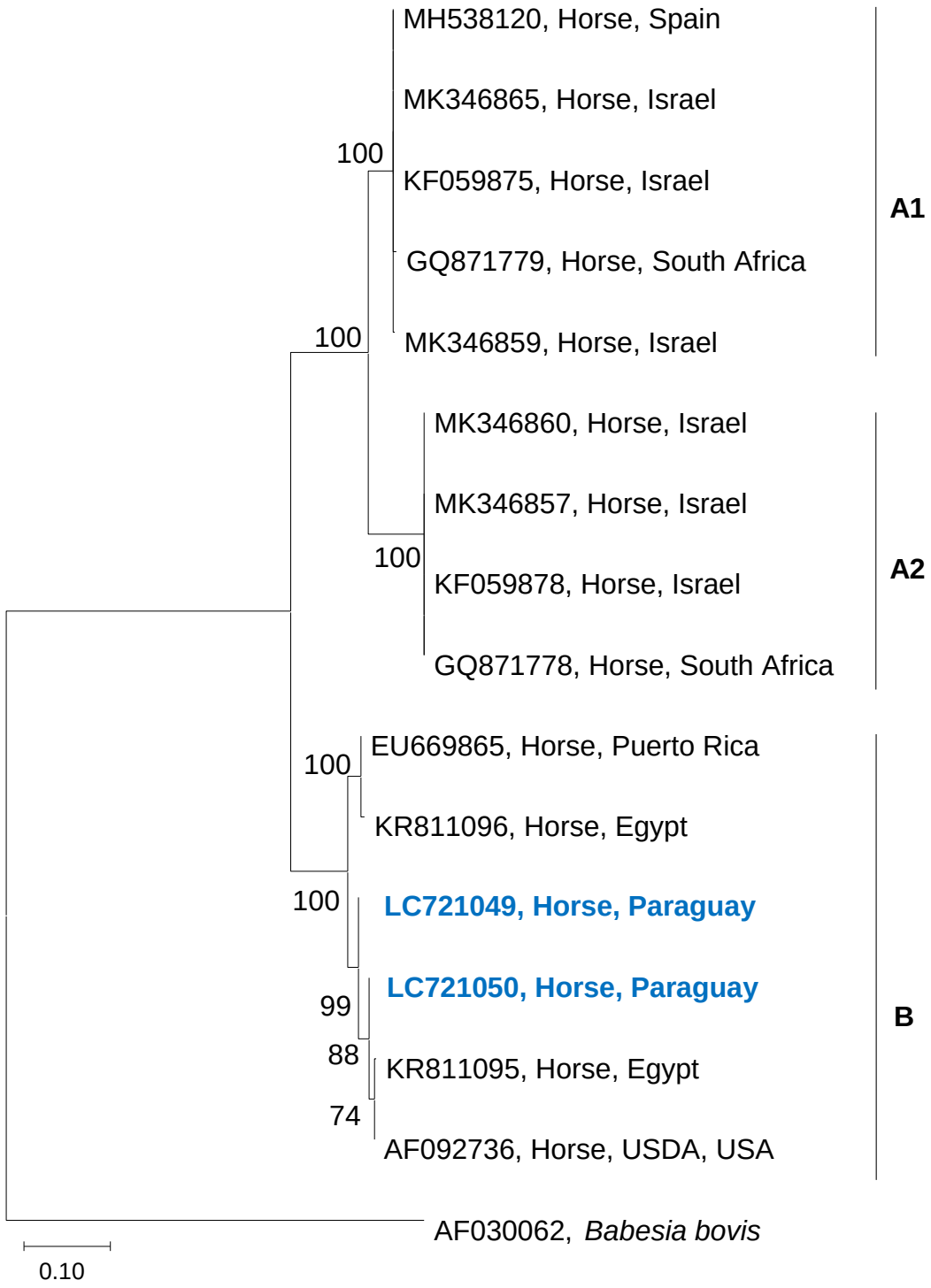

Fig. 15. Phylogenetic analysis of *Babesia caballi* RAP-1 sequences. The *B. caballi* 18S rRNA sequences determined in the present study and those retrieved from the GenBank were used to construct a maximum likelihood phylogenetic tree. The Paraguayan *B. caballi* sequences (highlighted in blue) occurred in clades B

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Chapter 3

Development and evaluation of specific PCR assays for detecting *Theileria equi* genotypes

3-1. Introduction

Equine piroplasmosis (EP) is an infectious disease caused by *Theileria equi* and *Babesia caballi* in horses and other equids, including mules, donkeys, and zebras, with significant economic impact on the equine industry (Knowles et al., 1992; Ueti et al., 2005; Uilenberg 2006). Both the parasites have a complex life cycle that involves both equine hosts and tick vectors. In the equine host, *T. equi* and *B. caballi* reproduce asexually within infected red blood cells (RBCs), causing a massive haemolysis, leading to severe anaemia and other clinical symptoms, such as fever, jaundice, haemoglobinuria, icterus, weight loss, and sometimes death (Ueti et al., 2005). In general, *T. equi* infection causes a more severe form of EP, as compared to that of *B. caballi* Rothschild, 2013; Wise et al., 2013). Furthermore, although *B. caballi*-infected animals naturally clear the infection within four years, *T. equi*-infected animals become lifelong carriers, from which the infection can be spread to other equines via tick vectors (Heim et al., 2007; Zobba et al., 2008; Rothschild, 2013; Wise et al., 2014). Consequently, the *T. equi*-infected carrier animals impede international equine trade, because importing countries, particularly those classified as free from EP, impose stringent regulations on equine imports (Friedhoff et al., 1990; Rothschild, 2013). Hence, the control of *T. equi* infection is crucial for a sustainable equine industry.

The currently available methods to control *T. equi* infection are heavily influenced by the genotypic diversity of parasite. On the basis of ribosomal RNA of 18S sub-unit (18S

rRNA) sequences, *T. equi* is classified into five genotypes, named A, B, C, D, and E (Bhoora et al., 2009; Knowles et al., 2018; Tirosh-Levy et al., 2020c), which can affect the diagnostic results, clinical outcome of infection, and therapeutic efficacy (Ueti et al., 2012; Sears et al., 2020; Bhoora et al., 2010). The World Organization for Animal Health (WOAH) recommends competitive enzyme-linked immunosorbent assay (cELISA) and indirect fluorescent antibody test (IFAT) (Madden and Holbrook, 1968; Knowles et al., 1991; Kappmeyer et al., 1999; WOAH, 2021) for the serodiagnosis of *T. equi* infection. The cELISA is widely used, because of its availability as a kit, predetermined cut-off value that makes the interpretation of test results simple, and suitability for analysis of a large number of samples. The cELISA has been developed based on the equi merozoite antigen 1 (EMA-1) of *T. equi* genotype A (Knowles et al., 1991; Kappmeyer et al., 1999). However, a new *Theileria* species, *T. haneyi*, which belongs to genotype C of *T. equi*, lacks the *ema-1* gene (Knowles et al., 2018). Therefore, it is unlikely that the cELISA can detect the antibodies in animals infected with the genotype C. The absence of *ema-1* in genotype C also implies that PCR assays based on this gene are not suitable for detecting *T. equi* infection. Genotypic diversity of *T. equi* may also influence the outcome of infection, as previous studies have shown that genotype A is more commonly associated with clinical EP than the other genotypes (Manna et al., 2018; Tirosh-Levy et al., 2021). Moreover, the genotypic diversity of *T. equi* may be a determinant in drug-induced clearance of the parasite from infected horses, because a previous study demonstrated that imidocarb dipropionate eradicates *T. equi* genotype A, but not *T. haneyi* (Ueti et al., 2012; Sears et al., 2020). These diagnostic, clinical, and therapeutic implications highlight the importance of detecting the genotypes for managing *T. equi* infection and facilitating the safe international transportation of equines.

Currently, PCR-sequencing and real-time PCR assays are employed to determine *T. equi* genotypes (Kim et al., 2008; Coultous et al., 2019; Bhoora et al., 2020; Ahedor et al., 2022; Chen et al., 2022). However, these methods are not without limitations. In the PCR-sequencing approach, the 18S rRNA is amplified from *T. equi*-positive samples, sequenced, and phylogenetically analysed to detect the genotype of parasite. However, in co-infected animals (i.e., those simultaneously infected with more than one *T. equi* genotype), this method is likely to detect the dominant genotype, potentially leaving the minor genotypes undetected (Chandler et al., 1997; Vestheim et al., 2008). Real-time PCR assays can provide quick and quantitative results, but they require specialized equipment and expertise (Coultous et al., 2019). Additionally, the real-time PCR assays available for *T. equi* genotyping have not been validated for wide use in different geographic regions (Coultous et al., 2019; Bhoora et al., 2020). Because the real-time PCR assays amplify only short fragments of 18S rRNA, validating the findings by sequencing analysis is challenging (Coultous et al., 2019; Bhoora et al., 2020).

On the contrary, conventional PCR assays are more accessible and cost-effective, and can be performed in resource-limited laboratories. Furthermore, the findings can be verified through the subsequent sequencing analysis (Yokoyama et al., 2011; Liyanagunawardena et al., 2016). However, conventional genotype-specific PCR assays have not yet been developed for *T. equi*. Therefore, in the present study, I developed 18S rRNA-based PCR assays for the genotype specific detection of *T. equi*, and evaluated them using previously identified *T. equi*-positive equine DNA samples from Sri Lanka and Paraguay (Chapter 1 & 2).

3-2. Materials and Methods

Primer design for genotype-specific PCR assays

I retrieved the long sequences (~1,600 bp) of 18S rRNA representing each *T. equi* genotype and those of *Babesia caballi* and equine hosts from GenBank, and aligned them using Multalin, an online software (http://multalin.toulouse.inra.fr/multalin/multalin.html) (Corpet, 1988). On the basis of alignment, I designed a pair of forward and reverse primers specific to each *T. equi* genotype (Fig. 16a). Moreover, I assessed whether the PCR findings were verifiable through the subsequent sequencing analysis of PCR amplicons. Briefly, the 18S rRNA fragments targeted by the PCR assays specific to genotypes A, B, C, D, and E of *T. equi* were trimmed at both the 5′- and 3′- ends, and aligned. The resulting 687-bp alignment was then analysed for nucleotide polymorphisms (Leaché and Oaks, 2017).

Plasmids-containing 18S rRNA representing each *T. equi* genotype

I prepared five plasmids, respectively containing an insert of 18S rRNA sequence representing each *T. equi* genotype, which were used for the specificity testing of developed PCR assays. A long 18S rRNA fragment (1,591 bp) of genotype A was amplified from DNAs extracted from an *in vitro* culture (*T. equi* USDA strain) (Avarzed et al., 1998), using the forward primer Nbab_1F and reverse primer TB-rev (Matjila et al., 2008; Oosthuizen et al., 2008), as described previously (Ahedor et al., 2022). The 18S rRNA of genotype B was amplified from a DNA template synthesized based on a Sudanese sequence (GenBank accession number: AB515312). The resulting PCR amplicons were purified by using a QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany), and then cloned into a vector pCR™ 2.1-TOPO (Invitrogen, Carlsbad, CA,

USA). The plasmids were purified using a QIAprep Spin Miniprep Kit (Qiagen), and then sequenced. The sequencing analysis confirmed that the 18S rRNA inserts in the plasmids represented the respective genotypes. Plasmids with inserts of 18S rRNA that belong to genotypes C and D were obtained from a donkey survey in Sri Lanka (Ahedor et al., 2022), while a plasmid containing 18S rRNA of genotype E was prepared from a horse survey in Mongolia (unpublished data). In the aforementioned studies, the 18S rRNA sequences of genotypes C, D, and E were amplified using the primers Nbab_1F and TB-rev, and then cloned, following the same methodology as in the present investigation.

Specificity testing

I evaluated each PCR assay for its ability to differentially detect the target genotype from the remaining genotypes of *T. equi* and DNAs of *B. caballi* and noninfected horse blood. Briefly, plasmid DNA of each genotype, *B. caballi* DNA from an *in vitro* culture (USDA strain), and blood DNA from an uninfected horse were analysed in each of the genotype-specific PCR assays. Each PCR assay was performed in a 10-µl reaction mixture, which included 1 µl of 0.01 pg/µl plasmid DNA, 5 µl of 2× PCR buffer (KOD FX Neo, Toyobo Co., Ltd., Osaka, Japan), 1 µl of 2 mM dNTPs (Toyobo), 0.5 µl of 10 µM forward and reverse primers (Table 6), 0.1 µl of 1 U/µl KOD FX Neo DNA polymerase (Toyobo), and 1.9 µl of double distilled water (DDW). The PCR reaction mixture underwent an initial denaturation at 95°C for 5 min, followed by 45 cycles, each including a denaturing step at 95°C for 30 sec, an annealing step at the appropriate temperature (see Table 6) for 1 min, and an extension step at 72°C for 1 min. Following a final elongation step at 72°C for 7 min, the PCR products were separated on 1.5% agarose gels, stained with MIDORI Green Xtra (Nippon Genetics, Tokyo, Japan), and then illuminated under UV light. PCR amplicons of the expected band size (Table 6) were considered to be positive for the

respective genotype of *T. equi*. The resulting PCR products were cloned, and two clones per genotype were sequenced to confirm that each PCR assay had amplified the targeted fragment of 18S rRNA.

Validation of the PCR assays

A total of 270 *T. equi*-positive blood DNA samples from apparently healthy equines, including 92 donkeys from Sri Lanka (Ahedor et al., 2022) and 178 horses from Paraguay (Ahedor et al., 2023a), were used to validate the newly developed genotype-specific PCR assays. Previous studies had performed phylogenetic analyses, using 18S rRNA sequences prepared from a selected number of DNA samples, and detected the genotypes C and D in Sri Lankan donkeys in Chapter 1 and the genotypes A and C in Paraguayan horses in Chapter 2. In the present study, I screened all of the 270 DNA samples in each genotype-specific PCR assay. Randomly selected amplicons from Sri Lankan and Paraguayan samples were cloned for each PCR assay, and two clones per amplicon were sequenced.

Phylogenetic analysis

To perform the phylogenetic analysis, I aligned the Sri Lankan and Paraguayan 18S rRNA sequences derived from the amplicons from each *T. equi* genotype-specific PCR assay, along with those obtained from GenBank, using multiple alignment using fast fourier transform (MAFFT), an online software (https://mafft.cbrc.jp/alignment/server/) (Katoh et al., 2019). I further analysed the alignment using molecular evolutionary genetics analysis (MEGA) version X software (Kumar et al., 2018) to predict the best nucleotide substitution model based on the lowest Akaike information criterion value. A maximum-

likelihood phylogenetic tree was then constructed with MEGA, employing the Tamura-Nei substitution model (Tamura et al., 2013).

3-3. Results

Development of genotype-specific PCR assays for *T. equi*

In the present study, I developed five kinds of 18S rRNA-based PCR assays for the differential detection of *T. equi* genotypes A, B, C, D, and E (Fig. 16a; Table 6). My *in silico* analysis found that the 18S rRNA fragment amplified in each PCR assay is characterized by unique nucleotide polymorphisms, indicating that the results can be confirmed through the subsequent sequencing analysis of PCR amplicons (Fig. 16b). The specificities of genotype-specific PCR assays were evaluated using plasmids with inserts of 18S rRNA representing each *T. equi* genotype. I found that each PCR assay exclusively detected the target genotype, without any amplification observed when 18S rRNA of other genotypes, *B. caballi* DNA, or noninfected horse DNA were tested, thereby validating the specificity of genotype-specific PCR assays (Fig. 17).

Field evaluation of genotype-specific PCR assays using *T. equi*-positive equine DNA

I used the genotype-specific PCR assays to screen a total of 270 *T. equi*-positive DNA samples prepared from Sri Lankan donkeys (92 samples) and Paraguayan horses (178 samples). I found that the surveyed donkeys in Sri Lanka were infected with four genotypes of *T. equi*, including A, C, D, and E (Table 7). The most common genotype was D, detected in 88 (95.7%) samples, followed by genotypes A, C, and E, which were detected in 75 (81.5%), 40 (43.5%), and 6 (6.5%) samples, respectively. All 92 *T. equi*-positive donkey samples were positive for at least one genotype. Co-infections were

common: 82 (89.1%) donkeys were infected with two, three, or four genotypes (Table 8). Co-infection with genotypes A and D was the most frequently observed combination.

The genotype-specific PCR assays detected all five genotypes in Paraguayan horses. Genotype C was most prevalent, detected in 114 (64.0%) samples, followed by genotypes A, D, E, and B, which were detected in 76 (42.7%), 10 (5.6%), 8 (4.5%), and 5 (2.8%) samples, respectively. Of 178 *T. equi*-positive horses, 166 (93.3%) were positive for at least one genotype. Of the surveyed horses, 40 (22.5%) had co-infections with two or three genotypes of *T. equi* (Table 8); co-infection with genotypes A and C was the most common combination.

To validate the PCR results, I sequenced four randomly selected amplicons from each PCR assay, except for genotype E from Sri Lanka and genotype B from Paraguay for which three amplicons per genotype were sequenced. The resultant of 34 18S rRNA sequences, including 15 Sri Lankan sequences representing genotypes A, C, D, and E, and 19 Paraguayan sequences representing all five genotypes were registered with GenBank (Accession No. LC775884 – LC775917), and then used to construction the phylogenetic tree. All of the newly determined sequences from the respective PCR assays occurred in clades according to their genotype, together with known reference sequences (Fig. 18).

3-4. Discussion

In the present study, I successfully developed a set of five specific PCR assays capable of differentiating the genotypes of *T. equi*. These newly developed PCR assays exhibited high specificity toward their target genotypes, as evaluated using 18S rRNA templates representing each of the genotypes. Subsequently, I validated the assays using *T. equi*-positive DNA samples prepared from donkeys in Sri Lanka and horses in Paraguay, focusing on their sensitivity, specificity, and practical ability to detect co-infection of an equid with multiple genotypes of *T. equi*. I also investigated whether the findings were verifiable by subjecting the sequences of PCR amplicons to phylogenetic analysis.

I found that the genotype-specific PCR assays were highly sensitive in detecting the *T. equi* genotypes from field samples, because all and 93.3% of *T. equi*-positive samples from Sri Lanka and Paraguay, respectively, were positive for at least one genotype. However, a small number of field samples from Paraguay (12/178) tested negative in all five genotype-specific PCR assays. The field DNA samples used in the present study had tested positive in a diagnostic PCR assay using a pair of primers that had been designed based on highly conserved regions in 18S rRNA among all *T. equi* genotypes (Alhassan et al., 2005). Therefore, it is possible that the negative samples might have been co-infected with multiple genotypes, each with very low parasitaemia, leading to negative results in the genotype-specific PCR assays, while the combined 18S rRNA templates from each genotype could have been sufficient to generate positive results in the diagnostic PCR assay (Ahedor et al., 2023a).

In the PCR-sequencing approach, only a subset of positive samples is subjected to sequencing analysis, because of cost and time limitations. As a result, any additional genotypes present in untested samples may remain undetected. By contrast, the genotype-specific PCR assays offer a convenient alternative for analyzing a large number of positive

samples, potentially detecting all the genotypes that are present (Yokoyama et al., 2011; Liyanagunawardena et al., 2016). Previous studies using the PCR-sequencing approach identified genotypes C and D in the samples from Sri Lanka and genotypes A and C in those from Paraguay, as observed in Chapters 1 & 2. Notably, the genotype-specific PCR assays not only confirmed the presence of these genotypes, but also detected additional genotypes A and E in the samples from Sri Lanka and genotypes B, D, and E in the samples from Paraguay, which were not previously reported in these countries as observed in Chapters 1 & 2. The detection of genotype A as the second most common genotype in both Sri Lanka and Paraguay is worrying, because this genotype is more commonly associated with clinical EP than the others (Manna et al., 2018; Tirosh-Levy et al., 2021). Similarly, the high detection rates of genotype C suggest that the currently available cELISA may not be suitable for the serodiagnosis of *T. equi* infection in these countries (Knowles et al., 2018).

The PCR assays successfully detected co-infections with multiple genotypes of *T. equi* in both Sri Lankan and Paraguayan DNA samples. The common occurrence of co-infections might have implications for the control of *T. equi*. For example, a previous study found that repeated treatment with imidocarb dipropionate clears the parasites from horses singly infected with genotype A, but not from horses co-infected with genotype A and *T. haneyi* (genotype C) (Sears et al., 2020). The ability of genotype-specific PCR assays to detect co-infections, as demonstrated in the present study, overcomes the limitation of the PCR-sequencing approach, which tends to detect only the dominant genotype in co-infected samples. Collectively, my findings demonstrate that the genotype-specific PCR assays are a potential alternative to the PCR-sequencing approach for *T. equi* genotyping.

The genotype-specific PCR assays require validation using a larger number of *T. equi*-positive samples from diverse geographic locations. I found that the 18S rRNA fragments targeted by the genotype-specific PCR assays contain unique nucleotide polymorphisms that can be readily used to verify the PCR results. Furthermore, I observed that the 18S rRNA sequences obtained from randomly selected amplicons from each of the genotype-specific PCR assays clustered within their respective phylogenetic clades. This finding not only confirmed the specificity of PCR assays, but also suggested that the genotype-specific PCR assays can be validated easily.

3-5. Summary

Theileria equi causes a more severe form of clinical equine piroplasmosis (EP), as compared to that of *B. caballi*, and consists of five genotypes, including A, B, C, D, and E, which have implications for the diagnosis, disease development, and control of equine EP. Limited progress has been made in research efforts to investigate these implications due to unavailability of type-specific PCR assays for *T. equi*. Therefore, I developed the PCR assays for *T. equi* genotyping. Evaluation of these PCR assays demonstrated exceptional sensitivity, detecting four genotypes in Sri Lankan samples and all five in Paraguayan samples. Co-infections with multiple genotypes were equally detected from the samples. Phylogenetic analysis reveal that the amplicons sequences occurred in the respective phylogenetic clades for each genotype, validating the specificity of PCR assays. These results indicate that the genotype-specific PCR assays are a reliable tool for differential detection of *T. equi* genotypes and can be used globally.

Table 6. Primers used in *Theileria equi* genotype-specific PCR assays

Genotypes	Primers (5′ - 3′) [a]	Annealing Tempt. (°C)	Amplicon sizes (bp)
A	F: CGTTGCGGCTTGGTTGGGTTTCGATTA R: GCAAAGTCCCTCTAAGAAGCGGA	70	692
B	F: GTGGTCCTTCGCTATGTCGAGTGGTCC R: CGCAAAGTCCCTCTAAGAAGCGATGGT	66	718
C	F: TGTATCGTTATCTTCTGCTTGACAGTTTGG R: GTCCCTCTAAGAAGCAGTGTAGAACATAAC	66	714
D	F: TGTATCGTTTTCCTCTGCTTGACAGTTGGA R: GTCCCTCTAAGAAGCAGTGTAGAACTAG	64	719
E	F: ATCGTGGTTCTTCGCTATGTCGAGTGATCT R: CGCAAAGTCCCTCTAAGAAGCGATAAC	72	722

[a] These primers were designed based on the 18S rRNA of the genotypes of *Theileria equi*.

Table 7: Detection of *Theileria equi* genotypes in *T. equi*-positive 92 donkeys in Sri Lanka and 178 horses in Paraguay

Genotypes	Sri Lankan donkeys		Paraguayan horses	
	No. of positives	**% (CI [a])**	**No. of positives**	**% (CI)**
A	75	81.5 (72.4-88.1)	76	42.7 (35.7-50.0)
B	0	0.0 (0.0-4.0)	5	2.8 (1.2-6.4)
C	40	43.5 (33.8-53.7)	114	64.0 (56.8-70.7)
D	88	95.7 (89.4-98.3)	10	5.6 (3.1-10.0)
E	6	6.5 (3.0-13.5)	8	4.5 (2.3-8.6)

[a] 95% confidence interval.

Table 8. Single and co-infections of *Theileria equi* genotypes in 92 Sri Lankan donkeys and 166 Paraguayan horses

Genotypes	**Number of positives (%)**	
	Sri Lankan donkeys	**Paraguayan horses**
Single genotype		
A	1 (1.1)	46 (27.7)
B	0 (0.0)	2 (1.2)
C	0 (0.0)	75 (45.2)
D	8 (8.7)	2 (1.2)
E	0 (0.0)	1 (0.6)
Total	**9 (9.8)**	**126 (75.9)**
Two genotypes		
A+B	0 (0.0)	1 (0.6)
A+C	2 (2.2)	22 (13.3)
A+D	40 (43.5)	0 (0.0)
C+D	8 (8.7)	4 (2.4)
C+E	0 (0.0)	6 (3.6)
D+E	1 (1.1)	0 (0.0)
Total	**51 (55.4)**	**33 (19.9)**
Three genotypes		
A+B+C	0 (0.0)	2 (1.2)
A+C+D	27 (29.3)	4 (2.4)
A+C+E	1 (1.1)	1 (0.6)
A+D+E	2 (2.2)	0 (0.0)
Total	**30 (32.6)**	**7 (4.2)**
Four genotypes		
A+C+D+E	2 (2.2)	0 (0.0)
Total	**2 (2.2)**	**0 (0.0)**
Grand Total	**92**	**166**

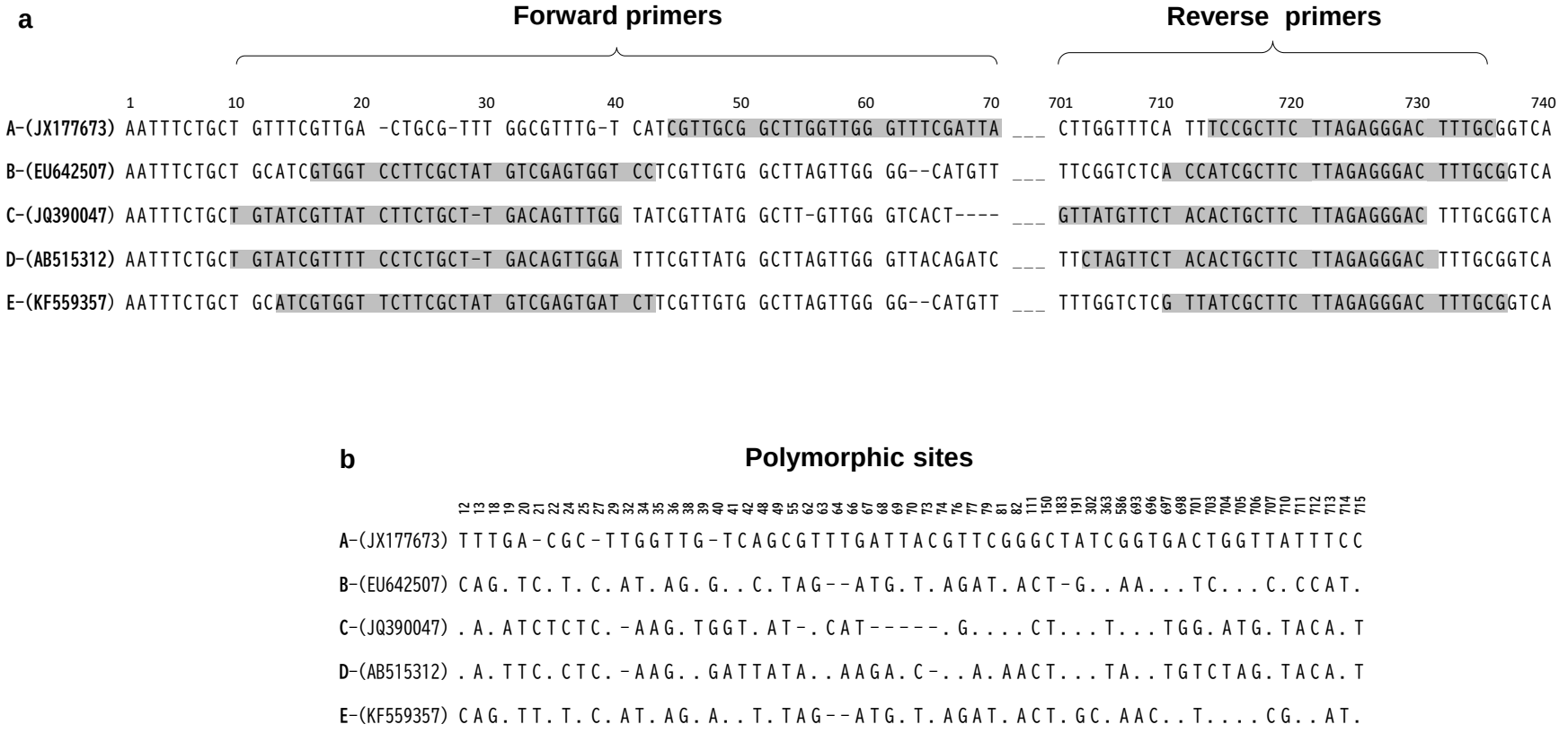

Fig. 16. Development of genotype-specific PCR assays for *Theileria equi*. **a**. Primer design for *T. equi* genotype-specific PCR assays. 18S rRNA sequences of *T. equi* were aligned, and a set of forward and reverse primers specific to each genotype was designed. The figure displays the specific binding regions (highlighted in gray) of the forward and reverse primers on the 18S rRNA sequences representing genotypes A (JX177673), B (EU642507), C (JQ390047), D (AB515312), and E (KF559357). The dashes represent gaps. **b**. Nucleotide polymorphisms among the 18S rRNA sequences targeted by the genotype-specific PCR assays. The 18S rRNA sequences targeted by the PCR assays specific to *T. equi* genotypes A, B, C, D, and E were trimmed and aligned, and the resultant 687-bp alignment was analysed for nucleotide polymorphisms. The dots and dash represent identical nucleotides and gaps, respectively, to those in the genotype A sequence. Conserved nucleotide polymorphisms in each genotype are shown.

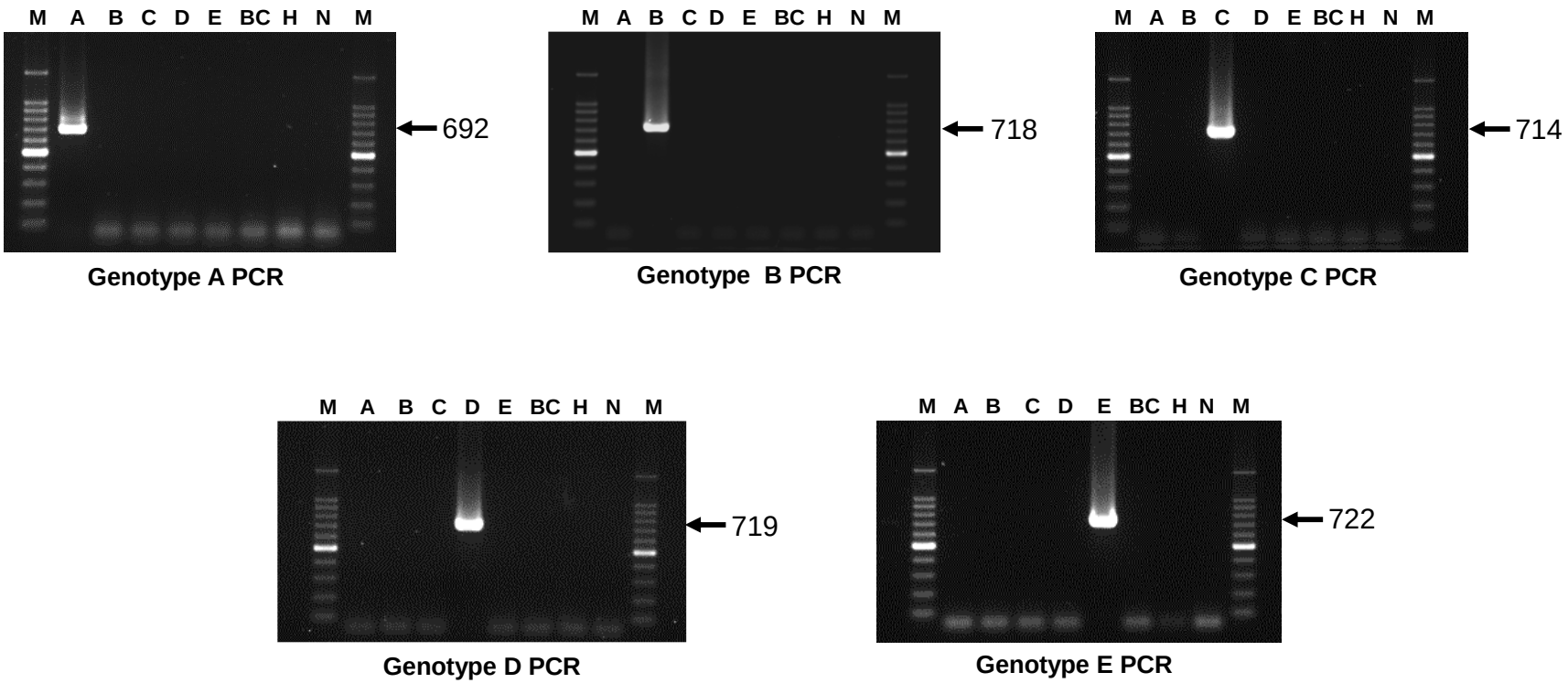

Fig. 17. Specificity of genotype-specific PCR assays for *Theileria equi*. Each genotype-specific PCR assay was evaluated for its ability to differentially detect the target genotype from the remaining genotypes and DNAs of *Babesia caballi* and noninfected horse blood. A, B, C, D, and E in each panel indicate plasmid DNAs with inserts of 18S rRNA from *T. equi* genotypes A, B, C, D, and E, respectively, while BC and H refer to DNAs of *B. caballi* and noninfected horse blood, respectively. N denotes the negative control (no template), and M indicates a 100-bp DNA ladder. The genotype-specific PCR assays were highly specific in detecting their target genotypes.

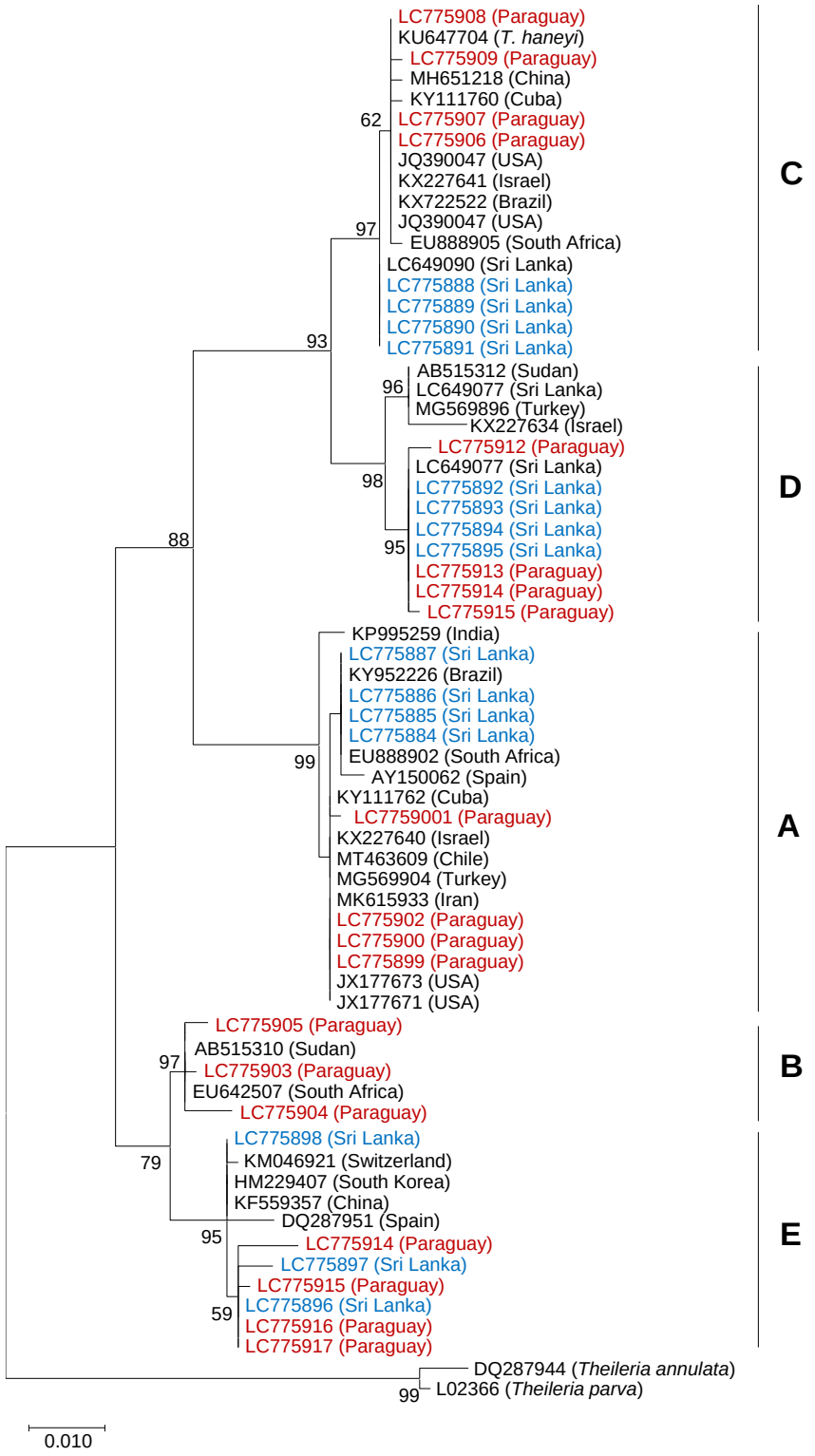

Fig. 18. Phylogenetic analysis of *Theileria equi* 18S rRNA. The 18S rRNA sequences amplified in the genotype-specific PCR assays, together with reference sequences representing each genotype, were used to construct a maximum-likelihood phylogeny with 1000 bootstrap replicates. The newly determined sequences from donkeys from Sri Lanka and horses from Paraguay (highlighted in blue and red, respectively) from the amplicons from each genotype-specific PCR assay clustered within the corresponding clades.

General discussion

Equine piroplasmosis (EP) is a globally distributed infectious disease that affects equines (Alhassan et al., 2007; Mans et al., 2015; Wise et al., 2013; Rothschild, 2013; Onyiche et al., 2019). EP leads to substantial economic losses from treatment costs, production losses, mortality, and trade restrictions (Mans et al., 2015; Wise et al., 2013; Rothschild, 2013; Wise et al., 2014). Currently, only a handful of countries have been confirmed to be EP-free (Tirosh-Levy et al., 2020c). However, the status of EP is still uncertain in various countries around the world. The unknown EP status has two implications for disease management: 1) controlling of EP is difficult in the countries without epidemiological data, and 2) there is a risk of introducing *T. equi* and *B. caballi* from countries with unknown EP status to EP-free regions, especially through international equine movement. Therefore, it is crucial to survey equines for *T. equi* and *B. caballi* infections in the regions with uncertain EP status.

In the course of my research, I examined a hypothesis that *T. equi* and *B. caballi* might be endemic in countries where potential tick vectors are prevalent. For these studies, I selected two different countries on two different continents with unknown epidemiological status. PCR-based surveys revealed *T. equi* infection in donkeys in Sri Lanka, and *T. equi* and *B. caballi* infections in horses in Paraguay. The findings further demonstrated that the majority of surveyed equines were infected in both countries. These findings validated my hypothesis that equines in countries with unknown EP status may be infected with *T. equi* and *B. caballi*. Therefore, prioritizing of the survey of *T. equi* and *B. caballi* infections is crucial in such countries, as the results will benefit the equine industry both locally and globally.

The horse industry plays a vital role in Paraguay's thriving agricultural sector, and contributes significantly to the national economy. However, various infectious diseases pose a threat to horse populations. Similarly, Sri Lanka has seen a concerning decline in donkey populations over recent years due to poaching and infectious diseases (Sivakumar et al., 2012; Zhyldyz et al., 2019; Inácio et al., 2019). Therefore, my research findings will be valuable in safeguarding equine populations in Sri Lanka and Paraguay. The EP in both the countries should be managed through tick control programs, surveillance, and chemotherapy. Although competent tick species are endemic in Sri Lanka and Paraguay, no studies have investigated if they could transmit *T. equi* and *B. caballi* in these countries. Consequently, it is now necessary to conduct additional studies in Sri Lanka and Paraguay to identify the tick vectors and determine their seasonal activities. This will enable the implementation of a systematic tick control strategy to minimize EP. Ongoing active surveillance is also crucial to predict changing epidemiological patterns in ever-changing climates. In addition, clinical EP should be diagnosed early and managed with anti-protozoan drugs. If valuable equines are to be exported, it may be worth considering the complete eradication of parasites using appropriate therapies. In other countries with an unknown EP status, if epidemiological surveys detect the *T. equi* and *B. caballi* in their equine populations, similar measures are also applicable to them.

In Paraguay, my epidemiological investigation revealed that the mean values for Hb, HCT, and RBC counts were comparable between the infected and non-infected horses. This observation represents a typical situation in the endemic countries; a large proportion of the infected equines remains asymptomatic. The apparently healthy status of infected animals does not mean that EP lacks clinical or economic significance. Despite the lack of clinical symptoms, animals chronically infected with *T. equi* and *B. caballi* act as a source of the infections (Mans et al., 2015; Wise et al., 2013; Rothschild, 2013; Tirosh-Levy et al.,

2020c), through which the parasites can be transmitted to susceptible animals, resulting in clinical EP. Equally, asymptomatic carriers hinder international horse trade, resulting in massive economic losses for the equine industry (Rothschild, 2013; Tirosh-Levy et al., 2020c). I observed that, although the haematological parameters were comparable between the non-infected and singly infected horses, two horses co-infected with *T. equi* and *B. caballi* were clinically anaemic, suggesting that the co-infection could potentiate the anaemia development (Sivakumar et al., 2012). This observation is inconclusive, as it only involved two co-infected horses, but justifies further epidemiological investigations using a larger number of co-infected horses.

The epidemiological surveys should preferably be followed by investigations to determine the genotypic diversity of the detected parasites, as the parasite genotypes may impact the clinical outcome of infection, diagnostic test results, and therapeutic clearance (Bhoora et al., 2010; Ueti et al., 2012; Rapoport et al., 2014; Manna et al., 2018; Tirosh-Levy et al., 2020b; Sears et al., 2020). The analysis of *T. equi* 18S rRNA sequences indicated that the Sri Lankan donkeys and Paraguayan horses were infected with genotypes C and D and genotype A and C, respectively (Ahedor et al., 2022; 2023a). *Babesia caballi* was detected only in Paraguay and consisted of only a single *rap-1* genotype B (Ahedor et al., 2023a). These genotypes were determined using a PCR-sequencing approach, which was the most commonly used method for genotyping. Because of high costs and time-consuming procedures, only a subset of positive samples is analysed by this method. This, together with its inability to identify all genotypes in co-infected samples, makes the PCR-sequencing approach less sensitive. Therefore, simple and sensitive assays became essential for the specific detection of *T. equi* and *B. caballi* genotypes. In particular, such assays are vital for *T. equi*, as each genotype might represent a distinct *Theileria* species. To address this issue, I developed five kinds of genotype-specific PCR assays for the

differential detection of *T. equi* genotypes A – E (Ahedor et al., 2023b). These highly specific PCR assays were able to detect four genotypes (A, C, D, and E) in Sri Lanka and all five genotypes in Paraguay. These findings suggest that the genotypic distribution of *T. equi* may have been underestimated, particularly in countries where PCR-sequencing approaches were employed.

A comprehensive understanding of the genotypes of *T. equi* will equip the veterinary authorities in endemic countries to formulate an effective EP management plan. For example, the prevalence of *T. equi* genotype A strongly suggests that clinical EP might be common among donkeys in Sri Lanka and horses in Paraguay. Therefore, equines in these countries should be monitored for the disease and treated promptly. The presence of *T. equi* genotype C suggests that the cELISA may not be effective in identifying sero-positive animals in Sri Lanka and Paraguay. Therefore, other sero-diagnostic assays capable of detecting antibodies to all *T. equi* genotypes should carefully be selected for the surveillance programs. The presence of genotype C and co-infections involving genotype C in Sri Lanka and Paraguay also highlight the challenges in drug-induced clearance of *T. equi*. Therefore, designing of EP management strategies become crucial for Sri Lanka and Paraguay in light of the genotypic diversity. These findings also highlight the importance of investigating or reinvestigating the genotypic diversity of *T. equi* in the endemic countries to identify all endemic genotypes, using efficient diagnostic tools, such as genotype-specific PCR assays developed in my studies.

The genotype-specific PCR assays developed in the present study have the potential to facilitate further research into the various implications of *T. equi* genotypes, such as for taxonomy, virulence, immunological cross-reactivity, infectious persistence, diagnosis, and transmission. A recent study that compared the morphology and whole genome data of genotypes A and C, concluded that the genotype C represents a novel

species named as *T. haneyi*, and suggested that several cryptic *Theileria* species are now collectively classified as *T. equi* (Knowles et al., 2018). Similar comparisons of morphology and genome data among all *T. equi* genotypes are essential to uncover novel *Theileria* species (WOAH, 2023). Moreover, the role of genotype as a risk factor for clinical EP in infected horses remains uncertain. Genotype A appears to be most associated with clinical cases (Manna et al., 2018; Tirosh-Levy et al., 2021), but to confirm this assumption, it would be necessary to comparatively evaluate the virulence of each genotype in experimental infections. The first step toward addressing the above research questions requires the identification of *T. equi* genotypes in the infected equines. The genotype-specific PCR assays developed in my studies will be a potentially useful tool in this regard.

In brief, the findings from my studies confirmed that *T. equi* and *B. caballi* are endemic globally, including in the regions with unknown EP status. Active surveillance programs coupled with studies to investigate the genotypic diversities of *T. equi* and *B. caballi* are now a priority both in endemic countries and in countries with unknown epidemiological status, in order to aid in formulating better management strategies for EP both locally and globally.

General summary

Equine piroplasmosis (EP), a serious infectious disease of equines caused by two protozoan parasites, *T. equi* and *B. caballi*. Infection with these parasites leads to intravascular haemolysis, resulting in various clinical symptoms, such as fever, anaemia, jaundice, haemoglobinuria, and eventually death. Economic losses associated with EP include the cost of treatment, production losses through abortions and death, and restrictions on international equine movement. Therefore, control of EP is crucial for sustaining the benefits derived from the equine industry and ensuring its socioeconomic viability. The currently available strategies for the control and prevention of EP include chemotherapy, tick control, and restricted movement of equines. However, the low efficacy and side effects of drugs, the emergence of acaricide-resistant ticks, and false-negative diagnostic test results render the control strategies less effective.

EP has a global distribution that coincides with the distribution of specific tick vectors capable of transmitting *T. equi* and *B. caballi*. In particular, EP is common among equines in the tropics, subtropics, and some temperate regions. The infected equines remain as persistent carriers; *T. equi* persists for life and *B. caballi* persists for up to four years. These persistent carriers act as a source of the infections, from which competent tick vectors acquire the infection and transmit the parasites to other equines. Due to this reason, EP-free countries impose stringent regulations when importing equines, to prevent the entry of *T. equi* and *B. caballi* into their countries. Currently, only a few countries, including Japan, Australia, Canada, Iceland, New Zealand, and Greenland, have been confirmed to be EP-free. However, there are several countries where the status of EP among their equine populations remains unknown, as no epidemiological surveys have been conducted to date. The unknown EP status in these countries has the potential to

cause huge economic losses to the domestic and global equine industry. Therefore, a survey of *T. equi* and *B. caballi* infections is important in countries with unknown EP status.

The epidemiological surveys should typically be followed by studies to investigate the genotypic diversity, as previous studies have demonstrated that the genotypes of *T. equi* and *B. caballi* have implications for the control of EP. In particular, the *T. equi* genotypes have clinical, diagnostic, and therapeutic significance. Therefore, the detection of *T. equi* genotypes is vital in endemic countries for successfully managing EP. The PCR-sequencing technique is the most commonly used approach to determine the genotypes, but it lacks sensitivity. Therefore, investigations using more simple, sensitive, and cost-effective genotyping assays are important to determine the true extent of the genotypic diversity of *T. equi*.

Through a series of studies, I have examined two hypotheses: 1) If the competent tick vectors are prevalent, *T. equi* and *B. caballi* are endemic in countries with unknown EP status, and 2) the genotypic diversity of *T. equi* is currently underestimated.

To test my first hypothesis, I conducted investigations in Sri Lanka and Paraguay, which are agricultural-based countries with large populations of livestock. However, tick infestations and tick-borne diseases are common among their livestock, as the local climate favours the tick activities and the farmers maintain their herds using extensive systems. Despite the presence of competent tick vectors, the epidemiological status of *T. equi* and *B. caballi* infections in equines was unknown, as no surveys have been conducted in Sri Lanka and Paraguay. Therefore, I conducted comprehensive survey of *T. equi* and *B. caballi* infections in donkeys in Sri Lanka and horses in Paraguay (Chapters 1 & 2) (Ahedor et al., 2022; 2023a).

To test my second hypothesis, I developed highly specific conventional PCR assays for the differential detection of each *T. equi* genotype, and used them to screen *T. equi*-positive DNA samples from donkeys in Sri Lanka and horses in Paraguay. The genotypic data obtained from the genotype-specific PCR assays were compared with those generated with the traditional PCR-sequencing approach (Chapter 3) (Ahedor et al., 2023b).

In Chapter 1, I prepared thin smears and DNAs from blood samples of randomly selected 111 donkeys in two districts of Sri Lanka. I subjected the blood smears and DNAs to microscopic examination and *T. equi*- and *B. caballi*-specific PCR assays, respectively. I found that the overall positive rates of *T. equi* were 57.7% and 85.6% by microscopy and PCR, respectively. All donkeys were, however, negative for *B. caballi* in both microscopy and PCR. The phylogenetic analysis of 18S rRNA sequences isolated from *T. equi*-positive DNA samples demonstrated that the Sri Lankan donkeys were infected with genotypes C and D. This study was the first to report genotype C in donkeys. The findings also suggested an African origin for *T. equi* in Sri Lanka, as genotype D, which is common in Africa, was the most prevalent genotype in Sri Lankan donkeys. The findings of my study highlighted the importance of addressing EP in the efforts to preserve the shrinking donkey population in Sri Lanka (Ahedor et al., 2022).

In Chapter 2, I surveyed horses in Paraguay for *T. equi* and *B. caballi* infections. Blood DNA samples were prepared from 545 horses in 16 out of 17 departments in Paraguay, and then screened to detect *T. equi* and *B. caballi* infections using specific PCR assays. Both the *T. equi* and *B. caballi* were detected with overall positive rates of 32.7% and 1.5%, respectively. Two horses (0.4%) were co-infected with *T. equi* and *B. caballi*. The infectious rates of *T. equi* were comparable between the horse breeds, male and female, and 1-3-year and >3-year age groups. The mean values of haematological parameters of horses infected either with *T. equi* or *B. caballi* were within the normal range and

comparable to those of non-infected horses. By contrast, the two co-infected horses were anaemic. The phylogenetic analysis indicated that Paraguayan horses were infected with *T. equi* 18S rRNA genotypes A and C and *B. caballi rap-1* genotype B. The present study, which was the first to report *T. equi* and *B. caballi* infection in Paraguayan horses, suggests that EP should be included in the differential diagnoses of anaemic horses presented to veterinary clinics (Ahedor et al., 2023a).

In Chapter 3, I developed genotype-specific PCR assays for the five *T. equi* genotypes (A – E). The PCR assays were evaluated on 270 *T. equi*-positive equine blood DNA samples (92 from donkeys in Sri Lanka and 178 from horses in Paraguay). I found that the genotype-specific PCR assays were highly sensitive and detected four genotypes (A, C, D, and E) in the Sri Lankan samples and all five genotypes in the Paraguayan samples. All of the Sri Lankan samples and 93.3% of the Paraguayan samples tested positive for at least one genotype, further emphasizing the sensitivity of the PCR assays. The assays also detected co-infections with different genotypes of *T. equi*, of various combinations in 90.2% and 22.5% of the Sri Lankan and Paraguayan samples, respectively. Furthermore, the sequences of the amplicons from genotype-specific PCR assays clustered with their respective phylogenetic clades, validating the specificity of the PCR assays. These findings confirmed that the genotype-specific PCR assays are reliable tools for the differential detection of *T. equi* genotypes (Ahedor et al., 2023b).

The findings from my epidemiological surveys in donkeys in Sri Lanka and horses in Paraguay showed that countries with unknown EP status are likely to be endemic for *T. equi* and *B. caballi* infections if competent tick vectors are prevalent there. Active surveillance of *T. equi* and *B. caballi* in the regions with uncertain EP status will facilitate the effective management of EP on a global scale. The control strategies for EP will be more effective, if they are designed in light of the parasite's genotypic diversity, using

appropriate assays, such as the genotype-specific PCR assays that I developed for *T. equi*. In brief, the series of studies that I conducted and the results that I obtained will serve as a framework for conducting meaningful epidemiological surveys in unexplored regions and will eventually aid in the management of EP, globally.

Abstract in Japanese

和文要旨

馬ピロプラズマ症（EP）は、*Theileria equi* と *Babesia caballi* による 2 種類のマダニ媒介性赤血球内寄生性原虫によって引き起こされる馬の重篤な感染症である。これらの原虫に感染すると血管内で溶血が起こり、感染馬に発熱、貧血、黄疸、血色素尿などの臨床症状を引き起こし、最終的には死に至る場合がある。EP に関連する経済的損失には、治療費、流産や死亡による生産性損失、国際的な馬の移動の制限などが挙げられる。そのため、馬産業の利益を維持・確保するためには EP の疾病制御が極めて重要となる。現在の EP の治療・予防戦略には、化学療法、マダニ駆除、馬の移動制限などが必要となる。しかし、治療薬の効果の低さや副作用、薬剤耐性マダニの出現、診断テストの不確かさなどにより、これらの戦略はあまり有効ではない。

EP は、*T. equi* と *B. caballi* を媒介できる特定マダニ種の生息分布と一致して世界的に広く発生が見られる。感染馬は持続的に原虫を保有することが知られており、*T. equi* は終生、また *B. caballi* は最長 4 年間持続感染する。これらの持続感染馬は重要な感染源となり、マダニの媒介により他の馬に感染を広げていく。このことから、EP 清浄国である日本、オーストラリア、カナダ、アイスランド、ニュージーランド、グリーンランドでは馬を輸入する際に厳しい規制を設けて *T. equi* および *B. caballi* の持ち込みを監視している。一方で、EP の発生状況が依然不明な国が多く存在する。これらの国々の状況は、その国内および世界の馬産業に甚大な経済的損失をもたらす危険性がある。そのため、EP の発生が不明な国々における *T. equi* と *B. caballi* の感染疫学調査は重要である。

本研究では、スリランカとパラグアイで疫学調査が行われた。農業を基盤する両国は家畜動物の数も多い。また、その気候がマダニの活動に適しており、かつ大規模な牛群を飼育していることから、マダニの蔓延とマダニ媒介性疾患の発生は家畜動物で一般的と考えられている。しかしながら、媒介可能なマダニ種が存在するスリランカとパラグアイでは EP に関する疫学調査が実施されていなかった。そこで、スリランカではロバを、パラグアイでは馬における *T. equi* および *B. caballi* の感染疫学調査を行った。

第 1 章では、スリランカで野生化した計 111 頭のロバから血液を採取し、血液塗抹標本を作製するとともに、その DNA も抽出した。血液塗抹標本は顕微鏡検査に供試し、DNA サンプルを用いて *T. equi* と *B. caballi*-特異的 PCR 法によるスクリーニング診断を行った。その結果、顕微鏡検査と PCR 診断による *T. equi* の陽性率は、それぞれ 57.7% と 85.6%となった。一方で、すべてのロバは顕微鏡検査と PCR 診断の両方で *B. caballi*

は検出されなかった。*T. equi* 陽性 DNA サンプルから分離された 18S rRNA 配列の系統学的解析から、スリランカのロバは *T. equi* の遺伝子型 C と D に感染していることが判明した。本研究の成果から、スリランカで減少しつつあるロバ個体群を保全する取り組みにおいて EP に対処することの重要性が明らかとなった。

第 2 章では、パラグアイで飼育されている計 545 頭の馬を対象に *T. equi* と *B. caballi* の感染について疫学調査を行った。その結果、PCR 診断による両種の検出率はそれぞれ 32.7%と 1.5%となった。そのうち 2 頭（0.4%）の馬が *T. equi* と *B. caballi* の両方に共感染していた。*T. equi* の感染率は、馬の品種間、雌雄間、1〜3 歳と 3 歳以上の年齢層間で有意差は認めれらなかった。また、*T. equi* または *B. caballi* に単独感染した馬の血液学的指標の平均値は正常範囲内であり、非感染馬のそれと同等であった。一方で、2 頭の共感染馬はその血液学的指標で明らかな貧血を示し、EP は貧血馬の鑑別診断項目に含めるべきであることが示唆された。系統学的解析の結果から、パラグアイの馬は *T. equi* 18S rRNA 遺伝子型 A と C、また *B. caballi* rap-1 遺伝子型 B に感染していたことが明らかとなった。

第 3 章では、5 つの *T. equi* 遺伝子型（A〜E）に対する遺伝子型特異的 PCR 法を開発し、スリランカのロバおよびパラグアイの馬から採取した計 270 の *T. equi* 陽性血液 DNA サンプルについて解析を行った。その結果、開発された遺伝子型特異的 PCR 法を用いることで、スリランカのロバサンプルから 4 つの遺伝子型（A、C、D、E）が、またパラグアイのサンプルから 5 つの遺伝子型すべてが検出されることが示された。さらに、スリランカでは全サンプル、パラグアイでは 93.3%のサンプルで少なくとも 1 つの遺伝子型の感染が確認され、この PCR 法の感度の高さが証明された。本 PCR 法は、スリランカとパラグアイのそれぞれ 90.2%と 22.5%のサンプルにおいて、様々な組み合わせの共感染も検出した。また、遺伝子型特異的 PCR 法で得られたアンプリコンの遺伝子配列から、本 PCR 法の特異性も証明された。これらの結果から、本遺伝子型特異的 PCR 法は、*T. equi* の遺伝子型を区別して検出できる有益なツールであることが確認された。

一連の調査研究の結果は、EP の発生状態が不明であっても媒介可能なマダニ種が蔓延している国であれば、*T. equi* および *B. caballi* 感染が流行している可能性が高いことを示唆している。EP の状態が不明な地域でこれらの感染疫学調査を積極的に行うことは、効果的な疾病制御を促進する上で重要となる。また、*T. equi* 用に開発された遺伝子型特異的 PCR 法を用いて、その遺伝子型の多様性を考慮した防疫戦略を策定すれば、EP に対する効果的な疾病制御に繋がる。結論として、本研究の成果は世界的な EP の制御に役立つものとなった。

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