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EQUINE PIROPLASMOSIS IN GREECE

Η ΠΙΡΟΠΛΑΣΜΩΣΗ ΤΩΝ ΙΠΠΟΕΙΔΩΝ ΣΤΗΝ ΕΛΛΑΔΑ

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Dedicated to my family

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Summary

A cross sectional survey was performed to explore the epidemiological status of equine piroplasm infection (EPI) in Greece, with a view to providing a basis for disease control strategies. The investigation, which covered various areas of Greece, focused on: a) the identification of the causative agents of EPI, b) the exploration of the genetic diversity of the identified agents, c) the determination of the risk factors associated with the infection, d) the modeling of the geographic distribution of the disease agents, and e) the detection of areas with significant clusters of infection.

A total of 796 equids were used in the study. A total of 787 blood films were examined for evidence of piroplasm stages in stained red blood cells while 544 sera were processed using the competitive inhibition ELISA (cELISA) to detect antibodies to piroplasms. The genetic divergence of piroplasms was explored from 787 DNA samples, using the reverse line blot hybridization (RLB) assay and sequence analysis. The maximum entropy (Maxent) modeling algorithm and the spatial scan statistic were utilized as geospatial tools to derive the potential geographic range and detect clusters of infections, respectively.

No piroplasm was detected microscopically. However, the overall seroprevalence of piroplasm infection was 11, 6 %, with 11% for *Theileria equi* and 2.2% for *Babesia. caballi*. The emerging profile of equids at risk of significantly high seropositivity level of infection involved mule species, farming activities and residence in the region of Thessaly. A higher seroprevalence was observed in local animals in comparison to imported equids, indicating that the disease is enzootic in Greece. Two *Theileria* genotypes (*T. equi* and *T. equi*-like) and one *Babesia* genotype (*Babesia caballi*-like) were distinguished. The prevalence was 43.96 %, 46. 25% and 0.38 % for *T. equi*, *T. equi*-like and *B. caballi*-like respectively. Seven piroplasm-positive samples could not be differentiated neither at species nor at genotype level. A partial sequence of 509 bp of the V4 region of the 18 S rRNA gene of a *T. equi*-like isolate was obtained and found to be 99 % similar with the reference *T. equi*-like from Northern Spain from which the detecting probe used in this study was designed, but showed 100 % similarity with the *T. equi*-like variant from Southern Spain. This indicated some degree of polymorphism within the population of *T.*

equi-like. No unusual parasites previously reported in horses, such as *B. canis canis* and *B. bovis* were detected in this study. The values of the bioclimatic variables were very similar between the geographic locations for *T. equi* and *T. equi-like* genotypes, suggesting the two are not yet different species as hypothesized by some authors but are possibly undergoing a speciation process within *Theileria* genotypes. Alternatively, convergence of both genotypes into a single species is possible. The potential geographic range of all the piroplasms covered the entire country but high probability areas for the presence of piroplasm infections concentrated in the Eastern half of mainland Greece. Significant clusters were detected for *B. caballi* and *T. equi* infections in North and Central regions of Greece respectively which have significant equine populations. Two competent tick vectors, common transmitters of both *T. equi* and *B. caballi* were identified. These consisted of *Rhipicephalus bursa* and *Rhipiecphalus sanguineus* collected from horses and dogs respectively at the time of sampling.

The present study showed that EPI and their required tick vectors are well established in the country, implying the need for control measures.

Περίληψη

Σκοπός της παρούσας έρευνας ήταν η διενέργεια επιδημιολογικής έρευνας χρονικού σημείου για να μελετηθεί η κατάσταση της πιροπλάσμωσης των ιπποειδών (ΠΙ) στην Ελλάδα, με στόχο τη συλλογή πληροφοριών για τη κατάρτιση στρατηγικών ελέγχου της ασθενείας. Η έρευνα, που κάλυψε διάφορες περιοχές της Ελλάδας, είχε ως σκοπό : α) τον προσδιορισμό των αιτιολογικών παραγόντων της ΠΙ, β) τη διερεύνηση της γενετικής ποικιλομορφίας των πιροπλασμάτων που ανιχνεύθηκαν, γ) το προσδιορισμό των παραγόντων επικινδυνότητας που συνδέονται με τη μόλυνση, δ) τη διαμόρφωση προτυποποίησης της γεωγραφικής κατανομής των πιροπλασμάτων, και ε) την ανίχνευση των περιοχών με σημαντικές εστίες της μόλυνσης.

Συνολικά από τα 796 ιπποειδή που περιλήφθηκαν στη μελέτη, 787 δείγματα αίματος εξετάστηκαν με τη μέθοδο της παρατήρησης στο οπτικό μικροσκόπιο μετά από χρώση επιχρίσματος αίματος και 544 δείγματα ορού υποβλήθηκαν σε προσδιορισμό αντισωματών κατά των πιροπλασμάτων χρησιμοποιώντας την ανοσοενζυμική δοκιμασία (cELISA). Η γενετική ποικιλομορφία των πιροπλασμάτων διερευνήθηκε σε 787 δείγματα DNA, χρησιμοποιώντας την αντίστροφη δοκιμή υβριδοποίησης στιγμάτων των γραμμών (RLB) και συμπληρώθηκε με την μελέτη της νουκλεοτιδική τους αλληλουχίας. Η προτυποποίηση της γεωγραφικής κατανομής με χρήση του αλγορίθμου της μέγιστης εντροπίας (Maxent) και η χωρική στατιστική ανίχνευση χρησιμοποιήθηκαν ως γαιοχωρικά εργαλεία για να παραχθεί η πιθανή γεωγραφική κατανομή και να ανιχνευθούν οι εστίες των μολύνσεων, αντίστοιχα.

Κανένα παράσιτο δεν ανιχνεύθηκε κατά τη μικροσκοπική παρατήρηση. Εντούτοις, ο συνολικός ορολογικός επιπολασμός της μόλυνσης της ΠΙ ήταν 11, 6% και ειδικότερα 11% για το *T. equi* και 2.2% για το *B. caballi*. Ο υψηλότερος επιπολασμός των ιπποειδών βρέθηκε στους ημίονους στη περιοχή της Θεσσαλίας. Επίσης, παρατηρήθηκε στα ζώα της ίδιας περιοχής υψηλότερος επιπολασμός σε σύγκριση με τα εισαγόμενα, δείχνοντας ότι η ασθένεια είναι ενζωοτική στην Ελλάδα. Ανιχνεύθηκαν δύο γενότυποι του είδους *Theileria* (*T. equi* και *T. equi-like*) και ένας γενότυπος του είδους *Babesia* (*Babesia caballi-like*). Ο επιπολασμός ήταν 43.96%, 46. 25%, και 0.38% για το *T. equi, T. equi-like,* και *B. caballi-like* αντίστοιχα. Επτά δείγματα που βρέθηκαν θετικά δεν ήταν δυνατόν να ταυτοποιηθούν σε επίπεδο είδους ή γενοτύπου. Μια μερική αλληλουχία αποτελούμενη από 509 ζεύγη βάσεων της V4 περιοχής του γονιδίου 18S rRNA του *T. equi-like,* απομονώθηκε και βρέθηκε ότι παρουσιάζει ομοιότητα 99% με το

στέλεχος αναφοράς του *T. equi-like* από τη Βόρεια Ισπανία και 100% με στέλεχος αναφοράς *T. equi-like* από τη Νότια Ισπανία. Αυτό έδειξε κάποιο βαθμό πολυμορφισμού μέσα στον πληθυσμό του *T. equi-like*. Κανένα ασυνήθιστο παράσιτο που έχει αναφερθεί σε άλλες μελέτες στα άλογα, όπως το *B. canis canis* και το *B. bovis* δεν ανιχνεύθηκε στη παρούσα έρευνα. Οι τιμές των βιοκλιματικών μεταβλητών ήταν παρόμοιες μεταξύ των γεωγραφικών θέσεων των γενοτύπων *T. equi* και *T. equi-like*, οπότε δεν πρόκειται για δυο διαφορετικά είδη όπως προτείνουν μερικοί ερευνητές αλλά ενδεχομένως οι γενότυποι να υποβάλλονται σε μια διαδικασία διαφοροποίησης μέσα στο ίδιο είδος του *Theileria*. Η πιθανή γεωγραφική κατανομή όλων των πιροπλασμάτων κάλυψε ολόκληρη τη χώρα αλλά οι περιοχές με την υψηλότερη πιθανότητα για τη παρουσία των μολύνσεων συγκεντρώθηκαν στο ανατολικό μισό της ηπειρωτικής χώρας. Σημαντικές εστίες των *T. equi* και *B. caballi* ανιχνεύθηκαν στις βόρειες και στις κεντρικές περιοχές της Ελλάδας όπου υπάρχει σημαντικός πληθυσμός ιπποειδών. Προσδιορίστηκαν δυο ενδιάμεσοι ξενιστές του *T. equi* και του *B. caballi*. Πρόκειται για τους κρότωνες *R. sanguineus* και *R. bursa* που συλλέχθηκαν από άλογα και σκύλους αντίστοιχα κατά την διάρκεια της δειγματοληψίας.

Η παρούσα μελέτη έδειξε ότι η ΠΙ και οι απαιτούμενοι ενδιάμεσοι ξενιστές (κρότωνες) βρίσκονται στη χώρα, οπότε είναι αναγκαία να ληφθούν μέτρα ελέγχου για την αντιμετώπιση της μόλυνσης στα ιπποειδή.

Résumé

Une étude cross-sectionelle visant à explorer le statut épidémiologique de la piroplasmose equine (PE) en Grèce a été menée afin de poser les bases pour des futures stratégies de control de la maladie. Les enquêtes couvraient plusieurs zones du pays et se sont focalisées sur les points suivants : a) l'identification des agents pathogènes responsable de la maladie; b) l'exploration de la diversité génétique des agents pathogènes identifiés; c) la détermination des factors de risque associés à l'infection; d) la modélisation de la répartition géographique des agents pathogènes identifiés; e) la détection des zones du domaine d'étude caractérisées par les agrégats d'infections.

Au total, 796 équidés étaient impliques dans cette étude. Un total de 787 frottis sanguins en couche mince ont été réalisés et examinés pour la recherche des piroplasmes dans les globules rouges et 544 échantillons de sérum testés par le test diagnostique cELISA (competitive inhibition enzyme-linked immunoabsorbant assay) pour l'évidence d'anticorps dirigés contre les piroplasmes équins. La divergence génétique des piroplasmes a été explorée chez 787 échantillons d'ADN par le biais de la technique RLB (reverse line blot hybridization), accompagnée du séquençage d'ADN. L'algorithme dit d'entropie maximale ainsi que la statistique spatiale de scan ont été respectivement utilisés pour dériver la carte de répartition géographique potentielle et détecter les agrégats spatiaux d'infection à piroplasmes.

Aucun piroplasme n'a été détecté par l'examen microscopique des frottis. Cependant, la seroprevalence globale des infections à piroplasmes était de 11.6 %, soit 11 % pour l'infection à *Theileria equi* et 2.2 % en ce qui concerne *Babesia caballi*. Le profile des équidés à haut risque de séropositivité pour ces infections qui a émergé était d'être une mule, être impliqué dans les activités agronomiques et être résident de la région de Trikala. Une haute prévalence a été observée chez les animaux locaux en comparaison à ceux qui sont importés, indiquant que la piroplasmose équine est enzootique en Grèce. Deux génotypes du genre *Theileria* (*T. equi* et *T. equi*-like) et un génotype appartenant au genre *Babesia* (*B. caballi*-like) ont été distingués. La prévalence se situait respectivement autour de 43.96 %, 46.25 % et 0.38% pour *T. equi*, *T. equi*-like et *B. caballi*-like. Sept échantillons ont été testés positive pour les infections à piroplasmes mais ces piroplasmes n'ont pu être différentiés ni à l'échelle de l'espèce, ni à l'échelle du génotype. Une séquence partielle de 509 pb de la région V4 du gène de l'ARNr 18S d'un isolat

de *T. equi*-like obtenu à révélé une similarité de 99 % avec *T. equi*-like de référence du Nord de l'Espagne à partir duquel la sonde employée dans cette étude a été créée. Cette séquence partielle a aussi montre une similarité de 100 % avec l'autre variant de *T. equi*-like au sud de l'Espagne, ce qui indique un certain degré de polymorphisme génétique au sein de la population de *T. equi*-like. Aucun parasite inhabituel précédemment décrit chez les chevaux tel que *B. canis canis* et *B. bovis* n'a été détecté dans cette étude. Les valeurs des variables bioclimatiques étaient très similaires entre les locations géographiques des génotypes *T. equi* et *T. equi*-like, suggérant que les deux génotypes ne sont pas encore deux espèces différentes d'après l'hypothèse émise par certains auteurs mais seraient plutôt en cours de spéciation. Alternativement, la convergence des deux génotypes en une seule espece est aussi possible. L'aire de distribution potentielle de tous les piroplasms couvrent toute l'étendue de territoire national mais les zones à forte probabilité de présence des infections à piroplasmes sont concentrées dans la moitie Est de la Grèce continentale. Les agrégats spatiaux statistiquement significatifs d'infections à *B. caballi* et *T. equi* ont été repérés au Nord et dans les régions du centre de la Grèce, lesquelles contiennent une proportion importante de populations d'équidés. Enfin deux espèces de tiques, à la fois compétent vecteurs de *T. equi* et *B. caballi* ont été identifiées. Il s'agit de *Rhipicephalus bursa* et *Rhipicephalus sanguineus,* respectivement récoltée sur les chevaux et les chiens.

Cette étude à montré que la PE et les tiques vecteurs sont bien établies dans le pays, ce qui invite à la mise en place des mesures adéquates de control.

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Introduction

Equine piroplasmosis (EP) is a tick-borne protozoan infection of equids (horse, pony, donkey, and mule) occurring in most tropical and subtropical regions of the world as well as in temperate zones. EP is endemic in many parts of Asia, Arabia, South and Central America, Africa and Europe. Within Europe, it is more prevalent in France (Leblong et al., 2005), Portugal (Bashiruddin et al., 1999), Spain (Camacho et al., 2005), Italy (Moretti et al., 2009) and Turkey (Karatepe et al., 2009). EP is considered the most important tick–borne diseases of horses in tropical and sub tropical areas (Schein, 1988). The symptoms of disease range from fever, inappetance, jaundice anemia, hepatomegaly, splenomegaly, chronic weigh lost, and poor exercise tolerance to haemoglobinuria which sometimes results in death (Brüning et al., 1997; Friedhoff, 1982; De Wall, 1992; Schein 1988). However, the clinical manifestations are variable, and subclinical infections do occur (Blood and Radostits, 1989). The etiologic agents of EP are *Babesia caballi* and/or *Theileria equi* formerly (Syn: *Babesia equi*). However, *Babesia canis* of dogs as well as *B. bovis* of cattle have been recently reported in horses (Criado-Fornelio et al., 2003; Criado et al., 2006). *T equi* is considered more pathogenic and a more consistent cause of haemoglobinuria and death than *B caballi* which causes a more persistent syndrome characterized by fever and anaemia (Zaugg and Lane, 1992). The genera of ticks that transmit piroplasmids in equids include *Dermacentor, Hyalomm*a, *Rhipicephalus*, *Boophilus* (Battsetseg et al., 2001) and *Haemophysali*s. In southern Europe, *T equi* is endemic and infections may occur asymptomatically, and more frequently than those due to *B caballi* (Friedhoff et al., 1990; Bashiruddin et al., 1999).

EP is of worldwide importance due to the fact that the disease- causing agents can be readily transmitted by carrier animals or by infected ticks into originally piroplasms-free countries. In this respect many countries such as the United States of America, Canada, Australia and Japan have established strict importation regulations restricting the movement of piroplasm-seropositve horses across their borders (Kuttler, 1988; Friedhoff et al., 1990). In Europe, awareness of the economic losses due to equine piroplasmosis is increasingly perceived (Habela et al., 2005; Camacho et al., 2005). Moreover, EP is among the listed diseases of the World Organization for Animal health (OIE), notifiable within 72 hours. Long-term monitoring of the

prevalence of infection therefore is very important in areas where the disease has been reported, given the international importance of the disease and the potential impact on the horse industry. Knowledge of the prevalence of infection is essential to set up efficient control measures.

However, the literature on the epidemiology of EP in Greece is quite scant with only one report documenting the isolation of the sporozoites of *B. caballi* and *T. equi* in *Rhipicephalus sanguineus and Hyalomm*a *plumbeum* respectively (Haralabidis, 2001). There are a few published reports on the occurrence of other piroplasm species in ruminants in Greece (Papadopoulos et al., 1996; Papadopoulos et al., 1999; Theodoropoulos et al., 2006). The animal population susceptible to infection is diversified in Greece, as well as the potential tick vectors. Officially, there are about 32000 equids throughout the country in 51 departments (HSA, 2007). The larger herd of horses is located in the equestrian center of Markopoulo in Attica region, approximating 1500 individuals. The competent vectors reported in Greece include *R. sanguineus*, *H. plumbeum* (syn: *H. marginatum*) and *H. anatolicum excavatum* (Soulsby et al., 1986, Haralabidis, 2001). The occurrence of both the vectors and the extent of host range raises the question of how far the disease is present in the country. Horses are internationally more involved in equine industry especially in sports and show events than other equid species. However, this latter group of equids plays a double role first to their owners and secondly on the epidemiology of EP. Mules, donkeys and ponies are draught, recreation (pleasure-riding), entertainment and pasture animals to their owners. Epidemiologically, they constitute a natural reservoir of transmission of infection to horses. Knowledge of the status of EPI is thus of great importance to Greece which harbors one of the largest equestrian center in southern Europe and most specially, hosted the equestrian Olympic Games in 2004.

I. LITERATURE REVIEW

I.2. History

Laveran in 1901 was the first to describe an intraerythrocytic parasite which he discovered in peripheral blood smears taken from South African horses. He accordingly named it *Piroplasma equi* (Schein, 1988). Nuttall and Strickland (1910) noticed another pathogenic organism, *Babesia caballi* which was morphologically distinct from *B. equi* but likewise caused piroplasmosis in the horse. They named the smaller species in the blood smears previously described by Lavaran (1901) *Nutallia equi* and retained the larger species ressembling *Babesia bigemina* in the genus *Babesia* which were then called *Babesia* (*Piroplasma) caballi* (Schein, 1988). The genus "*Nuttalia*" has already been assigned to a North American bivale shell species, (Nomen occupatum), making the former designation "*Nutallia equi*" obsolete and consequently the organism was classified under the genus *Babesia* (Schein, 1988).

The taxonomic position of *B. equi* to the genus *Babesia* was questioned again by Schein and co-workers (1981) in an investigation pertaining to the developmental cycle in horse where they demonstrated that *B. equi* exhibits characteristic of the *Theileria* in their morphology and development in the equine host as well as in the tick vector. It was not until 1998 that Mehlhorn and Schein thoroughly redescribed *B. equi* as *Theileria equi*. Thus former *B. equi* was reclassified to the genus *Theileria* and since then *Theileria equi* has been used in the literature.

Other piroplasma species, *Babesia canis* of dogs (Criado-Fornelio et al., 2003; Hornok et al., 2007) as well as *B. bovis* of cattle (Criado et al., 2006) have been reported in horses recently but no clinical signs attributable to this parasite have been documented in equids.

I.3.The tick vectors of piroplasmosis in horses.

Ticks transmit a greater variety of pathogenic micro-organisms, protozoa rickettsia, spirochaette and virus than any other arthropod vector group and are among the most important

vectors of diseases affecting livestock, humans and companion animals (Jogejang and Uilenberd, 2004). Of the 867 species currently known (Jogejang and Uilenberd, 2004), more than 19 belonging to 5 genera transmit equine piroplasmosis (Table 1).

Table 1. Tick vectors capable of transmitting EP with other diseases (compiled from Baker, 1999; Becker, 2002; Hillyard, 1996; Jonjejan and Uilenberg, 2004; Soulsby, 1982

Tick species	Borne agent			Geographical Distribution
	T. equi	*B. caballi*	Other agents or affection	
Boophilus microplus	+	-	*Babesia bovis*, B. *begemina* (bovine babesiosis); *Anaplasma marginale* (Bovine anaplasmosis).	South-East Asia, Tropics, Australia, East and Sourthen Africa, South and Central America.
Dermacentor nitens	-	+		Florida, Panama
D. reticulatus	+	+	*Babesia canis* (canine babesiosis), *B divergens* (bovine babesiosis), *B .ovis*, *Theileria ovis* (babesosis and theileriosis of small ruminants), *Francisella tularensis* (tularemia of cattle)	Eurasia, Cental Africa
D. marginatus	+	+	*Babasia canis*, *B divergens*	Moroco, Spain, Italy, Southern France, Switzerlan,Western Germany, Poland Easwards to Central Asia.
D. nutalli	+	+	*Rickettsia sibirika* (human rickettsiosis)	Mangolia(Central Asia)
D. albipictus[1]	-	+		USA
D. variabilis[1]	-	+		USA
Haemaphylasis spp	-	+		USA
Hyalomma anatolicum anatolicum	+	+	*Theileria annulata (bovine tropical theileriosis),* Anaplasma marginale, *Trypanosoma theileri infections, Theileria lestoquardi* (theileriosis of small ruminants), at least 5 arbovirus, vector of Crimean-Congo hemorrhagic fever virus to humans.	Northern Africa, portion of the Middle and Near East Arabia, Asia Minor, Southern Europe, Russia, India
H. anatolicum excavatum	+	+	*Theileria annulata*	Northern Africa, portion of the Middle and Near East Arabia, Asia Minor, Southern Europe, Russia, India
H. dromadarii	+	+	*Theileria annulata*	North Africa, India
H. marginatum (H plumbeum plumbeum)	+	+	*Babesia canis, Babesia ovis, Rickettsia aeschlimanii (spotted fever in humans)* , Crimean- Congo Haemorrhagic fever (CCHF)virus , *Coxiella burnetii* (Q-fever conorii),	Asia, Southern Europe and Africa, North western Europe
H. scupense	+	+	*Theileria annulata*	Ukraine
Rhipicephalus bursa	+	+	*Babesia bovis,B begemina ,Anaplasma marginal, B ovis, Anaplasma ovis (*anaplasmosis of small ruminants*),Coxiella burnetti,* Narobi sheep disease and Crimean-Congo haemorrhagic fever viruses	Southern Europe, the Near East, Maghreb.
R. evertsi evertsi	+	+	*Anaplasma marginale toxin (*tick paralysis in animal*)*	South Africa to Northern Sudan and Yemen
R. sanguineus	+	+	*Babesia canis, B. vogeli (*canine babesiosis*), Ehrlichia canis Hepatozoon canis (canine hepatozoonosis), Rickettsia Conorii (*Tick bite fever in humans*. Anaplasma marginale, Coxiella burnetii,*	Worldwide
R. turanicus	+	-		Ex Soviet Union

[1] Experimental transmission of *Babesia caballi* under laboratory conditions has been reported using *Dermacentor albipictus* and *D. variabilis* (Kuttler, 1984).

I.3.1.Taxonomy of ticks

The tick vectors of equine piroplasmosis fall into the phylum Arthropoda, class Arachnida, order Acarina, suborder Ixodoidea, family Ixodidae (hard ticks) including the genera *Biophilus* Curtice , 1891, *Dermacentor* Koch, 1844, *Hamaphysalis* Koch, 1844 , *Hyalomma* Koch 1844 and *Rhipicephlus* koch,1844 (Soulsby, 1982). The family ixodidae contains almost all the species of tick of veterinary importance. The second family, the Argasidae (the soft ticks) contains a relatively low number of species of veterinary importance, none of them bearing equine piroplasms.

I.3.1.1. Morphology of Ixodid ticks

I.3.1.1.1.Family identification.

The morphological characteristics of the Ixodidae and the constituting genera were described from Richard et al. (1997), Urquhart et al. (1996) and Soulsby (1982). Ixodid ticks are relatively large, ranging between 2 and 20 mm in length. The body of the unfed tick is flattened dorsoventrally and is similar in structure to that of mites, being divided into only two sections, the anterior gnathosoma or (capitulum) and the posterior idiosoma, which bears the legs. The mouthparts carried on the capitulum are anterior and visible from the dorsal surface (Fig. 1A). They are structurally similar to those of mites consisting of:

-a pair of four-segmented palps which are simple sensory organs;

-a pair of heavely sclerotised, two segmented appendages called chelicerae, housed in cheliceral sheaths between the palps; the chelicerae are capable of moving back and forth and its tooth-like digits are used to cut and pierce the skin of the host animal during feeding;

- a basis capituli, the enlarged, fused coxae of the palps which varies in shape in the different genera from rectangular or hexagonal to triangular (Fig. 1B);

-a hypostome, the anterior and ventral extension of the lower wall of the basis capituli lies below the chelicerae; the hypostome does not move, but in larvae, nymphs and adults females is armed with rows of backwardly directed ventral teeth used to attach the tick securely to its host (Fig. 1B)

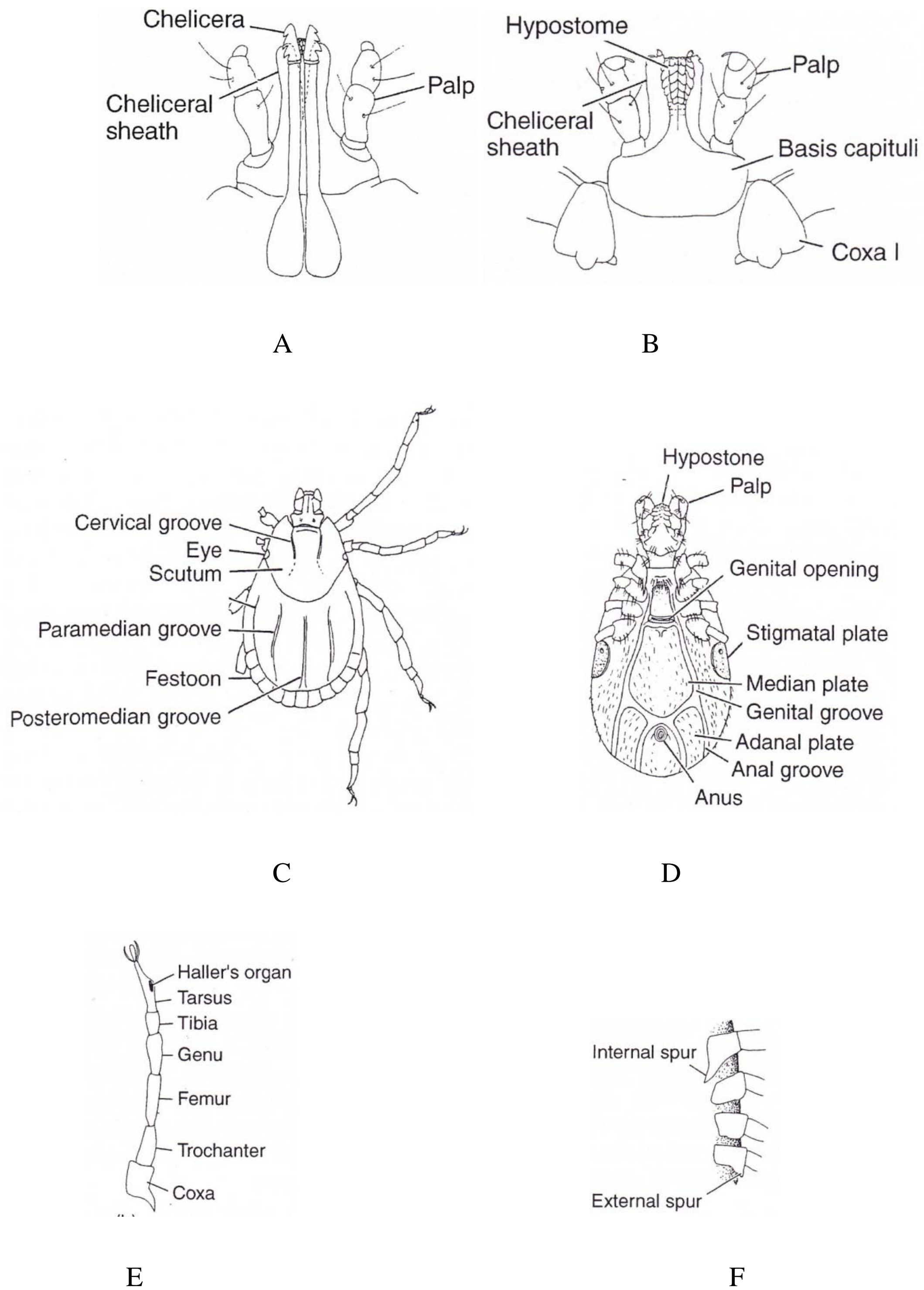


Fig. 1. Parts of a generalized Ixodid tick: dorsal (A) and ventral (B) views of the mouthpart; dorsal view of a male (C); ventral view of a female (D); segments of the leg (E); ventral view of the coxae (F) (adapted from Wall and Shearer, 1997).

The immatures stages of ticks are very similar morphologically to the adults. The larvae, sometimes known as seed ticks, are six-legged whereas nymphs and adults have four pairs of legs (Fig. 1C, D). The region of the idiosoma which carries the legs is called the podosoma and the region behind the legs is the opisthosoma. Each leg is attached to the body at the coxa. The coxa may be armed with internal and external ventral spurs, and their number, size and shape may be important in species identification (Fig. 1F). Following the coxa are the trochanter, femur, genu, tibia, and tarsus (Fig. 1E). On the tarsi of the first pair of legs is located a pit known as Haller's organ which is packed with chemoreceptor setae and used in host location. Chemoreceptors are also present on the palps, chelicerate and scutum.

Ixodid ticks possess a rigid, chitinous dorsal shield or plate on the idiosoma known as a scutum which covers the entire dorsal surface of the adult male. In the adult female and in the larva and nymph, it extends for only a small area which permits the abdomen to swell after feeding. The ixodidae are called hard ticks because of the presence of the scutum. Some ticks with coloured enamel-like areas (grey and white on a dark background) on the body called ornate ticks. Inornate ticks have not.

Ticks do not possess antennae and when eyes are present, are simple and consist of one pair situated on the lateral margin of the scutum. There are a series of grooves on the scutum and body, the number and presence of which may be important in species identification. Other distinguishing features are a number of rectangular regions (a row of notches) on the posteror border or margin of the body called festoons (Fig. 1C). Chitinous plates are sometimes present on the ventral surface of the male.

The genital opening is in the ventral mid-line and the anus is posterior. Sexual differentiation is usually not obvious in the larva and nymph, and immature ixodids generally look like small female but without the genital opening. In the adult, the genital opening, the gonopore is a transverse slit situated at the level of the second pair of legs (Fig. 1D).

I.3.1.1.2. Genus identification

One of the distinctive characteristics of the ixodid tick at genus level lies on the morphology of the gnathosoma (Fig. 2).

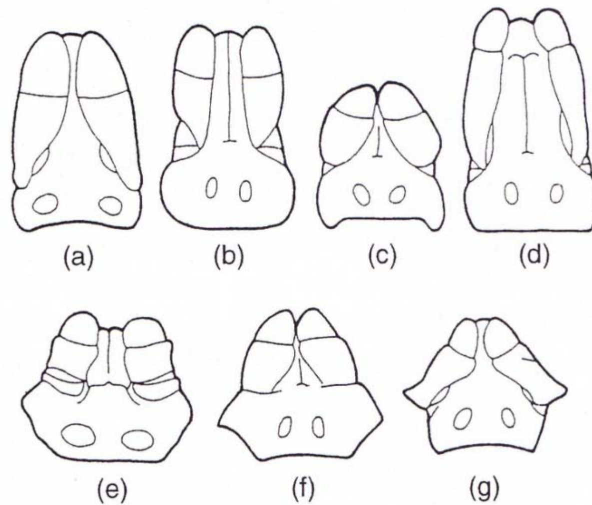
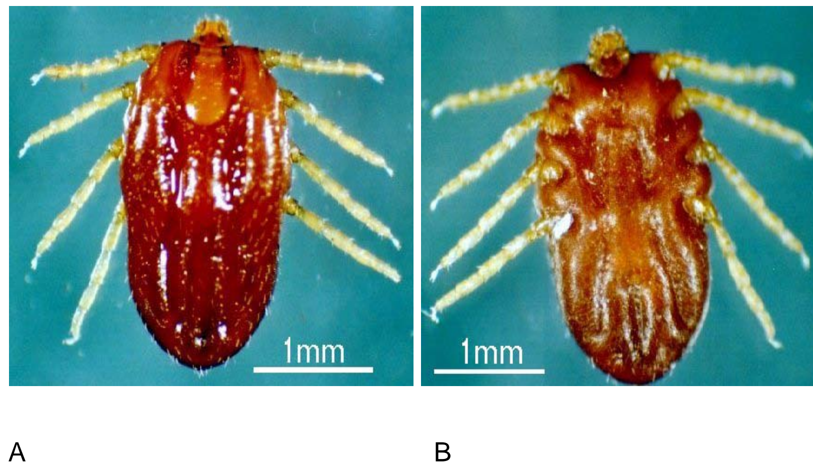


Fig.2. Digrammatic dorsal view of the gnathosoma of seven genera of Ixodid ticks: Ixodes (a), *Hyalomma* (b), *Dermacentor* (c), *Amblyomma* (d), *Boophilus* (e), *Rhipicephalus* (f) and *Haemaphysalis* (g) (reproduced from Wall and Shearer, 1997).

I.3.1.1.2.1. Genus *Boophilus* Curtice, 1891

Example: *Boophilus decoratus* (Fig. 3)



A B

Fig. 3. Boophilus decoratus female: dorsal (A) and ventral (B) views (adapted from http://webpages.lincoln.ac.uk/fruedisueli/FR-webpages/parasitology/Ticks/TIK/tick-key/boophilus_adult.htm)

Ticks of this genus are inornate, with eyes present and festoons absent. The palps and the hypostome are short; the basis capituli is hexagonal (Fig. 2). The male have adanal or accessory ventral shields. These tick known as “blue ticks” are one-host ticks and the most important vectors of *Babesia* spp and *Anaplasma marginal* in cattle in subtropical and tropical countries.

I.3.1.1.2.2. Genus *Dermacentor* koch, 1844

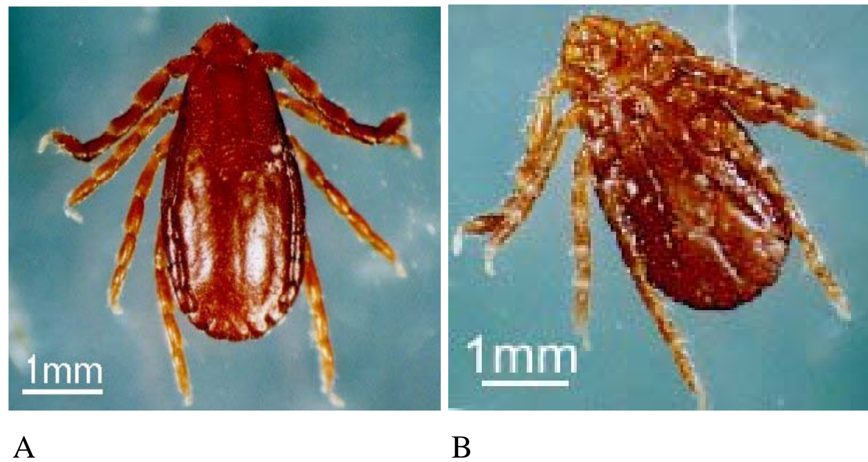
Example: *Dermacentor reticulatus* (Fig. 4)

Fig.4. *Dermacentor reticulatus* female (left) and male (right) (adapted from www.weissepfoten.de/HOS/Zecke.htm)

Members of this genus are medium-sized to large ticks, usually with ornate patterning. The mouthparts are short and the basis capituli is rectangular (Fig. 2). Eyes and festoon are present .The males lack ventral plates and the fourth coxae are greatly enlarged. Most species of this genus are three-host ticks, but few are one-host ticks.

I.3.1.1.2.3. Genus *Haemaphysalis* Koch, 1844

Example: *Haemaphysalis leachi* (Fig. 5)



A B

Fig. 5. *Haemaphysalis leachi* female:dorsal (A) and ventral (A) views (adapted from http://webpages.lincoln.ac.uk/fruedisueli/FR-webpages/parasitology/Ticks/TIK/tick-key/haemaphysalis_adult.htm)

These are unornamented ticks with festoons present and eyes absent. The sensory palps are short and broad with the second segment extending beyond the basis capituli which is rectangular) (Fig. 2). The male have no ventral shields (chitinous plates) and the anal groove contours the anus posteriorly. These inhabit humid, well-vegetated habitats in Eurasia and tropical Africa. They are three-host ticks.

I.3.1.1.2.4. Genus *Hyalomma*

Example: *Hyalomma plumbeum plumbeum*

Syn: Hyalomma marginatum (Fig.6)

A **B**

Fig.6. *Hyalomma marginatum* female: dorsal (A) and ventral (B) view (adapted from http:// webpages.lincoln.ac.uk/.../hyalomma_adult.htm)

Members of this genus are usually inornate but few are ornamented.species medium-size to large ticks, with eyes and long mouth parts. Festoons present or absent. The males have ventral plates on each side of the anus (adanal shields). *Hyalomma* spp are usually two-host ticks, with the larvae and nymphs feeding on birds and small mammals and the adults on ruminants and equids, though some species may use three hosts.

I.3.1.1.2.5. Genus *Rhipicephalus*

Example: *Rhipicephalus bursa* (Fig. 7)

A B

Fig.7. *Rhipicephalus bursa* female: Dorsal view (A) and ventral view (specimens found and identified in the present study)

Species of this genus are usually inornate, provided with eyes and festoons. The mouthparts are short and the basis capituli is hexagonal dorsally (Fig. 2) and, in male, paired plates are found on each side of the anus. The first coxa has two strong spurs. Spiracles are comma-shaped, short in the female and long in the male. Most species are three-host ticks but some species are two- host ticks.

I.3.2. Ecology and life cycle of tick species found in Greece

Three species have been incriminated as vectors of equine piroplasmosis in Greece (Soulsby, 1982; Haralabidis, 2001) but their geographical distribution is unknown. These include; *Hyalomma plumbeum plumbeum, Hyalomma anatolicum excavatum* and *Rhipicephalus sanguineus*, the brown down tick. Another tick, *R. bursa* joint vector of both *T. equi* and *B. caballi* was also described in the present study.

I.3.2.1. Ecology

These ticks present the major ecological features of the ixodidae. They are temporary parasites which lay their small, spherical, and yellowish-brown to dark brown eggs in sheltered spots under stones and clods of soil or in crevice of walls and cracks of wood near the ground. The female lays all her eggs in one batch and may produce several thousands. The subsequent development to the adult stage is greatly influenced by the prevailing temperature, cold weather

causing marked prolongation of the different stages, especially hatching of the eggs and the pre-ovoposition period of the engorged female (Soulsby, 1982). Generally, ticks are more active during the warm season provided there is sufficient rainfall (Urquhart et al., 1996). Hard ticks seek host by an interesting behaviour known as "questing". To obtain blood, the newly hatched larvae or seed tick and any field –inhabiting tick climb on to grass and shrubs, usually at a height appropriate to their host and wait there until a suitable host passes, to which they attach themselves with their claws. The ticks detect the approach of the host using cue such as carbon dioxide and semiochemicals emitted by the host, which they sense using the chemoreceptors, particularly those packed into Hallers's organs on the tarsi of the first pair of the legs; vibration, moisture, heat and passing shadows may be important cues in host recognition (Wall et Shearer, 1997). Once the host brushes past, the ticks transfer to it and attach to the preferred attachment sites. The males remain much longer on the host than the females and consequently they accumulate on the host. Though all stages are capable of survival on a variety of hosts, the larvae and nymphs are usually most successful on smaller animals. Consequently, when both larger and smaller potential host species are in close proximity, the higher survival of the immature stages may result in particularly large tick population and severe infestations (Wall et Shearer, 1997).

The host location behavior of ticks represents in itself a risky way of finding a host animal and many die without ever feeding. To compensate for the risk involved, tick have developed a variety of complex life-cycle and feeding strategies which reflect the nature of the inhabited habitat and the probability of contact with an appropriate host.

For ixodid ticks, which inhabit forests and pastures where there is relatively plentiful supply of hosts and in habitats where conditions are suitable for good survival during the off-host phase, a three-host life –cycle has been adopted (Wall et Shearer, 1997), as is the case with *Rhipicephalus sanguineus.* Three-host ticks do not moult on the host; the engorged larva drop off, moults to a nymph which then has to find a second host animal on which it engorges and drops off again to moult to the adult stage, which attaches to a third final host animal (Jongejan and Uilenberg, 2004).In contrast, ticks inhabiting areas where hosts are scarce and the seasonal periods of unfavorable climate are long, have evolved two and one-host feeding strategies. One-host ticks moult twice on the same host animal from larva to nymph and from nymph to adult, while two-host ticks moult once on the host from the larval to the nymph stage; the engorged

nymph drops off, moults off the host and the resulting adult has to find a second host (Jongejan and Uilenberg, 2004).

I.3.2.2. Life cycle

The life cycles (Fig.8) of ixodid ticks involves four instars: egg, six-legged larva, eight-legged nymph and eight legged adult. During the passage through these stages, ixodid ticks take a large number of blood meals interspersed by lengthy free-living periods and the time spent on the host may occupy as little as 10% of the life of the tick (Wall et Shearer, 1997).

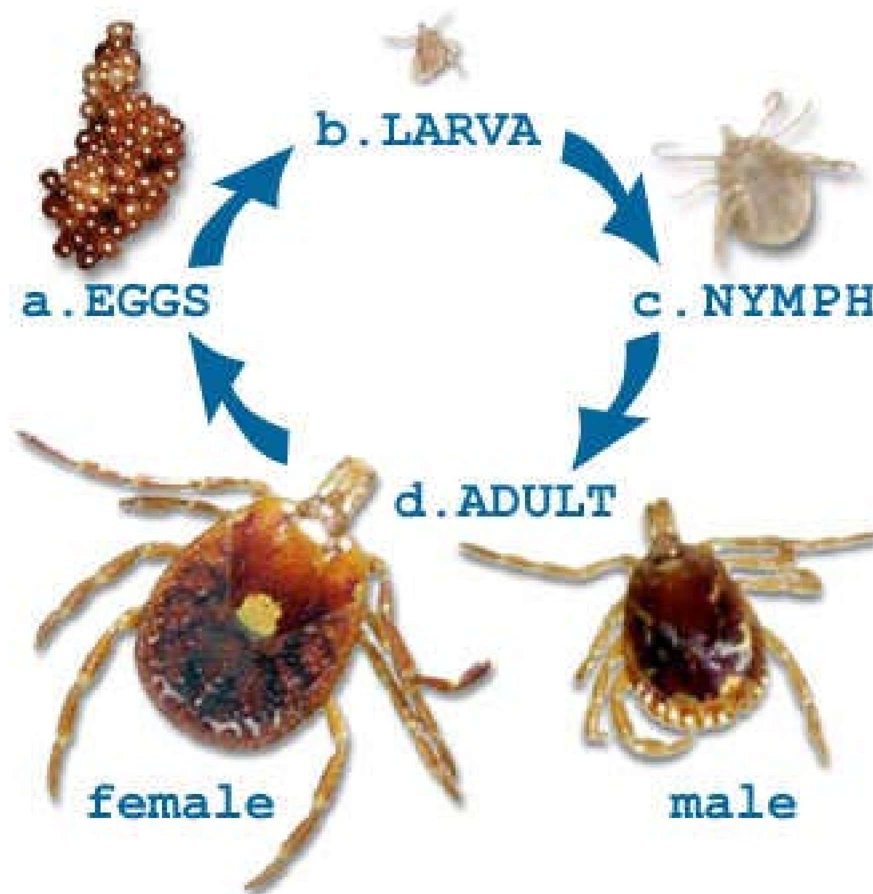


Fig. 8. Life-cycle of an ixodid Tick (reproduced from (www.ticktexas.org).

I.3.2.2.1. *Hyalomma anatolicum excavatum*

This is a three-host tick. Immature stages chiefly infest rodents but birds and reptiles may serve as host to them; adults target camels, cattle, horses and smaller mammals like hares

(Goddard, 2000). This tick species is also an avid parasite of humans (Goddard, 2000). The preoviposition period varies between 37 to 59 days, the oviposition 37 to 59 days and the entire life cycle can be completed within 121 days (Soulsby, 1982)

I.3.2.2.2. Hyalomma *plumbeum plumbeum*

(Syn: *H. marginatum*)

This is a two-host tick. Larvae and nymphs target small wild mammals, lizards and birds while adults feed on domestic animals including horses, deers, camels, dogs, cattle and sheep; humans are mostly attacked by adults stages (Baker, 1999; Hillyard, 1996). Following attachment to the host, the female adults are fully engorged after 5 to 6 days and the pre-ovoposition period lasts 4 to 12 days, after which they ovoposit batches of eggs within about 37 to 59 days (Soulsby,1982). The entire life cycle from egg to adult can be completed within fourteen weeks minimum (Baker, 1999, Hillyard, 1996).

I.3.2.2.3. *Rhipicephalus sanguineus*

Commonly known as the “brown dog tick”, this species, thought to be the most widely distributed tick species in the world (Wall et Shearer, 1997) is a three-host tick with a very wide range of hosts. Though it occurs on a wide variety of mammals (equids, cattle, sheep…etc) and birds (Soulsby, 1982), it is particularly associated with dogs in kennels. It feeds on dogs during all the three life stages, but drops off and reattaches during each stage. Eggs are deposited in the cracks of kennel walls or other areas used frequently by dogs, in batches of up to 5000. Larvae hatch from the egg within 20 to 30 days and begin to quest shortly after. Larvae, nymphs and adults may infest the same host. Under favorable conditions, the life cycle may last 63 days but adults have been found to survive for up to 568 days without feeding (Wall et Shearer, 1997).

I.3.2.2.4. *Rhipicephalus bursa*

This is a three-host tick, feeding once in each of the larval, nymphal and adult life cycle stages. They occur in a wide host range including cattle, sheep, horses, dogs (Soulsby, 1982, Goddard, 2000). After engorging, as a larva and nymph, it drops from the host and then moults,

before locating a further host. After engorging, adult females drop to the ground, lay their eggs and then die.

I.4. Etiological agents of equine piroplasmosis

Two different protozoa of the genus *Babesia* and *Theileria* are known to cause piroplasmosis in equids. They are *Babesia caballi* and *Theileria equi* respectively. However, *Babesia canis canis* of dogs (Criado-Fornelio et al., 2003, Hornok et al., 2007) as well as *B. bovis* of cattle (Criado et al., 2006) have also been reported in horses. .

I.4.1. Taxonomy and morphology

I.4.1.1 *Theileria equi* Mehlhorn, Schein 1998

Synonyms: *Piriplasma equi, Nuttallia equi, N. asini, N. minor, Nicollia equi*, *Babesia equi.*

The taxononic classification of *Theileria* spp. places them in the phylum Apicomplexa (Sporozoa), class Aconoidasida (Piroplasmea), order Piroplasmida, and the family Theileriidae (Soulsby, 1982; Mehlhorn et al., 1998).

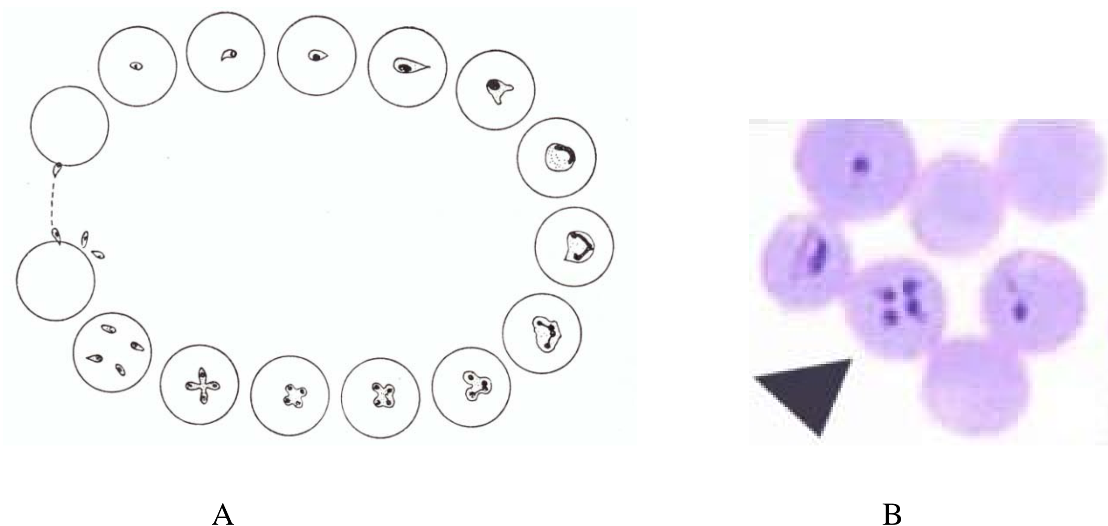
Piroplasms are characterized by intraerythrocytic forms which can be pear-shaped. They have apical complex organelles (including rhoptries and micronemes), a merogonic stage within the vertebrate host erythrocytes, and sexual development and sporozoite formation within the invertebrate host.

The *Theileriidae* are round, ovoid, rod-like or irregular forms, found in lymphocytes, histiocytes and erythrocytes; they do not produce pigment and are transmitted by ixodid ticks (Soulsby, 1982).

Theileria species multiply by schizogony in lymphocytes and finally invade erythrocytes. The development in the tick vector is quite distinctive as the *Theileria* zygotes unlike *Babesia*, do not multiply but invade the hemolymph of the tick and then go towards the salivary glands; there is no invasion of other organs, and no passage through the ovaries and the eggs. The zygotes are much larger but less numerous than the vermicules of *Babesia* and are commonly called ookinetes. When the next stage of the tick vector attaches to a new host, sporogony and maturation of the sporozoites in the salivary glands occur, and transmission takes place by the

injection of infected saliva. Moreover, the tick loses its Theilerial infection after having transmitted it; the infection does not persist to the next stage let alone the next generation (Uilenberg, 2006)

Morphologically, *Theileria equi* sporozoites are ovoid or spindle-shape, 3 to 4 x 1 to 2 µm in size, while merozoites within the lymphocyte measure 1.5 to 2 µm. Merozoites within red blood cells are spherical (when they just entered erythrocytes), pyriform measuring 2 to 3 µm-long (after the first stage of binary fission) or pear-shaped measuring about 2 µm in length. Usually, four pear-shaped stages form a tetrad in a form of a Maltese cross (Fig.9 B) in the red cells, characteristic of the species. Besides, spherical, or ovoid stages, as large as about 2-3 µm in diameter, looking like the ring forms (Fig. 9A) in malaria parasites occur and are considered to represent gamonts (Melhorn et Schein, 1998)



A B

Fig 9. Various forms of *T. equi* seen during multiplication in the red blood cells (reproduced from Soulsby et al., 1982) (A) and the characteristic Maltese cross in a stained red blood cell (B).

I.4.1.2. *Babesia caballi* Nuttall, 1910

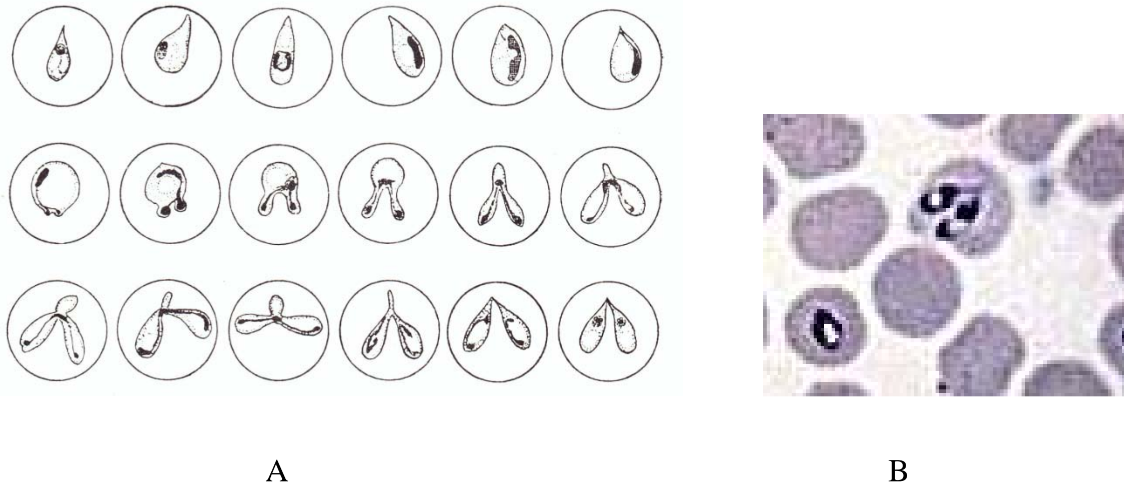
Synonym: *Piroplasma caballi*

This species falls into the family of *babesiidae* within the order *Piroplasmida.* Members of the family *Babesiidae* are relatively large organisms. They are round, pyriform or amoeboid forms occurring in the erythrocytes (Soulsby, 1982).

Organisms of the genus *Babesia* multiply in the erythrocytes by asexual division, resulting in two or four non-pigmented daughter cells (Soulsby, 1982) which leave the previous red cells and enters each into another erythrocyte (Uilenberg, 2006). Within the tick vector, *Babesia* zygotes multiply, and the "vermicules" which result invade numerous organs of the tick, including the ovaries. In this way, the infection passes through the ovary and the eggs to the next tick generation. This is called transovarial transmission. It is usually the female tick that becomes infected and sporogony takes place in the salivary glands of larval, nymphal and/or adult ticks of the next generation. When the tick attaches to a new host, maturation of the sporozoites takes place and the host is infected with saliva from the tick. Certain species of *Babesia* can persist over several tick generations, even without new infections (Uilenberg, 2006).

Babesia are grouped informally into the small *Babesia* (trophozoites are 1.0 to 2.5µm) and large *Babesia* (trophozoites measure 2.5 to 5.0 µm; species include *Babesia caballi* among others). These morphological classification are generally consistent with the phylogenetic characterisation based on the nuclear small subunit ribosomal DNA (nss-rDNA) sequences, which show that the large and small *Babesia* fall in two phylogenetic clusters, with the small *Babesia* being more related to *Theileria spp* than the large (Homer et al., 2000).

Babesia caballi (Fig.10) is a large species which morphologically resembles *B. bigemina*. Organisms commonly occur as pairs, are pyriform and measure 2.5 to 4 µm in length; the angle formed by the organisms is acute (Fig. 10B). Round or oval forms, 1.5 to 3 µm in diameter may also occur



A B

Fig 10. Various forms of *B. caballi* seen during multiplication in red blood cells (reproduced from Soulsby et al., 1982) (A) and the characteristic pair of joint pyriform organisms forming an acute angle in a stained red blood cell (B)

I.4.2. Life cycle

I.4.2.1. *Theilera equi*

The tick vectors of *T. equi* encompass 4 genera of ixodid tick namely: *Dermacentor, Boophylus*, *Hyalomma* and *Rhipicephalus* (Table 1). The host range consists of horses, mules, donkeys, zebras, pony but human infections have been reported in the USA (Ash and Orihel, 1990). Schizogony takes place in the vertebrate host, and gamogony and sporogony occur in the tick.

Schizogony (asexual reproduction in the vertebrate host) starts after injection via tick saliva of the ovoid or spindle-shaped sporozoites in the vertebrate host organism. Therein, the sporozoites penetrate into the lymphocytes, where the formation of the large macroschizonts and microschizonts occurs, giving rise to about 200 merozoites per infected cell. These merozoites enter erythrocytes, become spherical, and start reproducing by binary fission, thus forming 2- to 3 µm -long, pyriform erythrocytic stages. In several erythrocytes, asexual division elicits four pear-shaped stages forming a characteristic arrangement called Maltese cross (Fig. 9B). After

rupture of the red blood cells, the erythrocytic merozoites enter other red blood cells, become spherical therein, and another phase of asexual reproduction starts. Nevertheless, some of these penetrated stages become spherical or ovoid, reaching a diameter of about 2-3 µm. They resemble the ring form in malaria parasites (Fig. 11) and are thought to represent gamonts (Melhorn et Schein, 1998).

Gamogony (formation and fusion of gametes inside the tick gut) occurs soon after ingestion by the ticks of gamonts which persist unchanged in the intestine during the first 2 days. Beginning at 48-72 h after infestation of the ticks, these gamonts grow rapidly, reaching diameters of about 2-3 µm and then start nuclear reproduction and formation of protrusions, thus appearing as ray bodies. These multinuclear, about 5-to 6- µm long stages, which divide into thread-like stages, are considered to be microgamonts, the thread-like stages representing microgametes. Concurrently, some unchanged spherical (ring-shaped) stages occur and are considered to be the macrogamonts or macrogametes. The microgametes and the macrogametes form conglomerations, within which two stages always fuse to form zygotes. Inside the growing zygote a single, club-shaped kinete measuring 14 µm in length (range 11-18 µm) is quickly formed reaching a width of 4-7 µm at its anterior end. From day 5-7 of infestation, some of the kinetes are free in the hemolymph of the tick (Mehlhorn et Schein, 1998).

Sporogony (asexual reproduction in salivary glands) begins after penetration of the kinete into the salivary gland cells (type III) from days 7-8 of infestation. At first, large multinuclear sporogonic masses (sporonts) form inside the hypertrophying host cells. These sporonts divide into several spherical, multinuclear sporoblasts at the periphery of which the ovoid-to-spindle-shaped sporozoites form. The latter reach a size of about 3-4 x 1-2 µm and have an intensively stained spherical nucleus. The full development is completed between day 6 and day 24 after tick repletion (Mehlhorn et Schein, 1998). The complete life cycle is depicted in Fig. 11.

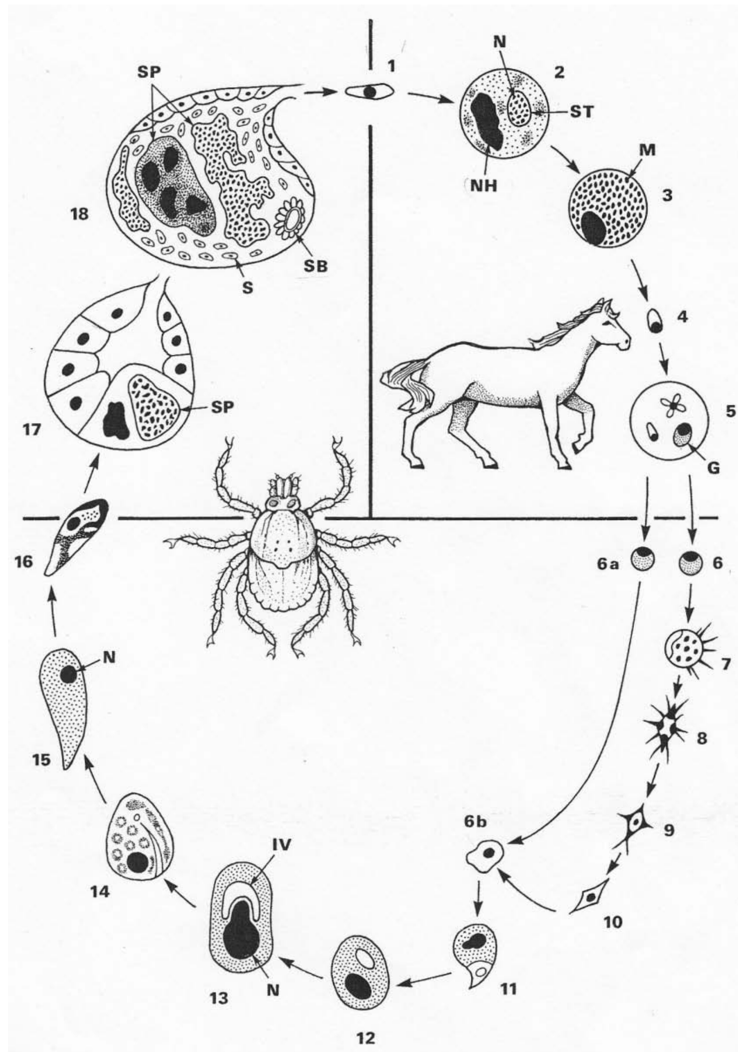


Fig.11 .Diagrammatic representation of the life cycle of *T. equi*. G: gamont; M: merozoite; N: nucleus/nuclei; NH; nucleus of the host cell; S: sporozoites; SB: sporoblast; SP: sporont; ST schizont; [1 Sporozoite injected with tick saliva; 2 young schizont in a lymphocyte; 3 late schizont in a lymphocyte during the formation of merozoites; 4 free merozoite; 5 reproduction inside erythrocytes – note the occurrence of Maltese-cross-like dividing stages and the presence of spherical stages (gamonts); 6 after engorgement of ticks the ovoid/spherical gamonts undergo further development within the blood masses inside the intestine (mostly inside the blood cells; 7-10 by divisions, some ray-like microgametes (10) are produced by microgamonts (7-8); 11 fusion (symgamy) of gametes; 12-16 inside the zygote (12) a slender, motile, club-shaped kinete is developed, which leaves the intestinal cells and enters via hemolymph the salivary cells of the tick after their moult (larva to nymph or nymph to adult female) and their attachment to another host; 17 penetrated kinetes grow up inside the salivary gland cells and give rise to multinucleated

sporonts; 18 the multinucleated sporonts become divided into numerous small sporoblasts, which form sporozoites by a budding process at their periphery; during the next sucking period the sporozoites are injected with the saliva into the new host] (adapted from Mehlhorn and Schein, 1998).

I.4.2.2. *Babesia caballi*

More than four genera of ixodid tick harbor and transmit *B. caballi* to vertebrate hosts on feeding (Table 1).Vertebrate hosts include horses, donkeys, mules, ponies. Like *T. equi*, *Babesia* organisms go through three main stages of reproduction in their life cycle: schizogony in the vertebrate host, gamogony and sporogony in the tick.

Schizogony. After their transmission via tick saliva in the host, the sporozoites of *Babesia* spp infect the red cells; invasion of the host erythrocytes is through a process of invagination forming a parasitophorous vacuole (Homer et al., 2000). The vacuole membrane gradually disintegrates, and the parasite is left with the defining piroplasm feature of a single membrane, in contrast to plasmodium species, which invade by similar mechanism but retain the host membrane (Homer et al., 2000). Within the erythrocytes, the sporozoites grow and become trophozoites which divide by binary fission to produce more merozoites. These latter lyses the cell and go on to infect additional cells, the process being repeated until a large number of erythrocytes are parasitized. Rapid reproduction destroys the host cell and leads to haemoglobinuria (Homer et al., 2000). There is a controversy about the occasional status of some cells showing a multiple infections, which is thought to be a result of binary fission of trophozoites rather than a multiple invasion of the cell (Soulsby, 1982). In *B. caballi* infections, atypical blood forms may occur following multiple infections of erythrocytes (Fig. 12). Some trophozoites however do not reproduce, increase in size and become potential gametocytes (Homer et al., 2000). Later on in the tick gut, these gametocytes will develop into gametes prior to leaving the erythrocytes.

Gamogony. All stages of *Babesia* are still detectable within the consumed erythrocytes in ticks 10 h after the latter begin to feed on an infected host (Homer et al., 2000). Only the gametocytes develop new organelles, most notable of which is an arrowhead-shaped organelle called Strahlenkorper or ray bodies at the anterior end of the organism. The arrowhead structures

are likely involved in the fusion of the gamete (Homer et al., 2000). The resulting zygote uses the arrowhead to enter the epithelial cells of the tick gut where the parasites move to the acini via the hemolymph (Rudzinska et al., 1983).

Sporogony. Sporozoites development within the salivary gland can be divided into three stages. The parasites first expand and fill the hypertrophied host cell, forming a multinucleate sporoblast which is relatively undifferentiated, three- dimensional, branching meshwork from which the sporozoites will bud. Then, the specialized organelles of the future sporozoites (micronemes, rhoptries, and double membrane segment beneath the plasma membrane) develop within the meshwork. Finally, the mature sporozoites form through a budding process. Mature sporozoites are pyriform and contain a smooth endoplasmic reticulum, free ribosome, mitochondrion-like organelle, a single anterior rhoptry, and several micronemes.(Homer et al., 2000).

In large *Babasia* like *babesia caballi*, transovarian transmission occurs. Once in the hemolymph, the zygotes which first differentiate into "kinetes" may invade other cells, such as fat body cells or nephrocytes, and undergo a second cycle of division (Telford et al., 1993). These secondary kinetes penetrate the gut wall and attach to the cells of various organs, especially the ovaries of the tick. In these cells a multiple division occur again with the formation of a third generation of kinetes which at this stage penetrate the eggs of the tick. After the larvae have hatched and are attached to the host, during the suction phase some of the kinetes penetrate the salivary glands. The remaining kinetes penetrate other cells and infect the salivary glands first in the nymph or adult stage. The last phase of multiplication takes place in the salivary glands in which a large number of small spherical or pear-shaped 2.5 to 3-μm-sized sporozoites evolve. In addition to the typical transovarial transmission, *B. caballi* can be transmitted transtadially, from the larvae to the nymph and from the nymph to the adult tick. Because of the multifarious propagation of *B. caballi*, the tick vectors represent an infection reservoir and can remain infectious for horses over many generations (Schein, 1988)

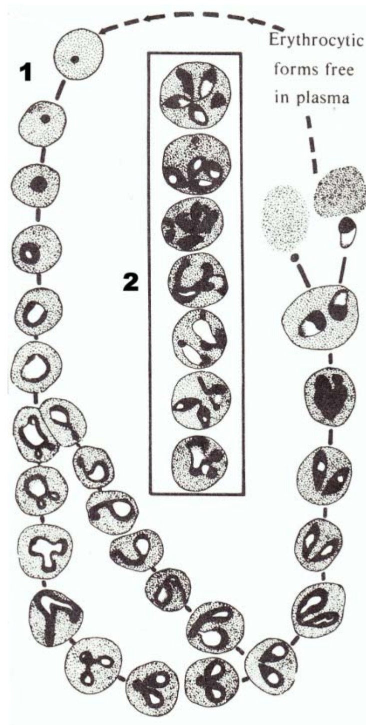


Fig.12. Life cycle of *B. caballi* in erythrocytes of the vertebrate host (horse): multiplication of *Babesia* organism occurs in the erythrocytes by budding process called schizogony to form two, four or more trophozoites which are liberated to invade other cells; the process is repeated until a large number of blood cells are parasitized (1); occasionally a cell show multiple infection with a large number of trophozoites but it is considered that this represent a series of binary fission rather than a multiple invasion of the blood cell (2) (adapted from Soulsby, 1982).

I.5. Genetic diversity of equine piroplasms

Data on the morphology, anatomy, host affiliation, infection sites and life cycle of parasites do not provide sufficient information for the identification, differentiation and genotype of species. In addition, the epidemiology and pathogenicity of a species and the evolutionary relationship within species are only partially understood based on the aforementioned data alone. The genetic data have been extensively used in various fields to address the problem posed by *Theileria* and *Babesia* species to livestock (Kappmeyer, 1999).

1.5.1. Genes commonly targeted.

1.5.1.1. In *T. equi* species

A number of genes have been targeted in the identification, differentiation and characterization of *T. equi*, including the merozoite antigen-1(EMA-1) gene (Kappmeyer, 1993, 1999), the merozoite antigen-2 and 3(EMA-2, EMA-3) genes (Ikadai et al., 2006), the 16S rRNA gene (Bashiruddin, 1998) and the 18S rRNA gene (Nicolaiewsky et al., 2001; Rampersad et al., 2003; Criado et al., 2006). The 18S rRNA is most commontly employed among other genes as it is present in multiple copies through the genome (Alhassan et al., 2005), contains highly repeated sequences and is also the most conserved (Hillis et al., 1996; Prichard, 1997). In addition, this gene tends to be conserved between different members of the same species, so that intraspecific variation is very low compared with interspecific variation (Prichard, 1997). A useful locus conferring on this gene a great importance in epidemiological investigations and genetic diversity includes the V4 hypervariable region of the small subunit (SSU) of the gene (Gubbels et al., 1999 Bhoora et al., 2009). In addition it is more convenient to use the 18S rRNA gene in DNA-based studies due to the availability of DNA sequences in molecular databases (Criado-Fornelio et al., 2003; Nagore et al., 2004; Bhoora, 2009).

1.5.1.2. In *Babesia caballi* species

Various genetic markers have been employed for the differentiation and characterization of *B. caballi*, comprising the BC48 and 134 protein genes (Tomaki et al., 2004), the rhoptry-associated protein (RAP-1) gene (Kappmeyer et al., 1999), the 16 S rRNA gene (Bashiruddin et al., 1999) and 18S rRNA gene (Nagore et al., 2004). Of all these genes, the 18S rRNA gene remained the most common target gene in various investigations.

I.5.2. Diversity within *T. equi* species

On the basis of the 18S rRNA gene and using the polymerase chain reaction (PCR)-based techniques followed by sequencing, different genetic variants of *T. equi* (isolate Spain1 and Spain 2) differing by four bases and sharing 99% identity with the African genotype have

been described (Criado-Fornelio et al., 2003). With the aid of the reverse line hybridization (RLB) assay, two *Theileria* genotypes *(T. equi* and *T. equi*-like) showing 96.8 % similarity between their 18S rRNA gene sequences were differentiated in Spain (Nagore et al., 2004). *T. equi* genotype was 99.5 % identical to the previously described *T. equi* Spain 1 and Spain 2 and regarded as a strain variation of these isolates. The other genotype, *T. equi*-like sharing 96.8% similarity with *T. equi* 18S rRNA gene is considered more pathogenic (Nagore et al., 2004). Evidence of genetic polymorphism occurring in the 18S rRNA gene of Spanish *T. equi*-like has been reported whereby *T. equi*-like isolates in southern Spain shared 99.4 % identity with the northern *T. equi*-like isolates (Criado et al., 2006). Moreover, the isolate from southern Spain was more closely related (99.8%) to *T. equi* from Swiss tick than *T. equi*-like from Northern Spain (Criado et al., 2006). In South Africa, twelve distinct *T. equi* 18rRNA gene sequences have been documented and grouped in three clades identified as group A, B and C (Bhoora et al., 2009). Group A contained the previously published African *T. equi* and all the Spanish *T. equi* strains. Group B contained new isolates from Zebra and *T. equi*-like isolate from Spainish horses (Nagore et al., 2004). Group C encompassed the new African *T. equi* 18S rRNA sequences distinct from previously published 18S rRNA sequences of *T. equi*.

1.5.3. Diversity within *B. caballi* species

Based on the sequences of the 18S rRNA gene, and using PCR-based techniques followed by sequencing, two genetic variants of *B. caballi* have been reported in Spanish horses (Criado-Fornelio et al., 2004). The first variety, isolate Spain1 showed a relatively low similarity with the African genotype (97% identity) while the second variety represented by isolate Spain 2 and 3 showed a high genetic similarity with the African genotype (97.7-100% identity). Using the RLB assay followed by sequencing, investigation of the V4 region of the SSU of the 18S r RNA gene revealed the occurrence of two *B. caballi* genotypes in Spain, one of which was very similar to the African genotype and identified as such (Nagore et al., 2004). The other genotype, *B. caballi*-like showed lower similarity (97.4%) with typical *B. caballi* from South Africa. Using the same techniques and targeting the same markers of the 18S rRNA gene, two main genetic groups of *B. caballi* 18S rRNA gene sequences have been documented in South Africa (Bhoora et al., 2009). Group A contained *B. caballi* Spain 1 and *B. caballi*-like genotype from Spain (Criado-Fornelio et al., 2004, Nagore et al., 2004) as well as *B. caballi* USDA reference strain

(*B. caballi.* Ames, Iowa) (Kappmeyer et al., 1999). Group B comprised the newly identified isolates and the original African *B. caballi* genotype (Allsopp et al., 1994).

There is a high level of genetic diversity in the *T. equi* and *B. caballi* 18 S rRNA gene, both in Europe and Africa which may have a great influence in their control since DNA sequence differences between various strains of a single species have been pointed out as an ultimate cause of incomplete immunisation after vaccination trials, impaired immunologic diagnosis or poor chemotherapeutic results (Criado et al., 2004).

I. 6. Equine piroplasmosis

I.6.1. Distribution and transmission

EP is established in tropical, subtropical and temperate regions of the world ((Mehlhorn and Schein, 1998). EP is endemic in many parts of Asia, Arabia, South and Central America and Europe (Fig. 13). In the latter area it is more prevalent in France (Leblong et al., 2005), Portugal (Bashiruddin et al., 1999), Spain (Camacho et al., 2005), Italy (Moretti et al., 2009) and Turkey (Karatepe et al., 2009). The disease is transmitted to the vertebrate host via bites of infected immature and adult ticks. However, accidental transmission through contaminated needles, syringes or any device in routine surgery, castration or vaccination procedure (iatrogenic transmission) can occur. Though infected ticks, contaminated surgical and vaccination materials readily ensure the disease propagation, another mode of disease spread, not the least, includes the importation or introduction of piroplasm-positive animals and/or exotic tick in disease-free areas where competent hosts and tick vectors coexist (Martin, 1999; Butler et al., 2005).

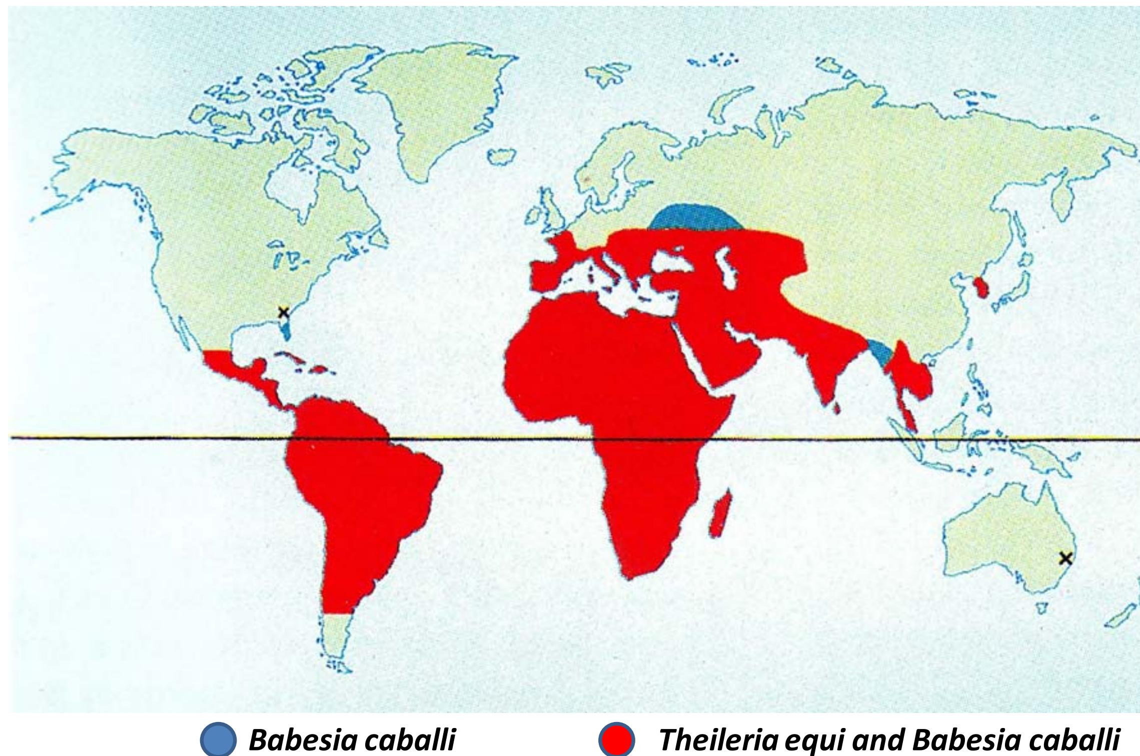


Fig.13. Geographic distribution of equine piroplasmosis. The crosses mark localities where isolated outbreaks occur (adapted from Kaufmann, 1996). EP outbreak has been documented in several states in the USA since October 2009 (http://www.thehorse.com/ViewArticle.aspx?ID=15300).

I.6.2. Host susceptibility

All breeds of horses are equally susceptible to *B. equi* infection, although the disease is more marked in older horses (Soulsby, 1982). Similarly, there is no difference in susceptibility to *T. equi* and *B. caballi* for male and female hosts (Asgarali, 2006). Various determinants are involved in the severity of the disease manifestation. Among those identified in horse are mixed infections with the two causative agents (Soulsby, 1982), strenuous exercise (Hailat, 1997) and stress.

I.6.3. Clinical presentation

The incubation period varies according to the parasite in presence. With *B. caballi*, it ranges between 1 to 3 weeks (Maslin et al., 2004) and varies from 2-10 days up to 21 days depending on the vector tick (Mehlhorn et Schein, 1998) in *T. equi* infection

The infection may be asymptomatic but the clinical manifestations vary a great deal depending on the parasite involved.

In *B. caballi* infection, the course may be acute or chronic, mild or severe and in some cases leads to death. In acute form, following the incubation, the disease starts with a sudden and important increase in body temperature (41-42 °C) which lasts 1 to 2 days and then persists in plateau for 8 to 10 days. These high temperatures are associated with the following clinical signs: typhus, anorexia, congestion of the mucous membrane, polypnea and tachycardia. Anemia with icterus may occur a few days later as well as a more retarded haemoglobinuria. As in all *Babesia*, atypical forms may be observed, with various gastrointestinal manifestations. In absence of treatment, this classical acute form leads to death within 1 to 4 weeks after the onset of clinical signs (Maslin et al., 2004). Disturbances of the central nervous system (CNS) are common and may result in posterior paralysis (Soulsby, 1982). Restlessness, nervousness, and walking in cycle with incoordination are the clinical signs associated with the CNS disorder. In chronic form, persistent anemia correlated to the maintenance of the parasites in the host occurs. The carrier state of the host for *B. caballi* may last several months or years (Maslin et al., 2004). The time of persistence of *B. caballi* was shown to vary between 1 to 4 years (Hourrigan and Knowles, 1979; Schein, 1998; de Wall et van Heerden, 1994) and decrease with increasing age (Rüegg et al., 2007).

With infection due to *T. equi*, clinical signs at the beginning are as follow: a slight increase in body temperature from 39 to 40 °C (Maslin et al., 2004), listlessness, depression, marked thirst, inappetence, watering of the eyes and swelling of the eyelids (Mehlhorn and Schein, 1998). The rise in body temperature coincides with the appearance of the organisms in the circulating blood (Soulsby, 1982). In acute cases, the disease process lasts 8 to 10 days and if recovery is to occur there is a fever crisis around the tenth day; thereafter, the body temperature falls to normal (38°C) and animal recovers and becomes carrier (Soulsby, 1982). Without treatment, mortality may reach up to 50% in older horses experiencing an initial infection (Shkap, 1998). During this acute phase the host presents with intermittent fever, significant anemia with lymphocytosis and constant icterus; there may also be heamoglobinuria, hard feces

covered with yellowish mucus, oedema and petichiae of the mucous membrane. In peracute cases, death may occur in one to two days after the onset of the clinical signs and 30 to 90 % of erythrocytes may be infected (Maslin et al., 2004). The subacute infection develops more slowly and is more prolonged, followed by a recovery that may take several weeks or months (Soulsby, 1982). Nervous disturbances and posterior paralysis observed in *B. caballi* infections are absent. Chronic form may be found, characterized by a moderate anemia, subicterus, lack of performance, body weight loss and non- specific signs; the residual parasitemia may last many years.

The pathological changes readily seen include enlargement of the spleen and liver, flabby kidney with petichial hemorrhages in acute cases and oedema of the lungs and terminal pneumonia in severe cases (Soulsby, 1982). *T. equi* produces an acute tick-borne hemolytic anemia in susceptible horses that can be followed by a chronic carrier state and result in reduced oxygen-carrying capacity which causes decreased performance of racehorses (Homer et al., 2000).

I.6.4. Diagnosis

The diagnosis of piroplasmosis is based on the clinical manifestations, the presence of tick vectors, the history of the area, and the demonstration of either the parasites or the antibodies to the parasites in the host. However, for purpose of confirmation or specific diagnosis, the demonstration of the parasites or the antibodies to the parasite is more often achieved, using several techniques.

I.6.4.1. Haematology

Examination of blood smear for the presence of parasites within the erythrocytes is useful in diagnosing acute infection with both *T. equi and B. caballi.* Trophozoites of *B. caballi* are apparent within the red blood cells as round, oval or elliptical structures but commonly occur as paired, pyriform organisms joined at their posterior ends that discriminate them from *T. equi* organisms (Fig. 10B*). T equi* trophozoites appear as oval, round, elliptical or spindle-like structures .Very most frequently, *T equi* erythrocytic merozoites cluster to form a typical,

characteristic “Maltese cross” (Fig.9B) which distinguishes *T. equi* to *Babesia caballi* organisms. The most satisfactory site to obtain blood sample is the skin of ear, the first drop of blood usually having the greatest number of parasites (Soulsby, 1982). Blood examination is best done during the fever periods as subsequently organisms became scarce and scant in the blood (Soulsby, 1982). Therefore in the event of low parasitemia when fever has subsided, blood smear technique becomes inappropriate and results in inaccurate diagnosis. Other detection techniques exist that rely both on the immunological factors induced by the parasites in the host and the parasite genome.

I.6.4.2. Serology

The serological diagnosis of disease based on the detection of circulating antibodies is one of the techniques available for the identification of current and previous exposure to infectious agents. A range of tests to detect antigen/antibody reactions has been developed over the past decades for *Theileria equi* and *Babesia caballi* infections including: the complement fixation test (CFT) (Friedhoff, 1982; Donnelly et al., 1982; Weiland et al., 1986), the card test (Amerault et al., 1979), the indirect fluorescent antibody test (IFAT) (Callow et al., 1979; Donnelly et al., 1980), the enzyme-linked immunoabsorbant assay (ELISA) (Bruining et al., 1997); Kappmeyer et al.,1999), a monoclonal-antibody (Mo.Ab) based inhibition ELISA or competitive ELISA (cELISA) (Knowles et al., 1991; Shkap et al., 1998), the immunochromatographic test (BeICT) (Huang et al., 2004).

For many years, the reference technique for serodiagnosis and epidemiological studies recommended by the “Office International des Epizooties”(OIE), the world organization for animal health and the United States Department of Agriculture (USDA) were IFA and CFT but cross reactions between species have been reported (Bruining, 1996; Papadopoulos et al., 1996,).The c-ELISA was demonstrated to be of great value (Shkap et al., 1998), validated and recommanded by the OIE and approved by the USDA as the official diagnostic assay (Goff et al., 2006; OIE, 2008). The main disadvantage of the IFAT is that the antigen is prepared by the spreading of blood on slides with various percentages of infected erythrocytes, and interpretation is subjective. Alternatively, the c-ELISA is performed by coating plates with known amounts of antigen, thus providing improved standardization above that obtained with IFAT. Moreover the c-ELISA requires small amount of recombinants antigen and monoclonal antibody, both

available in vitro and in contrast to IFAT, the result is recorded automatically, allowing the testing of large number of samples simultaneously.

I.6.4.3. Molecular biological techniques

The use of immunodiagnostic methods has certain limitations since the demonstration of antibodies against a parasite provides little information on whether the parasite is still present in the animal's body or not. Moreover, if the parasite is in primary infections, the disease will develop well before antibodies become detectable. Therefore the need to be able to detect the parasites directly has led to the development of highly sensitive and specific techniques based on the demonstration of parasites' nucleic acid sequences.

Early detection of equine piroplasms by molecular biology techniques was achieved with probes which detect higher number of carrier animals than microscopic methods (Posnett and Ambrosio, 1989; Posnett et al., 1991). Repetitive DNA probes constructed for the detection of *T. equi* (Posnett et Ambrosio, 1989) were shown to be specific as they did not hybridize to DNA from horse blood infected with *B. caballi* (Posnett et al., 1991). However, these probes were found to be equivalent in sensitivity to Giemsa-stained thin blood smears (Böse et al., 1995). For detection of parasite nucleic in blood with high sensitivity and specificity, the polymerase chain reaction (PCR) assay (Saiki et al., 1985, 1988a) has largely superseded DNA probes. PCR tests with high sensitivity for *T. equi* and *B. caballi* have been developed, which detect the parasite genome in blood with an approximated parasitemia of 0.000083% (Bashiruddin et al., 1999) up to 0.000006 %(Nicolaiewsky et al., 1999). Improved PCR assays have been constructed for the routine detection of *T. equi* and *B. caballi*, such as the nested PCR (Rampersad et al., 2003) and the single round and multiplex PCR (Alhassan et al., 2005).The nested PCR was shown to be both superior to Giemsa- stained films and the primary PCR methods; the single round and multiplex PCR simultaneously detect *B. caballi* and *T. equi* with improved sensitivity and rapidity and was documented to be simpler and more rapid than the nested PCR. Though these assays accurately detect and differentiate between *T. equi* and *B. caballi*, they remain limited as they cannot detect unknown piroplasms present in equine blood.

Indeed these molecular assays target known species that were early detected and identified based on morphological parameters and host specificity. It is suspected, however that many of the early description were of similar or identical species that the traditional methods

could not distinguish (Persing et al., 1995a, b). Many arguments are in favor of this suspicion: different parasites in the same host may appear to be morphologically similar (*Babesia caballi* and *Babesia canis* in horse (Purnell, 1981;Criardo et al, 2003), *plasmodium* and some *Babesia* species); the same parasite may have different microscopic appearance in different hosts, probably due to host- specific factors such as splenic function and immunological predisposition (*Babesia divergence* has its characteristic appearance in bovine erythrocytes but in humans, it exhibits extensive pleomorphism which complicate it diagnosis; the classification of *Babesia* and *Theileria* based on host specificity proves less useful than once thought, since certain extensively studied species such as *Babesia microti* have been shown to have a broad host specificity (Brandt et al., 1977, Etkind et al., 1980; Spielman et al.,1981). Similarly *T. equi* have been documented besides its traditional hosts, in humans (Ash and Orihel, 1990; Goddard, 2000) and in dogs (Criardo-Fornelio et al., 2003).

Among the current molecular- biological methods, the reverse line blotting (RLB) hybridization assay appears to be the most versatile technique as it allows a simultaneous detection of all piroplasm parasites in the host (Gubbels et al.,1999; George et al., 2001; Nagore et al., 2004) with a sensitivity comparable to the nested PCR protocol (Nagore et al., 2004). The RLB was initially developed as a reverse dot blot assay for the diagnosis of sickle cell anemia (Saiki et al 1988b) but the essence of both techniques is the hybridization of PCR products to specific probes immobilized on a membrane in order to identify differences in the amplified sequences. In the “line” approach, multiple samples can be analyzed against multiple probes to enable simultaneous detection (Gubbels et al., 1999). This approach initially developed for the identification of streptococcus serotypes (Kaufhold et al., 1994) was used in cattle (Gubbels et al., 1999), in equids (Nagore et al., 2004) and in small ruminants (Altay et al., 2007).

I.6.4.4. Alternatives techniques

In order to address problems posed by the aforementioned techniques, some alternatives are often used. In cases of false- positives or false-negative reactions encountered in serological tests (Donnelly et al., 1980; Herr et al., 1985), the passage of blood from a suspected animal to a disease-free host will help determine the true status of an animal. Large quantities of blood (500ml for instance) are transfused into a susceptible, preferably splenectomised horse and then, the animal is kept under close observation for clinical signs of disease (O.I.E, 2008). Diagnosis is

confirmed by the presence of parasites in its red blood cells. Alternatively, a specific clean tick vector is fed on a suspect animal and the organism can then either be identified in the tick or through the transmission of the organism by the tick to another susceptible animal. Another alternative supplement to the previous methods is the *in vitro* culture of *T. equi* and *B. caballi* for the identification of carrier animals (Holman et al., 1993) Zweygarth et al., 1995, 1997). Thus *B. caballi* parasites were successfully cultured from the blood of two horses tested negative by the CFT (Holman et al., 1993). Similarly *T. equi* could be cultured from horses that did not show any patent parasitemia at the time of the initiation of the cultures (Zweygarth et al., 1995, 1997).

I.6.5. Differential diagnosis

There are a number of conditions that should be considered and which resemble equine pirolasmosis. These include: surra caused by Trypanosomes, equine infectious anemia caused by a retrovirus, dourine caused by *Trypanosoma equiperdum*, Africa horse sickness caused by an Orbivirus, purpura hemorrhagica (purpura associated with a reduction in circulating blood platelets which can result from a variety of factors) and various plant and chemical toxicities (CFSPH, 2008).

I.6.6. Prognosis

Early diagnosis and successful treatments generally elicit favorable prognosis in young animals exhibiting for the first time an acute form of disease. In contrast, it becomes more guarded for cases displaying clinical symptoms for several days, in old animals with past history of piroplasm infections or suffering from another disease, such as renal failure (Maslin et al., 2004).

I.6.7. Immunity

All mammalian host examined have been able to develop immunity to *Babesia* species either after an episode of infection and recovery or after prophylactic immunization (Homer et al., 2000). Both humoral and cellular factors are involved in immunity to babesiosis. However the humoral component of the immune system is currently of limited importance in protection

against babesial infections as the protective role of antibodies seems to be restricted to a short window of time between the moment that the parasite gains access to the bloodstream and the time it invades the target cells (Homer et al., 2000)

In general, horses born and raised in endemic areas are more resistant to piroplasmosis than those originating from disease-free regions. Like cattle in endemic areas, horses in their younger age are protected by the premunition immunity and considered non receptive until 8 months of age. Thereafter, successive infections may occur, giving rise to the so-called co-infection immunity which does not prevent new parasitic infections but nullifies the clinical expression of the disease (Maslin et al., 2004). There is no cross-immunity between *B. caballi* and *T. equi* (Soulsby, 1982).

I.6.8. Treatment

Chemotherapy is generally effective and constitutes the choice method for the treatment of equine piroplasmosis. Both *B. caballi* and *T. equi* respond to the bactericidal drug but *T. equi* is more refractory to treatment than *B. caballi*.

Imidocarb appears to be the drug of choice, administrated at a dosage of 2.2 mg/kg two times at a 24-hour interval. This regimen is effective against *B. caballi*-infected horses but as for *T. equi* infections which are resistant to treatment, a dose of 4 mg/kg is given four times at a 72-hour interval. Side effects characterized by restlessness, abdominal pain, sweating, rolling and heavy breathing are not uncommon following imidocarb treatment at these higher levels and may even lead to death by toxicosis unless atropine sulfate is administered (Schein, 1988).

Importantly, it has recently be shown that a high-dose regimen of imidocarb dipropionate (a dose of 4.0 mg per kg of body weight four times at 72- hour intervals via injection) clears *Babesia caballi* infections from persistently infected asymptomatic horses and/or eliminates transmission risk (Schwint et al., 2009)

To date, efficacy of imidocarb against *T. equi* is rather controverted as it seems only to be effective against the intraerythrocytic form and further more when given at higher doses. In contrast, oxytetracycline is a drug which given at a dose of 12mg/kg/day for seven days proves successful in treating *T. equi* infections (Maslin et al., 2004). Other drugs available for the treatment of EP include for instance diminazene diaceturate effective in the chemosterilization of *B. caballi* and in the elimination of clinical signs in *T. equi* infections (Bruning, 1996)

A noteworthy observation is that *T. equi* resistance to the commonly used antibabesiacidal drug (imidocarb) and its succeptibility to many antitheilericidal drug other than oxytetracycline such as halofuginone and parvoquinone (Kuttler, 1988) are parts of arguments for a reclassification of *Babesia equi* as *Theileria equi* (Mehlhorn and Schein, 1998).

Antitheilericidal drugs such as buparvaquone have been demonstrated to be effective in combatting disease due to *B. equi* and may, in combination with imidocarb also eliminate the parasite (Bruning, 1996).

I.6.9. Prevention

Preventive measures against piroplasms infections usually include vaccination, tick control, sanitation and disinfection

I.6.9.1. Vaccination

Although efforts have been invested to develop vaccines for cattle and other animals resulting in effective attenuated vaccines (Callow, 1977 Lawrence, 1997) and recombinant vaccines (Wright et al., 1992 Palmer et al., 1995) there is still no efficacious vaccine available against equine piroplasms. However, there is a report of a successful killed vaccine, utilizing a blood-based antigen, which reduces the severity of subsequent challenge infections in equids (Singh et al., 1981)

However, in highly endemic areas chemoprophylaxis using imidocarb at a dose of 2mg/kg conferrers a 4 to 6- week protection on equine and cattle (Maslin et al., 2004). This chemo preventive dose is to be repeated during the “at-risk” seasons.

I.6.9.2. Tick control

Tick control is an essential part in the fight against tick-borne disease in general. Elimination of tick would eliminate the transmission of pathogens which over time would lead to its eradication. On horses ticks are commonly found in the ears, between the legs, at the tail base but also can be seen everywhere on the body surface. The control of ticks is based on:

- the chemical acaricides applied either by total immersion in a dipping bath or in the form of spray, shower or spot-on

- integrated strategies that consider the host resistance to ticks and the disease they transmit, the seasonal dynamic of ticks infestation and the tick ecology (Young et al., 1988)
- the use of newly developed anti-ticks vaccine (Willadsen et Jongejan 1999, Fragaso et al, 1998; Pipano et al, 2003).

Many biological agents are currently being investigated in laboratories as tick control agent including fungus (Arruda et al., 2005), virus (Assenga et al., 2006) and tick pheromones (Soneshine, 2006).

1.6.10. Zoonotic potential of piroplasm

Human piroplasmosis (babesiosis) is a tick-borne zoonosis commonly caused by *B. microti*, *B. duncani, B. divergens, B. divergens*-like organisms, and *B. venatorum* (Vannier and Krause, 2009). Human infection with equine piroplasms is not well documented and very rare. Unlike *B. caballi*, *T. equi* infection has been reported in North and South America (Ash and Orihel, 1990). Symptoms associated with this infection may encompass fever, hemolytic anemia and hemoglobinuria.

I.7. Overview of the diagnostic and geospatial techniques used in this study

I.7.1. Diagnostic techniques

I.7.1.1. Microscopy

The direct detection of blood parasites based on their morphology relies on blood smear technique. There are two kinds of blood smears available: the thick blood smear and the thin blood smear. The first allows a more efficient detection of parasites with increased sensitivity (Ash and Orihel, 1990) but do not permits a full discrimination of the parasite morphology. The second is more reliable for species identification and was used in this study. To prepare a thin blood film, a small drop of blood is spread over a slide in a thin layer as illustrated in Fig. 14.

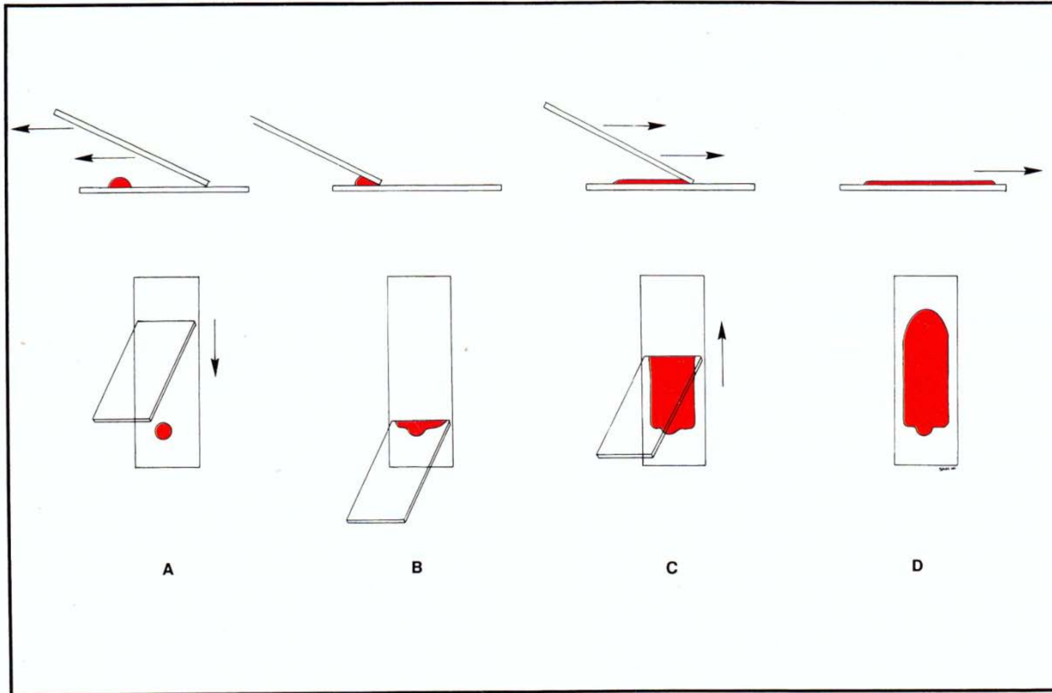


Fig.14. Demonstration of the thin blood smear preparation (adapted from Hendrix and Robinson, 2006.)

Following fixation and staining, the blood components (erythrocytes, white cells, and platelets) are intact. At the terminal, feathered end of the smear, the cells are only one layer thick, allowing easy visualisation of parasites infecting red blood cells.

Absolute methanol is routinely used to fix the dry film while Giemsa's stain is the preferred stain for demonstrating blood parasites in thick and blood film (Ash and Orihel, 1990). Giemsa stain is a mixture of stains based on methylene blue and eosin. The cells are fixed to prevent their explosion due to the osmotic shock in presence of the staining fluid with lower saline concentration. The erythrocytes are transparent and leucocytes are too pale so that staining is required if they are to be observed. Basic component of the cells such as haemoglobin or certain inclusion or granules, which will unite with the acidic portion of the stain (eosin) are said to be eosinophilic. They are stained varying shade of pink or red. Acidic cell component such as nucleic acid, reactive cytoplasm, etc. take up the basic dye component, methylene azure and stain blue or purple. For best staining results, it recommended to adjust the pH of the stain to pH 7.2 for mammalian blood and pH 6.7 for avian blood (Soulsby, 1982), and to process the blood films preferably within 24 hours of preparation (Ash and Orihel, 1990)

Examination of the stained blood film for the presence of protozoan parasites is made at high magnification, using the 40 X objective or the oil immersion objective (100X objective) for greater details (Zajac and Convoy, 2006).

1.7.1.2.a Serology: Enzyme-Linked Immunosorbent Assay (ELISA)

The enzyme-linked immunosorbent assay (ELISA) is a solid-phase immunoassay which employs an enzyme-linked anti-immunoglobulin, as opposed to the solid phase radioimmunoassay (SPRIA) which employs radioactive anti-immunoglobulins (Benjamini and Leskowitz 1988). ELISA tests are immunoassay techniques used for the detection or quantification of a substance based on an immunological reaction (Kemeny, 1991). In diagnostics, ELISA is used to evaluate either the presence of antigens or antibodies associated with an infectious agent in a sample. The first effective enzyme-labeled assay was described by Engvall (1971). ELISA relies on the following principles outlined by Crowther (1995).

1) Proteins (antibodies, antigens) and carbohydrates can be passively attached (adsorption) to solid surfaces such as plastic, referred to as solid phase in the assay

2) The separation of bound and free reagents after the incubation period is easily made by simple washing procedure, because one of the components is attached to a solid phase by passive adsorption.

3) One of the reagents can be conjugated to an enzyme which subsequently allows obtaining the ELISA results as a color reaction that may be observed by eye and read by dedicated machines. The diagrammatic representation of ELISA is depicted in Fig 15.

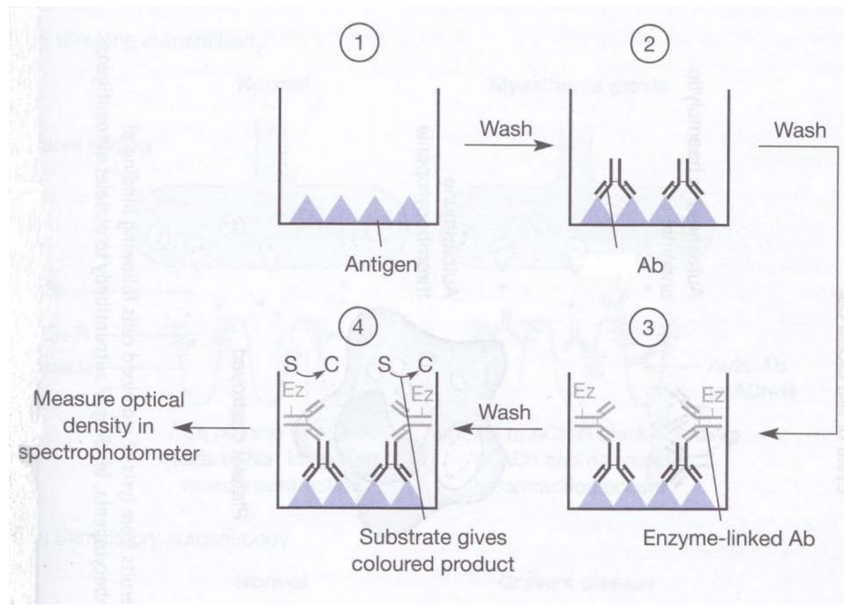


Fig 15. Schematic representation of ELISA principle (adapted from Wood, 2006). Antigen is bound to a plastic well (1); serum is added to the well and if antibody to the antigen is present it will bind to the antigen in the well (2). An enzyme-linked anti-immunoglobulin is added to the well and will bind to any antibody bound to the antigen (3). A colorless substrate (S) is added to the well and this will be converted into a colored product (c) by the enzyme (4). The amount of colored product produced can be measured using a spectrophotometer. The amount of product is proportional to the amount of enzyme which in turn is proportional to the amount of antibody that bound to the antigen.

The use of passive adsorption allows a great deal of flexibility in assay design. Therefore ELISA may be classified under four headings: direct, indirect, sandwich and competition. The choice of a particular design depends first on the test feasibility, given the availability of the reagents and the desired goal; secondly on the validation of the test showing that the test is stable and is evaluated over time and under different conditions; thirdly on standardization or quality control establishing that the test is precise and can be used by different workers in different laboratories (Crowther, 1995). Whatever the ELISA designs, the following stages are involved in performing the assay:

1) The adsorption of antigen or antibody to the solid-phase. The solid phase is the 96-well microtiter plate manufactured from polyvinyl chloride (PVC, flexible plates) or polystyrene (inflexible, rigid plates). Plates with flat-bottomed wells are recommended

where spectrophotometric reading is employed whereas round-bottomed wells can be used where visual assessment of ELISA is made (Crowther, 1995). Immobilization of antigen or antibody to the plate is carried out by coating, using coating buffers which neutralize repulsive forces between proteins and the solid-phase, thus ensuring greater binding of proteins.

2) The addition of the test sample and subsequent reagents. To avoid nonspecific adsorption of proteins to wells from samples added after the coating of the solid phase, it is imperative to “block” any uncoated plastic surface ((Benjamini and Leskowitz 1988). Such blocking ensures that the antibodies only bind to the antigen, not to the free plastic surfaces and is achieved by coating the plastic surface with a high concentration of an unrelated protein or immunologically inert substances.
3) The incubation of the reactants. The reaction between antigens and antibodies depends on their distribution, time, temperature and buffering conditions (pH) at which the incubation steps take place. Two types of incubation conditions are used to enable maximum contact of molecules in liquid phase with those on the solid phase: a) the incubation of stationary plates and b) the incubation of rotating plates with shaking. Mixing in rotation incubation ensures that potentially reactive molecules are continuously in contact with the solid phase. In contrast mixing during stationary incubation occurs only by diffusion of reagents and therefore requires greater time.
4) The separation of bound and free reactants by washing. Washing involves emptying of plate wells or reagents followed by the addition of liquids, usually buffered in order to maintain isotonicity since most antigen-antibody reactions are optimal under such conditions (Crowther, 1995).
5) The addition of enzyme-label reagent. In order to quantify the assay, reagents (antibodies or antigens) are conjugated to an enzyme that will react with the appropriate substrate to yield a colored product.
6) The addition of enzyme detection system (substrate). The rate of color development is proportional to the amount of enzyme conjugate present. Further enzymatic reaction is interrupted by addition of a stopping reagent at a time determined in a specific assay. The stopping solution is added at a time when the enzyme-substrate product is in a linear phase (Crowther, 1995).

7) The visual or spectrophotometric reading of the assay. Since the product of the substrate is colored, it can be read in two ways. ELISAs can be designed to use by-eye inspection or a spectrophotometer under different conditions and controls included in the test. However the interpretation of by-eye test can vary from operator to operator, giving rise to subjective results (Crowther, 1995). In machine reading system, the product of the substrate catalysis by enzyme is measured by transmitting light of a specific wavelength through the product and measuring the amount of adsorption of that light.

1.7.1.2.b Serology: competitive inhibition Enzyme-Linked Immunosorbent Assay (cELISA)

Unlike direct, indirect or sandwich Elisa which are direct binding assays, the competitive inhibition ELISA (cELISA) allows for the inhibition of enzyme-labelled antibody (secondary antibody) by the test sera antibody. Enzyme-labelled antibodies are blocked by the test sera antibodies reacting with common antigenic sites so that, where all sites are blocked, there is no color development since no enzyme-labelled antibodies are attached to antigen. Thus strong color development implies strongly positive sample in direct, indirect and sandwich ELISA whereas, weak color development in cELISA indicates a strongly positive sample. The advantage of cELISA is the ability to use relatively crude antigens for coating and still ensures a high specificity of the assay (Crowther, 1995).

I.7.1.3. Molecular tests

I.7.1.3.1. PCR amplification.

The polymerase chain reaction (PCR) (Fig.16) is the amplification of specific DNA sequences in vitro in a short period of time (Reece, 2004). PCR repetitively copies a section of the DNA to yield sufficient amounts of the specific section that can be used for various purposes (cloning, sequencing, hybridization, restriction mapping etc.). To set up a PCR reaction, a DNA template is mixed with a supply of nucleotides, buffer, thermostable polymerase and primers. Primers are short synthetic DNA sequences usually between 17 and 30 nucleotides in length that are complementary to the outer extremes of the target DNA sequence being amplified (Caldwell et al., 2006). A PCR is initiated when the double-stranded DNA template is separated by heating the sample. This step is called “denaturation” or “denaturing”. The temperature is then lowered

so that the primers can bind to the complementary sequences found on the single-stranded template (known as annealing). The thermostable polymerase, which is not damaged by the denaturation step, then extends the DNA strands by adding nucleotides to the ends of the primers (known as extension). The result is now two copies of the DNA target sequence. Prior to the denaturation step, a longer step referred to as "predenaturation" is necessary to ensure that the double-stranded DNA is fully separated and is performed once at the very beginning of PCR. The PCR continues with a repetitive series of these denaturation, annealing and extension steps, resulting in a logarithmic expansion in the amount of the target sequence. The last step is a final extension to ensure that all newly synthesized strands are fully extended before ending the PCR reaction (Caldwell et al., 2006).

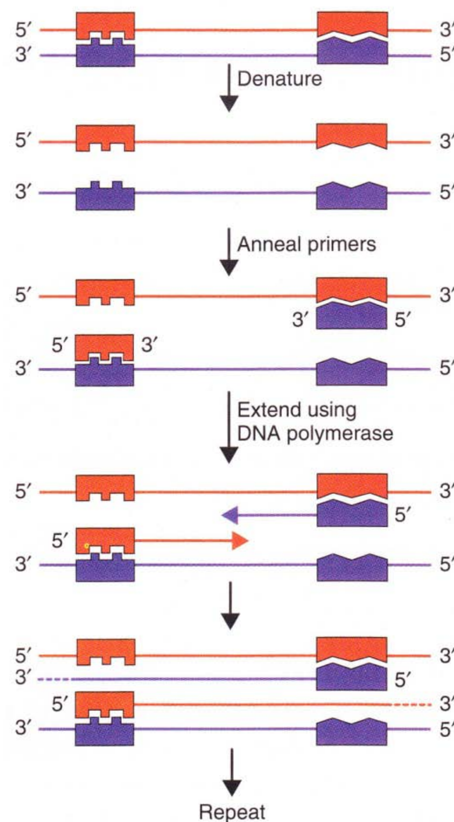


Fig. 16. The steps of a PCR experiment. The two DNA strands of the target DNA molecule, shown in red and blue to differentiate them, are denatured, or separate by heating. The boxed regions depict unique sequences within the target DNA to which the oligonucleotide primers will bind. Once the strands are separated, they are then cooled in the presence of oligonucleotides that are complementary to each strand. This results in the annealing of the oligonucleotides to their complementary DNA sequence. The oligonucleotides are then extended using DNA polymerase

in the presence of four deoxynucleotide triphosphates. This cycle of denaturing, annealing and extension is repeated 20 to 40 times to result in a massive amplification of the DNA in between the two oligonucleotide binding sites (adapted from Reece, 2004).

I.7.1.3.2 Gel electrophoresis

Gel electrophoresis (Fig. 17) is a powerful and versatile method to resolve mixture of different nucleic acid molecules and allows the fractionated molecules to be viewed directly, to be recovered in pure form or to be characterized directly by hybridization (Tijssen, 1993). Separation of molecules is based on their size during the application of an electrical current. In general, polyacrylamide gels are used for small molecules (<700 bp) and agarose gels for larger molecules (100-25000 bp) whereas pulse-field gel electrophoresis may be used for larger molecules, up to about 5- 10 000 kbp (Tijssen, 1993). There is an intermediate size range (70-1000 bp for double stranded DNA) for which choice of polyacrylamide or agarose is available. For analytical work, the prime concentration will be the rapidity and ease of the technique, and for this reason, horizontal agarose minigel is widely preferred. For preparative work, polyacrylamide offers many advantages, principally ease of elution and lack of coeluted inhibitors of subsequent enzymatic reactions (Berger and Kimmel, 1987).

At pH near neutrality, DNA is negatively charged and migrates from cathode to anode with a mobility dependant primarily on fragment size. Normally smaller linear DNA fragment migrates faster than larger ones (Berger and Kimmel, 1987). The resolving power of agarose gels is function of the concentration of dissolved agarose, and the migration rate of nucleid acids through agarose gels is dependent upon the molecular size (for linear fragment), conformation (circular, nicked circle, or linear) and voltage gradient (Berger and Kimmel, 1987). The effective range of separation for agarose gels of various percentages is shown in Table 2

Table 2. Range of separation for agarose gels (illustrated from Berger and Kimmel, 1987)

Agarose (%)	Optimal range of separation of linear DNA (kb)
0.3	60-5.0
20	20-1.0
0.7	10-0.8
0.9	7-0.5
1.2	6-0.4
1.5	4-0.2
2.0	3-0.1

Typical applications of agarose gels are analysis of restriction enzyme digests of clone DNA, preparation of cut vectors and fragments for cloning and direct sequencing, sizing large DNA and RNA, S1 mapping, visualizing PCR products, and analysis of cDNA cloning intermediates.

The horizontal position is the preferred position for agarose gel as it has the advantages of simplicity in pouring, loading and handling, versatility in size using the same apparatus, and support from below, important for low percentage gel (Berger and Kimmel, 1987). For non-denaturing agarose gels, agarose powder are available in many grades but high-quality, low melting temperature agarose should be chosen as it melts at 65ºC and sets at 30ºC, therefore allows DNA to remain double stranded and also allows many enzymes to be used for subsequent manipulations of DNA (Berger and Kimmel, 1987). Horizontal agarose gels are prepared as followed: (1) Powder agarose is added to the desired electrophoresis buffer (0.5X) to give the correct percentage. It is common to make 100 ml of gel solution but a typical minigel apparatus will require 25ml. The agarose is dissolved completely by heating, most conveniently in a microwave oven and checking so that the solution is homogenous. (2) The solution is allowed to cool to approximately 50 to 60º then poured into a buffer-containing mold (known as a gel tray or gel track) where it solidifies into a porous substrate in about 20 to 30 minutes (Caldwell et al., 2006). Before pouring, there is an option of adding ethidium bromide (EtBr), which allows the gel run to be monitor during the run by transmitted UV light. However to minimize damage to the DNA-ethidium complex by light, it is recommended that gels be run without inclusion of EtBr (Berger and Kimmel, 1987). (3) After setting, the gel should look uniformly opalescent.

Then the tape and comb are gently removed. If the gel was not stained before pouring, staining is achieved after electrophoresis in electrophoresis buffer containing 0.5 μg/ml EtBr for 30 minutes.

The most widely used buffers in gel electrophoresis of nucleic acids are Tris/acetate/EDTA (TAE) and Tris/borate/EDTA (TBE). TBE has the best buffering capacity though the use of TAE tends to result in somewhat sharper bands (Tijssen, 1993). Samples for the gels are conveniently loaded after addition of a loading dye containing glycerol (which weighs down the DNA, ensuring that it does not float out of the well) and bromophenol blue serving as a tracking dye. Bromophenol blue migrates not according to size, but is indicator of migration (Tijssen, 1993). A DNA molecular size marker is usually included in the gel in a lane adjacent to the samples to serve as a ruler to determine the size of the DNA band being examined. These markers include suitable restriction digests (e.g., EcoRI plus HindIII digest of lamda DNA) or ligation ladders.

Agarose for most purposes are run at room temperature, exceptions being the low percentage agarose gels (<0.5%) which are easier to handle in the cold, and low melting agarose gels which may melt if run too fast a room temperature (Berger and Kimmel, 1987). The best results, in terms of resolution and accurate sizing, are obtained by running agarose gels very slowly (<5V/cm). Generally large duplex DNA fragment are best resolved at low-voltage gradient whereas small fragment are run faster to limit diffusion. A balance between the consideration of fragment length, resolution required and available time is often determined empirically by each experimenter in each situation.

Detection, visualization and photography of the nucleic acid require UV transilluminators supplied with an UV-pass, visible blocking filter. The fluorescence of EtBr stacking in duplex DNA is 10 times greater than that of free EtBr (Berger and Kimmel, 1987). By running DNA through EtBr-treated agarose gel and visualizing it with UV light, distinct DNA bands become visible.

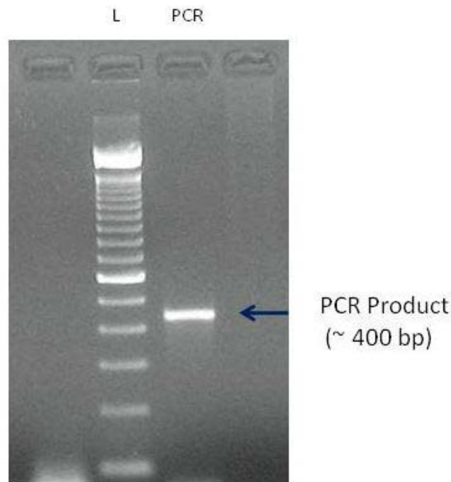


Fig.17. Ethidium bromide-stained agarose gel. (L) DNA size ladder (Gel run in the present study).

I.7.1.3.3. Reverse line blot hybridization assay

The Reverse line blot hybridization assay (RLB) assays is a non radio-active hybridisation of DNA samples (up to 43 simultaneously) against different oligonucleotides probes in a single assay (Gubbels et al., 1999). In this method, oligonucleotide probes deduced from a PCR- amplified region of a specific gene of related parasites are covalently linked to a membrane in parallel line using a miniblotter. After binding of the probes, the membrane is revolved at an angle of 90º in the miniblotter, so that the slots of the miniblotter are perpendicular to the probes lines (Fig.18). The slots are then filled with biotin-labelled PCR products. Hybridization takes place in the miniblotter and is visualised using a streptavidin-peroxydase conjugate which interacts with the biotin of the PCR products, followed by chemiluminescence detection (Fig. 18 and 19) The membrane with hybridized PCR products can be stripped and reused several times (Gubbels et al., 1999; Nagore et al., 2004).

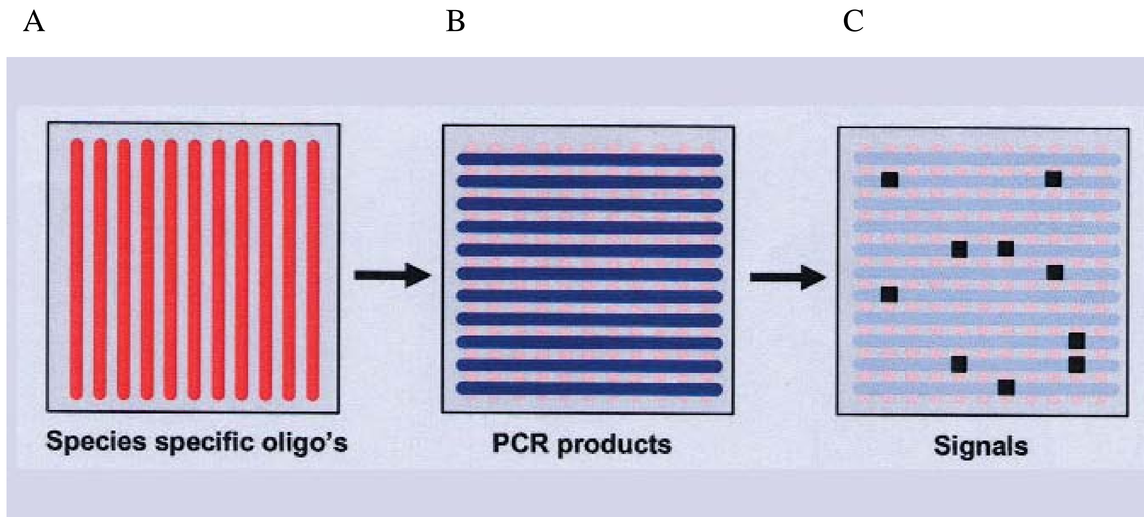


Fig.18. Schematic representation of the RLB assay (adapted from www.isogen-lifescience.com
Oligonucleotide probes are covalently linked to a membrane in parallel line (A). Then the membrane is rotated 90º in the miniblotter, so that the slots of the miniblotter are penpendicular to the probes lines; the slots are then filled with PCR products (B). After stringent washing to remove unbound PCR products, the hybridized PCR products are visualised by chemiluminescence detection (C).

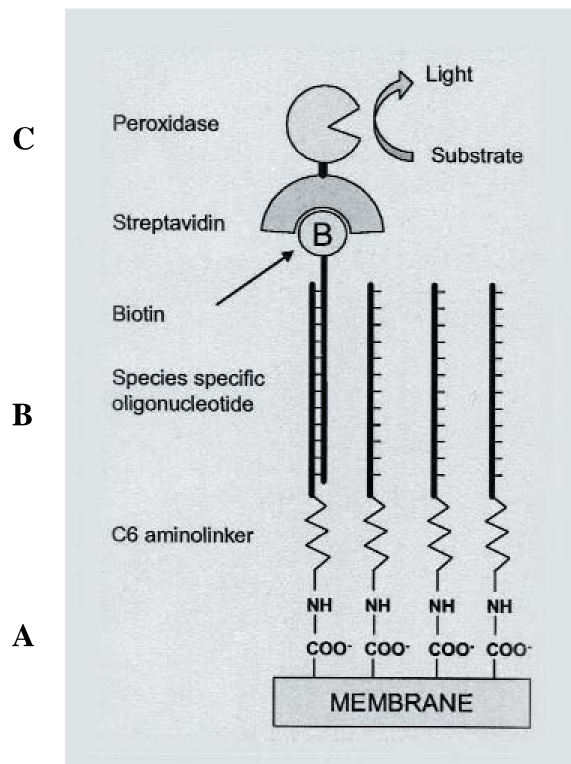


Fig.19 . Diagrammatic representation of the hybridization principle (Adapted from www.isogen-lifescience.com)

The species-specific probes deduced from the region of a gene amplified in the PCR step and attached to a C6 amino group are covalently linked to the membrane (A). Then PCR products in the hybridization process binds to the specific probe (B) and is detected by chemiluminescence using the streptavidin-peroxydase conjugate which interact with biotin of the PCR products (C).

I.7.1.3.4. Sequencing

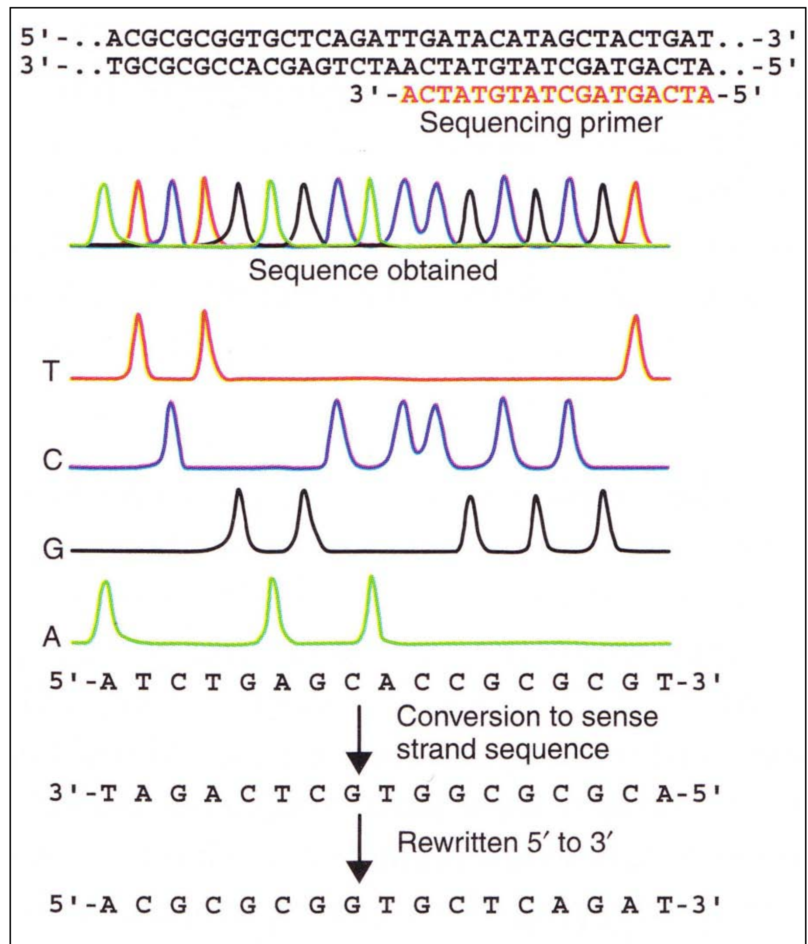
DNA sequencing is the technique used to determine the precise order of the bases within a nucleic acid. There are two alternative, basic approaches to sequencing which are the Maxam-Gilbert or chemical method and the Sanger method referred to as chain-termination method. Briefly, in the chemical method, chemicals are used to cleave the sugar-phosphate backbone of the radio-labelled DNA fragment at specific bases (Maxam and Gilbert, 1977). Specific chemicals are used to modify individual DNA bases or set of bases prior to cleavage of the sugar-phosphate backbone with piperidine at the modified bases (Maxam and Gilbert, 1980). The separation of the cleaved products is achieved with the aid of high-resolution polyacrylamide gel electrophoresis that allows unequivocal assignment of individual bases within a DNA sequence. This method is nevertheless limited by the length of the DNA that can be sequenced during a single reaction (approximately 100 bases) and by the use of harsh chemicals required to modify and cleave the DNA (Reece, 2004).

The Sanger alternative sequencing technique also called dideoxy method is based on the faithful replication of the DNA using a DNA polymerase. DNA synthesis occurs at the primer configuration, one in which the double stranded DNA ends with a 3’-OH group on one strand and the other strand continues as a single stranded DNA. The dideoxy method creates a primer configuration of the DNA to be sequenced and enables replication to proceed. A trick, using chain-terminating nucleotides, stops DNA synthesis at known positions. These chain-terminating

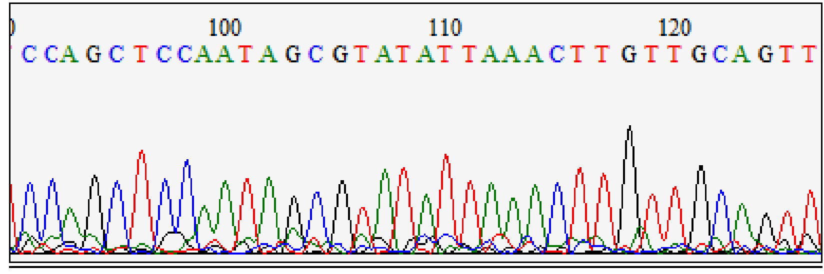
nucleotides are formed of sugar lacking a hydroxyl group (-OH) at both the 2' and 3' carbons (hence the term dideoxy). Without 3'-OH group, a dideoxynucleotide cannot be used for further DNA polymerisation. Chain terminating nucleotides permits synthesis to be stopped at a known base. The sample to be sequenced is elongated separately in four different reaction mixtures, each having all four normal nucleotides, radio-labelled so that newly synthesised DNA could be identified. In addition each reaction mixture also has a proportion of one of the chain-terminating nucleotides (approximately 1/10 of the deoxynucluotide counterpart). For instance, if the pool of the thymine-containing triphosphate nucleotides contains a portion of the dideoxythymidine triphosphate molecules, then the synthesis of the growing strand is sometimes terminated when adenine (the complement of thymine) appears on the template, creating fragments that end in thymine. Similar reactions are carried out in separate test tubes for each of the nucleotides, producing fragments that terminate when the respective complementary nucleotide is present. Since many DNA molecules are produced at the same time, this process results in the formation of a population of partially synthesised radioactive DNA molecules, each having a common 5'-end, but varying in length to a specific base at the 3'-end. After DNA synthesis is completed, the sequencing primer and the template are removed, leaving only the synthesised fragments. Newly replicated segments of various lengths from each reaction mixture are placed in separate slots then electrophoresed on polyacrylamide gels to determine the length of the fragments and read the sequences. Since only newly synthesised DNA segments are radioactive, autoradiography is used to keep tract of newly synthesised DNA. Usually, lanes are repeated for easier identification of the bands of the autoradiograph of a dideoxy sequencing gel. Also, the sequencing accuracy can be verified by sequencing the complementary strand and checking for agreement.

To increase the throughput of the Sanger method, some innovations are currently available that involve the use of fluorescent dyes, each fluorescing at a different length (505, 512, 519, and 526 nm) (Tamarin, 2004). Each of the four dideoxynucleotides has a different dye attached. After the newly synthesised fragments are isolated the products from all four reactions are run together in the same lane of the polyacrylamide gel. The gel is then scanned with an argon laser that excites the dye molecules. The intensity and wave length of the fluorescent emission is measured as the DNA fragment move past over a laser and fluorescence detector located at the bottom of the gel. This information is fed directly into a computer so that the resulting sequence can be automatically assigned and stored. This method, known as the dye-

terminator sequencing (Fig. 20) greatly simplifies sequencing since it is automated and also obviate the need for radioactive tags. As many as 1000 bases can be read automatically from a single reaction, although the sequence obtained from within 500 bp of the primer is generally more reliable than that further away (Reece, 2004). In addition, the detection methods used during automated sequencing are far more reliable than sequence interpretation from autoradiography (Reece, 2004). Automated sequencing is not even so, infallible as when long continuous runs of the same nucleotide can become compressed together as they travel through the gel, which may result in multiple overlapping peaks on the fluorescent trace that need to be deconvoluted manually. In spite of the high accuracy of the automating sequencing, error is likely to occur whereby bases are incorrectly assigned. It is therefore necessary to sequence the same section of the DNA many times to ensure that accuracy levels are as high as possible.



A



B

Fig.20. Dye-terminator (automated) sequencing. (A) A sequencing primer is used to initiate DNA synthesis that is terminated using a fluorescently labeled dideoxynucleotide. The series of peaks obtained has been separated into individual fluorescent components and the sequence assembled based on the data obtained (adapted from Reece, 2004). (B) The printout of an actual sequence obtained from an ABI Prism 3130XL sequencing machine (sequence obtained in the present study

I.7.2. Geospatial method

I.7.2.1. Species distribution modeling

An alternative approach to compensate for the lack of a systematic sampling in order to obtain a geographic distribution map is the modeling of species distribution. The models relate the biological response of a species to environmental factors using a statistical model and referred to as habitat models (Guisan and Zimmermann, 2000), habitat suitability models (Hirzel et al., 2001), species distribution models (Guisan et Thuillier, 2005), ecological niche models (Philips et al., 2004). The basic concept underlying species' distribution modeling arises from the definition of the ecological niche of a species, which is the conjunction of environmental conditions within which a species can maintain populations without immigration (Yun-sheng et al., 2007).

Different modeling algorithms explore the association of species occurrence and environmental data to produce species distribution models which translate geographically into a species distribution maps. The algorithms include the Logistic Regression (LG), Classification and Regression Tree (CART), Genetic Algorithm for Rule-Set Prediction (GARP), Maximun Entropy method (Maxent) and more (Elith et al., 2002, Mingyang et al., 2008). Maxent model has been shown to outperform the other modeling methods and to give better predictions (Yun-Sheng et al., 2007; Mingyand et al., 2008). Maxent model is implemented in MaxEnt program (Philips et al., 2006) (available from http://www.cs.princeton.edu/~schapire/maxent/).

Maxent model calculates the distribution that is close to uniform with regard to a number of constraints (Philips et al., 2006; Phillips and Dudik, 2008). The model gives continuous results over the study area with results ranging from 0 to 100, providing a map showing the probability gradient for the species potential distribution (Philips et al., 2006) which is translated into host suitability maps (predicted species geographic distribution). The result map displays the variation in the habitat suitability in percentages. The higher the probability, the greater the suitability of the habitat for a given species. The prediction result of Maxent is evaluated using the threshold-independent test. The test is obtained by using the receiver operating characteristic (ROC) curves (Zweig and Cambell, 1993). The ROC analysis is based on the area under the curve (AUC) which provides a single measure of model performance (Hanley and McNeil, 1982). The higher

the value of the AUC is, the stronger the relationship between the presence location and environmental variables, which indicates a better performance of the model. AUC ranges from 0.5 for random predictions to 1.0 for models with a perfect predictive ability. A score>0.9 indicates a very good discrimination, a score between 0.8-0.9 is good and a score >0.7 is acceptable. The main advantage of ROC analysis is that AUC provides a single measure of model performance independent of any particular choice of threshold. Maxent also calculates a threshold-dependent statistic where probabilities above a threshold (e.g. minimum training presence) predict presence, and probabilities below the threshold predict absence. P-values are calculated based on the null hypothesis that the test data are predicted no better than a random prediction.

To measure the contributions of individual variables on the models, Maxent performs a jackknife test in which the model is run multiple times: 1) using all variables, 2) dropping one variable at a time, and 3) running the model using only one variable. Variables which produce the highest training gains or reduce the training gain when left out of the model are considered to be the most important variables.

An important advantage of Maxent is that "presence-only" data indicating the occurrence of a species is used while other models requires both "species presence" and "species absence" data.

I.7.2.2. Spatial scan statistic

The spatial scan statistic is a cluster detection test in a multi-dimensional point process (Kulldorff, 1997). The test detects both the location of clusters and their statistical significance. The theory behind the spatial scan statistic is the generalization of a test proposed by Turnbull and co-workers (1990).

The statistic uses a circular window of variable radius that moves across the map. The radius of the window varies continuously in size from zero to some upper limit specified by the user. As the window moves across the map, it creates an infinite number of distinct geographical cycles with different sets of neighboring locations within them. Each cycle is a candidate cluster. If the window contains the centroid of a location, the whole location is included in the window (Kulldorff and Nagarwalla, 1995). The cluster assessment is performed by comparing the number of cases within the window with the number expected if cases are randomly distributed

in the space. The test of significance of the identified cluster is based on the likelihood ratio (Kulldorff and Nagarwalla, 1995) whose p-value is obtained through Monte Carlo hypothesis testing (Dwass, 1957). As the statistic gradually scans a window across space and noting the number of observed and expected observations inside the window at each location, multiple window sizes are used. The window with the maximum likelihood is the most likely cluster which is the cluster least likely to be due to chance. A p-value is assigned to this cluster. The cut-off value of p is often set to 0.05 for the most likely cluster. But for the secondary non-overlapping cluster, the cut-off value is lower (0.01) because the secondary cluster from the same data set is compared with the most likely cluster and the test is therefore conservative (Kulldorff and Nagarwalla, 1995, Kulldorff, 1997).

In disease surveillance, the statistic tests the risk of disease within and outside the window, under the null hypothesis of equal risk and produces the relative risk for each cluster. The spatial scan statistic is implemented in the SaTScan software (Kulldorff, 2009) (available from http://www.satscan.org/) that analyses spatial, temporal and space time data using the spatial, temporal or space-time scan statistics. SaTScan has been found to be the best equipped package for use in an automated disease surveillance system in comparison with ClusterSeer GeoSurveillance and R-Package 'Surveillance' programs (Robertson and Nelson, 2010). The spatial scan statistic offers several advantages: it corrects for multiple comparisons, adjusts for the heterogeneous population densities among the different areas in the study, detects and identifies the location of the clusters without prior specification of their suspected location or size thereby overcoming pre-selection bias, and the method allows for adjustment for covariates (Kulldorff and Nagarwalla, 1995, Kulldorff, 2001).

II. OBJECTIVES OF THE STUDY

The main objective of this study was to investigate the epidemiology of equine piroplasm infection (EPI) in Greece. To reach this goal, the specific objectives were:

- To identify the infectious agents of EPI in Greece
- To determine the prevalence of infection using hematological, serological and molecular tools
- To assess the genetic diversity of the identified infectious agents
- To determine the host and environment-related risk factors associated with the infection
- To explore the tick fauna, vectors of the equine piroplasms occurring in Greece, on the sampled equids and their associated animals.
- To assess whether or not Greece should be regarded as an enzootic area for EPI.
- To model the geographic distribution of any identified species and genotypes of piroplasms.
- To detect equine piroplasm infection clusters in Greece, if any.

III. MATERIALS AND METHODS

III.1. Animals and collection of samples

Animals belonging to the genus *Equus* were randomly sampled, after permission of their owners, between 2007 and 2008 in different regions of Greece including Attica, Macedonia, Thessaly, Central Greece and Peloponnese. All the animals but one horse in Attica region were clinically healthy at the time of sampling. Whole blood samples intended for molecular and microscopy examinations were obtained via jugular venipuncture in sterile Vacutainer® tubes containing ethylenediamine tetraacetic acid (EDTA). Thin blood smears were prepared from the whole blood upon arrival at the laboratory and the remaining blood was stored at -4° C until molecular processing. Blood samples for serum were collected in the same way in anticoagulant-free tubes, allowed to clot, and then centrifuged at 2500 rpm for 15 minutes. Sera were then collected and stored at -20° C until processing. Questionnaires on zoographic data (gender, age, and species), origin, as well as the type of activity of the equids were completed by the investigators at host location. In addition, each animal host as well as pastoral or domestic dogs were diligently searched for the presence of ticks at the time of sampling for subsequent identification using keys from Papadopoulos et al. (1996), Walker et al. (2000) and Pavlidou et al. (2008).

III.2. Microscopy

Two thin blood smears were prepared from fresh blood (Appendix 1), fixed in absolute methanol for five minutes, stained with Giemsa for 30 min and observed with light microscope. At least 200 fields per smear were examined under oil immersion lens (100X) for evidence of intracellular forms morphologically compatible with the shape of *Theileria*/ *Babesia* species.

III.3. Serological examination

The indirect detection of *T. equi* and *B. caballi* infections in equids was performed using commercial cElisa kits for *Babesia equi* and *Babesia caballi*, purchased from VMRD, Inc

(Pullman, WA. 99163, USA). *B. equi* cElisa test kit detects serum antibodies that target the surface EMA-1 protein on *T. equi* merozoite (Knowles et al., 1992). *B. caballi* cElisa assay specifically detects serum antibodies to the rhoptry-associated protein (RAP-1) of *B. caballi* (Kappmeyer et al., 1999). The sensitivity of *T. equi* and *B. caballi* cELISA is higher than that of the complement fixation text (CF) whereas the specificity of *T. equi* and *B. caballi* cELISA is 99.2- 99.5% (OIE, 2008).

The test was conducted according to the manufacturer's instructions (Appendix 2). Cinquante microliter of each serum sample were used to coat the plate. An automatic plate reader (Infinite 2000, Tecan) was used with the OD set at 630 nm. A sample was considered positive when the percent inhibition was ≥40%.

III.4. Molecular test

III.4-1. Preparation of samples for RLB assay

III.4.1.2. DNA extraction

Two different kits (NuleoSpin® Blood, Macherey-Nagel GmbH and Co. KG, Germany and Jetquick Blood & Cell Culture DNA Spin Kit, Genomeh GmbH, Germany) were used to extract genomic DNA from whole blood according to the manufacturers's instructions (Appendix 3). 200 µl of whole blood per sample were used for each kit. The eluted DNA obtained was stored at -20 ºC until used.

III.4.1.3. PCR amplification.

III.4.1.3.1. Oligonucleotide primers

Primers used were RLB-F (5'-GACACAGGGAGGTAGTGACAAG-3') and RLB-R (biotin-5'-CTAAGAATTTCACCTCTGACAGT-3') as adapted by Georges et al. (2001). Primer RLB-F and RLB-R target the hypervariable V4 region of the 18S rRNA gene of the genera *Theileria* and *Babesia* and generate bands of approximately ~430 and ~390 bp. Primer RLB-R was chemically labelled with biotin for hybridization purposes. The biotinylated primer will be detected using the streptavidin-enzyme conjugate in the RLB assay, which will allow the identification of positive samples.

III.4.1.3.2. Procedure

The PCR program used in this work was as previously described by Nagore and co-workers (2004). Reactions were performed in a final volume of 25 µl containing 2.5 µl of PCR buffer, 0.75 µl of $MgCl_2$, 0.5µl of dNTP, 0.3ul of Tag polymerase and 1 µl of each primer. The cycling conditions were: an initial step of 4 minutes at 94ºC, followed by 40 cycles of 0.35s at 94ºC, 0.35s at 51ºC 0.35s at 72ºC and a final extension step of 10 minutes at 72ºC. The detailed protocol is presented in Appendix 4.

Positive controls of different sources were used in the experiment. *T. equi* and *B. caballi* positive blood samples were kindly supplied by Prof. Panayiotis Karanis (Medical and Molecular Parasitology Laboratory, University of Cologne, Medical School, Center of Anatomy, Institute II, Cologne, Germany). *T. equi* and *B. caballi* DNAs extracted from whole red blood cells cultures were provided by Dr Alvin Gajadhar (Center for Food-borne and Animal Parasitology, Canadian Food Inspection Agency, Canada). Dr Ana Hurtado (Deparment of Animal health, Instituto Vasco de Investigación y Desarrollo Agrario, Spain) kindly supplied cloned *B. canis canis* and *B. vogeli* DNAs controls.

III.4.1.4. Gel electrophoresis

The amplification success was assessed by running an agarose electrophoresis gel of the PCR products. 5µl-aliquot of PCR product and DNA size ruler were run on 2 % agarose gel stained with ethidium bromide as detailed in Appendix 5.

III.4.2. RLB assay

III.4.2.1. Oligonucleotide probes

The oligonucleotide probes (Table 3) were selected from the literature and synthesised with a 5' terminal group, MMT C6 linker 6-(4-Monomethoxytritylamino) hexyl-[(2-cyanoethyl)-(N,N-diisopropyl)]-phosphoramidite (Isogen Life science, Maarssen, Netherlands).

Table 3. Sequence, melting temperature (T_{m}) and concentration (C) of the oligonucleotide probes covalently linked to the membrane

Probe	Sequence (5'-3'	Tm (ºC)	C (µM)	Reference
Catchall[a]	TAATGGTTAATAGGA(A/G)C(A/G)GTTG	54.7[b]	8	Gubbels et al., 1999
T. equi	GTTTCGATTATTCGTTTCCCGG	58.4	16	Nagore et al., 2004
T. equi-like	GGGGCATGTTTTCATGACTCGA	60.3	8	Nagore et al., 2004
B. caballi	GTTGCGTTGTTCTTGCTTTTTGCTT	59.7	32	Nagore et al., 2004
B. caballi-like	CGGGTTATTGACTTCGCTTTTTCTT	59.7	8	Nagore et al., 2004

[a]Catchall is a probe specifically designed to hybridize to any piroplasm that may be present in a sample.

[b]Tm for the degenerate oligonucleotide (Catchall) is an approximate value.

III.4.2.2. Membrane preparation

The preparation of RLB membrane was carried out as previously described by Gubbels and co-workers (1999) (Appendix 6). After preparation, the membrane was stored unsealed in at least 125ml of 20mM EDTA pH 8 at 5±3º to avoid dehydration.

III.4.2.3. Hybridization with PCR amplicons and detection

Hybridization was carried out as previously described by Nagore and co-workers (2004)(Appendix 7) with the following modifications in detection method: after hybridization the membrane was incubated in 10ml of BM Chemiluminescence Blotting Substrate for one minute,

followed by exposure to an X ray film (Kodak BioMax Film) for two to 40 minutes depending on the signal.

III.5. Sequencing

III.5.1. Preparation of PCR products

III.5.1.2. PCR amplification

III.5.1.2.1. Oligonucleotide primers

Samples subject to sequencing were amplified using primer pair RLB-F2 (GAG GTAGTGACAAGAAATAACAATA) and RLB-R2 (TCTTCGATCCCCTAACTTTC) (Gubbels et al., 1999) that amplify the hypervariable V4 region of the 18S rRNA gene of *Theileria* and *Babesia*, generating fragments of approximately 460 to 520 bp.

III.5.1.2.2. Procedure

The same program and procedure used on RLB samples were applied on samples subject to sequencing, with a sight modification on the reactions conditions as presented in Table 4

Table 4. PCR conditions for samples subject to sequencing (reaction volume=30µl)

Reagents	Concentration	1 reaction
10 x PCR buffer	1X	2.5 µl
50 mM Mgcl$_2$	1.5mM	0.75 µl
10mM dNTPs	200µM	0.5 µl
5U/µl DreamTaq polymerase	1.5 U	0.3 µl
20 pmol RLB-F2	0.67 µM	1 µl
20 pmol RLB-R2	0.67 µM	1 µl
Water (autoclaved)		13.95 µl

III.5.1.3. Purification of PCR amplicons from Agarose gel

DNA fragments were extracted from 1.5% agarose gel using a commercial kit (Jet quick Gel Extraction Spin Kit/50, Genomed) according to the manufacturer's instructions (Appendix 8).

III.5.2. Sequencing of PCR amplicons

Eluted DNA samples were sent to a commercial subcontractor for automatic sequencing using the PCR primers in an ABI Prism 3130 x L sequencing machine from Applied Biosystems. Fragments from three PCR reactions were sequenced for the same sample. ChromasPro Program version 1.5 (TechnelySium Pty) was used to edit the sequences based on the forward and reverse strands simultaneously.

III.6. Species geographic range modelling.

The potential geographic distribution of piroplasms was predicted using a maximum entropy algorithm based on ecological niche modelling concept and implemented in Maxent software (Phillips et al., 2004; Phillips et al., 2006, Phillips and Dudik, 2008) available from http://www.cs.princeton.edu/~schapire/maxent/).

III.6.1. Species' location geographic coordinates

The geographic coordinates of the presence location for each species or genotype (as determined by cELISA or RLB assay) were recorded at each host' location

III.6.2. Source of environmental data

Three types of environmental data were obtained for this study: climate, elevation, and land cover data. These data sets were converted using ArcGis, version 9.3, to a common projection, map extent and resolution prior to use in the Maxent modeling program.

Bioclimatic variables with a spatial resolution of 1 km were downloaded from WorldClim version 1.4 dataset (http://www.worldclim.org). WorldClim provides data as 50-year means for each month for precipitation, minimum temperature, and maximum temperature. The data are further processed into a series of bioclimatic variables (Table 5).

Elevation data were also downloaded from the WorldClim website. WorldClim processed this data set from NASA Shuttle Radar Topography Mission (SRTM) data to have the same projection and resolution as the other WorldClim layers.

Land cover data were obtained from the U.S Geological Survey's (USGS) Global Land Cover Characteristics Database version 2 Global (http://edcsns17.cr.usgs.gov/glcc/). The USGS developed the land cover from 1-km Advanced Very High Resolution Radiometer (AVHRR) satellite data acquired during April 1992–March 1993. The USGS website provides the data in several classification schemes; the Global Ecosystems land cover classification was selected for this study. This land cover classification contains 100 classes, 10 of which corresponded to the occurrence localities of the piroplasm genotypes (Table 6). To simplify the statistical analysis in the investigation of habitat similarities, however, the original 10 classes were combined into 3 generalized classes, as listed in the second column of Table 6.

Table 5. Bioclimatic variables used in this study

Variable	Description
Bio1	Annual Mean Temperature
Bio2	Mean Diurnal Range (Mean of monthly (max temp - min temp))
Bio3	Isothermality (P2/P7) (* 100)
Bio4	Temperature Seasonality (standard deviation *100)
Bio5	Max Temperature of Warmest Month
Bio6	Min Temperature of Coldest Month
Bio7	Temperature Annual Range (P5-P6)
Bio8	Mean Temperature of Wettest Quarter
Bio9	Mean Temperature of Driest Quarter
Bio10	Mean Temperature of Warmest Quarter
Bio11	Mean Temperature of Coldest Quarter
Bio12	Annual Precipitation
Bio13	Precipitation of Wettest Month
Bio14	Precipitation of Driest Month
Bio15	Precipitation Seasonality (Coefficient of Variation
Bio16	Precipitation of Wettest Quarter
Bio17	Precipitation of Driest Quarter
Bio18	Precipitation of Warmest Quarter
Bio19	Precipitation of Coldest Quarter

Table 6. Global Ecosystems land cover legend associated with the piroplasms' occurrence localities

Original land cover classes (10).	Generalised land cover classes
Urban	Urban/Crop
Crops and Town	Urban/Crop
Deciduous Tree Crop	Urban/Crop
Mixed Forest	Forest
Deciduous Broadleaf Forest	Forest
Forest and Field	Forest
Mediterranean Scrub	Savanna
Semi Desert Shrubs	Savanna
Fields and Woody Savanna	Savanna
Woody Savanna	Savanna

III.6.3. Modeling methodology

Twenty-five percent of the occurrence points (testing or evaluation points) were used for testing the model accuracy and the remaining 75 % were used for building the model (training or

calibration points). Training and testing points are randomly selected by the Maxent program. The GIS software ArcGIS ver. 9.3 was used to display the prediction results and represent the sampled localities.

III.6.4. Model performance evaluation

The model accuracy was assessed using two methods provided by Maxent : 1) Maxent calculates the test statistic known as AUC (the Area Under the Receiver Operating Characteristic Curve) which is a threshold-independent measure of predictive performance (Hanley and McNeil, 1982). The test is derived from the Receiver Operating Characteristic Curve (ROC) which is a plot of the sensitivity versus 1-specificity (Swets, 1988; Fielding and Bell 1997). Random predictions have AUC values of 0.5; the best performing models have values of 0.9 and above. 2) Maxent also calculates a threshold-dependent statistic where probabilities above a threshold (e.g. minimum training presence) predict presence, and probabilities below the threshold predict absence. P-values are calculated based on the null hypothesis that the test data are predicted no better than a random prediction.

III.7. Assessment of habitat similarities between different piroplasm genotypes

In order to support the assignment of taxonomic entities to piroplasms genotypes, similarities between habitats associated with the genotypes were assessed, using environmental variables which included bioclimatic variables and land cover.

III.7.1. Localities coordinates

The geographical locations of the piroplasm genotypes were recorded as the latitude and longitude coordinates of the location of the hosts of origin. The piroplasm genotypes were determined by the RLB assay described in the present study.

III.7.2. Environmental data and Methodology

The same bioclimatic variables and land cover data employed for model building were used to assess habitat similarities between genotypes.

The bioclimatic variable values associated with the presence localities were compared between related piroplasm genotypes in a first step. Then presence and absence locatities were compared for each individual piroplasm genotype in a second step. Finally, the preferred land cover for each genotype was determined.

III.8. Spatial cluster analysis

Clusters of piroplasms were investigated using the spatial scan statistic (Kulldorff, 1997) implemented in SaTScan software, version 8.1.1 (Kulldorff, 2009) available from http://www.satscan.org/.

III.8.1. Cases, controls and coordinates files

All test-positive hosts were regarded as cases whereas test-negative hosts were considered as controls. The geographical coordinates were recorded as longitude and latitude coordinates of each host's location, in decimal number of degrees (DND) format.

III.8.2. Probability model, Monte Carlo hypothesis testing and cluster size.

The Bernouilli probability model was chosen for the analysis. The number of permutation selected was 9999 for Monte Carlo testing and the cluster size was set to 5% and 11% for *Babesia* and *Theileria* respectively to reflect the prevalence previously observed (Kouam et al., 2010).

III.9. Statistical analysis

The Chi-square and Fisher's exact tests were used alternatively to analyse the difference in the seroprevalence of *T. equi* and *B. caballi* species between the host species. The same tests were used to analyse the influence of all the examined factors as independent categorical variable on piroplasm prevalence. For significant factors, the relative risk (RR) was computed with the associated 95 % confidence interval. Fisher' exact test was used to compare the difference in prevalence for each genotype infection within host group and to assess the difference in the prevalence of occurrence locations for each piroplasm genotype in the land cover classes. The Kruskal-Wallis test was used to compare the values of the bioclimatic variables associated with the examined localities for each piroplasm genotype. All the parameters were computed using Epi Info, Version 3.5.1. Variables were considered significant for p-value <0.05.

IV. RESULTS

IV. 1. Hosts and Sampling areas

A total of 796 equids consisting of 776 horses, thirteen mules and seven ponies were sampled in various geographic areas of Greece as presented in Fig. 21 and Table 7. The equids were located in the equestrian racing complex in Markopoulos Mesogeas and in privately-owned premises spread over 32 localities.

Fig.21. Geographic locations where equid hosts were sampled. The star symbol(★) approximately represents the sampling areas.

IV.2. Microscopical findings

A total number of 787 whole blood samples were collected from 772 horses, eight mules and seven ponies and examined microscopically. None of the samples was found positive with either *Theileria* or *Babesia* infection.

IV.3. Serological findings

A total of 544 sera were examined from 524 horses, thirteen mules and seven ponies. Mules and ponies were very difficult to locate and contributed to their low sample size. Samples were identified on the basis of host species, animal host age, host gender, host type of activity, host origin and host region group. The results of the serological findings are summarized in table 8.

IV.3.1. Seroprevalence of *Theileria equi* and *Babesia equi*

The seroprevalences of both species is given in Table 9. The seroprevalence of piroplasms in all the examined animals in the survey was 11.6 % (9.1-14.6%), with 11% (8.6-14%) for *T. equi* and 2.2 % for *B. caballi*. Co-infection with both *T. equi* and *B. caballi* was recorded in a small portion of the examined equids (1.7% (0.8-3.2%).

IV.3.2. Factors associated with seroprevalence

The seroprevalence of *T. equi* and *B. caballi* was significantly affected by the species of the equid host ($p<0.0001$ in both cases). The seroprevalence levels were significantly higher in mules than in horses for *T. equi*, *B. caballi* and mixed *T. equi* and *B. caballi* infections ($p<0.0001$ for all cases) but not than in ponies (Table 8). No significant difference in seroprevalence was observed between ponies and horses, and between ponies and mules.

The gender of hosts was not found significant for any of the infections investigated (Table 8)

The seropositivity level was significantly higher in older than in younger equids for *T. equi* ($p<0.05$) but not for *B. caballi* (Table 8). In addition, the seroprevalence to *T. equi* in animals over five years old was significantly higher than in animals between two and five years old ($p< 0.01$). Antibodies were not detected in animals less than one year old for *T. equi* infection and in animals less than 2 years old for *B. caballi* infection (Table 8).

Mixed infection with both parasites involved only animals born in Greece. Besides, the infection level was significantly higher in local animals compared to imported animals ($p<0.05$) (Table).

The type of activity significantly affected the seroprevalence to *T. equi*, *B. caballi* and mixed infection ($p<0.01$) (Table 8). The seroprevalence was significantly higher in farm equids than in race and recreation equids for *T. equi*, *B. caballi* and mixed infection ($p<0.01$ for all cases) and lower in race than in recreation equids for *B. caballi* and mixed infection ($p<0.05$ in both cases).

The geographical location of hosts (regional level) was significantly associated with *T. equi*, *B. caballi* and mixed infection ($p<0.01$ in all cases). The highest level of seroprevalence among animal hosts for *T. equi* (38.8%) and *B. caballi* (6.1%) was observed in the region of Thessaly. The seroprevalence for *T. equi* was significantly higher in Thessaly than in Peloponnese or Macedonia ($p<0.01$ in both cases) but not for *B. caballi*. Also, the prevalence of antibodies to *B. caballi* was significantly lower in Attica than in Peloponnese or Macedonia ($p<0.05$ in both cases) (Table 8). Co-infection of hosts with both *T. equi* and *B. caballi* was observed in all regions except Attica.

IV.3.3. Analysis of the relative risk (RR) of infection

The relative risk (RR) of infection was found to be eight-fold and thirty-three-fold higher in mules than in horses in regard to *T. equi* and *B. caballi* infection, respectively ($p<0.01$for both) (Table 10). Additionally, the risk for mixed infection was forty times higher in mules than horses ($p<0.01$) (Table 10).

The risk of infection was three times greater in farm equids compared with recreation equids for *T. equi* and 0.14 times lower in race equids than recreation equids for *B. caballi* ($p<0.05$) (Table 10).

The estimated RR for *T. equi* and *B. caballi* comparing Thessaly with Attica was 4.70 and 10.01 respectively ($p<0.05$ in both cases) (Table 10). Comparing Peloponnese and Macedonia in relation to Attica region, the risk of *B. caballi* infection was six times and twelve times higher, respectively ($p<0.05$ for both cases).

IV.4. Genomic analysis

IV.4.1. RLB assay

IV.4.1.1. PCR amplification of the target gene

Fig. 22 presents the amplicon resulting from the PCR amplification of the V4 region of the 18S rRNA gene.

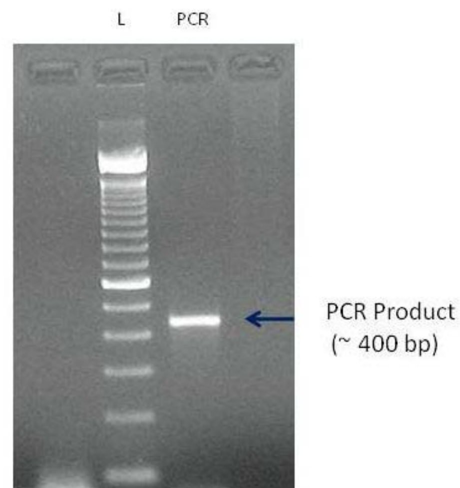


Fig.22. PCR amplification of the V4 region of the 18S rRNA gene from *Theileria/Babesia* genomic DNA. (L) DNA size ladder.

IV.4.1.2. Hybridisation of PCR products

Fig. 23 shows the results of hybridization of the target gene fragment against the specific probes covalently linked on a RLB nylon membrane.

IV.4.1.3. Genotypic analysis by RLB

A total of 787 equids were examined including 772 horses, eight mules, and seven ponies. All the animals but one horse was clinically healthy. The summary of RLB results are presented in Table 11.

Three genotypes, comprising two *Theileria* (*T. equi* and *T. equi*-like) and one *Babesia* (*B. caballi*-like) were identified. Fig. 1 shows strong signals observed for *T. equi* and *T. equi*-like genotypes and faint signals for *B. caballi*-like genotype. As presented in Table, 47.14% of the samples tested hybridised to catchall probe, 43.96% to *T. equi* probes, and 46.25% to *T. equi*-like probe, 0% to *B. caballi* probe and 0.38% to *B. caballi*-like probe. Horse blood samples tested positive for both *Theileria* and *Babesia* genotypes while mules and a single pony were infected by the *Theileria* genotype only. However, no significant difference in prevalence among hosts was found for all the genotypes ($p>0.05$ in all cases). The symptomatic horse which died later was co-infected with both *T. equi* and *T. equi*-like.

Seven samples from horses yielded faint signals with the catchall probe only, indicating the occurrence of novel piroplasm species, or a novel genotype or a known genotype occurring at a very low level of parasitemia. Due to the very low level of piroplasm parasitemia of these samples, the target gene could not be amplified and sequenced in order to clarify the piroplasm identity.

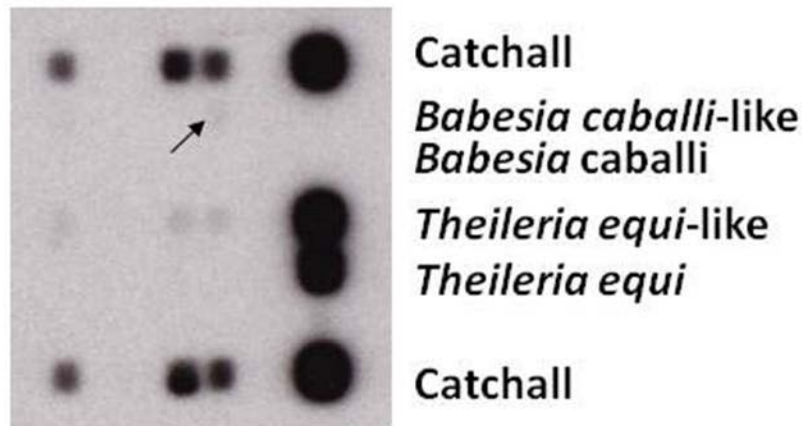


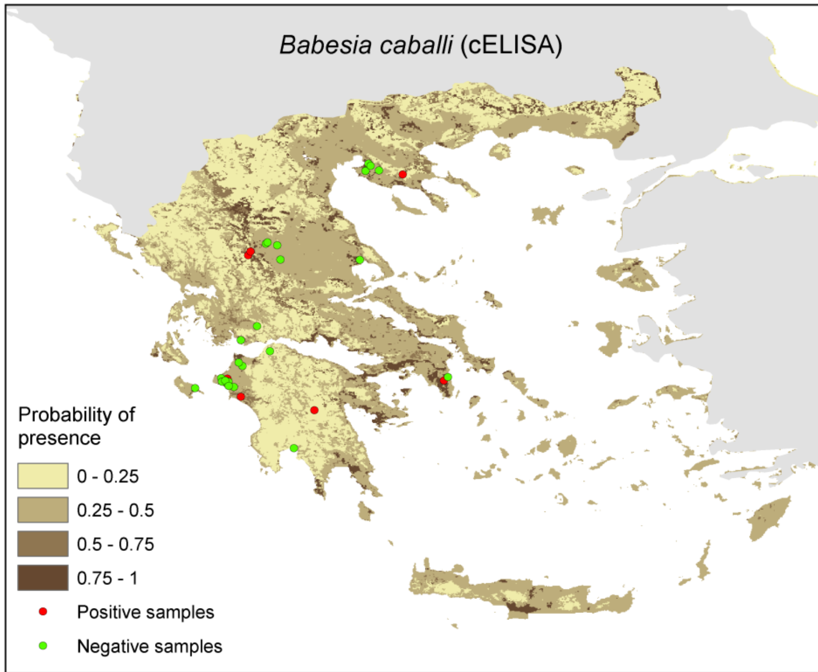
Fig. 23. RLB assay results: hybridization of 4 field samples (columns) on Catchall, *B. caballi*-like (faint signal indicated by arrow), *T. equi*-like and *T. equi* probes (rows).The signals represent the hybridization of PCR products generated by amplification of genomic DNA from blood samples naturally infected with equine piroplasms. The empty spaces between column signals (positive samples) represent piroplasm- negative samples. None of the samples hybridized to *Babesia caballi* probe.

IV.4.2. DNA Sequencing

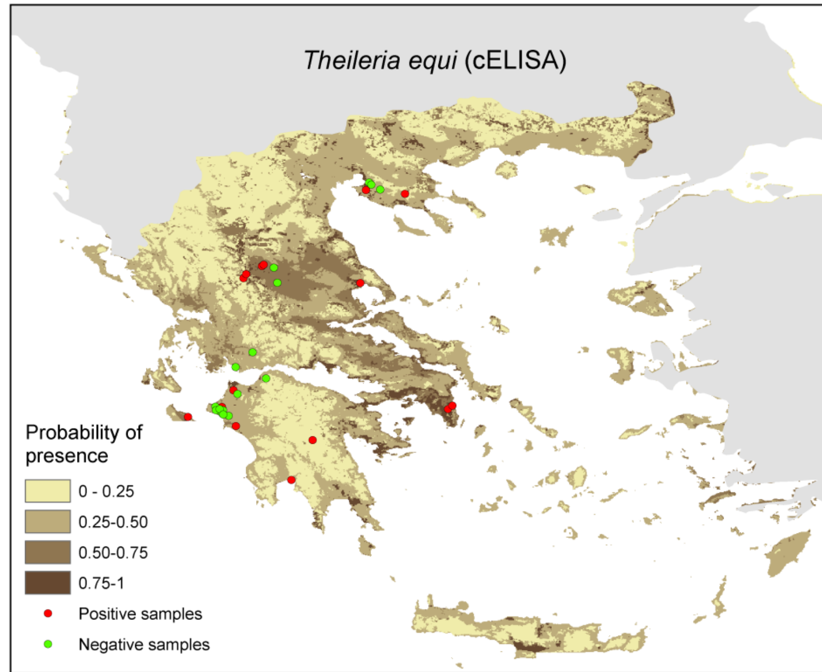
All *T. equi* positive samples were co-infected with *T. equi*-like while *B. caballi*-like occurred at a very low parasitemia. For these reasons, a partial sequence of 509 bp from a representative sample of *T. equi*-like only could be obtained. Blast similarity search (Table 12) revealed that *T. equi*-like isolates shared 100% similarity with the Southern Spain *T. equi*-like isolates (DQ287951) (Criado et al., 2006) and 99% similarity with *T. equi*-like isolates from Northern Spain (AY534882)(Nagore et al., 2004).

IV.5. Predicted geographic range of piroplasms

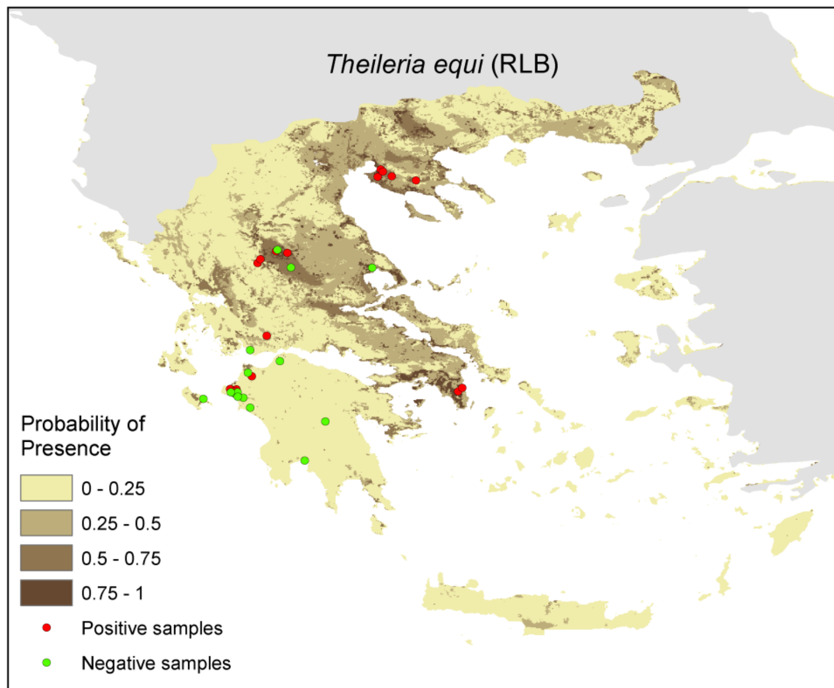
Table 13 presents the number of equid tested positive per host location and Fig. 24 displays the potential geographic distribution of the piroplasms predicted by Maxent program.



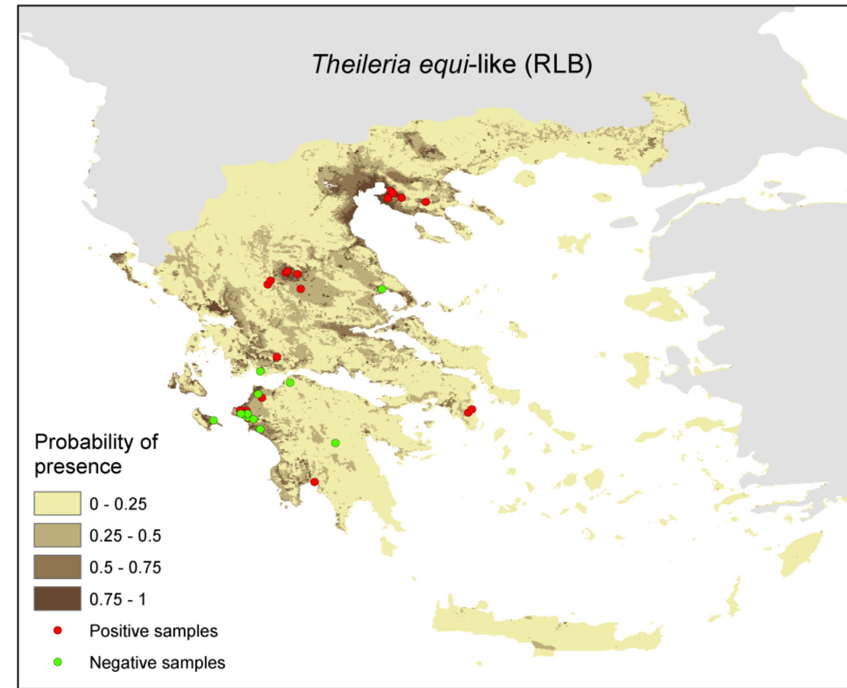
A



B



C



D

Fig.24. Predicted geographic distribution ranges for equine piroplasms. A: *Babesia caballi* species; B: *Theileria equi* species; C: *Theileria equi* genotype; D: *Theileria equi*-like genotype.

As shown in Fig. 24A, the highest level of probability (p>0.75) for the geographic range of *B. caballi* species covers small areas spreading from north to south in the eastern half of mainland Greece and Peloponnese and extends downwards to the south coast of the island of Crete. The regions with a considerable concentration of potential equid hosts (Table 14) and characterized by a high probability of presence for *B. caballi* include the regions of Macedonia, Thessaly and the Aegean islands.

High probability (p>0.75) areas for *T. equi* species mainly cover the eastern part of continental Greece from north to south, in the western coastlines as well as the southermost coast of the country at the island of Crete as shown in Fig.24B. Areas with a significant concentration of equids and with higher probability of presence for *T. equi* species infection consist of the regions of Thessaly, Macedonia and Peloponnese (Table 14).

The areas with the highest probability (p>0.75) of occurrence of *T. equi* genotype are mostly circumscribed in the eastern half of mainland Greece (Fig. 24C). The regions characterised by an important concentration of potential equid hosts and with a high probability of presence for *T. equi* genotype encompass the regions of Macedonia and Thessaly (Table 14).

The geographic range for *T. equi*-like genotype characterized by a high probability (p>0.75) of presence concentrates in the central part of mainland Greece from the western coast bordering the Ionian island sea to the eastern coast lining the Aegean sea and extends northwards towards central Macedonia as shown in Fig. 24D. The regions with a high probability of presence of *T. equi*-like infection, which exhibit a significant concentration of equids consist of the regions of Macedonia and Thessaly (Table 14).

The AUC for the models range from 0.822 to 0.977, indicating that the models are good to very good (Table X). The p-value using minimum training presence as the threshold is high for *Theileria equi* RLB (p=0.09) indicating that the model may not be useful. The other models have lower p-values (Table 15), with *Theileria equi*-like having the lowest (p=0.001), indicating the best prediction.

Different categories of environmental variables contributed significantly to model building. For *Babesia* and *Theileria* spp., land cover was the variable that achieved the highest training gain when used to build a model with no other variables (Figure 25). Land cover was

also the variable that reduced the training gain the most when dropped from the model. By these measures, land cover is the single most important variable in modelling these species. Land cover types that occurred at the species sampling areas are shown in Table 16. Forest is the primary land cover.

Variable	Babesia caballi	Theileria equi eELISA	Theileria equi RLB	Theileria equi-like RLB
BIO1	0.013	0.216	0.1571	0.2703
BIO2	0	0	0	0.0063
BIO3	0.0082	0	0.0549	0
BIO4	0	0	0.0772	0.1847
BIO5	0.0296	0.2606	0.3449	0.4146
BIO6	0.0088	0.1043	0.0181	0.0834
BIO7	0	0	0.0602	0.61
BIO8	0	0.026	0.0003	0.0198
BIO9	0.028	0.2571	0.2754	0.4093
BIO10	0.0209	0.2638	0.3137	0.4685
BIO11	0.0108	0.1465	0.0497	0.1378
BIO12	0	0.0021	0.0616	0.1019
BIO13	0	0	0.0601	0.1804
BIO14	0.0128	0.1464	0.065	0.1699
BIO15	0	0.0079	0.0097	0.0791
BIO16	0	0	0.0657	0.0796
BIO17	0.0031	0.0949	0.0283	0.1026
BIO18	0.0026	0.0902	0.0376	0.3122
BIO19	0	0	0.0486	0.025
Elevation	0	0.0583	0.0903	0.2214
Land cover	0.4762	0.315	0.5328	0.7526

Figure 25. Training gain achieved by models using single variables. Length of bar represents the training gain value. A longer bar represents a higher training gain.

Other variables that show training gain when modelling with only a single variable included: annual mean temperature (BIO1), maximum temperature of the warmest month (BIO5), mean temperature of the driest quarter (BIO9), and mean temperature of the warmest quarter (BIO10) (Figure 25). Plots from Maxent (not reproduced here) showed that the probability of the species occurrence increases as these temperature variables increase.

Precipitation has a smaller effect on *Babesia* and *Theileria* spp. in the model. For *Theileria equi*-like RLB, the precipitation variable that achieved the highest training gain was precipitation of

the warmest quarter (BIO18). Precipitation seems to have less effect on the presence of *Babesia caballi* and *Theileria equi*.

IV.6. Habitat similarities between related genotypes

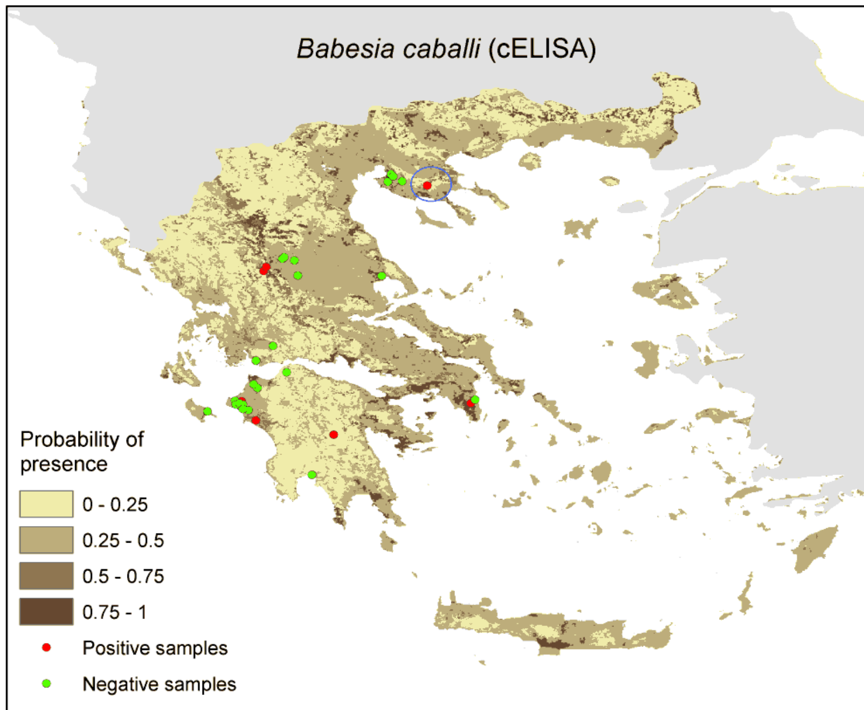
Environmental variables did not differ significantly between the presence locations of *T. equi* and *T. equi*-like genotypes (Table 17). Comparing the presence and absence locations for each genotype, thirteen of 19 bioclimatic variables were significant ($p<0.05$) for *T. equi,* while 10 of the same 13 variables were significant ($p<0.05$) for *T. equi*-like (Table 18).

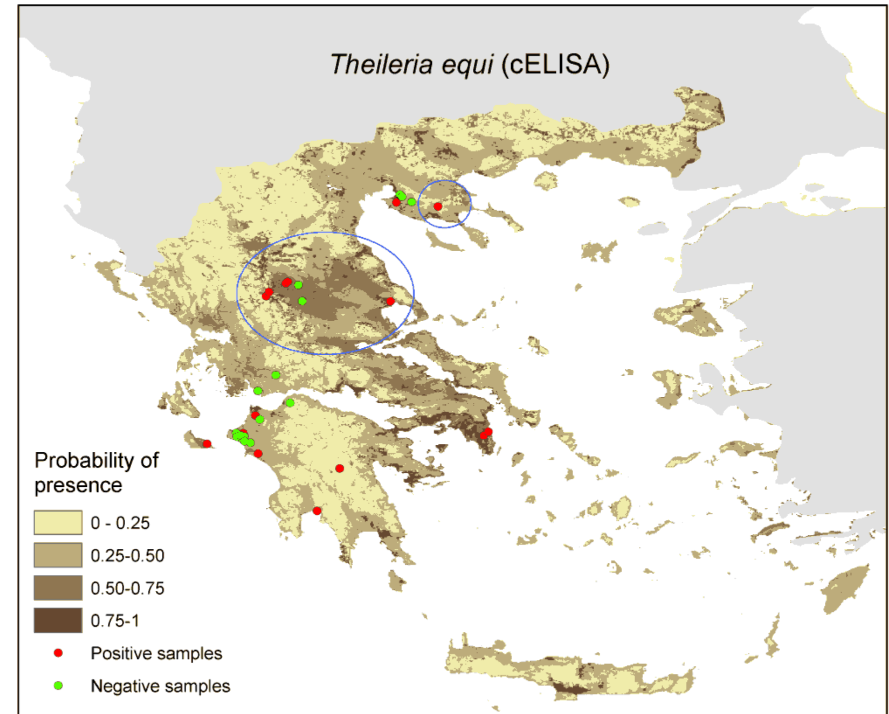
The occurrence locations for *T. equi* and *T. equi*-like were most predominant in forest type land cover class (Table 19). The prevalence of occurrence localities for *T. equi* and *T. equi*-like were significantly higher in forest than in urban and crop plus savanna combined ($p< 0.05$ in both cases), and lower in savanna than in urban and crop plus forest combined ($p< 0.05$ in both cases).

IV.7. Detection of clusters of piroplasm infection

Host geographic location and the number of equids tested positive per location and for each piroplasm are presented in Table 13. The results of the spatial scan statistic analyses are presented in Table 20. Figure x displays the geographic locations of clusters detected.

A significant cluster of infected equids was detected for the species *B. caballi* ($p=0.0001$) as presented in Table 20 and Fig. 26 A. Besides, one most likely cluster ($p=0.0001$) and one secondary cluster ($p=0.003$) of infected animals were identified for the species *T. equi* (Table 20 and Fig. 26 B). No significant clusters were detected for the genotypes *T. equi* and *T. equi*-like.





A **B**

Fig. 26. Significant clusters of equine piroplasms in Greece. (A) Most like cluster of *B. caballi* infection (circle). (B) Most likely cluster (bigger circle) and secondary cluster (smaller circle) of *T. equi* infection.

IV.8. Identification of ticks collected

A total of 13 ticks (5 adults and 8 nymphs) were collected from stray dogs roaming the equestrian centre and from privately owned dogs and horses in a stud farm in Attica region. No ticks were found in other regions at the time of sampling, including Thessaly region where the highest seroprevalence of piroplasm species was recorded. The adult ticks were identified as *Rhipicephalus bursa* (2 females) (Fig. 27) and *R. sanguineus* (2 males and 1 female) (Fig 28) recovered on horses and dogs respectively. Nymphs (8) collected from dogs were identified as *R. sanguineus*.

Fig. 27. *R. bursa* (female)

A B C

Fig 28. *R. sanguineus*. A) Female; B) Male; C) Nymp

Table 7. Number of host sampled and prefectures where hosts where sampled.

Prefectures	**Hosts**			**Total**
	Horses	**Mules**	**Ponies**	
Achaea	10	0	0	10
Aetolia-Arcanania	1	0	0	1
Arcadia	1	0	2	3
Chalkidiki	1	6	0	7
East Attica	575	0	0	575
Ilia	38	0	1	39
Karditsa	8	0	0	8
Magnessia	1	0	0	1
Messinia	5	0	0	5
Pirgos	5	0	3	8
Thessaloniki	84	0	0	84
Trikala	46	7	1	54
Zakynthos	1	0	0	1
Total	776	13	7	796

Table 8. Number (n) of equids tested for infection with *Theileria equi* and/or *Babesia caballi* and seroprevalence (%) of infection within hosts groups and associated factors. Values significantly different ($p<0.05$) between host groups or associated factors are labelled with the same letter ([a, b or c]) in the same column.

Factor	N	*T. equi*		*B. caballi*		*T. equi* and *B. caballi*	
		n	%	n	%	n	%
Animal species							
Horse	524	48	9.2 [a]	6	1.1 [a]	4	0.8 [a]
Mule	13	10	76.9 [a]	5	38.5 [a]	4	30.8 [a]
Pony	7	2	28.6	1	14.3	1	14.3
Gender							
Male	153	20	13.1	2	1.3	0	0
Female	210	23	11	5	2.4	5	2.4
Gelding/mule	181	17	9.4	5	2.8	4	2.2
Age							
<1 year	8	0	0	0	0	0	0
>1-2 years	30	2	6.7	0	0	0	0
>2-5 years	272	19	7[a]	3	1.1	1	0.4
> 5	227	34	15[a]	5	2.2	5	2.2
Origin							
Greece	282	29	10.3	8	2.8	6[a]	2.1
Imported	213	12	2.6	1	0.5	0[a]	0
Unknown	49	-	-	-	-	-	-
Activity							
Racing	327	27	8.3 [a]	2	0.6 [a,b]	0	0 [a,b]
Recreation	91	6	6.6 [b]	4	4.4 [a]	3	3.3 [a]
Farming	126	27	21.4 [a,b]	6	4.8 [b]	6	4.8 [b]
Location							
Attica	327	27	8.3 [a]	2	0.6 [a,b,c]	0	0 [a,b,c]
Thessaly	49	19	38.8 [a,b,c]	3	6.1 [a]	3	6.1 [a]
Peloponnesse	77	8	10.4 [b]	3	3.9 [b]	3	3.9 [b]
Macedonia	91	6	6.6 [c]	4	4.4 [c]	3	3.3 [c]

Table 9. Total number (n) of equid tested positive for all the piroplasms, *T. equi* only, *B. caballi* only, dual *T. equi* and *B. caballi* and seroprevalence (%) of infections with the associated 95% confidence interval (CI).

Total number of equid examined	**Piroplasm**			***T. equi***			***B. caballi***			***T. equi/B. caballi***		
	n	%	95%CI	n	%	95%CI	n	%	95% CI	n	%	95%CI
544	63	11.6	9.1-14.6	60	11[a]	8.6-14	12	2.2[b]	1.2-3.9	9	1.7	0.8-3.2

[a,b]: the superscripts indicate that the values are significantly different ($p<0.05$).

Table 10. Relative risk (RR) of infections computed for significant factors.

Factors	***T. equi***				***B. caballi***				***T. equi* and *B. caballi***			
	n[a] (%)[b]	**RR**	**95%CI[c]**	**p[d]**	**n (%)**	**RR**	**95%CI**	**p**	**n (%)**	**RR**	**95%CI**	**p**
Animal species												
Horse	48 (9.2)	1			6 (1.1)	1			4 (0.8)	1		
Mule	10 (76.9)	8.39	5.61-12.54	0.00	5 (38.5)	33.58	11.74-96.14	0.00	4 (30.8)	40.31	11.30-143.31	0.00
Pony	2 (28.6)	3.11	0.93-10.37	0.13	1 (14.3)	12.47	1.72-90.49	0.08	1 (14.3)	18.71	2.33-146.00	0.06
Activity												
Recreation	6 (6.6)	1			4 (4.4)	1			3 (3.33)	1		
Racing	27 (8.3)	1.25	0.54-2.94	0.60	2 (0.6)	0.14	0.03-0.07	0.02	0	-	-	0.01
Farming	27(21.4)	3.25	1.39-7.54	0.00	6 (4.8)	1.08	0.31-3.72	0.58	6 (4.8)	1.44	0.37-5.62	0.43
Location												
Attica	27 (8.3)	1			2 (2.06)	1			0			
Thessaly	19 (38.8)	4.70	2.84-7.78	0.00	3 (6.1)	10.01	1.71-58.40	0.01	3.61	-	-	0.00
Peloponnese	8 (10.4)	1.25	0.59-2.66	0.34	3 (3.9)	6.37	1.08-37.46	0.04	3 (3.9)	-	-	0.00
Macedonia	6 (6.6)	1.40	0.6-3.3	0.28	4 (4.4)	12.17	2.35-68.00	0.00	3 (3.3)	-	-	0.00
Age group												
≤1	0				-	-	-	-	-	-	-	-
>1-2 years	2(6.7)	1			-	-	-	-	-	-	-	-
>2-5 years	19 (7.0)	1.04	0.25-4.2	0.64	-	-	-	-	-	-	-	-
>5 years	34 (15.0)	2.24	0.57-8.89	0.17	-	-	-	-	-	-	-	-

[a]: number of positive; [b]: prevalence; [c]: confidence interval; [d]: p value

Table 11. Number (n) of equids tested positive and prevalence of infection (%) within hosts. No significant difference in prevalence was found within hosts for any infection (p>0.05).

		Probe									
		Catchall		***T. equi***		***T. equi-like***		***B. caballi***		***B. caballi-like***	
Host	N	n	%	n	%	n	%	n	%	n	%
Horse	772	364	47.20	340	44.00	357	46.20	0	0	3	0.40
Mule	8	6	75.00	5	62.50	6	75.00	0	0	0	0
Pony	7	1	14.30	1	14.30	1	14.30	0	0	0	0
Total	787	371	47.14	346	43.96	364	46.25	0	0	3	0.38

Table 12. Blast similarity search results for the partial 18S rRNA gene sequence of *T. equi*-like.

Genotype sequence (509 bp)	Closest GenBank entry/Origin	Percent identity
Theileria equi-like/Greece	DQ287951.1/ Spain AY534882.1/Spain	100 % 99%

Table 13. Number of equid tested positive (P) or negative (N) for each piroplasm infection in different localities.

B. caballi species			*T. equi* species			*T. equi* genotype			*T. equi*-like genotype		
P	**N**	**Locality**	**P**	**N**	**Locality**	**P**	**N**	**Locality**	**P**	**N**	**Locality**
1	19	Andravida	1	19	Andravida	1	19	Andravida	1	19	Andravida
1	2	Tripoli	1	4	Kalamata	1	0	Neochori	1	4	Kalamata
1	7	Pirghos	1	0	Volos	3	4	Riolos	1	0	Neochori
4	3	Chalkidiki	1	0	Zakinto	1	0	Kavalissas	1	7	Karditsa
2	297	Hippodrome	1	2	Tripoli	5	33	Mikra	3	4	Riolo
2	5	Ropoto	1	1	Lapas	1	14	Thermi	1	0	Kavalissas
1	11	Kotroni	2	6	Pirghos	2	14	Vassilika	1	1	Roviata
			1	37	Mikra	2	13	Ikem	5	33	Mikra
			5	2	Chalkidiki	5	2	Chalkidiki	2	13	Thermi
			23	276	Hippodrome	311	236	Hippodrome	2	14	Vasslika
			4	24	Vavrona	7	21	Vravrona	2	13	Ikem
			3	2	Trikalla	2	6	Trikalla	5	2	Chalkidiki
			4	3	Ropoto	2	5	Ropoto	317	230	Hippodrome
			7	14	Loggaki	1	1	Petrochori	9	19	Vravrona
			5	7	Kotroni	1	2	Faneromeni	2	6	Trikalla
						1	3	Kotroni	4	3	Ropoto
									1	1	Petrochori
									1	2	Faneromeni
									4	17	Loggaki
									1	3	Kotroni

Table 14. Distribution of equid population in regions in Greece by decreasing order of importance. Adapted from the Hellenic Statistical Authority (HSA. 2007).

Regions	**Percentage (%)**
Central Macedonia	15.06
Thessaly	12.95
North Aegean	11.105
East Macedonia and Thrace	10.80
Peloponnese	10.73
West Greece	10.57
South Aegean	10.53
Central Greece	5.30
Epirus	4.20
West Macedonia	3.05
Crete	2.54
Ionian islands	2.45
Attica	0.77

Table 15. Statistical evaluation of the model.

Genotype	**Number* of presence records (Training/Testing)**	**AUC for Training Data**	**AUC for Test Data**	**P-value Using Minimum Training Presence as Threshold**
Babesia caballi cELISA	6/2	0.946	0.946	0.01
Theileria equi cELISA	12/3	0.923	0.954	0.01
Theileria equi RLB	12/4	0.963	0.822	0.09
Theileria equi-like RLB	15/5	0.977	0.857	0.001

*Duplicate presence records for the same localities were removed from the model.

Table 16. Number of sample localities within different land cover categories of the USGS Global Ecosystems land cover classification.

Land cover (class number)	***B. caballi***	***T.equi* ELISA**	***T.equi* RLB**	***T.equi*-like RLB**
Urban (1)	-	2	1	2
Mixed forest (24)	-	1	4	5
Deciduous broadleaf forest (26)	1	1	1	1
Crops and Town (31)	-	2	3	4
Mediterranean Scrub (46)	-	1	-	-
Semi desert Shrubs (51)	-	1	-	-
Forest and Field (56)	4	4	4	4
Fields and Woody Savanna (58)	-	1	1	1
Woody Savanna (91)	2	2	1	2
Deciduous Tree Crop (96)	-	-	1	1

Table 17. Comparison of the bioclimatic variables between occurrence locations for the two *Theileria* genotypes (Temperature values are in ^{o}C; Precipitation values are in mm). No significant difference ($p<0.05$) was found.

Bioclimatic variable	***Theileria equi* occurrence location n=16**		***Theileria equi*-like occurrence location n=20**		
	Mean	**SD[1]**	**Mean**	**SD**	**p value**
Annual Mean Temperature	15.7815	2.1902	16.0100	2.0367	0.7127
Mean Diurnal Range (Mean of monthly (max temp - min temp))	9.8625	1.3321	9.9000	1.2872	0.9488
Isothermality (P2/P7) (* 100)	3.3500	0.2191	3.4000	0.2340	0.5718
Temperature Seasonality (standard deviation *100)	667.6125	68.1426	662.0950	75.5039	0.8235
Max Temperature of Warmest Month	31.7063	1.7650	31.9550	1.8254	0.8606
Min Temperature of Coldest Month	2.7750	3.3575	2.9800	3.2728	0.8603
Temperature Annual Range (P5-P6)	28.9313	3.3226	28.9750	3.5824	0.9363
Mean Temperature of Wettest Quarter	8.8813	3.0023	9.0450	3.0123	0.8734
Mean Temperature of Driest Quarter	24.1938	1.9699	24.3450	1.7957	0.9109
Mean Temperature of Warmest Quarter	24.4313	1.9227	24.5750	1.7603	0.9617
Mean Temperature of Coldest Quarter	7.4375	2.8598	7.8900	2.8049	0.6094
Annual Precipitation	647.5000	189.4504	665.3500	174.2053	0.8986
Precipitation of Wettest Month	103.0625	41.3996	108.4000	39.8568	0.7377
Precipitation of Driest Month	13.4375	7.4563	12.5500	7.1780	0.6879
Precipitation Seasonality (Coefficient of Variation)	51.1875	20.0772	53.2000	19.6029	0.7129
Precipitation of Wettest Quarter	274.8750	111.5442	286.6000	107.2756	0.7740
Precipitation of Driest Quarter	53.5000	29.0815	51.4500	28.3595	0.7738
Precipitation of Warmest Quarter	62.5000	25.2824	61.1500	23.5825	0.6785
Precipitation of Coldest Quarter	252.3750	102.6690	260.5000	95.2678	0.8483

[1] Standard deviation

Table 18. Comparison of the bioclimatic variables between occurrence and absence locations for the two *Theileria* genotypes (Temperature values are in °C; Precipitation values are in mm).

Bioclimatic variable	*Theileria equi* genotype					*Theileria. equi*-like genotype				
	Occurrence location (n=16)		Absence location (n=16)			Occurrence location (n= 20)		Absence location (n= 12)		
	Mean	SD[1]	Mean	SD	p value	Mean	SD	Mean	SD	p value
Annual Mean Temperature	15.7875	2.1902	17.0625	1.6613	0.0492*	16.01	2.0367	17.0917	1.8549	0.0726
Mean Diurnal Range (Mean of monthly (max temp - min temp))	9.8625	1.3321	9.7063	1.1958	0.5705	9.9	1.2872	9.4	0.9234	0.204
Isothermality (P2/P7) (* 100)	3.35	0.2191	3.5688	0.1852	0.0153*	3.4	0.2340	3.5583	0.1881	0.0824
Temperature Seasonality (standard deviation *100)	667.6125	68.1426	604.05	61.5438	0.0039*	662.095	75.5039	592.0583	35.2687	0.0054*
Max Temperature of Warmest Month	31.7063	1.7650	31.9875	1.5466	0.6351	31.955	1.8254	31.6667	1.3248	1
Min Temperature of Coldest Month	2.775	3.3575	5.1313	2.6007	0.0342*	2.98	3.2728	5.575	2.3538	0.017*
Temperature Annual Range (P5-P6)	28.9313	3.3226	26.8563	3.0796	0.0246*	28.975	3.5824	26.0917	1.8118	0.0192*
Mean Temperature of Wettest Quarter	8.8813	3.0023	11.025	2.4912	0.0329*	9.045	3.0123	11.475	2.1085	0.014*
Mean Temperature of Driest Quarter	24.1938	1.9699	24.7688	1.4827	0.3446	24.345	1.7957	24.7083	1.6941	0.2575
Mean Temperature of Warmest Quarter	24.4313	1.9227	24.8875	1.5323	0.6487	24.575	1.7603	24.8	1.7341	0.6662
Mean Temperature of Coldest Quarter	7.4375	2.8598	9.625	2.1265	0.018*	7.89	2.8049	9.8667	2.0513	0.0348*
Annual Precipitation	647.5	189.4504	783.75	100.5369	0.1415	665.35	174.2053	799.4167	107.4891	0.0644
Precipitation of Wettest Month	103.0625	41.3996	143	27.9094	0.0128*	108.4	39.8568	147.4167	27.8844	0.012*
Precipitation of Driest Month	13.4375	7.4563	7.625	3.9476	0.0379*	12.55	7.1780	7.1667	3.5887	0.0849
Precipitation Seasonality (Coefficient of Variation)	51.1875	20.0772	68.3125	12.7055	0.0169*	53.2	19.6029	70.6667	10.5515	0.0243*
Precipitation of Wettest Quarter	274.875	111.5442	380.1875	77.4556	0.0194*	286.6	107.2456	395.75	72.2774	0.0141*
Precipitation of Driest Quarter	53.5	29.0815	32.6875	19.0953	0.0687	51.45	28.3595	29.1667	15.4204	0.0715
Precipitation of Warmest Quarter	62.5	25.2824	48.625	10.4363	0.0221*	61.15	23.5825	46.25	6.9429	0.0464*
Precipitation of Coldest Quarter	252.375	102.6690	341.6875	65.9644	0.0194*	260.5	95.2678	357.9167	62.9422	0.0068*

[1] Standard deviation

*Significant difference ($p<0.05$)

Table 19. Number (n) and prevalence (%) of occurrence locations by generalized land cover class associated with each *Theileria* genotype based on RLB hybridization assay. Each land cover class was contrasted against the grouping of the other two land cover classes. A similar letter ([a, b, c, d]) indicates a significant difference (p<0.05).

Land cover class vs.	**Grouping of the other two land cover classes**	**N**	***T. equi*** n	***T. equi*** (%)	***T. equi*-like** n	***T. equi*-like** (%)
Urban/Crop vs.		9	5	56.6	7	77.8
	Forest plus Savanna	23	11	47.8	13	56.5
Forest vs.		11	9	81.8[a]	10	90.9[c]
	Urban/Crop plus Savanna	21	7	33.3[a]	10	47.6[c]
Savanna vs.		12	2	16.7[b]	3	25.0[d]
	Urban/Crop plus Forest	20	14	70.0[b]	17	85.0[d]

Table 20. Significant clusters of piroplasm infections.

Species	**Cluster**	**Locality/Region**	**Number of cases**	**Expected cases**	**Relative risk**	**p-value**
B. caballi	Most likely	Chalkidiki/Central Macedonia	4	0.24	24.93	0.0001
T. equi	Most likely	Kotroni, Ropoto, Trikala, Loggaki, Volos/Thessaly	21	6.30	4.59	0.0001
	Secondary	Chalkidiki/Central Macedonia	5	0.92	5.84	0.003

V. DISCUSSION

V. 1. Microscopy

Equine piroplasmosis (EP) is routinely diagnosed by microscopic examination of stained blood smear owing to its simplicity and rapidity. Microscopy detection method is generally the test of choice for symptomatic animals that need an immediate veterinary intervention.

In the present study, none of the animals examined tested positive, which did not necessarily mean that the equids tested were infection-free. The major drawback of light-microscopy is its inability to reliably detect equid piroplasms during low parasitemias (Quintao-Silva and Ribeiro, 2003; Krause, 2003) and even in acute cases at the onset of the disease (Mehlhorn and Schein, 1998). Similar results have been reported in Spain where clinically healthy free-ranging horses all tested negative to EP by microscopy examination but 50.8% of the same animals tested positive by RLB (Nagore et al., 2004).

Though the peripheral blood from the skin of the equid ear has been shown to contain the greatest number of piroplasms (Soulsby et al., 1982), studies using blood collected via jugular venipunture to investigate piroplasm infection by microscrospic examination of blood smears are frequently reported (Dumanli et al., 2005; Birkenheuer et al., 2006; Servinc et al., 2008). Aditionally, routine detection of piroplasms in large mammals by light microscopy is usually based on jugular vein blood (Dumanli et al., 2005; Servinc et al., 2008). Therefore, the negative results observed by microscopic examination of thin blood smears cannot be attributed to the use of jugular blood.

The microscopy method, despite its low sensitivity remains more accessible but should be supplemented in case of routine screening by other more sensitive detection systems including serological and molecular methods.

V.2. Serology

V.2.1. Seroprevalence of *T. equi* and *B. caballi* infections

This is the first study on the epidemiology of *T. equi* and *B. caballi* infections in Greece, using serology. The cELISA used in the present study has been shown to be a suitable serological assay for the detection of antibodies to *T. equi* and *B. caballi* in equids, and it is internationally recommended for certification purposes (OIE, 2008)

Previous studies carried out in other countries indicated a wide range of seroprevalence for equine piroplasmosis. Prevalences reported include 59 % for *T. equi* and 45 % for *B. caballi* by IFAT and CFT in Brazil (Heuchert et al., 1999), 40 % for *T. equi* and 28.3 % for *B. caballi* in Spain (Camacho et al., 2005). In Italy, a seroprevalence of 68.4 % by IFAT was found with 12.4 % of animals *T. equi*-positive and 17.9 % *B. caballi* positive (Moretti et al., 2009). In Turkey, one of Greece's nearest neighbour country, the infection rates ranged from 0 to 100 % for *T. equi* and 0 to 33.33 % for *B. caballi*, using IFAT and CFT (Karatepe et al., 2009). In the present study, the overall seroprevalence was found to be 11.6 % for all piroplasms, 11 % for *T. equi* and 2.2 % for *B. caballi,* which is low compared to other countries. The seroprevalence in this study also varied by geographical region within Greece ranging from 6.6 % in Macedonia to 38.8 % in Thessaly for *T. equi* and 4.4% to 6.1% for *B. caballi*. The difference in the prevalence of equine piroplasmosis among countries may be due to differences in sensitivity of the diagnostic tests used, the occurrence and abundance of competent vectors, the activity of the equids and the presence and effectiveness of any control measures. Similarly, the reasons for the variation in prevalence levels among regions may be related to the host activity, the management practices and the difference in prevalence of suitable tick vectors. In Thessaly, where higher prevalences occurred, equids are daily involved in farm activities and consequently, are continuously exposed to ticks infestations. In Macedonia and Peloponnese, equids are used mainly for recreation purposes and therefore have limited access to pasture. Horses in Attica are primarily involved in competition events and are well groomed. However, the presence of dogs with ticks inside the paddock as well as the frequent movement of horses between the hippodrome and different regions of Greece might explain the level of seroprevalence found in Attica. In addition, the horses from the stud farm (representing 8.6 % of the animals examined in Attica) are exposed to tick bites that increase the risk of acquiring piroplasms parasites in the region. The following ticks that transmit both *T. equi* and *B. caballi* occur in Greece: *Hyalomma plumbeum plumbeum* (syn=*H. marginatum*), *H. anatolicum excavatum* and *Rhipicephalus sanguineus* (Soulsby, 1982). *R. bursa*, known to occur in Bulgaria (Soulsby, 1982) and *R. sanguineus* were found in the

region of Attica on horses and dogs, respectively, during the present study. This tick collection is indicative of the tick fauna in Greece. Climatic factors such as temperature, humidity and rainfall influence the habitat of ticks, hence the difference in their prevalence which probably impacts the dynamic of transmission of EP. This is supported by the occurrence of three climatological areas in Greece namely Mediterranean, alpine, and mid-European. The alpine type covers Thessaly and Peloponnese whereas Macedonia belongs to the temperate climate. Attica is located in the transition area between the Mediterranean and the alpine climate.

The seroprevalence of *T. equi* infection was significantly higher than that of *B. caballi*, both at national and regional level in spite of the occurrence of common tick vectors to both piroplasm species in the country. There are reports that *T. equi* is more common than *B. caballi* in endemic countries (Barbosa et al., 1995; Brüning, 1996). This may be associated with the fact that infected animals which survive an initial infection completely eliminate *B. caballi* from their circulation after 1 to 4 years, whereas *T. equi* remains as a life-long infection (de Waal and Van-Heerden, 1994).

V.2.2. Host-related Factors associated with the infections

In regard to host species, the infection rates were significantly higher in mules than in horses for both parasites. This finding may be linked to the outdoor living conditions of mules which were occupied with farm activities, mainly the daily transportation of wood from the forest. This extended exposure to pasture probably increased the likelihood of tick bites. Due to the small sample size of mules and ponies, the significantly higher prevalence of infection in mules compared to horses should not be generalised until a large number of mules and ponies is considered in future studies.

The type of activity of equids significantly impacted the seroprevalence. The level of infection was significantly higher for both piroplasms in farm animals than in recreation or race equids. Farm animals kept under poor environmental conditions are more likely than other equids to be exposed to tick vectors, so the risk of infection is increased (Shkap et al, 1998). Occurrence of piroplasms in race horses that are well groomed may be related to the presence of dogs infested with ticks in their surroundings or to subclinical infections.

Equids less than 2 years old did not show any seropositivity to *B. caballi* infections but exhibited a low prevalence with *T. equi* infections. This finding is not consistent with a previous report from Trinidad where Asgarali et al. (2006) found a higher frequency of *B. caballi* than *T. equi* infection in foals and yearlings. The contradictory findings may be associated with differences in the level of infection with each parasite in the two countries or differences in the managements of age groups. Antibodies to *B. caballi* were more frequent than those to *T. equi* in Trinidad (68.8% and 33.3%, respectively) whereas in Greece, *T. equi* seropositivity was five times higher (11%) than that of *B. caballi* (2.2%). *T. equi*-seropositivity was observed to be significantly higher in older equids and increased with the age of the animals. These results are in agreement with other findings (Asgarali et al., 2006; Rüegg et al., 2007). The reason for the increased prevalence of *T. equi*-positive sera according to age group may be the persistence of *T. equi* in their hosts after recovery from a primary or acute infection (Hourrigan and Knowles, 1979; Brüning, 1996). However, it has been shown that offsprings of preimmunized mares are naïve at birth and acquire passive immunity through colostrum (de Waal and Van Heerden, 1994). Nevertheless, this immunity is transitory and wanes after a period of time (Kumar et al., 2008). Higher seropositivity in older animals may also be due to lower transmission levels which require longer exposure for the acquisition of infection. As for *B. caballi*, the converse may occur whereby formerly infected animals clear the parasites within 1-4 years regardless of the treatment (Holman et al., 1993; de Waal and Van- Heerden, 1994; Rüegg et al., 2007Rüegg et al., 2007).

The seropositivity levels were not significantly different between genders of hosts. This observation agrees with the results of Asgarali et al., (2006) and Karatepe et al., (2009). However, Shkap et al. (1998) found that stallions were significantly less affected than mares and geldings in Israel, but this was due to the fact that stallions, maintained under strict control for breeding were less exposed to tick infestation.

The present study found that the prevalence of seropositive equids for both *T. equi* and *B. caballi* was much higher in native animals than those imported. No mixed infection was recorded in imported equids. The difference in prevalence between native and imported animals in regard to mixed infections was significant. These findings clearly establish that EPI is enzootic in Greece. The enzootic status of EPI in Greece is supported by the occurrence of four tick vectors,

namely *R. sanguineus*, *R. bursa*, *Hyalomma plumbeum plumbeum*, *H. anatolicum excavatum* which are suitable vectors for both *T. equi* and *B. caballi* in the country (Soulsby, 1982). Since most of the imported horses came from countries where the disease has been documented, such as France (Leblong et al., 2005), Italy (Moretti et al., 2009) and Spain (Camacho et al., 2005), the source of the infection in equids in Greece is unknown. Greece is geographically part of the Balkan Peninsula which is an enzootic area for EPI (Schein, 1988).

Canine piroplasmida, *B. canis* has been detected in horses both by molecular (Criado-Fornelio et al, 2003) and serological methods (Hornok et al., 2007). The diagnostic test used in this study (cELISA) has been shown to be of high specificity, ranging from 99.2 to 99.5 % for both *T. equi* and *B. caballi*. Therefore, the chance of cross-reactions to occur between *B. caballi* and *B. canis* is very low.

V.3. Genomic analysis

The prevalence levels of *Theileria* infections in equids in the present study were much higher than in a previous report (Kouam et al., 2010). It is worth mentioning that the samples in the present investigation were collected from the same areas as in the aforementioned study. This difference may be explained by the high sensitivity of the RLB assay (Nagore et al., 2004); furthermore it is not known whether or not the competitive-inhibition ELISA test (cELISA) used in the previous study was able to detect both *Theileria equi* and *Theileria equi*-like genotypes, since the similarity of the merozoite antigene-1 (EMA 1) gene used for the cElisa antigen (Knowles et al., 1992) has not yet been explored between the two genotypes. Further studies are needed to see if there is a difference between the two genotypes in terms of immunogenicity and not just within the 18S rRNA gene. Likewise, the similarity of the rhoptry-associated protein (RAP-1) gene used for the cELISA antigen to detect antibodies against *B. caballi* (Kappmeyer et al., 1999) has not been investigated between *B. caballi* and *B. caballi*-like genotypes. The low prevalence of *Babesia* recorded in the present work (0.38%) is comparable to the prevalence observed in the previous study (2.2%). This finding reinforces the observation that in the Mediterranean region, equine *Theileria* infections are more frequently diagnosed than *Babesia* infections (Schein, 1988; Bashiruddin et al., 1999; Criado-Fornelio et al., 2003). It has been reported that parasitemia in *Babesia* infections in equids generally tend to be very low, rarely

exceeding 1% (Hanafusa et al., 1998). Therefore, the low prevalence generally observed in *Babesia* infections may be related to a very low parasitemia level of the parasite.

Two genetically distinct *Theileria* and one *Babesia* genotypes were clearly identified in the present study. This is the first report on the use of RLB assay for genotype identification of equine piroplasms in Greece. These genotypes were first described in Spain (Nagore et al.,2004) and recently reported in South Africa within 3 heterogeneous groups for *Theileria* (Group A, B and C) and 2 for *Babesia* (Group A and B) (Bhoora et al., 2009). Under this nomenclature, *T. equi* and *T. equi*-like genotypes reported in this study were assigned to Group A and Group B, respectively within the *Theileria* Group, whereas *Babesia caballi*-like belongs to group A within the *Babesia* Group. Within *Theileria*, Group A, B, C have been recently reported in Sudan, besides a fourth group designated as Group D (Salim et al., 2010). The occurrence of *T. equi* and T. *equi*-like genotypes in Spain, Greece, Sudan and South Africa and *Babesia equi*-like genotype in Spain, Greece and South Africa shows that these genotypes are wide-spread. In the present study *T. equi* always occurred as a mixed-infection with *T. equi*-like genotype but the latter occurred predominantly alone in eighteen samples. The predominance of *T. equi*-like over *T. equi* has been reported in Spain as well (Nagore et al., 2004). The *T. equi*-like genotype has been reported to be more pathogenic than the *T. equi* genotype and it was even associated with the death of a horse in Spain (Nagore et al., 2004). In the present study, the only case of horse death reported was associated with a mixed *T. equi*-like and *T. equi* infection. Therefore, the predominance of *T. equi*-like in Greek equids should be a matter of great concern for the stakeholders of the equine industry in Greece.

A partial sequence of the V4 region of the 18S rRNA gene of a *T. equi*-like isolate showed only 99 % similarity with the reference *T. equi*-like isolates from Northern Spain (Nagore et al., 2004) from which the detecting probe used in the present study was designed and 100% identity with the *T. equi*-like variants from Southern Spain (Criado et al., 2006). This finding is an indication of a measureable degree of polymorphism within the *T. equi*-like genotype, and was previously observed by Criado and co-workers (2006).

Only the *B. caballi*-like genotype was identified in this study, but this finding does not preclude the presence of the *B. caballi* genotype in Greece. Bhoora et al., (2009) reported in their

study the inability of RLB assay to detect all the *Babesia caballi* infections, an observation that was attributed to the very low *Babesia caballi* parasitemia.

No unusual parasites previously reported in horses such as *B. canis canis* (Criado-Fornelio et al.,2003, Hornok et al., 2007) and *B. bovis* (Criado et al., 2006) were detected in the present work. However, seven samples gave faint signals with the catchall probe which detects all possible *Theileria/Babesia* genotypes in a sample, and consequently acts as a guarantee that no new piroplasm species or a new strain of a known piroplasm species will pass undetected. Usually, the signal of group-specific probes in a RLB assay is much stronger compared to species- or genotype-specific probes as a result of a multiple reaction with all or multiple parasite variants in a sample (Gubbels et al., 1999, Schnittger et al., 2004). Therefore, the occurrence of a faint signal with a group-specific probe such as the catchall probe is interpreted as the possible presence, in a very low amount, of a known or unknown piroplasm species or genotype in Greece. As mentioned above, no sequence information could be obtained from the V4 region of the 18S r RNA gene of the parasites in these samples. Consequently, no clear-cut conclusions on the real status of these piroplasm-positive samples could be made.

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Bioclimatic variables, which are relevant to the geographic distribution of a species (Estrada-pena et al.,2008), were similar between *T. equi* and *T. equi*-like genotypes' geographical locations and slightly differed when the presence and absence of locations were compared for each genotype raising the question of the real taxonomic position of these closely-related genotypes. Considering the divergence of *T. equi*-like with respect to *T. equi* genotype, Nagore and co-workers (2004) hypothesised that they might be dealing with a new piroplasm species but indicated that additional non-genetic features would be necessary to be conclusive. Bhoora and co-workers (2009) could not rule out the possibility that the different groups of *T. equi* and *B. caballi* identified in their study on the basis of the heterogeneity of the 18S rRNA gene sequence represented different parasite species, though it is not possible to use the 18S rRNA gene sequence variation to classify the organisms as different species (Chae et al., 1999, Criado-Fornelio et al., 2004). Given the difficulty in assigning a taxon to an organism based on the heterogeneity of the 18S rRNA gene, there a need for the scientific community to agree by how much the 18S rRNA sequences must differ from the source organisms to be considered

different species rather than merely strain or genotype variations. Additionally, sequences from new target genes that can complement findings from the 18S r RNA gene may be useful. In the present study, the occurrence locations for *T. equi* and *T. equi*-like genotypes shared the same relevant bioclimatic variables and when occurrence and absence locations were compared for each genotype, both genotypes shared the same significant variables and differed only in three. This finding is evidence that both genotypes are closely related ecologically and should not be regarded as different species yet but as organisms undergoing a speciation process. Alternatively, species convergence is a possibility due the fact that both *T. equi* and *T. equi*-like occur in the same site (red blood cells) in the vertebrate host (Nagore et al., 2004). Further studies involving both the vertebrate (equids) and the invertebrate (ticks) hosts will be helpful to elucidate the possibility of convergence phenomenon. Nagore and co-workers (2004), and Criado-Fornelio and co-workers (2004) pointed out that in order to assign a taxon to a newly reported piroplasm genotype, a possible differential feature should be a distinct geographic distribution, which is unlikely in this case since the same host types (equids for *T. equi* and *T. equi*-like) were co-infected with both sister genotypes. The nature of the hypothesized speciation is therefore thought to be sympatric rather than allopatric. Additionally, one of the conditions of sympatric speciation in parasitology is when a parasite is able to choose its hosts actively such as ticks and fleas (McCoy, 2003). Since equine piroplasms are tick-borne parasites, the speciation underway between the sister genotypes is regarded as sympatric.

The preferred land cover class for both *T. equi* and *T. equi*-like occurrence locations was the forest. This is expected since vegetation is known to provide the tick vectors adequate protection and infallible support for hosts questing (Wall and Shearer, 2001; Torina et al., 2008)

V.4. Geographic distribution of equine piroplasms

The present study reports for the first time clusters of piroplasm infections as well as the potential geographic range of different piroplasms species and genotypes that occur in the country.

The relatively small number of presence locations (7 to 20) used to model *Babesia* and *Theileria* spp. may have resulted in poorer models as the lower p-values indicate. In other research, the precise number of samples needed for an accurate model varies. Stockwell and Peterson (2002) found that the accuracy of models was best using 50 data points in the GARP modeling program. Papes and Baubert (2007) found an agreement between GARP and Maxent models starting at around 15 records which may indicate the minimum number of records needed for accurate modeling. Pearson et al. (2007) found that models built with as few as 5 records showed some statistical significance using Maxent. However, by experimentally dropping records from a model, they found that the predicted distribution was affected by which records were kept.

Additional collection sites for *Babesia* and *Theileria* spp. may change the predicted distribution of these species. Therefore, it needs to be stressed that these models should be regarded as a first attempt at predicting the location of these species and not as a final product. Future research should attempt to collect data in new locations to improve the current models. The models at this stage may be useful for guiding the collection of data in future studies.

The response of *Babesia* and *Theileria* spp. to land cover and temperature in the model may be a reflection of the environmental requirements of the tick vectors of these parasites. Ticks have been found to survive for longer periods of time in forested areas, particularly bottomland forests with regulated temperatures and higher humidities, compared to open meadows (Semtner et al., 1971). Tick oviposition rate and hatching success are affected by temperature and humidity (Chilton and Bull, 1994; Despins, 1992). In addition to the effect of temperature on tick development, temperature has been shown to affect the development rate of *Theileria* in ticks (Young and Leitch, 1981). Whether the importance of temperature in the ecological niche model is a reflection of the tick environmental requirements or the piroplasm environmental requirements is unknown. Future research and modeling of tick distribution in Greece might improve our understanding of the distribution of these piroplasms.

Although humidity plays a role in tick development (Chilton and Bull, 1994; Despins, 1992), humidity data were not available for developing the current model. Precipitation which was available for modelling is associated with humidity but appears to play a lesser role in disease development than temperature.

Significant clusters of infection for both piroplasm species were detected in the regions of Central Macedonia and Thessaly which are characterized by a considerable size of equine populations, 15.06 % and 12.95% of the total equine population in Greece, respectively. The occurrence of the most likely cluster of *B. caballi* infection and the secondary non overlapping cluster of *T. equi* infection in the same area is consistent with a possible involvement of a common risk factor, probably a common tick vector. Common tick vectors of *B. caballi* and *T. equi* have been detected in Greece (Papadopoulos et al., 1996; Kouam et al., 2010). The most likely cluster for *T. equi* infection did not coincide with that of *B caballi* infection, suggesting that other risk factors might be important for clusters of *Theileria equi*. Since both *T. equi* and *B. caballi* occur in the same hosts, a possible discriminating factor might be the presence of *Theileria equi*-specific tick vectors. The distribution range of tick species may well vary between high risk areas associated with both significant clusters as a result of the heterogeneity of climates and landscapes. It has been demonstrated that the habitat of ticks is more set by abiotic factors such as vegetation and climate (which determine tick development and survival rates on the ground) rather than by host-related factors (Randolph, 2000). The significant clusters detected might be helpful in investigating the underlying causes of increased risk in the identified areas, the landscape attributes, and the climate variables characteristic of high risk areas.

The clusters identified in this study fall in areas with high probability ($p>0.75$) of presence for both *Theileria* and *Babesia* species predicted by Maxent program and which concentrates in the eastern half of the country. Furthermore, the regions with significant concentration of potential equid hosts are located in the high probability predicted areas. This observation suggests that the eastern part of Greece should be considered as a priority area for detailed epidemiological investigations and regular surveillance for equine piroplasmosis.

VI. CONCLUSION AND PERSPECTIVES

This study demonstrated that Greece should be regarded as an enzootic region for equine piroplasm infections. Two competent tick vectors (*Rhipicephalus. sanguineus*, "the brown dog tick" and *Rhipicephalus bursa)* for *Theileria equi* and *Babesia caballi* were unequivocally identified. The fact that R. *sanguineus* was collected from stray dogs within an equestrian centre paddock and from a neighbouring stud farm should alert veterinarians and horse owners of the potential danger to the horse industry in the Attica region. Nationally, there is a need for further research on the dynamics of tick transmission and the geographical distribution of the four resident vector species.

Two equine *Theileria* genotypes and one *Babesia* genotype occur in Greece. The Greek *T. equi*-like isolate is 100% similar to the southern Spanish isolates and shares 99 % similarity with the northern Spain isolates. The question of the predominance of *Theileria equi*-like over *Theileria equi* in Greece should be addressed seriously since the former genotype was reported to be more pathogenic than the latter one. Rather than regarding *Theileria equi* and *Theileria equi*-like genotype as distinct species as suggested by Nagore and co-workers, they should be regarded as sister genotypes, possibly undergoing a sympatric speciation or converging into a single species.

Further research is needed to compare different genotype isolates among regions, to identify the specific tick vector of each genotype, and to produce geographic distribution maps of equine piroplasms in Greece and elsewhere in order to better understand the epidemiology of equine piroplasmosis.

This study also demonstrated that clusters of equine piroplasms occurred in the regions of Macedonia and Thessaly in continental Greece. The eastern half of the country which covers areas with high probability of piroplasm presence and contains a significant number of potential equid hosts and clusters of piroplasms should be prioritized for further epidemiological

investigations. Finally, maximum entropy ecological niche modelling and spatial scan statistic have proved to be useful tools for the surveillance of animal diseases.

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VIII. APPENDICES

Appendix 1

Microscopic examination of equid blood samples

Purpose

To prepare Giemsa -stained thin blood smears with the view to detecting piroplasms by microscopic examination)

1.1. Reagents

Giemsa' s solution (Azur-eosin-methlylene blue solution for microscopy), Merck
Absolute methanol
DPX mountant for microscopy, BDH
Immersion oil for microscopy, Merck
Distilled water

1.2. Equipment

Glass microscope slides
Coverslips
Plastic Pasteur pipettes or wooden applicator stick
Staining jars (vertical staining jars which accommodate 8 slides, with a glass slid)
Microscope (Olympus BX50) connected to an image analysis system (Image Pro Plus, version 3.0.0.1.0.0 software)

1.3. Procedure

(1) Gently flick the blood sample tube a few times to mix the whole blood
(2) Dispense 5µl (1-2 mm) of blood near the label end of the microscope slide on a flat surface as illustrated in Fig. 1 A
(3) Angle a spreader (a second glass slide or a coverslip) about 40º and allow the spreader tip to contact the drop of blood, then allow the blood to spread across the spreader (Fig.1 B)
(4) In a smooth and quick motion, drag the spreader forward from the label end of the first slide at 30-40º, creating a thin blood smear (Fig. 1C-D).
(5) Allow smear to dry as rapidly as possible, in a dust free area (rapid drying produces a better result)
(6) Fix in absolute methanol in the staining jar for 5 minutes and air-dry
(7) Dilute stock Giemsa stain 1:20 with distilled water. Fresh stain should be prepared every two days
(8) Stain the blood film for 30-35 minutes by placing the slide in the staining jar containing an adequate amount of diluted stain. The solution should covered the blood film only, not the label end of the slide
(9) Wash stain away gently with tap water for 35-45s
(10) Air-dry;
(11) Place a drop of DPX mountant on the smear, then mount the coverslip. This step is not compulsory and is only necessary if one wants to keep the slide for a long time, to make a preparation permanent.
(12) Examine the blood film using the 100X (oil immersion) objective. Parasite cytoplasm will stain blue and nuclei will stain magenta. The characteristic feature of *B. caballi* is a joint pair of pyriform organisms forming and acute angle in stained red blood cells (Fig. 2A). The distinctive characteristic of *T. equi* species is four pear-shaped stages that form a tetrad in a form of a Maltese cross in stained red blood cells (Fig. 2B).
(13) Photograph the film using the image analysis software (eg. Image Pro Plus) for documentation and further uses.

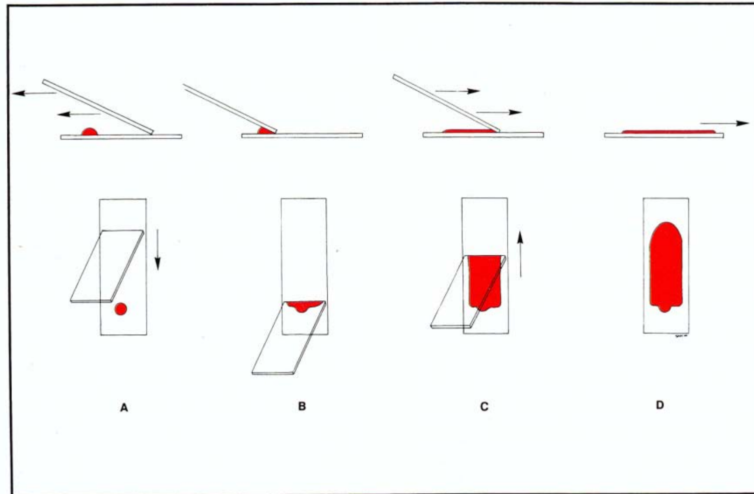
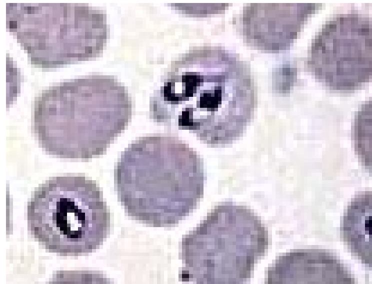
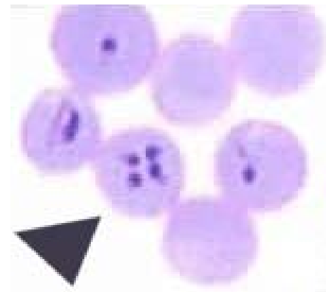


Fig.1. Demonstration of a thin blood smear preparation (adapted from Hendrix and Robinson, 2006).





A B

Fig. 2. Joint pyriform organisms forming a acute angle characteristic of *B. caballi* in a stained red blood cell (A) and four stages in the form of Maltese cross characteristic of *T. equi* (B) in a stained red blood cell.

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Appendix 2

Serological testing of equid sera

Purpose

To detect antibodies against *Theileria equi* and *B. caballi* antigens

The test procedure presented was adapted from the literature provided with the kits (*Babesia equi* Antibody Test Kit, cElisa and *Babesia caballi* Antibody Test Kit cElisa from VMRD, Inc).

2.1. Reagents

Kit contents (reagents stored at 2-7 °C (35- 45 °F).

Components		Amount/Number
Reagent Label	**Name**	
A	Antigen-coated plate	2 plates
B	Positive control	2 ml
C	Negative control	2 ml
D	100X Primary Antibody	300 µl
E	100X Secondary Antibody-Peroxydase conjugate	300 µl
F	Antibody Diluting Buffer	60 ml
G	Serum Diluting Buffer	9 ml
H	10X Wash Solution Concentrate	120 ml
i	Substrate Solution	30 ml
J	Stop solution	30 ml

2.2. Equipment

These were needed materials not included in the test kit.

- Single multichannel adjustable-volume pipettors.
- Disposables yellow plastic tips (1-200 µl).
- Non- antigen-coated transfer plates.
- Elisa microplate reader or spectrophotometer with 620, 630 or 650 nm filters.
- Deoinased or distilled water.
- Paper towels.
- Multichannel pipettor reservoirs.
- Graduate cylinder.
- Wash bottle.
- Timer (clock)

2.3. Procedure

2.3.1. Preparation of reagents

(a) Warm up reagents: bring the serum samples, reagents and plate(s) to room temperature prior to starting the test.

(b) Prepare controls and samples: the positive and negative controls (B&C) and test serum must be diluted with Serum Diluting Buffer (G) for use in the test. It is recommended that these dilutions be made in a non-antigen-coated transfer plate. Run Positive control (B) in duplicate and negative control (C) in triplicate, regardless of the number of serum samples to be tested. When the whole plate is used, it is best to put controls in wells on different parts of the plate. Controls must be run on every plate. Enter the control and serum sample IDs on a copy of the following set up record.

Setup record

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

(c) Prepare plates: remove the plate from the foil pouch and place the strips to be used in the frame and number the top of each strip to maintain orientation with the setup record. Always mark the strip in case they fall out of the frame during washing.

(d) Prepare primary antibody: prepare 1X primary antibody by diluting 1 part of the 100X primary antibody (D) with 99 Parts of antibody diluting buffer (F). Example: For 96 wells, mix 60 µl of 100X Primary antibody (D) with 5.940 ml of antibody diluting buffer (F) to yield 6 ml of 1X primary antibody. 50 µl are needed per well.

(c) Prepare secondary antibody-peroxidase conjugate: prepare 1X secondary antibody-peroxidase conjugate by diluting 1 part of the 100X secondary antibody_peroxdase conjugate (E) with 99 part of antibody diluting buffer (F). Example: for 96 wells, mix 60 µl of 100X secondary antibody- peroxidase conjugate (E) with 5.940 ml of antibody diluting buffer (F) to yield 6 ml of 1X secondary antibody- peroxidase conjugate . 50 µl are needed per well.

(f) Prepare wash solution: Prepare 1X wash solution by diluting 1 part of the 10X wash solution concentrate (H) with 9 part of deionized or distilled water. Approximately 1.8 ml are needed per well. Allow extra quantity for reservoir, tubing, pipetting, etc.

2.3.2. Test Protocol

(1) Load controls and serum samples: using a pipettor set at 50µl, transfer diluted controls and serum samples to the antigen-coated plate (A) according to the setup record. Tap the side of the loaded assay plate several times to make sure the samples coat the bottom of the well. Use care not to spill samples from well to well. Incubate the plate 30 minutes at room temperature (21-25°C, 70-77°F).

(2) Wash well: After the 30-minutes incubation, wash three times: for manual washing, dump contents of the wells into a sink and remove the remaining sera and control by sharply striking the inverted plate 4 times on a clean paper towel, striking a clean area each time. Immediately fill each well with 1X wash solution using a multichannel pipettor. Dump out the wash solution and strike the inverted plate sharply on a clean paper towel as above. Repeat the washing procedure 2 more times (3 wash total).

(3) Add primary antibody: add 50 µl of diluted (1X) primary antibody to each well. Tap the side of the loaded assay plate several times to make sure the primary antibody coats the bottom of the wells. Incubate for an additional 30 minutes at room temperature.

(4) Wash well: after the second 30-minutes incubation, wash the plate 3 times as in step 2.

(5) Add secondary antibody-peroxydase conjugate: add 50 µl (1X) secondary antibody conjugate to each well. Tap the side of the loaded assay several times to make sure the conjugate coats the bottom of the wells. Incubate for an additional 30 minutes at room temperature.

(6) Wash well: after the third 30-minute incubation, wash the plate 3 times as in step 2.

(7) Add substrate solution: add 50 µl of substrate solution (I) to each well. Tap the side of the loaded assay plate several times to make sure the substrate coats the bottom of the wells. Incubate for 15 minutes at room temperature. Avoid leaving the plate in direct sunlight. Do not empty wells.

(8) Add stop solution: add 50 µl of stop solution (J) to each well. Tap the side of the loaded assay plate several times to mix the substrate solution and the stop solution. Do not empty wells.

(9) Read and record the test results: immediately after adding the stop solution, the plate should be read on a plate reader. Set the optical density (O.D) reading wavelength to 620, 630 or 650 nm. Blank the reader on air and read plate(s). Some readers require an empty well on the plate for blanking. In this case, no reagents should be added to this well. An automatic plate reader (Infinite 2000, Tecan) was used in this study, with the OD set at 630 nm. The plate brand

available in the automatic plate reader compatible with the kit plate was “Corning 96 Flat bottom Transparent” plate.

(10) Return the remaining kit reagents to 2-7°C.

2.3.3. Test validation and interpretations of the results

The mean of the negative controls must produce an optical density> 0.300 and < 2.000.

The mean of the positive controls must produce an inhibition of >= 40 %.

Calculation of percent inhibition (%I):

%I = 100-[(Sample O.D. x 100) ÷ (mean negative control O.D)].

Interpreting the results

If a test sample produces >= 40% inhibition, it is positive.

If a test produces <40% inhibition, it is negative.

Appendix 3

DNA extraction from equid blood samples

Purpose

To extract parasite DNA from equid blood samples for use in molecular tests.

Two different kits (NuleoSpin® Blood, Macherey-Nagel GmbH and Co. KG, Germany and Jetquick Blood & Cell Culture DNA Spin Kit, Genomeh GmbH, Germany) were used to extract genomic DNA from whole blood.

The purification procedures presented were adapted from the literature provided with each test kit.

3.1. DNA purification using NuleoSpin® Blood kit

3.1.1. Reagents

Kit contents:

Reagents	Amount for 250 preps
Buffer B1	50 ml
Reagent B2	12.5 ml
Buffer B5 (concentrate)	2 x 20 ml
Buffer BW	2 x 75ml
Buffer BE	60 ml
Proteinase K (lyophilized)	2 x 75 mg
Proteinase Buffer	8 ml
NucleoSpin® blood columns and collection tubes	250
2 ml collecting tubes	500
Label for buffer B3	1

Other reagents needed but not included in the kit:

96-100% ethanol.

3.1.2. Equipment

Microcentrifuge

Microcentrifuge tubes, 1.5 ml, sterile

Incubator or watherbath

Vortex

Heating block

-20ºC freezer for storing crude genomic DNA samples.

3.1.3. Procedure

3.1.3.1 Preparation of the working solutions

(a) Prepare proteinase K solution: add 3.35 ml of Proteinase Buffer to dissolve lyophilised proteinase K. Proteinase k solution is stable at 4 ºC up to 6 months. Storage at -20 ºC is recommended if the solution will not be used up during this period.

(b) Prepare buffer B5: add 80 ml 96-100 % ethanol to buffer B5 concentrate. Store buffer B5 at room temperature (20-25 ºC) for up to one year.

(c) Prepare buffer B3: transfer buffer B1 to reagent B2 completely and mix very well. The resulting buffer B3 is stable for up to one year at room temperature.

3.1.3.2. Protocol

Before starting, set the incubator or the water bath to 70 ºC. Equilibrate buffer BE to 70 ºC.

(1) Lyse blood samples by pipetting 25 µl proteinase K and up to 200 µl blood into 1.5 ml microcentrifuge tube. Then add 200µl lysis buffer B3 to the samples and vortex the mixture vigorously (10-20 s). Incubate samples at 70ºC for 10-15 minutes using either the heating block or the incubator. Increase incubation time with proteinase K (up to 30 min) and vortex once or twice vigorously during incubation if processing older or clotted blood samples.

(2) Adjust DNA binding conditions by adding 210 µl ethanol (96-100%) to each sample, then vortex again.

(3) Bind DNA: for each preparation, take one NucleoSpin® Blood column placed in a 2ml centrifuge tube and load the sample. Centrifuge 1mn at 11, 000 x g. If the samples are not drawn through the matrix completely, repeat the centrifugation at higher g-force (< 15000 x g). Discard collecting tube with flow-through.

(4) Wash silica membrane: for the first wash, place the NucleoSpin® Blood column into a new 2 ml collecting tube and add 500 µl buffer BW. Centrifuge 1mn at 11,000x g. Discard collecting tube with flow-through. For the second wash, place the NucleoSpin® Blood column into a new collecting tube and add 600 µl buffer B5. Centrifuge 1 min at 11,000 x g. Discard flow-through.

(5) Dry silica membrane, placing the NucleoSpin® Blood column back to the collecting tube and centrifuge 1 mn at 11,000 x g. Residual alcohol is removed at this step.

(6) Elute highly pure DNA: place the NucleoSpin® Blood column in a 1.5 ml microcentrifuge tube and add 100 µl prewarmed elution buffer BE (70ºC). Dispense buffer directly onto the silica membrane. Incubate at room temperature for 1mn. Centrifuge 1min at 11,000 x g.

(7) Store the genomic DNA at – 20 ºC until needed.

The amount of DNA isolated was quantified spectrophotometrically and the typical concentration ranged between 21 to 89 g.

3.2. DNA purification using Jetquick Blood & Cell Culture DNA Spin kit.

3.2.1. Reagents

Kit contents:

RNase (dried form)
Buffer K1
Buffer KX
Buffer K2

Elution buffer, 10 mM Tris-HCL buffer (pH= 8.5)

Other reagents needed but not included in the kit:

Absolute ethanol

3.2.2. Equipment

The same items were required as in the previous kit.

3.2.3. Procedure

3.2.3.1. Preparation of the working solutions

(a) Prepare Genomeh-Protease solution: dissolve the protease in each vial in 1.1 ml water sterile, bidistilled water; this gives the working concentration of 20 mg/ml. Store the dissolve enzyme at -20ºC. Avoid multiple freezing/ thawing.

(b) Prepare RNase A solution: dissolve the Rnase A in 550 µl of sterile, bidistilled water; this gives a working concentration of 20 mg/ml. Store the dissolve enzyme at -20ºC. Avoid multiple freezing/ thawing.

(c) Reconstitute buffer KX: add 16 ml absolute ethanol to buffer KX.

(d) Reconstitute buffer K2: add 25 ml absolute ethanol to buffer K2.

3.2.3.2. Protocol

Before starting, set the heating block to 58ºC and the incubator or water bath to 70 ºC. Equilibrate the elution buffer to 70 ºC.

(1) Pipette into 1.5 ml microcentrifuge tube (Eppendorf) up to 200 µl of whole blood.

(2) Add 20 µl Genomed protease to the sample, and then mix very well either by vortexing or by inverting of the tube. Then, add 200 µl buffer k1 to this sample and

mix very thoroughly as previously indicated. For RNA-free genomic DNA, add 10 µl of RNAase A solution (20 mg/ml) prior to the addition of buffer K1.

(3) Incubate for 10 min at 58 ºC.

(4) Add 200 µl of absolute ethanol to the mixture and mix immediately and very thoroughly in order to prevent any precipitation of nucleic acids due to high local alcohol concentrations. Use only ethanol because other alcohols may cause inconsistent yields.

(5) Assemble a Jetquick micro-spin column with a 2 ml receiver tube. Apply the sample from step 4 into the Jetquick micro-spin column without moistening the rim of the micro-spin cup and centrifuge the sample for 1mn at 10.000 x g (approximately 10,600 rpm) through the silica membrane.

(6) Discard the flowthrough, re-assemble the micro-spin unit with the receiver tube and wash the Jetquick column by applying 500 µl buffer KX reconstituted with ethanol and centrifuge for 1 min at 10, 000 g.

(7) Discard the flowthrough, re-assemble the micro-spin unit with the receiver tube and wash the Jetquick column by applying 500 µl buffer K2 reconstituted with ethanol and centrifuge for 1 min at 10, 000 g.

(8) Discard the flowthrough, re-assemble the micro-spin unit with the receiver tube and centrifuge the empty tube again for 1 min at full speed (~ 13,000 rpm) in order to clear the silica membrane from residual liquid.

(9) Insert the Jetquick micro-spin tube into a new, sterile 1.5 ml reaction tube and elute the DNA from the membrane with 200 µl of prewarmed elution buffer (70 ºC) or simply water. The elution buffer should be pipetted directly onto the center of the silica membrane, making sure that the whole membrane comes into contact with the elution buffer.

(10) Incubate the spin column for 2 min at room temperature after application of the elution buffer and centrifuge for 2 min at 10,000 x g. The elution buffer now contains the pure DNA that can be further processed immediately.

DNA was stored at -20 ºC until use.

Appendix 4

PCR amplification of parasite DNA from equid blood sample

Puropose

To amplify the hypervariable V4 region of the 18S rRNA gene of *Theileria/Babesia* from equid blood samples to serve as target in the RLB assay.

4.1 Reagents

Water, DEPC treated, Molecular biology grade (autoclaved)
Taq DNA polymerase, recombinant, 5u/µl stock concentration, Invitrogen
10 X Taq polymerase buffer (Invitrogen)
50 mM $MgCl_2$ (invitrogen)
dNTP mixture, 10mM (contains dATP, dCTP, dGTP, and dTTP), Fermentas
20 pmol/µl forward primer (RLB-F), Thermo Scientific
20 pmol/µl reverse primer (RLB-R), Thermo Scientific
5µl (~250 ng) genomic DNA

4.2 Equipment

0.2-ml PCR thin-walled tubes (autoclaved)
1.5-ml microcentrifuge tubes
Thermocycler (MultiGene^{TM} II, Labnet International Inc) accepting 0.2-ml tubes

4.3. The PCR program

The program used in this work is presented in the table below

Table 1. PCR program for samples subject to RLB

Step	Temperature	Time	Function of each step
1	100 ºC	5-6 min	Preheating the lid
2	94ºC	4 min	Predenaturation of DNA
3	94ºC	0.35 s	Denaturation of DNA
4	51ºC	0.35 s	Annealing of primers
5	72 ºC	0.35 s	Extension
6	Repeat steps 3-5, 40 x		cycling
7	72 ºC	10 min	Final extension
8	4ºC	variable	Storage until removal

4.4. Protocol

(1) Remove the reagents and DNA samples from the freezer and allow them to thaw at room temperature or in the refrigerator

(2) Preheat the lid of the thermocycler by initiating the program outlined in table 1 above. When the temperature of the lid reached 100 ºC, pause the program

(3) Label one set of N 0.2-ml tubes corresponding to the number of samples to be analysed

(4) Prepare a master mix for PCR in a 1.5-ml reaction tube (Eppendorf) by using the recipe of Table 2 which has been calculated for one reaction in a total volume of 25µl. The master mix is equivalent to the number of samples (reactions) to be examined plus 3 extra for the negative control, the tubes and pipetting.

(5) Mix the component very well by repeated aspiration by pipette tip to ensure that the master mix is homogenous and then transfer 20 µl of master mix in each of 25 thin-walled tubes.

(6) Add 5 µl of each DNA sample in the corresponding thin-walled tube

(7) Take the reaction tubes into the thermocycler and close the lid. Resume the program by pressing the “pause” button. Remove the samples in the end of the program when the temperature displayed on the screen is 4ºC, representing the soak temperature and store them at 4ºC in the refrigerator.

(8) Run the gel as soon as possible to check for the success of the PCRs and store the samples at – 20ºC until needed.

Table 2. PCR conditions for samples subject to RLB (reaction volume 25µl)

Reagents	Concentration	One reaction
10 x PCR buffer	1 X	2.5 µl
50 mM $MgCl_2$	1.5 mM	0.75 µl
10mM dNTPs	200 µM	0.5 µl
5U/µl Taq polymerase	1.5 U	0.3 µl
20 pmol RLB-F	0.8µM	1 µl
20 pmol RLB-R	0.8µM	1 µl
Water (autoclaved)		13.95 µl

References

Nagore, D., Garcia-Sanmartin, J., Garcia-perez, A.L., Juste, R.A., Hurtado, A., 2004. Detection and identification of equine *Theileria* and *Babesia* species by reverse line blotting: Epidemiological survey and phylogenetic analysis. Vet. Parasitol. 123, 41-54.

Appendix 5

Agarose Gel electrophoresis of PCR products

Purpose

To visualize the PCR-amplified DNA fragments in order to confirm the success of the amplification reaction.

5.1. Reagents

Agarose (powder), Bio-Rad laboratory
Buffer solution stock (10x TBE, pH 8, 0), manually prepared
Ethidium bromide, 10 mg/ml, Research Organics
DNA molecular size marker (TrackIt^{TM} 100 bp DNA ladder, 0.1µg/µl, Invitrogen)
6X Gel loading dye, Fermentas

5.2. Equipment

Glass flask
Microwave
Parafilm
Laboratory tape
Gel comb
Gel tray
Gel electrophoresis apparatus
Power supply
UV illumination source
Gel documentation system (camera attached to UV illumination source)

5.3. Procedure

5.3.1. Preparation of 10X TBE buffer stock

5.3.2. Recipes

The recipes for the preparation of TBE buffer stock solution as well as the suppliers are presented in Table 1

Table x. Materials needed for TBE buffer

Reagents	Supplier	Recipes for 1 liter
1 kg Tris base	Research Organics	108 g Tris base
1kg Boric acid	Research Organics	55 g Boric acid
500 grm Disodium EDTA	Research Organics	9.3 g Disodium EDTA
Distilled, sterile water		

5.3.3. Buffer preparation steps

(a) Weigh out 108 g of Tris base, 55 g of Boric acid and 9.3 g EDTA and place them into a 2-l flask
(b) Dissolve the mixture in a final volume of 1 liter by stirring and heating
(c) Adjust the pH to 8.3 and store at room temperature.

5.3.4.. Gel electrophoresis protocol

(1) Weigh out 2g of agarose and place it into a 500-ml flask. Add 100 ml of 0.5X TBE buffer and microwave for 2-3 minutes, bringing the solution to the boil. Make sure that the agarose is completely dissolved otherwise, microwave again. Wearing gloves add 5µl of EtBr into the flask and swirl the solution to diffuse the EtBr
(2) Allow agarose to cool on the bench top for 10-15 minutes and pour into a gel tray that has been taped on the sides. Insert the gel comb at the dedicated notches on the sides of the gel tray
(3) Allow the gel to solidify for about 30 minutes

(4) Remove the tape from the gel tray and place the gel tray into an agarose electrophoresis apparatus. Orient the gel so that the wells are adjacent to the negative (black) electrode. Fill the gel chamber with 0.5XTBE buffer until it covers the gel by approximately 1cm. Carefully and evenly, pull the comb out of the gel using both hands

(5) Prepare PCR samples for loading by pipetting a 1-µl spot of 6 X gel loading buffer for each sample to be loaded onto a piece of parafilm spread on the benchtop. While noting the orientation of the samples, pipette 5 µl of each PCR sample into each spot of dye. Be sure to change pipette tips between samples and do not contaminate the PCR sample with gel dye. For each sample, mix up and down with the pipettor and directly load it all into a well of the agarose gel. Load 5µl of the DNA size ladder into an empty well

(6) Attach the electrode cover to the gel electrophoresis unit such that the positive (red) electrode is at the opposite end of the gel from the samples slots. DNA is negatively charged and moves towards the positive electrode. Plug the electrode into the power supply and set it for 100 volts. Start the electrical current. The run will take approximately 45 minutes to 1 hour. Stop the current before the front stain (yellow) of the marker dye migrates out of the gel

(7) Wearing gloves, remove the gel tray from the apparatus for documentation. Carefully take the gel tray to the UV illumination source. A gel may be carried in another container such as a plastic dish because it may easily slip out of the gel tray.

(8) Visualize the DNA bands under UV light, photograph with the camera attached to UV illuminator and print a copy of the gel picture. Record data and interpret the success of the PCRs

(9) Dispose of gel in an appropriate hazardous container.

References

Caldwell, G.A., Williams S. N., Caldwell K. A (Eds), 2006. Integrated genomics. A discovery-based laboratory course. John Willey & Sons, Ltd, West Sussex, 224 pp.

Appendix 6

Preparation of the RLB membrane

Purpose

To covalently link the oligonucleotide probes on the blotting membrane.

6.1. Reagents

1 kg $NaHCO_3$, SDS
10 g EDAC, Sigma
1kg NaOH, Merk
250 g EDTA, Alpha
20X SSPE, 4ltr, Life technologies
100 g SDS, Research Organics
Drawing pen ink
Biodyne C Membrane 0.45 µm, 24cm x 3 M, Pall Corporation
Oligonucleotide probes synthesized with a 5' terminal aminogroup, Isogen.

6.2. Equipment

Miniblotter MN 45 (Immunetics)
Foam cushions (Immunetics)
Plastic container
Hybridization oven equipped with a shaking platform
Vacuum aspirator
Blunt-ended forceps
Hybridization plastic bags

6.3. Procedure

6.3.1 Working solutions and preparation steps

0.5M NaHCO3, pH 8.4

Dissolve 10.5 g $NaHCO_3$ in 240 ml H_2O. Ajust to pH 8.4 with 2M NaOH. Add H_2O to a final volume of 250 ml.

16% (w/v) EDAC

Dissolve 1.6 g EDAC in 10 ml water.

100mM NaOH

Dissolve 1g NaOH in a final volume of 250 ml water. Store at room temperature for no longer than 1 month

125 ml 2xSSPE/0.1% SDS

First prepare 10% (w/v) SDS by dissolving 10 g SDS in a final volume of 100 ml by stirring and heating. This solution should be stored a room temperature for no longer than 1 month. Secondly, add 12.5 ml of 20x SSPE to 1.25 ml of 10% SDS, then complete with 111.25 ml of distilled water to a final volume of 125 ml.

1L 20mM EDTA

Dissolve 7.4 g EDTA in a final volume of 800 ml H_2O. Adjust the pH to 8 using 2M NaOH solution. Add H_2O to a final volume of 1L.

6.3.2. Protocol

(1) Dilute the oligonucleotides to the optimised concentrations into 150 µl final volume of 500 mM $NaHCO_3$ pH 8.4.

(2) Activate the Biodyne C membrane by 10 minutes incubation in freshly prepared 16 % (w/v) 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) in distilled water

(3) Rinse the membrane with distilled water and place it on a support cushion in a clean miniblotter system. Turn the screws hand-tight and remove residual water from slots by vacuum aspiration

(4) Fill the slots of the miniblotter with 150 µl of the diluted oligonucleotides solutions except the first and the last line (slot 1 and 45)

(5) Fill the first and the last line with drawing pen ink diluted 1: 100 in water or 2x SSPE. They will help to easily know the position of the probes

(6) After all samples are added, incubate at room temperature for at least one minute (5 -10 minutes)

(7) Remove the oligonucleotide solutions by vacuum aspiration in the same order as in which they were applied.

(8) Remove the membrane from the miniblotter using forceps then inactivate the membrane by incubation in 100 mM NaOH for no more than 8 minutes in a plastic container

(9) Wash the membrane in a plastic container under gentle shaking in 125 ml of 2x SSPE/0.1% sodium dodecyl sulphate (SDS) for 5 minutes at 60ºC. The membrane is ready for use

(10) If the membrane is not to be used straight away and is to be stored at this point, wash the membrane in a plastic container under gentle shaking in 100 mM 20 mM EDTA pH 8 for 15 minutes at room temperature

(11) Seal the membrane in plastic (hybridization bag) to avoid dehydration of the membrane and store at 4ºC or store unsealed membrane in 20mM EDTA pH 8 at 5±3º.

References

Gubbels, J.M., de Vos, A.P., Van der Weide, M., Viseras, J., Schouls, L.M., de Vries, E., Jongejan, F., 1999. Simultaneous detection of Bovine *Theileria* and *Babesia* species by reverse line blot hybridization. J. Clin. Microbiol. 37, 1782-1789.

Appendix 7

Hybridization and detection in the RLB assay

Purpose

To hybridize the amplified V4 region of the 18S rRNA gene against different oligonucleotide probes and visualise the signals arising from the hybridization reaction.

7.1. Reagents

20X SSPE, 4ltr, Life technologies
100 g SDS, Research Organics
250 g EDTA, Alpha
BM Chemiluminescence Blotting Substrate (solution A and B), Roche
Streptavidin-POD conjugate, Roche
X- Ray film (Kodak BioMax Film)

7.2. Equipment

The same equipment as in membrane preparation
Autoradiograph cassette (Kodak, X-Omatic^{TM})
X-ray film development machine
Thermocycler
Ice bucket
Plastic container
Aluminium foil
Blunt-ended forceps
Hybridization oven

7.3. Procedure

7.3.1 Working solution and preparation steps

All diluted buffer, prepared from concentrated stocks should preferably be stored for not longer than two days.

Buffer 1: 125 ml 2x SSPE/0.1% SDS
First prepare 10% (w/v) SDS by dissolving 10 g SDS in a final volume of 100 ml by stirring and heating. This solution should be stored at room temperature for no longer than 1 month. Secondly, add 12.5 ml of 20x SSPE to 1.25 ml of 10% SDS, then complete with 111.25 ml of distilled water to a final volume of 125 ml. Store buffer 1 at room temperature

Buffer 2: 250 ml 2x SSPE/0.5% SDS
Add 12.5 ml 10% SDS to 25 ml 20x SSPE, then complete with 212.5 ml water for a final volume of 250 ml. Prewarm to 52ºC before use
Buffer 3: 250 ml 2x SSPE/0.5% SDS. Prewarm to 42ºC before use
Buffer 4: 250 ml 2xSSPE
Measure 25ml 20x SSPE and complete to 250 ml with 225ml distilled water.
1% 10 ml SDS
Add 12.5 ml of 10% SDS to 112.5 ml water for a final volume of 125 ml
1L 20mM EDTA
Dissolve 7.4 g EDTA in a final volume of 800 ml H_2O. Adjust the pH to 8 using 2M NaOH solution. Add H_2O to a final volume of 1L.

7.3.2. Protocol

All buffer solutions should be prewarmed before use

(1) Dilute 20-25 µl PCR products with Buffer 1 (2x SSPE/0.1%) to an end volume of 150 µl.

(2) Heat-denature the diluted PCR products for 10 minutes at 100ºC using a thermocycler and cool on ice immediately

(3) Incubate the membrane for 5 minutes at room temperature in 125ml Buffer 1 in a plastic container

(4) Place the membrane onto the foam cushion into the miniblotter, such that the slots are perpendicular to the line pattern of the applied oligonucleotides (Fig. 1) and close the miniblotter

(5) Remove residual fluid from the slots of the miniblotter by vacuum aspiration

(6) Fill the slots with the diluted PCR product, avoiding air bubbles. Fill empty adjacent slots with 2x SSPE/0.1% SDS to prevent cross flow. Hybridize for 60 minutes at 42ºC on a horizontal surface. Avoid cross- flow to the neighbouring slots (no rocking or shaking)

(7) Remove the samples from the miniblotter by vacuum aspiration and take the membrane from the miniblotter using forceps

(8) Wash the membrane twice with shaking in 125ml buffer 2 (2x SSPE/0.5 SDS) for 10 minutes each time at 52ºC in a plastic container

(9) Incubate the membrane with 10ml of 1:4000 diluted SA-POD (peroxidase labelled streptavidin conjugate) in 2xSSPE/0.5% SDS for 45 minutes at 42ºC in a plastic container. For the dilution, pipette 2.5µl SA-POD to 9.9975 ml of 2x SSPE/0.5%

(10) For chemiluminescence detection, the substrate consists of two solutions (A and B). Add 150 µl of solution B to 15 ml of solution A. Store at room temperature in the dark (Wrap transparent device with aluminium foil) for at least 30 minutes before use

(11) Wash the membrane twice in 125ml buffer 3 (2x SSPE/0.5% SDS) for 10 minutes each time at 42ºC under shaking

(12) Wash the membrane twice in 125 ml buffer 4 (2x SSPE) for 5 minutes each time at room temperature. Remove excessive water by briefly blotting the membrane on a blotting paper

(13) Incubate the membrane for 1 minute in 15 ml of BM chemiluminescence Blotting substrate (solution A+B). Remove excessive liquid by briefly blotting the membrane on a blotting paper

(14) Seal the membrane in a transparent plastic folder (hybridization bag). Expose an X-ray film on top of the sealed membrane in an autoradiograph cassette for 2 to 40 minutes depending on the signal. Automatically develop the film using the X- ray machine.

(15) For membrane stripping, wash the membrane twice in 1% SDS at 80ºC for 30 minutes each time. Rinse the membrane in fresh solution of 20 mM EDTA pH 8 for 15 minutes. Seal the membrane in a plastic to avoid dehydration and store at 4º C until use or soak the unsealed membrane in at least 125 ml 20mM EDTA, pH 8 at 4°C.

A B C

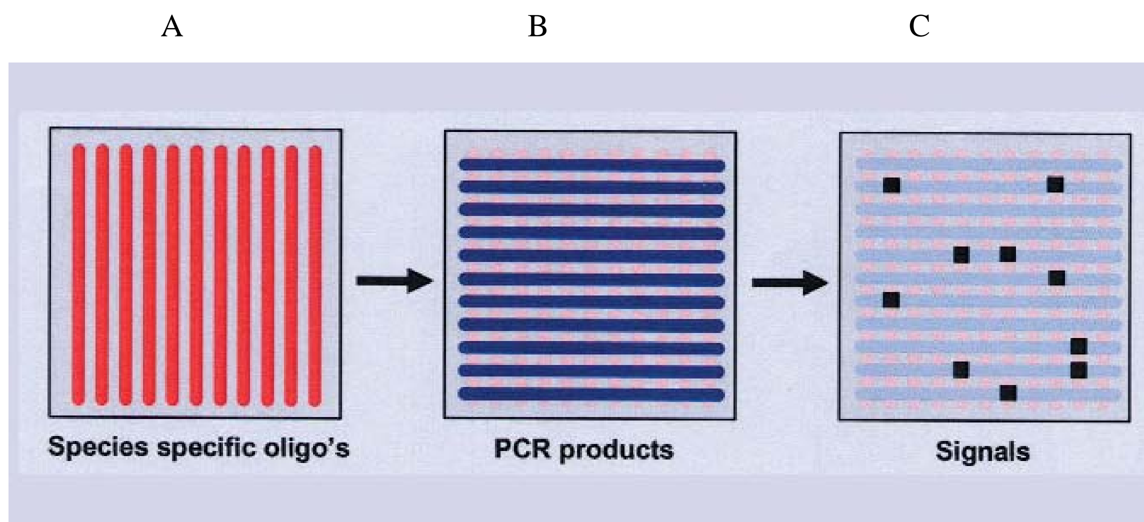

Fig. 1. RLB protocol steps. Detecting probes are covalently linked to a membrane in parallel line (A). Then the membrane is rotated 90º in the miniblotter, so that the slots of the miniblotter are penpendicular to the probes lines; the slots are then filled with PCR products (B). After stringent washing to remove unbound PCR products, the hybridized PCR products are visualised by chemiluminescence detection (C).

References

Gubbels, J.M., de Vos, A.P., Van der Weide, M., Viseras, J., Schouls, L.M., de Vries, E., Jongejan, F., 1999. Simultaneous detection of Bovine *Theileria* and *Babesia* species by reverse line blot hybridization. J. Clin. Microbiol. 37, 1782-1789.

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Appendix 8

Clean-up of PCR amplicons for sequencing

Purpose

To purify the desired PCR amplicons from other PCR components for direct sequencing.

The procedure presented was adapted from the literature supplied with the Genomed Jet Quick Gel extraction kit.

8.1. Reagents

Kit contents

Solution L1 (Gel solubilisation) stored at room temperature. Contains $NaClO_4$, sodium acetate and TBE-solubilizer

Solution L2 (Wash), reconstituted with 96-100% abslolute ethanol and stored at room temperature. Contains ethanol, NaCl, EDTA and Tris-HCL

TE buffer (DNA elution)

JETQUICK columns

2 ml receiver tubes

Other reagent not included in the kit:

Absolute ethanol

8.2. Equipment

Razor blades, new

1.5 ml microcentrifuge test tubes

Scale/ Balance

Microcentrifuge

Incubator

Vortex

Heating block

8.3. Protocol

Before starting set the incubator to 70ºC to preheat the elution buffer and set the heating block to 50 ºC.

(1) Record the weight of two empty 1.5-ml microcentrifuge tubes by writing the weight on the tube. Label the tube with the name of the fragment that will be isolated.
(2) Place gel (1.5%, TBE agarose gel run with the entire PCR reaction volume for a single slot) on transilluminator and, wearing gloves and protective glasses, cut out the band(s) of interest using clean razor blade while viewing with UV light. It is desirable to do this quickly to avoid nicking the DNA in the presence of UV light. Immediately turn off the UV light when the bands have been excised.
(3) Place the DNA-containing agarose into a preweighed tube. Weigh the tube again, record the weight and subtract it from the original tube weight to determine the weight of the gel slice.
(4) *Solubilisation of agarose*. For each 100 mg gel slice, add 300 µl of solution L1 and incubate at 50ºC for 15 min. Mix every 3 min by flicking or vortexing the tube to ensure that the agarose is completely solubilised. Larger gel slices (≥300mg) and gel of higher concentration (≥ 2%) take longer to dissolve and need the incubation time to be increased to 20-30 min. The maximum amount of agarose gel per JETQUICK column is 400 mg.
(5) *Column loading*. Place a JETQUICK spin column into a 2 ml receiver tube and load the mixture from step one into the prepared spin column. Centrifuge at 12,000 x g for 1min. Discard the flowthrough. Spin column can be loaded with 600 µl. For larger samples volumes multiple loadings are necessary. If the weight of the initial agarose

gel slice is > 250 mg it is recommended to insert an additional washing step with solution L1. Therefore, add 500µl of solution L1 to the JETQUICK spin column, let stand for 1 min at room temperature and centrifuge at 12,000 x g for 1 min. This additional step will yield better results if the eluted DNA is subsequently used for direct sequencing, in vitro transcription or microinjection.

(6) *Column washing*. Reinsert the spin column into the receiver tube and add 500 µl of reconstituted buffer L2 into the spin column. Centrifuge at >12,000x g for 1 min. Discard the flowthrough. Place the JETQUICK column back to the same receiver tube, and centrifuge again at maximum speed for 1 min. This additional step assures that no residual ethanol will be carried over into the next step.

(7) *DNA elution*. Place the JETQUICK column into a new 1.5 microcentrifuge tube and add 50 µl of sterile water (or TE buffer or 10 mM Tris-HCL, pH 8.0) directly onto the center of the silica matrix of the Jetquick spin column. Centrifuge at 12,000x g for 2 min. Higher DNA concentration can be obtained if the elution buffer is carried out with only 30 µl of elution buffer preheated to 65- 70ºC. DNA eluted with water should be stored at -20ºC.

IX-PUBLICATIONS

1- Kouam, M.K., Kantzoura, V., Gajadhar, A.A., Theis, J.H., Papadopoulos, E., Theodoropoulos, G., 2010. Seroprevalence of equine piroplasms and host-related factors associated with infection in Greece. Vet. Parasitol. 169, 273-278.

2- Kouam, M.K., Kantzoura, V., Masuoka, P.M., Gajadhar, A.A., Theodoropoulos, G., 2010. Genetic diversity of equine piroplasms in Greece with a note on speciation within *Theileria* genotypes (*T. equi* and *T. equi*-like). Infect. Genet. Evol. 10, 963-968.

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1- Kouam, M.K., Kantzoura, V., Gajadhar, A.A., Theis, J.H., Papadopoulos, E., Theodoropoulos, G., 2010. Seroprevalence of equine piroplasms and host-related factors associated with infection in Greece. Vet. Parasitol. 169, 273-278.

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Seroprevalence of equine piroplasms and host-related factors associated with infection in Greece

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ABSTRACT

Serum samples were collected from a total of 544 equids that included 524 horses, 13 mules, and 7 ponies from various regions of mainland Greece and were examined by competitive-inhibition ELISA (cELISA) to evaluate the level of exposure of Greek equids to *Theileria* (*Babesia*) *equi* and/or *Babesia caballi*, the causative agents of piroplasmosis. Association between seropositivity and host-related factors of species, gender, age, origin, activity and location were investigated. The overall seroprevalence was 11.6% (9.1–14.6%) with 95% confidence limit. The seroprevalence for *T. equi* and *B. caballi* was found to be 11% (8.6–14%) and 2.2% (1.2–3.9%), respectively. The animal-related factors significantly linked with seropositivity were the species, activities of farming, racing, recreation, and geographic location in Attica, Macedonia, Peloponnese and Thessaly region ($p < 0.05$). The relative risks for the presence of *T. equi*, *B. caballi* and mixed infection in mules compared to horses was 8.39, 33.58 and 40.31, respectively. The infection level for *T. equi*, *B. caballi* and mixed infection were significantly higher in farm equids than in racing equids ($p < 0.05$). Also, the rate of infection of *T. equi* was higher in farm equids than recreational equids ($p < 0.05$). The relative risk of *T. equi* infection between farming equids and equids used only for recreation activity was 3.25–1, while the relative risk of *B. caballi* infection was 0.14–1 for racing animals relative to recreation animals. The region with the highest level of infection to both parasites was Thessaly (38.8% *T. equi* and 6.1% B. *caballi*), followed by Peloponnese (10.4% *T. equi* and 3.9% *B. caballi*), Attica region (8.3% *T. equi* and 0.6% *B. caballi*) and finally Macedonia the region with the lowest prevalence (6.6% *T. equi* and 4.4% *B. caballi*). A higher seroprevalence rate was found among local animals compared to imported equids, indicating that equine piroplasm infection is enzootic in Greece. *T. equi* seroprevalence was significantly different and higher among increasing age groups of equids, suggesting persistent infections or lower transmission levels whereby animals may need to be exposed longer before acquiring the infection. Competent tick vectors *Rhipicephalus bursa* and *Rhipicephalus sanguineus* for the transmission of equine piroplasmosis were recovered from horses and dogs, respectively.

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1. Introduction

Equine piroplasmosis (EP) is a tick-borne disease of equids, caused by two species of apicomplexan protozoa,

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Theileria (syn. *Babesia*) *equi* and *Babesia caballi*. *Babesia canis canis* of dogs has been reported in horses (Criado-Fornelio et al., 2003) but no clinical signs attributable to this parasite have been documented in equids. *T. equi* and *B. caballi* are intra-erythrocytic parasites that occur in most tropical, subtropical and temperate areas of the world and may be found together when a common tick vector is present (Mehlhorn and Schein, 1998). EP is endemic in many parts of Asia, Arabia, South and Central America, Africa and Europe. Within Europe, it is more prevalent in France (Leblong et al., 2005), Portugal (Bachiruddin et al., 1999), Spain (Camacho et al., 2005), Italy (Moretti et al., 2009) and Turkey (Karatepe et al., 2009). Ixodid ticks of the genera *Dermacentor, Rhipicephalus*, *Hyalomma, Boophilus* and *Haemaphysalis* transmit *T. equi* and *B. caballi*, which can result in infections characterized by fever, anaemia, icterus, haemoglobinuria, bilirubinuria and sometimes, death. In addition, intrauterine infections with *T. equi* may result in abortion and neonatal death (Potgieter et al., 1992). In some cases of acute or chronic disease, mortality can reach up to 50% (de Waal, 1992). Clinical signs of the infection are not pathognomonic, especially in endemic areas. Infected animals that recover from acute or primary infection of *T. equi* remain life-long carriers, whereas horses infected with *B. caballi* may remain carriers for up to 4 years (de Waal and Van Heerden, 1994). Carrier hosts maintain the life cycle of the parasites by serving as a source of transmission for ticks.

Traditionally, piroplasms are detected and identified by microscopic examination of thin blood smears collected from acutely infected animals. Several serological assays such as the complement fixation test (CFT), the indirect fluorescent antibody test (IFAT), the enzyme-linked immunosorbent assay (ELISA) as well as the competitive-inhibition ELISA (cELISA) have been developed mostly for large scale studies and to monitor infections during the latent stage characterized by microscopically undetectable parasitemia (Brüning et al., 1997; Shkap et al., 1998; Ikadai et al., 2000). The cELISA is currently the test of choice recommended by the World Organization for Animal Health (OIE, 2008). Direct detection methods using molecular tools have recently been developed and are considered reliable (Caccio et al., 2000; Nagore et al., 2004).

While reliable estimates of the numbers of cases of equine piroplasmosis and the related economic losses are not readily available, reports of disease and deaths at equestrian centres or stud farms are not uncommon when piroplasm-free adult horses are introduced into enzootic areas (Kuttler, 1988). The importance of EP lies in the constraints on travel of horses and its effect on the horse racing industry. EP is also a list B disease of the OIE, notifiable within 72 h of diagnosis. Only seronegative horses for both *T. equi* and *B. caballi* are qualified for importation to the United States, Canada, Australia and Japan (Friedhorff et al., 1990). Since testing of horses for EP is mandatory for the international movement of horses either for participation in international events or for export, the disease is important to Greece which has one of the largest equestrian centres in southern Europe, and moreover, hosted the 2004 equestrian Olympic Games.

Despite the importance of equine piroplasmosis, the literature on this disease in Greece is limited, with only one report of finding *B. caballi* and *T. equi* sporozoites in *Rhipicephalus sanguineus* and *Hyalomma plumbeum*, respectively (Haralabidis, 2001). Therefore, a cross-sectional, serological survey was conducted to evaluate the level of exposure of equids to piroplasms in various regions of Greece in terms of seroprevalence and the risk factors associated with the infections.

2. Materials and methods

2.1. Animals and sampled areas

Blood samples were collected at random from clinically healthy equids by venipuncture into sterile, anticoagulant-free tubes after obtaining the agreement of their owners in various regions of mainland Greece during 2007–2008. At the time of blood collection, the equids and the associated farm dogs and also the stray dogs wandering close to the equids were inspected for the presence of ticks which were collected for subsequent identification (Papadopoulos et al., 1996; Walker et al., 2000; Pavlidou et al., 2008). Data on the characteristics of the sampled animals (species, gender, age, origin, activity, location) were collected through questionnaires completed by the investigators on location during sample collection.

2.2. Sera preparation and cElisa

Sera were obtained from clotted blood samples by centrifugation and stored at −20 °C until used. Serum samples were tested for the presence of antibodies to *T. equi* and *B. caballi* using a commercial cElisa test kit (VMRD Inc., Pullman, WA, USA) according to the manufacturer's instructions. The assay detects serum antibodies against EMA-1 surface protein on merozoites of *T. equi* (Knowles et al., 1992), and rhoptry-associated protein (RAP-1) of *B. caballi* (Kappmeyer et al., 1999). Samples associated with percent inhibition values $\geq$40 were considered positives. The sensitivity of *T. equi* and *B. caballi* cELISA is higher than that of the complement fixation text (CF) whereas the specificity of *T. equi* and *B. caballi* cELISA is 99.2–99.5% (OIE, 2008). The optical density values were obtained using an automatic plate reader (Infinite M200, Tecan).

2.3. Statistical analysis

The seroprevalence relative to the various characteristics (animal species, gender, age, origin, activity, and location) was calculated with an associated 95% confidence interval. Differences in prevalence between these various groups were assessed by the two-sided Chi-square and the Fischer's exact test. A p value of <0.05 was considered significant. The relative risk (RR) of the presence of infection was computed for the characteristics found significant. All the parameters were computed using Epi Info software (version 3.5.1). Each time an expected value in the Chi-square test was less than 5 as indicated by Epi-info software in an rxc contingency table, SISA-tables program was used to calculate the exact test p value.

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3. Results

A total of 544 equids, including 524 horses, 13 mules, and 7 ponies from various regions of mainland Greece were sampled during 2007–2008 (Table 1). The overall seroprevalence for equine piroplasmosis was 11.6% (9.1–14.6%), with 11% (8.6–14%) for *T. equi* and 2.2% (1.2–3.9%) for *B. caballi*. A small portion of equids (1.7%) was seropositive for both *T. equi* and *B. caballi*.

Table 2 shows the seroprevalence levels according to equid species and associated host factors. The species of the animal was found to be a significant factor for the presence of *T. equi* and *B. caballi* infections ($p < 0.0001$ for all cases) (Table 2). The infection level was significantly different between horses and mules for the two parasites and mixed infections ($p < 0.0001$ for all cases). Furthermore, the risk of infection was eight-fold and thirty-three-fold higher in mules than in horses for *T. equi* and *B. caballi*, respectively (Table 3). For mixed infection (*T. equi* and *B. caballi*), the risk was forty times higher in mules compared to horses (Table 3). No significant differences could be found in the piroplasmosis seropositivity between ponies, mules or horses. Mules and ponies were difficult to locate and obtain permission for sampling, and contributed to their small sample size.

No significant differences in infection levels between genders were observed for both parasites (Table 2).

Analysis of the results according to age (Table 2) showed that *T. equi*-positive animals occurred in all age groups except those less than 1 year of age whereas *B. caballi* infection was only present in animals greater than 2 years old. The prevalence of *T. equi* infection was significantly higher in older than younger animals ($p < 0.05$) in contrast to that of *B. caballi* infection which did not significantly differ among age groups. Seroprevalence of *T. equi* infection in animals over five years old was significantly higher compared to animals between two and five years old ($p < 0.01$).

Infections involving both parasite species occurred only in animals born in Greece and the difference in seropositivity was significant between imported and local animals ($p < 0.05$) (Table 2).

The type of activity was a significant animal-related factor for the presence of piroplasmosis ($p < 0.01$ in all cases) (Table 2). The seropositivity was significantly higher in farm horses than in race horses in regard to *T. equi*, *B. caballi* and mixed infection ($p < 0.01$ for all cases). The risk of infection was three times greater in farm equids compared to recreation equids for *T. equi* (Table 3). The seropositivity was significantly lower in race equids relative to recreation equids for *B. caballi* and mixed infection ($p < 0.05$ in both cases) (Table 2). The estimated RR of *B. caballi* infections between race horses and recreation equids was 0.14 (Table 3). No mixed infection was found among race horses (Table 2).

The highest seropositivity among equids for *T. equi* (38.8%) and *B. caballi* (6.1%) was observed in the region of Thessaly. The infections in horses between geographical regions were significantly different for *T. equi, B. caballi,* and mixed infections with *T. equi* and *B. caballi* ($p < 0.01$ in all cases) (Table 2). Comparing Thessaly to Attica, the risk of infection was estimated as being 4.70 times and 10.01 times greater for *T. equi* and *B. caballi* respectively (Table 3). The seroprevalence for *T. equi* was significantly higher in Thessaly than in Peloponnese or Macedonia ($p < 0.01$ in both cases) but not for *B. caballi*. Also, the prevalence of antibodies against *B. caballi* was significantly lower in Attica than in Peloponnese or Macedonia ($p < 0.05$ in both cases). Thus, the risks of *B. caballi* infections in Peloponnese and Macedonia relative to Attica were more than six fold and twelve fold higher, respectively (Table 3). Mixed infections with both parasites occurred in all regions except in Attica.

A total of 13 ticks (5 adults and 8 nymphs) were collected from stray dogs roaming the equestrian centre and from privately owned dogs and horses in a stud farm in

Table 1
Location and species of sampled equids ($n = 544$).

Location	Number of animals		
	Horse	Mule	Pony
Attica	327	–	–
Thessaly	42	7	–
Peloponnese	70	–	7
Macedonia	85	6	–
Total	524	13	7

Table 2
Number (n) of equids tested for infection with *Theileria equi* and/or *Babesia caballi* and seroprevalence (%) of infection within hosts groups and associated factors.

Factor	N	*T. equi*		*B. caballi*		*T. equi* and *B. caballi*	
		n	%	n	%	n	%
Animal species							
Horse	524	48	9.2[a]	6	1.1[a]	4	0.8[a]
Mule	13	10	76.9[a]	5	38.5[a]	4	30.8[a]
Pony	7	2	28.6	1	14.3	1	14.3
Gender							
Male	153	20	13.1	2	1.3	0	0
Female	210	23	11	5	2.4	5	2.4
Gelding/mule	181	17	9.4	5	2.8	4	2.2
Age							
<1 year	8	0	0	0	0	0	0
>1–2 years	30	2	6.7	0	0	0	0
>2–5 years	272	19	7[a]	3	1.1	1	0.4
>5	227	34	15[a]	5	2.2	5	2.2
Origin							
Greece	282	29	10.3	8	2.8	6[a]	2.1
Imported	213	12	2.6	1	0.5	0[a]	0
Unknown	49	–	–	–	–	–	–
Activity							
Racing	327	27	8.3[a]	2	0.6[a,b]	0	0[a,b]
Recreation	91	6	6.6[b]	4	4.4[a]	3	3.3[a]
Farming	126	27	21.4[a,b]	6	4.8[b]	6	4.8[b]
Location							
Attica	327	27	8.3[a]	2	0.6[a,b,c]	0	0[a,b,c]
Thessaly	49	19	38.8[a,b,c]	3	6.1[a]	3	6.1[a]
Peloponnesse	77	8	10.4[b]	3	3.9[b]	3	3.9[b]
Macedonia	91	6	6.6[c]	4	4.4[c]	3	3.3[c]

Values significantly different ($p < 0.05$) between host groups or associated factors are labelled with the same letter ([a, b or c]).

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Table 3
Relative risk of infections computed for significant factors.

Factors	*T. equi*				*B. caballi*				*T. equi* and *B. caballi*			
	n[a] (%)[b]	RR	95%CI[c]	*p*[d]	*n* (%)	RR	95%CI	*p*	*n* (%)	RR	95%CI	*p*
Animal species												
Horse	48 (9.2)	1			6 (1.1)	1			4 (0.8)	1		
Mule	10 (76.9)	8.39	5.61–12.54	0.00	5 (38.5)	33.58	11.74–96.14	0.00	4 (30.8)	40.31	11.30–143.31	0.00
Pony	2 (28.6)	3.11	0.93–10.37	0.13	1 (14.3)	12.47	1.72–90.49	0.08	1 (14.3)	18.71	2.33–146.00	0.06
Activity												
Recreation	6 (6.6)	1			4 (4.4)	1			3 (3.33)	1		
Racing	27 (8.3)	1.25	0.54–2.94	0.60	2 (0.6)	0.14	0.03–0.07	0.02	0	–	–	0.01
Farming	27(21.4)	3.25	1.39–7.54	0.00	6 (4.8)	1.08	0.31–3.72	0.58	6 (4.8)	1.44	0.37–5.62	0.43
Location												
Attica	27 (8.3)	1			2 (2.06)	1			0			
Thessaly	19 (38.8)	4.70	2.84–7.78	0.00	3 (6.1)	10.01	1.71–58.40	0.01	3.61	–	–	0.00
Peloponnese	8 (10.4)	1.25	0.59–2.66	0.34	3 (3.9)	6.37	1.08–37.46	0.04	3 (3.9)	–	–	0.00
Macedonia	6 (6.6)	1.40	0.6–3.3	0.28	4 (4.4)	12.17	2.35–68.00	0.00	3 (3.3)	–	–	0.00
Age group												
≤1	0				–	–	–	–	–	–	–	–
>1–2 years	2(6.7)	1			–	–	–	–	–	–	–	–
>2–5 years	19 (7.0)	1.04	0.25–4.2	0.64	–	–	–	–	–	–	–	–
>5 years	34 (15.0)	2.24	0.57–8.89	0.17	–	–	–	–	–	–	–	–

[a] Number of positive.
[b] Prevalence.
[c] Confidence interval.
[d] *p* value.

Attica region. No ticks were found in other regions at the time of sampling, including Thessaly region where the highest seroprevalence of piroplasms was recorded. The adult ticks were identified as *Rhipicephalus bursa* (2 females) and *R. sanguineus* (2 males and 1 female) recovered on horses and dogs respectively. Nymphs (8) collected from dogs were identified as *R. sanguineus*.

4. Discussion

Equine piroplasmosis presents a global problem for equestrian sports and trade. The international movement of horses for sport, show or related events has exacerbated the importance of equine piroplasmosis. Many countries including USA, Canada, Australia and Japan have established rigid control measures either to ban entry of seropositive horses into these countries or prevent movement of seropositive horses within countries in an effort to prevent the spread of the agents (Kuttler, 1988; Schein, 1988; Friedhorff et al., 1990).

This is the first report on the seroepidemiology of *T. equi* and *B. caballi* infections in Greece. The cELISA used in the present study has been shown to be a suitable serological assay for the detection of antibodies to *T. equi* and *B. caballi* in equids, and it is internationally recommended for certification purposes (OIE, 2008).

Previous studies carried out elsewhere indicated a wide range of seroprevalence for equine piroplasmosis. Prevalences reported include 59% for *T. equi* and 45% for *B. caballi* by IFAT and CFT in Brazil (Heuchert et al., 1999), 40% for *T. equi* and 28.3% for *B. caballi* in Spain (Camacho et al., 2005). In Italy, a seroprevalence of 68.4% by IFAT was found with 12.4% of animals *T. equi*-positive and 17.9% *B. caballi*-positive (Moretti et al., 2009). In Turkey, one of Greece's nearest neighbour country, the infection rates ranged from 0 to 100% for *T. equi* and 0 to 33.33% for *B. caballi*, using IFAT and CFT (Karatepe et al., 2009). In the present study, the overall seroprevalence was found to be 11.6% for all piroplasms, 11% for *T. equi* and 2.2% for *B. caballi,* which is low compared to other countries. The seroprevalence in this study also varied by geographical region within Greece ranging from 6.6% in Macedonia to 38.8% in Thessaly for *T. equi* and 4.4% to 6.1% for *B. caballi*. The difference in the prevalence of equine piroplasmosis among countries may be due to differences in sensitivity of the diagnostic tests used, the occurrence and abundance of competent vectors, the activity of the equids and the presence and effectiveness of any control measures. Similarly, the reasons for the variation in prevalence levels among regions may be related to the host activity, the management practices and the difference in prevalence of suitable tick vectors. In Thessaly, where higher prevalences occurred, equids are daily involved in farm activities and consequently, are continuously exposed to ticks infestations. In Macedonia and Peloponnese, equids are used mainly for recreation purposes and therefore have limited access to pasture. Horses in Attica are primarily involved in competition events and are well groomed. However, the presence of dogs with ticks inside the paddock as well as the frequent movement of horses between the hippodrome and different regions of Greece might explain the level of seroprevalence found in Attica. In addition, the horses from the stud farm (representing 8.6% of the animals examined in Attica) are exposed to tick bites that increase the risk of acquiring piroplasms parasites in the region. The following ticks that transmit both *T. equi* and *B. caballi* occur in Greece: *Hyalomma plumbeum plumbeum* (syn = *H. marginatum*), *H. anatolicum excavatum* and *Rhipicephalus sanguineus* (Soulsby, 1982). *R. bursa*, known to occur in Bulgaria (Soulsby, 1982) and *R. sanguineus* were

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found in the region of Attica on horses and dogs, respectively, during the present study. This tick collection is indicative of the tick fauna in Greece. Climatic factors such as temperature, humidity and rainfall influence the habitat of ticks, hence the difference in their prevalence which probably impacts the dynamic of transmission of EP. This is supported by the occurrence of three climatological areas in Greece namely Mediterranean, alpine, and mid-European. The alpine type covers Thessaly and Peloponnese whereas Macedonia belongs to the temperate climate. Attica is located in the transition area between the Mediterranean and the alpine climate.

The seroprevalence of *T. equi* infection was significantly higher than that of *B. caballi*, both at national and regional level in spite of the occurrence of common tick vectors to both piroplasm species in the country. There are reports that *T. equi* is more common than *B. caballi* in endemic countries (Barbosa et al., 1995; Bruning, 1996). This may be associated with the fact that infected animals which survive an initial infection completely eliminate *B. caballi* from their circulation after 1–4 years, whereas *T. equi* remains as a life-long infection (de Waal and Van Heerden, 1994).

In regard to host species, the infection rates were significantly higher in mules than in horses for both parasites. This finding may be linked to the outdoor living conditions of mules which were occupied with farm activities, mainly the daily transportation of wood from the forest. This extended exposure to pasture probably increased the likelihood of tick bites. Due to the small sample size of mules and ponies, the significantly higher prevalence of infection in mules compared to horses should not be generalised until a large number of mules and ponies is considered in future studies.

The type of activity of equids significantly impacted the seroprevalence. The level of infection was significantly higher for both piroplasms in farm animals than in recreation or race equids. Farm animals kept under poor environmental conditions are more likely than other equids to be exposed to tick vectors, so the risk of infection is increased (Shkap et al., 1998). Occurrence of piroplasms in race horses that are well groomed may be related to the presence of dogs infested with ticks in their surroundings or to subclinical infections.

Equids less than 2 years old did not show any seropositivity to *B. caballi* infections but exhibited a low prevalence with *T. equi* infections. This finding disagrees with a previous report from Trinidad where Asgarali et al. (2006) found a higher frequency of *B. caballi* than *T. equi* infection in foals and yearlings. The contradictory findings may be associated with differences in the level of infection with each parasite in the two countries or differences in the managements of age groups. Antibodies to *B. caballi* were more frequent than those to *T. equi* in Trinidad (68.8% and 33.3%, respectively) whereas in Greece, *T. equi* seropositivity was five times higher (11%) than that of *B. caballi* (2.2%). *T. equi*-seropositivity was observed to be significantly higher in older equids and increased with the age of the animals. These results are in agreement with other findings (Asgarali et al., 2006; Rüegg et al., 2007). The reason for the increased prevalence of *T. equi*-positive sera according to age group may be the persistence of *T. equi* in their hosts after recovery from a primary or acute infection (Hourrigan and Knwoles, 1979; Bruning, 1996). However, it has been shown that offsprings of preimmunized mares are naïve at birth and acquire passive immunity through colostrum (de Waal and Van Heerden, 1994). Nevertheless, this immunity is transitory and wanes after a period of time (Kumar et al., 2008). Higher seropositivity in older animals may also be due to lower transmission levels which require longer exposure for the acquisition of infection. As for *B. caballi*, the converse may occur whereby formerly infected animals clear the parasites within 1–4 years regardless of the treatment (Holman et al., 1993; de Waal and Van Heerden, 1994; Rüegg et al., 2007).

The seropositivity levels were not significantly different between genders of hosts. This observation agrees with the results of Asgarali et al. (2006) and Karatepe et al. (2009). However, Shkap et al. (1998) found that stallions were significantly less affected than mares and geldings in Israel, but this was due to the fact that stallions, maintained under strict control for breeding were less exposed to tick infestation.

The present study found that the prevalence of seropositive equids for both *T. equi* and *B. caballi* was much higher in native animals than those imported. No mixed infection was recorded in imported equids. The difference in prevalence between native and imported animals in regard to mixed infections was significant. These findings clearly establish that EP is enzootic in Greece. The enzootic status of EP in Greece is supported by the occurrence of four tick vectors, namely *R. sanguineus*, *R. bursa*, *Hyalomma plumbeum plumbeum*, *H. anatolicum excavatum* which are suitable vectors for both *T. equi* and *B. caballi* in the country (Soulsby, 1982). Since most of the imported horses came from countries where the disease has been documented, such as France (Leblong et al., 2005), Italy (Moretti et al., 2009) and Spain (Camacho et al., 2005), the source of the infection in equids in Greece is unknown. Greece is geographically part of the Balkan Peninsula which is an enzootic area for EP (Schein, 1988).

Canine piroplasmida, *B. canis* has been detected in horses both by molecular (Criado-Fornelio et al., 2003) and serological methods (Hornok et al., 2007). The diagnostic test used in this study (cELISA) has been shown to be of high specificity, ranging from 99.2 to 99.5% for both *T. equi* and *B. caballi*. Therefore, the chance of cross-reactions to occur between *B. caballi* and *B. canis* is very low.

In conclusion, this study demonstrated that Greece should be regarded as an enzootic region for equine piroplasms. Two competent tick vectors (*R. sanguineus*, "the brown dog tick" and *R. bursa*) for *T. equi* and *B. caballi* were unequivocally identified. The fact that *R. sanguineus* was collected from stray dogs within an equestrian centre paddock and from a neighbouring stud farm should alert veterinarians and horse owners of the potential danger to the horse industry in the Attica region. Nationally, there is a need for further research on the epidemiology of EP in Greece using molecular methods, including the dynamics of tick transmission and the geographical distribution of the four resident vector species.

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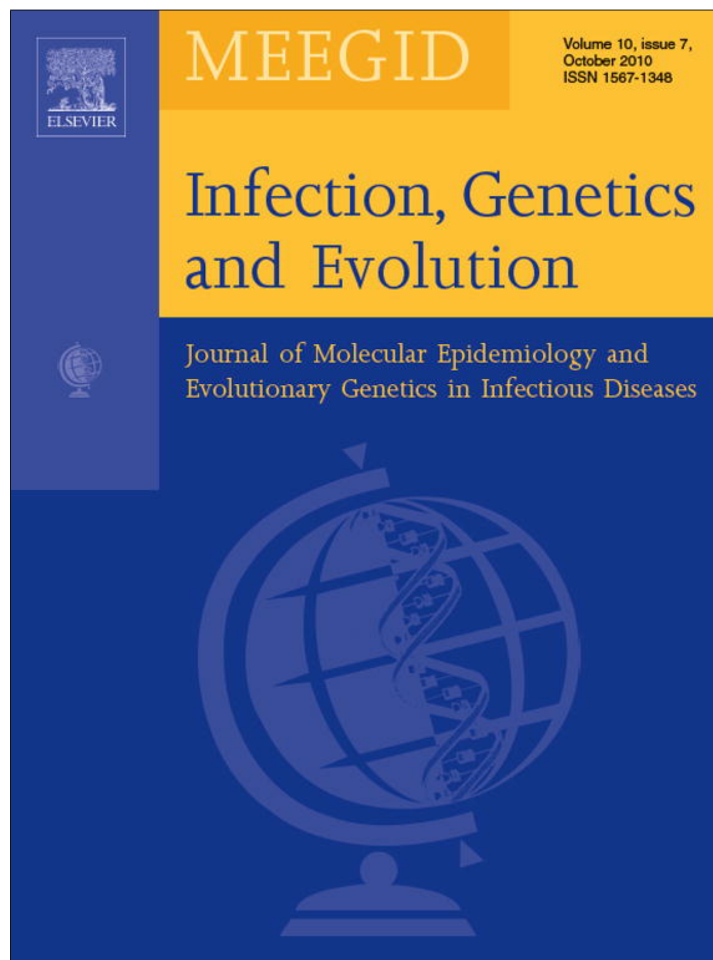

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Genetic diversity of equine piroplasms in Greece with a note on speciation within *Theileria* genotypes (*T. equi* and *T. equi*-like)

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ABSTRACT

Equine piroplasms in Greece were studied using the reverse line blot hybridization (RLB) assay. Three genotypes consisting of two *Theileria* (*T. equi* and *T. equi*-like) and one *Babesia* (*B. caballi*-like) were identified. Of 787 samples tested, 371 (47.14%) hybridised to catchall probe (probe specifically designed to capture any piroplasm species present in a sample), 346 (43.96%) to *T. equi* probe, 364 (46.25%) to *T. equi*-like probe, 0 (0%) to *B. caballi* probe and 3 (0.38%) to *B. caballi*-like probe. Seven samples gave faint signals with the catchall probe only, indicating the presence of known or unknown piroplasm species, or a novel genotype or a known genotype occurring at a very low level of parasitemia. A partial sequence (509 bp) of the V4 region of the 18S rRNA gene of a *T. equi*-like isolate showed only 99% similarity with the reference *T. equi*-like isolates from Northern Spain from which the detecting probe used in the present study was designed but showed 100% similarity with the *T. equi*-like variants from Southern Spain. This indicated a noticeable degree of polymorphism within the population of *T. equi*-like. No unusual parasites previously reported in horses, such as *B. canis canis* and *B. bovis* were detected in this study. The values of the bioclimatic variables were very similar between the geographic locations for *T. equi* and *T. equi*-like genotypes, suggesting the two are not yet different species as hypothesized by some authors but are possibly undergoing a speciation process within *Theileria* genotypes. Both *T. equi* and *T. equi*-like were found in predominantly forest type land cover.

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1. Introduction

Equine piroplasmosis is a disease of economic importance that is gaining global significance due to the international trade and the increased movement of horses all over the world (Bachiruddin et al., 1999; Nicolaiewsky et al., 2001). However, knowledge of the epidemiology of equine piroplasms in many areas of the world is limited. Various *Theileria* and *Babesia* genotypes in equids have been described in some countries, some of which were hypothesized to be new species (Nagore et al., 2004; Bhoora et al., 2009). A canine piroplasm, *B. canis canis* (Criado-Fornelio et al., 2003; Hornok et al., 2007) as well as a bovine piroplasm (*B. bovis*) (Criado et al., 2006) have been reported in horses. In Greece, a small number of studies documenting the occurrence of both *Theileria equi* and *Babesia caballi* have been reported (Haralabidis, 2001; Kouam et al., 2010). A recent serological survey of equids in Greece only had the capacity to detect infections with *T. equi* and *B. caballi* (Kouam et al., 2010). Therefore, it is necessary to use a molecular tool to detect and differentiate various species and strains in order to get a better insight into the genetic heterogeneity and diversity of equine piroplasms occurring in this country.

The objectives of the present study were: (i) to detect all possible equine piroplasm genotypes occurring in Greece using the reverse line blot hybridization (RLB) assay, (ii) to explore the genetic heterogeneity and diversity of these piroplasm genotypes, and (iii) to investigate possible associations between the genotypes and the bioclimatic variables that could support new taxa.

2. Materials and methods

2.1. Blood samples collection

A total of 787 blood samples were collected at random from 772 horses, 8 mules and 7 ponies in various regions of continental Greece during 2007–2008 after obtaining the agreement of their owners. One horse showed symptoms consistent with equine piroplasmosis (acute fever, inappetence, weight loss, anemia,

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jaundice) at the time of sampling. Blood samples were aseptically collected in tubes with EDTA and stored at 4 °C until subsequent DNA extraction.

2.2. DNA extraction

DNA was purified from 200 μl of whole blood with either Nucleospin Blood kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) or Jetquick Blood & Cell Culture DNA Spin kit (Genomeh GmbH, Löhne, Germany) according to the manufacturers' instructions. DNA was stored at −20 °C until used.

2.3. PCR amplification

Two sets of PCR experiments were performed to generate amplicons subject to RLB assay and sequencing, respectively. The primers used in the first PCR assay for samples subject to reverse line blot hybridization (RLB) assay were RLB-F (5′-GACACAGGGAGG-TAGTGACAAG-3′) and RLB-R (biotin-5′-CTAAGAATTTCACCTCTGA-CAGT-3′) (Thermo Scientific, Ulm, Germany) as adapted by Georges et al. (2001). These primers, specific for *Theileria* and *Babesia*, amplify the V4 hypervariable region of the 18S rRNA gene and generate fragments of approximately 430 bp for *Theileria* and about 390 bp for *Babesia*. The primers used in the second PCR for samples submitted to sequencing were RLB-F2 (GAGGTAGTGACAAGAAA-TAACAATA) and RLB-R2 (TCTTCGATCCCCTAACTTTC) (Thermo Scientific, Ulm, Germany) (Gubbels et al., 1999) that amplify the hypervariable V4 region of the 18S rRNA gene of *Theileria* and *Babesia*, generating fragments of approximately 460–520 bp, respectively. The amplification mixture and the PCR conditions for both PCR reactions have been described earlier (Nagore et al., 2004).

2.4. Reverse line blot hybridization assay

The PCR products were subjected to RLB assay as described by Nagore et al. (2004). The oligonucleotide probes used for the detection of all the piroplasms present in the samples are listed in Table 1. All but the Catchall probes are specific. All probes (Isogen Life science, Maarssen, Netherlands) contained a MMT C6 linker 6-(4-monomethoxytritylamino) hexyl-[(2-cyanoethyl)-(N,N-diisopropyl)]-phosphoramidite 5′ terminal group.

2.5. DNA sequencing

PCR amplicons were recovered from agarose gel using the Jetquick extraction Spin kit (Genomeh GmbH, Löhne, Germany) according to the manufacturer's instructions and submitted for bidirectional sequencing in an ABI (Applied Biosystems Inc., Foster City, CA, USA) automated sequencer. The PCR primers were also used for sequencing. Fragments from at least three PCR assays were sequenced for a representative sample of *T. equi*-like. The sequences were checked for accuracy using ChromasPro 1.5 program (TechnelySium Pty Ltd.), which allowed the nucleotide editing based on chromatograms from both forward and reverse strands.

Table 2
USGS global ecosystems land cover classification corresponding to the collection locations of piroplasm genotypes in Greece.

Original land cover classes	Generalised land cover classes
Urban	Urban/crop
Crops and town	Urban/crop
Deciduous tree crop	Urban/crop
Mixed forest	Forest
Deciduous broadleaf forest	Forest
Forest and field	Forest
Mediterranean scrub	Savanna
Semi-desert shrubs	Savanna
Fields and woody Savanna	Savanna
Woody Savanna	Savanna

2.6. Occurrence localities of piroplasm genotypes

The geographical locations of the piroplasm genotypes were recorded according to the latitude and longitude of the hosts of origin at the time of blood collection during the 2007–2008 survey. The piroplasm genotypes were determined by the RLB assay described in the present study.

2.7. Environmental variables

Environmental variables, including bioclimatic variables and land cover, were examined to determine similarities between habitats of the different genotypes. Bioclimatic variables with a spatial resolution of 1 km were downloaded from WorldClim version 1.4 dataset (http://www.worldclim.org). Land cover data were obtained from the U.S. Geological Survey's (USGS) Global Land Cover Characteristics Database version 2 Global (http://edcsns17.cr.usgs.gov/glcc/). The USGS developed the land cover from 1-km Advanced Very High Resolution Radiometer (AVHRR) satellite data acquired during April 1992 to March 1993. The USGS website provides data in several classification schemes; the global ecosystems land cover classification was selected for this study. This land cover classification uses 100 classes, 10 of which corresponded to the occurrence localities of the piroplasm genotypes. To simplify the statistical analysis, however, the original 10 classes were combined into 3 generalized classes, as listed in the second column of Table 2.

2.8. Statistical analysis

The Kruskal–Wallis test was used to compare the values of environmental variables relevant to the geographic distribution of each piroplasm genotype. Fisher's exact test was used to compare the difference in prevalence of infection within host group and to assess the difference in the prevalence of occurrence locations for each piroplasm genotype in the land cover classes. All

Table 1
Sequence, melting temperature (T_m), and concentration (C) of oligonucleotide probes covalently linked to the membrane in the RLB assay.

Probe	Sequence (5′–3′)	T_m (°C)	C (μM)	Reference
Catchall[a]	TAATGGTTAATAGGA(A/G)C(A/G)GTTG	54.7[b]	8	Gubbels et al. (1999)
T. equi	GTTTCGATTATTCGTTTCCCGG	58.4	16	Nagore et al. (2004)
T. equi-like	GGGGCATGTTTTCATGACTCGA	60.3	8	Nagore et al. (2004)
B. caballi	GTTGCGTTGTTCTTGCTTTTTGCTT	59.7	32	Nagore et al. (2004)
B. caballi-like	CGGGTTATTGACTTCGCTTTTTCTT	59.7	8	Nagore et al. (2004)

[a] Catchall is a probe specifically designed to hybridize to all piroplasm species that may be present in a sample.
[b] T_m for the degenerate oligonucleotide (catchall) is an approximate value.

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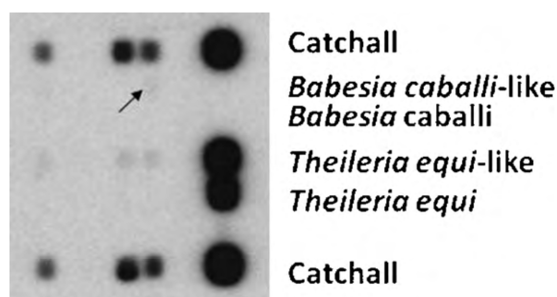

Fig. 1. RLB assay results: hybridization of 4 field samples (columns) on Catchall, *B. caballi*-like (faint signal indicated by arrow), *T. equi*-like and *T. equi* probes (rows). The probes were applied in row and the samples in column. The signals represent the hybridization of PCR products generated by amplification of genomic DNA from blood samples naturally infected with equine piroplasms. The empty spaces between column signals (positive samples) represent piroplasm-negative samples. The Catchall probe was used as control. It reacts with all the piroplasm parasites (Gubbels et al., 1999), ensuring that any piroplasm (known or unknown) will be detected. None of the samples hybridized to *Babesia caballi* probe.

the parameters were computed using Epi Info Software (version 3.5.1).

3. Results

Three genotypes consisting of two *Theileria* (*T. equi* and *T. equi*-like) and one *Babesia* (*B. caballi*-like) were detected. Fig. 1 shows strong signals observed for *T. equi* and *T. equi*-like genotypes and faint signals for *B. caballi*-like genotype. As presented in Table 3, 47.14% of the total samples tested hybridised to catchall probe, 43.96% to *T. equi* probe, 46.25% to *T. equi*-like probe, 0% to *B. caballi* probe, and 0.38% to *B. caballi*-like probe. Both *Theileria* and *Babesia* genotypes were detected in blood of horses while only *Theileria* genotypes were detected in blood of mules and ponies. However, the difference in prevalence within hosts was not significant for all the genotypes. The symptomatic horse which died later was co-infected with both *T. equi* and *T. equi*-like.

Seven samples from horses gave faint signals with the catchall probe only. The 18S rRNA gene from these samples with a very low piroplasm parasitemia could not be amplified to be sequenced in order to get an insight into the piroplasm identity. All *T. equi*-positive samples were co-infected with a *T. equi*-like genotype. For this reason, a partial sequence (509 bp) from *T. equi*-like 18S rRNA gene only could be obtained. Blast similarity search showed that *T. equi*-like isolates had 100% nucleotide similarity with Southern Spain *T. equi*-like isolates (DQ287951)(Criado et al., 2006) and 99% similarity with *T. equi*-like isolates from Northern Spain (AY534882) (Nagore et al., 2004). As the three cases of *B. caballi*-like positive samples exhibited a low parasitemia, the 18 S rRNA gene sequences from this genotype could not be retrieved.

Environmental variables did not differ significantly between the presence locations of *T. equi* and *T. equi*-like genotypes (Table 4). Comparing the presence and absence locations for each genotype, thirteen of 19 bioclimatic variables were significant ($p < 0.05$) for *T. equi*, while 10 of the same 13 variables were significant ($p < 0.05$) for *T. equi*-like (Table 5).

Table 3
Number (*n*) of equids tested positive and prevalence of infection (%) within hosts. No significant difference ($p < 0.05$) in prevalence was found within hosts for any infection.

Host	N[a]	Probe									
		Catchall		*T. equi*		*T. equi*-like		*B. caballi*		*B. caballi*-like	
		n	%	*n*	%	*n*	%	*n*	%	*n*	%
Horse	772	364	47.20	340	44.00	357	46.20	0	0	3	0.40
Mule	8	6	75.00	5	62.50	6	75.00	0	0	0	0
Pony	7	1	14.30	1	14.30	1	14.30	0	0	0	0
Total	787	371	47.14	346	43.96	364	46.25	0	0	3	0.38

[a] Number of animals examined.

Table 4
Comparison of the bioclimatic variables between occurrence locations for the two *Theileria* genotypes (temperature values are in °C; precipitation values are in mm). No significant difference ($p < 0.05$) was found.

Bioclimatic variable	*Theileria equi* occurrence location $n = 16$		*Theileria equi*-like occurrence location $n = 20$		*p* value
	Mean	SD[a]	Mean	SD	
Annual mean temperature	15.7815	2.1902	16.0100	2.0367	0.7127
Mean diurnal range (mean of monthly (max temp – min temp))	9.8625	1.3321	9.9000	1.2872	0.9488
Isothermality (P2/P7) (×100)	3.3500	0.2191	3.4000	0.2340	0.5718
Temperature seasonality (standard deviation × 100)	667.6125	68.1426	662.0950	75.5039	0.8235
Max temperature of warmest month	31.7063	1.7650	31.9550	1.8254	0.8606
Min temperature of coldest month	2.7750	3.3575	2.9800	3.2728	0.8603
Temperature annual range (P5–P6)	28.9313	3.3226	28.9750	3.5824	0.9363
Mean temperature of wettest quarter	8.8813	3.0023	9.0450	3.0123	0.8734
Mean temperature of driest quarter	24.1938	1.9699	24.3450	1.7957	0.9109
Mean temperature of warmest quarter	24.4313	1.9227	24.5750	1.7603	0.9617
Mean temperature of coldest quarter	7.4375	2.8598	7.8900	2.8049	0.6094
Annual precipitation	647.5000	189.4504	665.3500	174.2053	0.8986
Precipitation of wettest month	103.0625	41.3996	108.4000	39.8568	0.7377
Precipitation of driest month	13.4375	7.4563	12.5500	7.1780	0.6879
Precipitation seasonality (coefficient of variation)	51.1875	20.0772	53.2000	19.6029	0.7129
Precipitation of wettest quarter	274.8750	111.5442	286.6000	107.2756	0.7740
Precipitation of driest quarter	53.5000	29.0815	51.4500	28.3595	0.7738
Precipitation of warmest quarter	62.5000	25.2824	61.1500	23.5825	0.6785
Precipitation of coldest quarter	252.3750	102.6690	260.5000	95.2678	0.8483

[a] Standard deviation.

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Table 5
Comparison of the bioclimatic variables between occurrence and absence locations for the two *Theileria* genotypes (temperature values are in °C; precipitation values are in mm).

Bioclimatic variable	*Theileria equi* genotype					*Theileria equi*-like genotype				
	Occurrence location ($n=16$)		Absence location ($n=16$)		p value	Occurrence location ($n=20$)		Absence location ($n=12$)		p value
	Mean	SD[a]	Mean	SD		Mean	SD	Mean	SD	
Annual mean temperature	15.7875	2.1902	17.0625	1.6613	0.0492*	16.01	2.0367	17.0917	1.8549	0.0726
Mean diurnal range (mean of monthly (max. temp – min. temp))	9.8625	1.3321	9.7063	1.1958	0.5705	9.9	1.2872	9.4	0.9234	0.204
Isothermality (P2/P7) (×100)	3.35	0.2191	3.5688	0.1852	0.0153*	3.4	0.2340	3.5583	0.1881	0.0824
Temperature seasonality (standard deviation × 100)	667.6125	68.1426	604.05	61.5438	0.0039*	662.095	75.5039	592.0583	35.2687	0.0054*
Max temperature of warmest month	31.7063	1.7650	31.9875	1.5466	0.6351	31.955	1.8254	31.6667	1.3248	1
Min temperature of coldest month	2.775	3.3575	5.1313	2.6007	0.0342*	2.98	3.2728	5.575	2.3538	0.017*
Temperature annual range (P5–P6)	28.9313	3.3226	26.8563	3.0796	0.0246*	28.975	3.5824	26.0917	1.8118	0.0192*
Mean temperature of wettest quarter	8.8813	3.0023	11.025	2.4912	0.0329*	9.045	3.0123	11.475	2.1085	0.014*
Mean temperature of driest quarter	24.1938	1.9699	24.7688	1.4827	0.3446	24.345	1.7957	24.7083	1.6941	0.2575
Mean temperature of warmest quarter	24.4313	1.9227	24.8875	1.5323	0.6487	24.575	1.7603	24.8	1.7341	0.6662
Mean temperature of coldest quarter	7.4375	2.8598	9.625	2.1265	0.018*	7.89	2.8049	9.8667	2.0513	0.0348*
Annual precipitation	647.5	189.4504	783.75	100.5369	0.1415	665.35	174.2053	799.4167	107.4891	0.0644
Precipitation of wettest month	103.0625	41.3996	143	27.9094	0.0128*	108.4	39.8568	147.4167	27.8844	0.012*
Precipitation of driest month	13.4375	7.4563	7.625	3.9476	0.0379*	12.55	7.1780	7.1667	3.5887	0.0849
Precipitation seasonality (coefficient of variation)	51.1875	20.0772	68.3125	12.7055	0.0169*	53.2	19.6029	70.6667	10.5515	0.0243*
Precipitation of wettest quarter	274.875	111.5442	380.1875	77.4556	0.0194*	286.6	107.2456	395.75	72.2774	0.0141*
Precipitation of driest quarter	53.5	29.0815	32.6875	19.0953	0.0687	51.45	28.3595	29.1667	15.4204	0.0715
Precipitation of warmest quarter	62.5	25.2824	48.625	10.4363	0.0221*	61.15	23.5825	46.25	6.9429	0.0464*
Precipitation of coldest quarter	252.375	102.6690	341.6875	65.9644	0.0194*	260.5	95.2678	357.9167	62.9422	0.0068*

[a] Standard deviation.
* Significant difference ($p < 0.05$).

Table 6
Number (n) and prevalence (%) of occurrence in locations by generalized land cover class associated with each *Theileria* genotype based on RLB hybridization assay. Each land cover class was contrasted against the grouping of the other two land cover classes. A similar letter (a, b, c, and d) indicates significant difference ($p < 0.05$).

Land cover class vs.	Grouping of the other two land cover classes	N*	*T. equi*		*T. equi*-like	
			n	(%)	n	(%)
Urban/crop vs.		9	5	56.6	7	77.8
	Forest plus Savanna	23	11	47.8	13	56.5
Forest vs.		11	9	81.8[a]	10	90.9[c]
	Urban/crop plus Savanna	21	7	33.3[a]	10	47.6[c]
Savanna vs.		12	2	16.7[b]	3	25.0[d]
	Urban/crop plus forest	20	14	70.0[b]	17	85.0[d]

* Number of sampled localities.

The occurrence locations for *T. equi* and *T. equi*-like were most predominant in forest type land cover class (Table 6). The prevalence of occurrence localities for *T. equi* and *T. equi*-like were significantly higher in forest than in urban and crop plus Savanna combined ($p < 0.05$ in both cases), and lower in Savanna than in urban and crop plus forest combined ($p < 0.05$ in both cases).

4. Discussion

The RLB assay is a powerful tool which allows a simultaneous detection and differentiation of all possible piroplasm species in a sample and permits a strain or genotype identification of the piroplasms present in the samples as well (Gubbels et al., 1999; Nagore et al., 2004; Bhoora et al., 2009).

The prevalence levels of *Theileria* infections in equids in the present study were much higher than in a previous report (Kouam et al., 2010). It is worth mentioning that the samples in the present investigation were collected from the same areas as in the aforementioned study. This difference may be explained by the high sensitivity of the RLB assay (Nagore et al., 2004); furthermore it is not known whether or not the competitive-inhibition ELISA test (cELISA) used in the previous study was able to detect both *T. equi* and *T. equi*-like genotypes, since the similarity of the merozoite antigene-1 (EMA 1) gene used for the cElisa antigen (Knowles et al., 1992) has not yet been explored between the two genotypes. Further studies are needed to see if there is a difference between the two genotypes in terms of immunogenicity and not just within the 18S rRNA gene. The low prevalence of *Babesia* recorded in the present work (0.38%) is comparable to the prevalence observed in the previous study (2.2%). This finding reinforces the observation that in the Mediterranean region, equine *Theileria* infections are more frequently diagnosed than *Babesia* infections (Schein, 1988; Bachiruddin et al., 1999; Criado-Fornelio et al., 2003). It has been reported that parasitemia in *Babesia* infections in equids generally tend to be very low, rarely exceeding 1% (Hanafusa et al., 1998). Therefore, the low prevalence generally observed in *Babesia* infections may be related to a very low parasitemia level of the parasite.

Two genetically distinct *Theileria* and one *Babesia* genotypes were clearly identified in the present study. This is the first report on the use of RLB assay for genotype identification of equine piroplasms in Greece. These genotypes were first described in Spain (Nagore et al., 2004) and recently reported in South Africa within 3 heterogeneous groups for *Theileria* (Groups A, B and C) and

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2 for *Babesia* (Groups A and B) (Bhoora et al., 2009). Under this nomenclature, *T. equi* and *T. equi*-like genotypes reported in this study were assigned to Group A and Group B, respectively within the *Theileria* Group, whereas *Babesia caballi*-like belongs to group A within the *Babesia* Group. Within *Theileria*, Group A, B, C have been recently reported in Sudan, besides a fourth group designated as Group D (Salim et al., 2010). The occurrence of *T. equi* and *T. equi*-like genotypes in Spain, Greece, Sudan and South Africa and *Babesia equi*-like genotype in Spain, Greece and South Africa shows that these genotypes are wide-spread. In the present study *T. equi* always occurred as a mixed-infection with *T. equi*-like genotype but the latter occurred predominantly alone in eighteen samples. The predominance of *T. equi*-like over *T. equi* has been reported in Spain as well (Nagore et al., 2004). The *T. equi*-like genotype has been reported to be more pathogenic than the *T. equi* genotype and it was even associated with the death of a horse in Spain (Nagore et al., 2004). In the present study, the only case of horse death reported was associated with a mixed *T. equi*-like and *T. equi* infection. Therefore, the predominance of *T. equi*-like in Greek equids should be a matter of great concern for the stakeholders of the equine industry in Greece.

A partial sequence of the V4 region of the 18S rRNA gene of a *T. equi*-like isolate showed only 99% similarity with the reference *T. equi*-like isolates from Northern Spain (Nagore et al., 2004) from which the detecting probe used in the present study was designed and 100% identity with the *T. equi*-like variants from Southern Spain (Criado et al., 2006). This finding is an indication of a measureable degree of polymorphism within the *T. equi*-like genotype, and was previously observed by Criado et al. (2006).

Only the *B. caballi*-like genotype was identified in this study, but this finding does not preclude the presence of the *B. caballi* genotype in Greece. Bhoora et al. (2009) reported in their study the inability of RLB assay to detect all the *Babesia caballi* infections, an observation that was attributed to the very low *Babesia caballi* parasitemia.

No unusual parasites previously reported in horses such as *B. canis canis* (Criado-Fornelio et al., 2003; Hornok et al., 2007) and *B. bovis* (Criado et al., 2006) were detected in the present work. However, seven samples gave faint signals with the catchall probe which detects all possible *Theileria*/*Babesia* genotypes in a sample, and consequently acts as a guarantee that no new piroplasm species or a new strain of a known piroplasm species will pass undetected. Usually, the signal of group-specific probes in a RLB assay is much stronger compared to species- or genotype-specific probes as a result of a multiple reaction with all or multiple parasite variants in a sample (Gubbels et al., 1999; Schnittger et al., 2004). Therefore, the occurrence of a faint signal with a group-specific probe such as the catchall probe is interpreted as the possible presence, in a very low amount, of a known or unknown piroplasm species or genotype in Greece. As mentioned above, no sequence information could be obtained from the V4 region of the 18S r RNA gene of the parasites in these samples. Consequently, no clear-cut conclusions on the real status of these piroplasm-positive samples could be made.

Bioclimatic variables, which are relevant to the geographic distribution of a species (Estrada-Peña et al., 2008), were similar between *T. equi* and *T. equi*-like genotypes' geographical locations and slightly differed when the presence and absence of locations were compared for each genotype raising the question of the real taxonomic position of these closely related genotypes. Considering the divergence of *T. equi*-like with respect to *T. equi* genotype, Nagore et al. (2004) hypothesised that they might be dealing with a new piroplasm species but indicated that additional non-genetic features would be necessary to be conclusive. Bhoora et al. (2009) could not rule out the possibility that the different groups of *T. equi* and *B. caballi* identified in their study on the basis of the heterogeneity of the 18S rRNA gene sequence represented different parasite species, though it is not possible to use the 18S rRNA gene sequence variation to classify the organisms as different species (Chae et al., 1999; Criado-Fornelio et al., 2004). Given the difficulty in assigning a taxon to an organism based on the heterogeneity of the 18S rRNA gene, there a need for the scientific community to agree by how much the 18S rRNA sequences must differ from the source organisms to be considered different species rather than merely strain or genotype variations. Additionally, sequences from new target genes that can complement findings from the 18S r RNA gene may be useful. In the present study, the occurrence locations for *T. equi* and *T. equi*-like genotypes shared the same relevant bioclimatic variables and when occurrence and absence locations were compared for each genotype, both genotypes shared the same significant variables and differed only in three. This finding is evidence that both genotypes are closely related ecologically and should not be regarded as different species yet but as organisms undergoing a speciation process. Alternatively, species convergence is a possibility due the fact that both *T. equi* and *T. equi*-like occur in the same site (red blood cells) in the vertebrate host (Nagore et al., 2004). Further studies involving both the vertebrate (equids) and the invertebrate (ticks) hosts will be helpful to elucidate the possibility of convergence phenomenon. Nagore et al. (2004), and Criado-Fornelio et al. (2004) pointed out that in order to assign a taxon to a newly reported piroplasm genotype, a possible differential feature should be a distinct geographic distribution, which is unlikely in this case since the same host types (equids for *T. equi* and *T. equi*-like) were co-infected with both sister genotypes. The nature of the hypothesized speciation is therefore thought to be sympatric rather than allopatric. Additionally, one of the conditions of sympatric speciation in parasitology is when a parasite is able to choose its hosts actively such as ticks and fleas (McCoy, 2003). Since equine piroplasms are tick-borne parasites, the speciation underway between the sister genotypes is regarded as sympatric.

The preferred land cover class for both *T. equi* and *T. equi*-like occurrence locations was the forest. This is expected since vegetation is known to provide the tick vectors adequate protection and infallible support for hosts questing (Wall and Shearer, 2001; Torina et al., 2008).

In conclusion, two equine *Theileria* genotypes and one *Babesia* genotype occur in Greece. The Greek *T. equi*-like isolate is 100% identical to the southern Spanish isolates and shares 99% identity with the northern Spain isolates. The question of the predominance of *T. equi*-like over *T. equi* in Greece should be addressed seriously since the former genotype was reported to be more pathogenic than the latter one (Nagore et al., 2004). Rather than regarding *T. equi* and *T. equi*-like genotype as distinct species as suggested by some authors, *T. equi* and *T. equi*-like should be regarded as sister genotypes, possibly undergoing a sympatric speciation.

Further research is needed to compare different genotype isolates among regions, to identify the specific tick vector of each genotype, and to derive geographic distribution maps of equine piroplasms in Greece and elsewhere in order to better understand the epidemiology of equine piroplasmosis.

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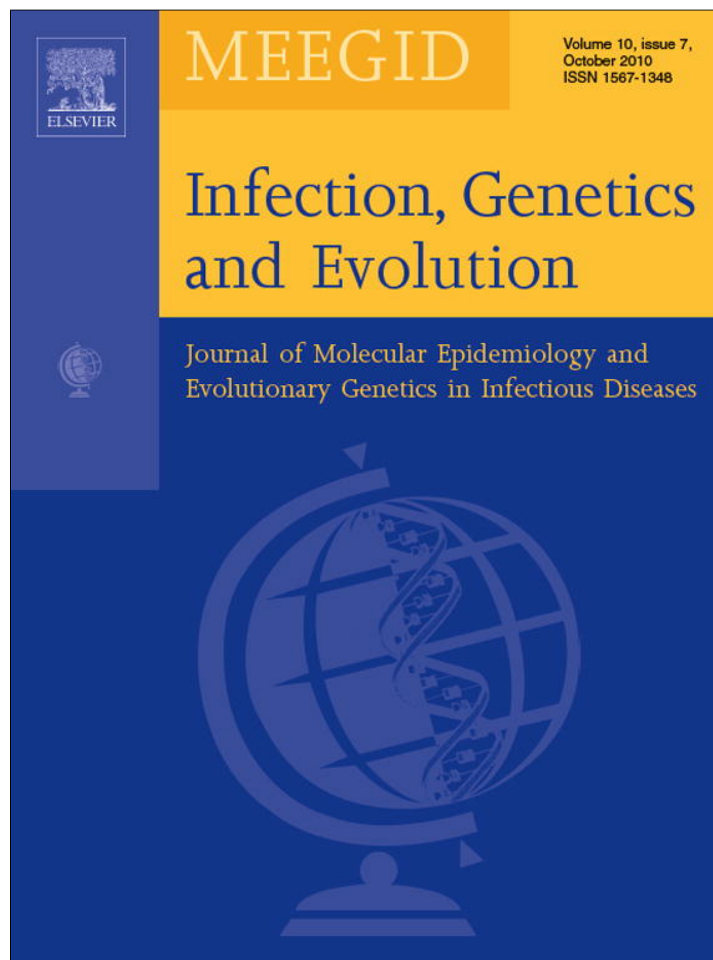

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Geographic distribution modeling and spatial cluster analysis for equine piroplasms in Greece

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ABSTRACT

Maximum entropy ecological niche modeling and spatial scan statistic were utilized to predict the geographic range and to investigate clusters of infections for equine piroplasms in Greece, using the Maxent and SaTScan programs, respectively. The eastern half of the country represented the culminating area with high probabilities ($p > 0.75$) of presence of equine piroplasms and encompassed most regions with high concentration of equid host populations. The most important environmental factor that contributed to the ecological niche modeling was land cover followed by temperature. Significant clusters ($p < 0.0001$) were detected for *Babesia caballi* and *Theileria equi* infections in North and Central regions of Greece, respectively, which have significant equine populations. Maximum entropy ecological niche modeling and spatial scan statistic have proved to be useful tools for the surveillance of animal diseases.

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1. Introduction

Theileria equi and *Babesia caballi* are both tick-borne parasites of equids restricting the international movement and trade of horses all over the world (Bashiruddin et al., 1999; Nicolaiewsky et al., 2001). Two main *Theileria* genotypes (*T. equi* and *T. equi*-like) and two main *Babesia* genotypes (*Babesia equi* and *B. equi*-like) have been described in Spain (Nagore et al., 2004) and reported in Greece (Kouam et al., 2010a) and in South Africa (Bhoora et al., 2009). *T. equi* and *T. equi*-like have been hypothesized to be separate species (Nagore et al., 2004) or to be undergoing a speciation process (Kouam et al., 2010a). Within *Theileria* genotype, *T. equi*-like has been reported to be more virulent than *T. equi* genotype (Nagore et al., 2004). *T. equi* and *B. caballi* occur in the Mediterranean basin where *T. equi* infection is more frequently diagnosed than *B. caballi* infections (Schein, 1988; Bashiruddin et al., 1999; Criado-Fornelio et al., 2003). These species occur in Greece with seroprevalence estimates of 1.2–3.9% and 8.6–14% for *B. caballi* and *T. equi*, respectively (Kouam et al., 2010). The region of host location has been found to be a significant risk factor for equine piroplasmosis in this country (Kouam et al., 2010). This observation indicates that significant geographic clusters might be occurring for each species.

Reports on piroplasm distribution are unavailable in Greece, even in horse industry hotspots such as the hippodrome of the region of Attica where the 2004 Olympic equestrian Games took place. Ecological niche modeling offers the opportunity to derive predictive distribution maps from species occurrence and environmental data (Phillips et al., 2006; Masuoka et al., 2009). For this reason, a cross sectional survey was conducted to collect information on the spatial distribution of equine piroplasms and to detect spatial clusters in Greece.

2. Materials and methods

2.1. Occurrence localities' database

Presence locations of piroplasms were obtained from a field survey of randomly selected clinically healthy equids (horses, mules and ponies) after obtaining the consent of their owners in various areas of mainland Greece between 2007 and 2008.

Piroplasm species (*T. equi* and *B. caballi*) were detected from 544 sera using species-specific competitive-inhibition ELISA (cELISA) as described by Kouam et al. (2010). Piroplasm genotypes were determined from 787 DNA samples by the reverse line blot hybridization (RLB) assay using strain-specific probes as previously described (Kouam et al., 2010a). Longitude and latitude coordinates of the investigated localities were recorded at each animal host location. Table 1 presents the positive number of piroplasm records and the number of associated localities. These

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Table 1
Occurrences and associated localities of equine piroplasm species and *Theileria* genotypes used for geographic distribution modeling and spatial cluster analysis.

Species/genotype	Occurrences		Localities	
	cELISA	RLB	cELISA	RLB
Piroplasm species				
Babesia caballi	12	1	7	1
Theileria equi	60	N/A[a]	15	N/A
Theileria genotypes				
Theileria equi	N/A	346	N/A	16
Theileria equi-like	N/A	364	N/A	20

[a] N/A: not applicable because ELISA was used only for species characterization and RLB was used only for genotype characterization.

records were utilized for ecological niche modeling and spatial scan statistic analysis.

2.2. Source of environmental data for species distribution modeling

Three types of environmental data were obtained for this study: climate, elevation, and land cover data. These data sets were converted to a common projection, map extent and resolution prior to use in the Maxent modeling program.

WorldClim version 1.4 climate data (Hijmans et al., 2005) were obtained from the WorldClim website (http://www.worldclim.org). WorldClim provides data as 50-year means for each month for precipitation, minimum temperature, and maximum temperature. The data are further processed into a series of bioclimatic variables (Table 2). The bioclimatic variables with an approximate resolution of 1 km were used for this project.

Elevation data were also downloaded from the WorldClim website. WorldClim processed this data set from NASA Shuttle Radar Topography Mission (SRTM) data to have the same projection and resolution as the other WorldClim layers.

Land cover data were downloaded from the United States Geological Survey's (USGS) Global Land Cover Characteristics Database, version 2 Global (http://www.edsns17.crusgs.gov/glcc/). The land cover data were developed from 1-km Advanced Very High Resolution Radiometer (AVHRR) satellite data from April 1992 to March 1993. Land cover data are available in multiple classification schemes; the Global Ecosystems land cover classification, which contains 100 classes, was used for the modeling work.

Table 2
List of WorldClim bioclimatic variables used in the model (Hijmans et al., 2005).

Bioclimatic variable	Description
BIO1	Annual mean temperature
BIO2	Mean diurnal range (mean of monthly (max temp – min temp))
BIO3	Isothermality (P2/P7) (×100)
BIO4	Temperature seasonality (standard deviation × 100)
BIO5	Max temperature of warmest month
BIO6	Min temperature of coldest month
BIO7	Temperature annual range (P5–P6)
BIO8	Mean temperature of wettest quarter
BIO9	Mean temperature of driest quarter
BIO10	Mean temperature of warmest quarter
BIO11	Mean temperature of coldest quarter
BIO12	Annual precipitation
BIO13	Precipitation of wettest month
BIO14	Precipitation of driest month
BIO15	Precipitation seasonality (coefficient of variation)
BIO16	Precipitation of wettest quarter
BIO17	Precipitation of driest quarter
BIO18	Precipitation of warmest quarter
BIO19	Precipitation of coldest quarter

2.3. Ecological niche modeling

Ecological niche modeling was performed with the Maxent program (available from http://www.cs.princeton.edu/~schapire/maxent/). Maxent uses a maximum entropy algorithm to predict a species' distribution using species' presence data and environmental data (Phillips et al., 2004, 2006; Phillips and Dudik, 2008). Maxent has been used for a number of studies and has been shown to be a high performing modeling program (Elith et al., 2006; Yunsheng et al., 2007; Mingyang et al., 2008).

For testing the model accuracy, 25% of the presence records were randomly selected and withheld from model building to use for testing the model. Maxent uses two methods to evaluate the models. Maxent calculates the area under the curve (AUC) for the receiver operating characteristic (ROC), a plot of the sensitivity versus 1-specificity (Swets, 1988; Fielding and Bell, 1997). Random predictions have AUC values of 0.5; the best performing models have values of 0.9 and above. Maxent also calculates a threshold-dependent statistic where probabilities above a threshold (e.g. minimum training presence) predict presence, and probabilities below the threshold predict absence. *p*-values are calculated based on the null hypothesis that the test data are predicted no better than a random prediction.

To measure the contributions of individual variables on the models, Maxent performs a jackknife test in which the model is run multiple times: (1) using all variables, (2) dropping one variable at a time, and (3) running the model using only one variable. Variables that produce the highest training gains or reduce the training gain when left out of the model are considered to be the most important variables.

The GIS software ARCGIS ver. 9.3 was used to display the prediction results and represent the sampled localities.

2.4. Spatial cluster analysis

The spatial scan statistic implemented in SaTScan software (version 8.0) was used to investigate geographic clusters of infection (available from http://www.satscan.org/). The concept of spatial scan statistic is based on the generalization of a test probability by Turnbull et al. (1990) and further developed by Kulldorff and Nagarwalla (1995) and Kulldorff (1997). The spatial scan statistic uses a circular window of variable radius that moves across the map to represent potential geographic clusters. The radius of the cluster varies from zero up to a specified maximum value. By gradually changing the circle centre and radius, the window scans the geographic areas for potential localised clusters without incorporating prior assumptions about their size and location and noting the number of observed and expected observations inside the window at each location. The assessment of a cluster is done by comparing the number of cases (infection) within the circle with the number of expected cases under the assumption that cases are randomly distributed in the space. The test of significance is based on the likelihood ratio test for which the window with the maximum likelihood is the most likely cluster (Kulldorff and Nagarwalla, 1995). The *p*-value is obtained through Monte Carlo hypothesis testing (Dwass, 1957). The spatial scan statistic adjusts for spatial variations in the density of the population in the study area, which is an asset since an apparent disease cluster in a particular area could be misleading when it is due to the cluster of the population itself (Kulldorff and Nagarwalla, 1995). The population in the Poisson probability model may be the actual count from a census or covariate adjusted expected counts from a statistical regression model, while in the Bernoulli model it is denoted as the total of cases (positives samples) and controls (negative samples) in the study area. SaTScan has been shown to be the best-equipped package for use in

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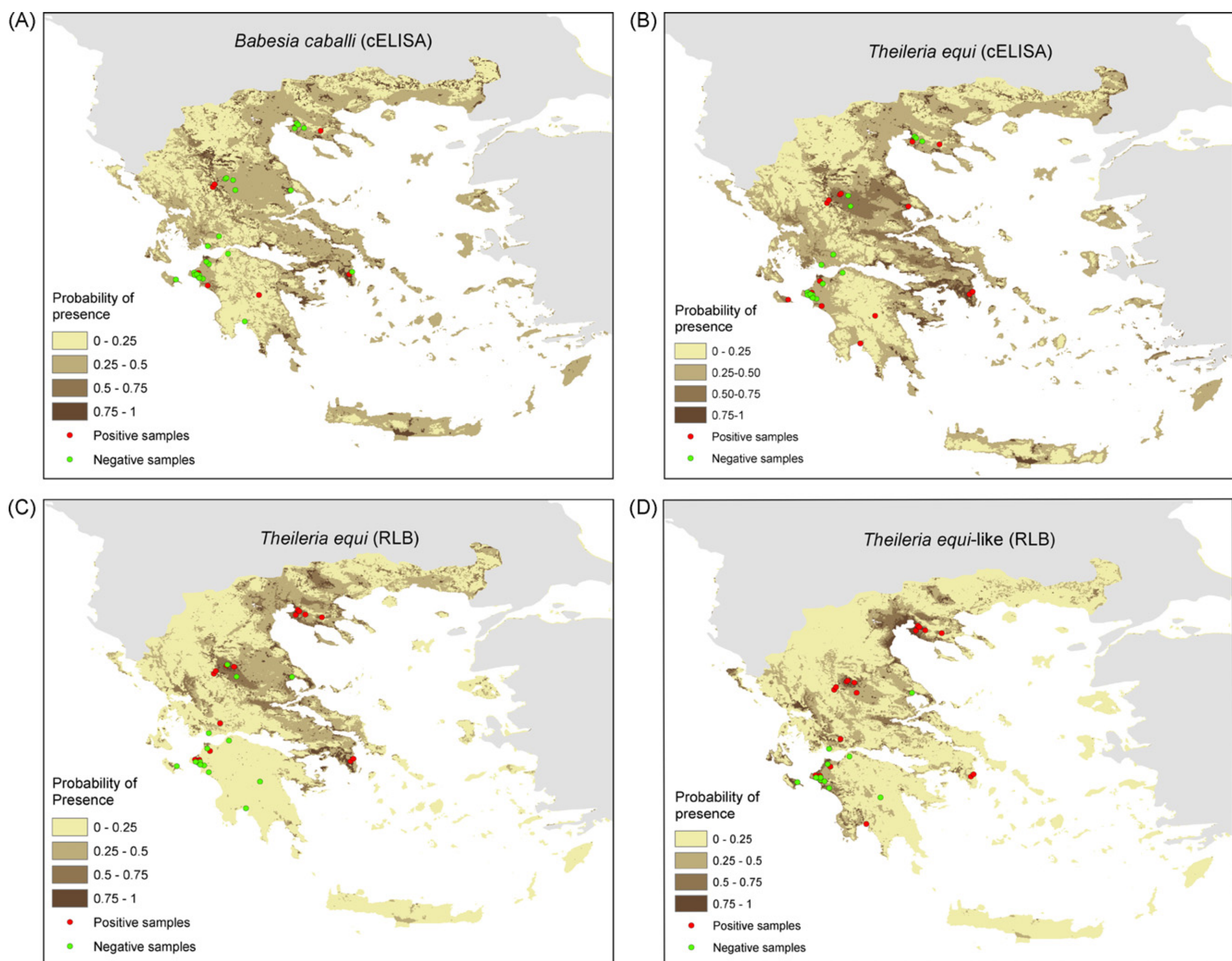

Fig. 1. Predicted geographic distribution ranges for equine piroplasms. (A) *Babesia caballi* species as determined by cELISA and RLB; (B) *Theileria equi* species; (C) *Theileria equi* genotype; and (D) *Theileria equi*-like genotype.

an automated surveillance system compared to other available programs such as ClusterSeer, GeoSurveillance and the R-Package 'Surveillance' (Robertson and Nelson, 2010).

The detection of clusters in the present study was performed under the Bernoulli probability model, using the maximum cluster size of 5% and 11% of the total population for *Babesia* and *Theileria* infections, respectively. The cluster size choice was based on the seroprevalence of these infections reported in a previous study (Kouam et al., 2010.). Test-positive equids were considered as cases while test-negative equids were regarded as controls. The number of simulations for Monte Carlo testing was set to 9999. For each window of varying position and size, the SaTScan program tested the risk of piroplasm infection within and outside the window, with the null hypothesis of equal risk.

3. Results

Fig. 1 displays the potential geographic distribution of piroplasms predicted by Maxent program. As shown in Fig. 1A, the highest level of probability ($p > 0.75$) for the geographic distribution of *B. caballi* covers small areas spreading from north to south in the eastern half of mainland Greece and Peloponnese and extends downwards to the south coast of the island of Crete. The regions with significant concentration of potential equid hosts (Table 3) and with a high probability of presence for *B. caballi* infection consist of the regions of Macedonia and Thessaly and the Aegean islands.

High probability ($p > 0.75$) areas for *T. equi* species mainly cover the eastern part of continental Greece from north to south, in the western coastlines as well as the southernmost coast of the country at the island of Crete (Fig. 1B). The regions of high probability for *T. equi* infection, with significant concentration of potential equid hosts include the regions of Thessaly, Macedonia and Peloponnese (Table 3).

The range with the highest probability ($p > 0.75$) of presence for *T. equi* genotype is restricted for the most part in the eastern half of

Table 3
Distribution of equid population in regions in Greece by decreasing order of importance.

Regions	Percentage (%)
Central Macedonia	15.06
Thessaly	12.95
North Aegean	11.05
East Macedonia and Thrace	10.80
Peloponnese	10.73
West Greece	10.57
South Aegean	10.53
Central Greece	5.30
Epirus	4.20
West Macedonia	3.05
Crete	2.54
Ionian islands	2.45
Attica	0.77

Adapted from the Hellenic Statistical Authority (2007).

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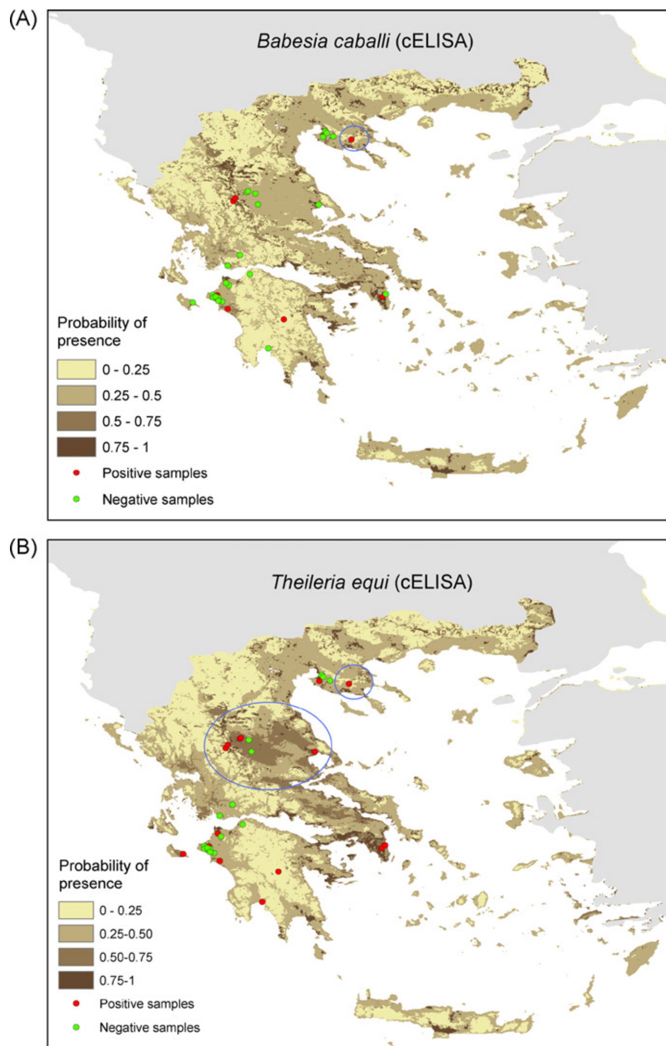

Fig. 2. Significant clusters of equine piroplasms in Greece. (A) Most likely cluster of *B. caballi* infection (circle). (B) Most likely cluster (big circle) and secondary cluster (small circle) of *T. equi* infection. The circles enclose the locations identified within each cluster.

mainland Greece (Fig. 1C). The regions of highest probability for the presence of *T. equi* genotype with significant concentration of potential equid hosts (Table 3) encompass the regions of Macedonia and Thessaly.

The geographic distribution for *T. equi*-like genotype with the highest probability of presence concentrates in central part of mainland Greece from the western coast bordering the Ionian Sea to the eastern coast lining the Aegean Sea and extends northwards towards central Macedonia (Fig. 1D). The regions with high probability of presence of *T. equi*-like infection with significant concentration of potential equid hosts consist of the regions of Macedonia and Thessaly (Table 3).

The results of the spatial scan statistic analyses showed one most likely cluster of infected animals for the species *B. caballi* ($p = 0.0001$). In addition, one most likely cluster ($p = 0.0001$) and one secondary cluster ($p = 0.003$) of infected animals were detected for the species *T. equi* (Table 4 and Fig. 2). No significant clusters were detected for the genotypes *T. equi* and *T. equi-like*.

3.1. Model validation

AUC values for the models range from 0.822 to 0.977, indicating that the models are good to very good (Table 5). The *p*-value using minimum training presence as the threshold is high for *T. equi* RLB ($p = 0.09$) indicting that the model may not be useful. The other models have lower *p*-values (Table 5) with *T. equi*-like RLB having the lowest (0.001) indicating the best prediction.

3.2. Effect of environmental variables on the model

For *Babesia* and *Theileria* spp., land cover was the variable that achieved the highest training gain when used to build a model with no other variables (Fig. 3). Land cover was also the variable that reduced the training gain the most when dropped from the model. By these measures, land cover is the single most important variable in modeling these species. Land cover types that occurred at the species sampling areas are shown in Table 6. Forest is a primary land cover.

Other variables that show training gain when modeling with only a single variable included: annual mean temperature (BIO1), maximum temperature of the warmest month (BIO5), mean temperature of the driest quarter (BIO9), and mean temperature of the warmest quarter (BIO10) (Fig. 3). Plots from Maxent (not reproduced here) showed that the probability of the species occurrence increases as these temperature variables increase.

Precipitation has a smaller affect on *Babesia* and *Theileria* spp. in the model. For *T. equi*-like RLB, the precipitation variable that achieved the highest training gain was precipitation of the warmest quarter (BIO18). Precipitation seems to have less effect on the presence of *B. caballi* and *T. equi*.

Table 4
Significant clusters of piroplasm infections.

Species	Cluster	Locality/region	Number of cases	Expected cases	Relative risk	*p*-value
B. caballi	Most likely	Chalkidiki/central Macedonia	4	0.24	24.93	0.0001
T. equi	Most likely	Kotroni, Ropoto, Trikala, Loggaki, Volos/Thessaly	21	6.30	4.59	0.0001
	Secondary	Chalkidiki/central Macedonia	5	0.92	5.84	0.003

Table 5
Statistical evaluation of the model.

Genotype	Number of presence records[a] (training/testing)	AUC for training data	AUC for test data	*p*-value using minimum training presence as threshold
Babesia caballi cELISA	6/2	0.946	0.946	0.01
Theileria equi cELISA	12/3	0.923	0.954	0.01
Theileria equi RLB	12/4	0.963	0.822	0.09
Theileria equi-like RLB	15/5	0.977	0.857	0.001

[a] Duplicate presence records for the same localities were removed from the model.

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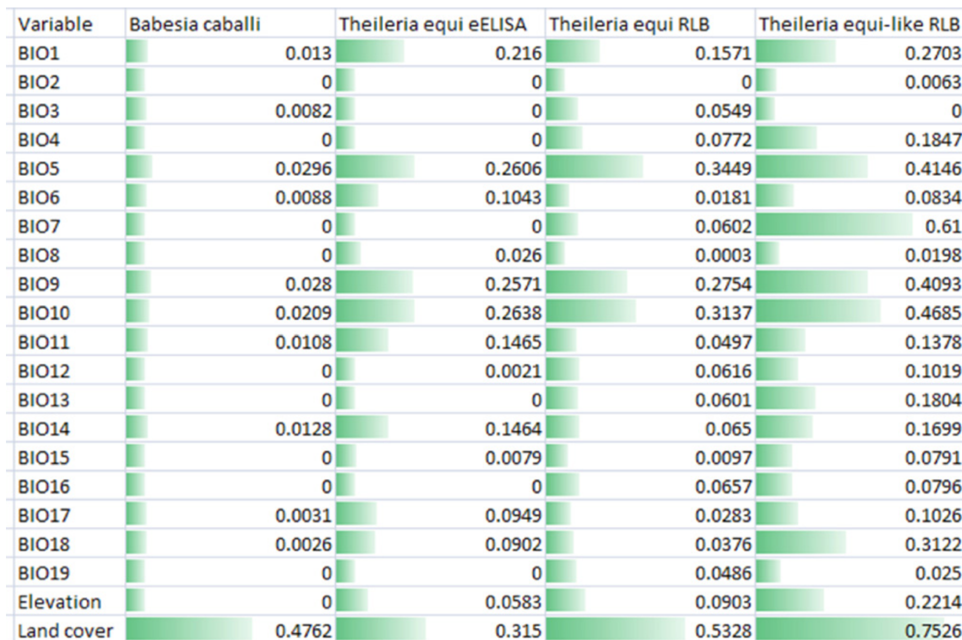

Variable	Babesia caballi	Theileria equi eELISA	Theileria equi RLB	Theileria equi-like RLB
BIO1	0.013	0.216	0.1571	0.2703
BIO2	0	0	0	0.0063
BIO3	0.0082	0	0.0549	0
BIO4	0	0	0.0772	0.1847
BIO5	0.0296	0.2606	0.3449	0.4146
BIO6	0.0088	0.1043	0.0181	0.0834
BIO7	0	0	0.0602	0.61
BIO8	0	0.026	0.0003	0.0198
BIO9	0.028	0.2571	0.2754	0.4093
BIO10	0.0209	0.2638	0.3137	0.4685
BIO11	0.0108	0.1465	0.0497	0.1378
BIO12	0	0.0021	0.0616	0.1019
BIO13	0	0	0.0601	0.1804
BIO14	0.0128	0.1464	0.065	0.1699
BIO15	0	0.0079	0.0097	0.0791
BIO16	0	0	0.0657	0.0796
BIO17	0.0031	0.0949	0.0283	0.1026
BIO18	0.0026	0.0902	0.0376	0.3122
BIO19	0	0	0.0486	0.025
Elevation	0	0.0583	0.0903	0.2214
Land cover	0.4762	0.315	0.5328	0.7526

Fig. 3. Training gain achieved by models using single variables. Length of bar represents the training gain value. A longer bar represents a higher training gain.

Table 6
Number of sample localities within different land cover categories of the USGS Global Ecosystems land cover classification.

Land cover (class number)	*B. caballi*	*T. equi* ELISA	*T. equi* RLB	*T. equi*-like RLB
Urban (1)	–	2	1	2
Mixed forest (24)	–	1	4	5
Deciduous broadleaf forest (26)	1	1	1	1
Crops and Town (31)	–	2	3	4
Mediterranean Scrub (46)	–	1	–	–
Semi desert Shrubs (51)	–	1	–	–
Forest and Field (56)	4	4	4	4
Fields and Woody Savanna (58)	–	1	1	1
Woody Savanna (91)	2	2	1	2
Deciduous Tree Crop (96)	–	–	1	1

4. Discussion

Equids are an important source of income for the Greek economy, with approximately 32,000 heads spreading all over the country (HSA, 2007). They are subject to an intensive breeding to satisfy increased demands for draft, carriage, show events and racing needs. Unfortunately, equine piroplasmosis represents one of the major obstacles to equine industry in the country. The present study reports for the first time clusters of piroplasm infections as well as the potential geographic range of different piroplasms species and genotypes that occur in the country.

The relatively small number of presence locations (8–20) used to model *Babesia* and *Theileria* spp. may have resulted in poorer models as the lower *p*-values indicate. In other research, the precise number of samples needed for an accurate model varies. Stockwell and Peterson (2002) found that the accuracy of models was best using 50 data points in the GARP modeling program. Papes and Baubert (2007) found an agreement between GARP and Maxent models starting at around 15 records, which may indicate the minimum number of records needed for accurate modeling. Pearson et al. (2007) found that models built with as few as 5 records showed some statistical significance using Maxent. However, by experimentally dropping records from a model, they found that the predicted distribution was affected by which records were kept.

Additional collection sites for *Babesia* and *Theileria* spp. may change the predicted distribution of these species. Therefore, it needs to be stressed that these models should be regarded as a first attempt at predicting the location of these species and not as a final product. Future research should attempt to collect data in new locations to improve the current models. The models at this stage may be useful for guiding the collection of data in future studies.

The response of *Babesia* and *Theileria* spp. to land cover and temperature in the model may be a reflection of the environmental requirements of the tick vectors of these parasites. Ticks have been found to survive for longer periods of time in forested areas, particularly bottomland forests with regulated temperatures and higher humidities, compared to open meadows (Semtner et al., 1971). Tick oviposition rate and hatching success are affected by temperature and humidity (Chilton and Bull, 1994; Despins, 1992). In addition to the effect of temperature on tick development, temperature has been shown to affect the development rate of *Theileria* in ticks (Young and Leitch, 1981). Whether the importance of temperature in the ecological niche model is a reflection of the tick environmental requirements or the piroplasm environmental requirements is unknown. Future research and modeling of tick distribution in Greece might improve our understanding of the distribution of these piroplasms.

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Although humidity plays a role in tick development (Chilton and Bull, 1994; Despins, 1992), humidity data were not available for developing the current model. Precipitation, which was available for modeling, is associated with humidity but appears to play a lesser role in disease development than temperature.

Significant clusters of infection for both piroplasm species were detected in the regions of Central Macedonia and Thessaly that are characterized by a considerable size of equine populations, 15.06% and 12.95% of the total equine population in Greece, respectively. The occurrence of the most likely cluster of *B. caballi* infection and the secondary non-overlapping cluster of *T. equi* infection in the same area is consistent with a possible involvement of a common risk factor, probably a common tick vector. Common tick vectors of *B. caballi* and *T. equi* have been detected in Greece (Kouam et al., 2010). The most likely cluster for *T. equi* infection did not coincide with that of *B caballi* infection, suggesting that other risk factors might be important for clusters of *T. equi*. Since both *T. equi* and *B. caballi* occur in the same hosts, a possible discriminating factor might be the presence of *T. equi*-specific tick vectors. The distribution range of tick species may well vary between high-risk areas associated with both significant clusters as a result of the heterogeneity of climates and landscapes. It has been demonstrated that the habitat of ticks is more set by abiotic factors such as vegetation and climate (which determine tick development and survival rates on the ground) rather than by host-related factors (Randolph, 2000). The significant clusters detected might be helpful in investigating the underlying causes of increased risk in the identified areas, the landscape attributes, and the climate variables characteristic of high-risk areas.

The clusters identified in this study fall in areas with high probability ($p > 0.75$) of presence for both *Theileria* and *Babesia* species predicted by Maxent program and which concentrates in the eastern half of the country. Furthermore, the regions with significant concentration of potential equid hosts are located in the high probability predicted areas. This observation suggests that the eastern part of Greece should be considered as a priority area for detailed epidemiological investigations and regular surveillance of equine piroplasmosis.

In conclusion, the study demonstrated that clusters of equine piroplasms occurred in the regions of Macedonia and Thessaly in continental Greece. The eastern half of the country, which covers areas with high probability of piroplasm presence and contains a significant number of potential equid hosts and clusters of piroplasms, should be prioritized for further epidemiological investigations. Finally, maximum entropy ecological niche modeling and spatial scan statistic have proved to be useful tools for the surveillance of animal diseases.

Conflict of interest statement

None declared.

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