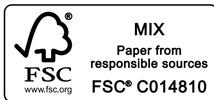
Equine tick-borne infections in the Netherlands

by

Catharine Michelle Butler

2012



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EQUINE TICK-BORNE INFECTIONS IN THE NETHERLANDS

Teken gebonden infecties bij
paarden in Nederland

(met een samenvatting in het Nederlands)

Proefschrift

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door

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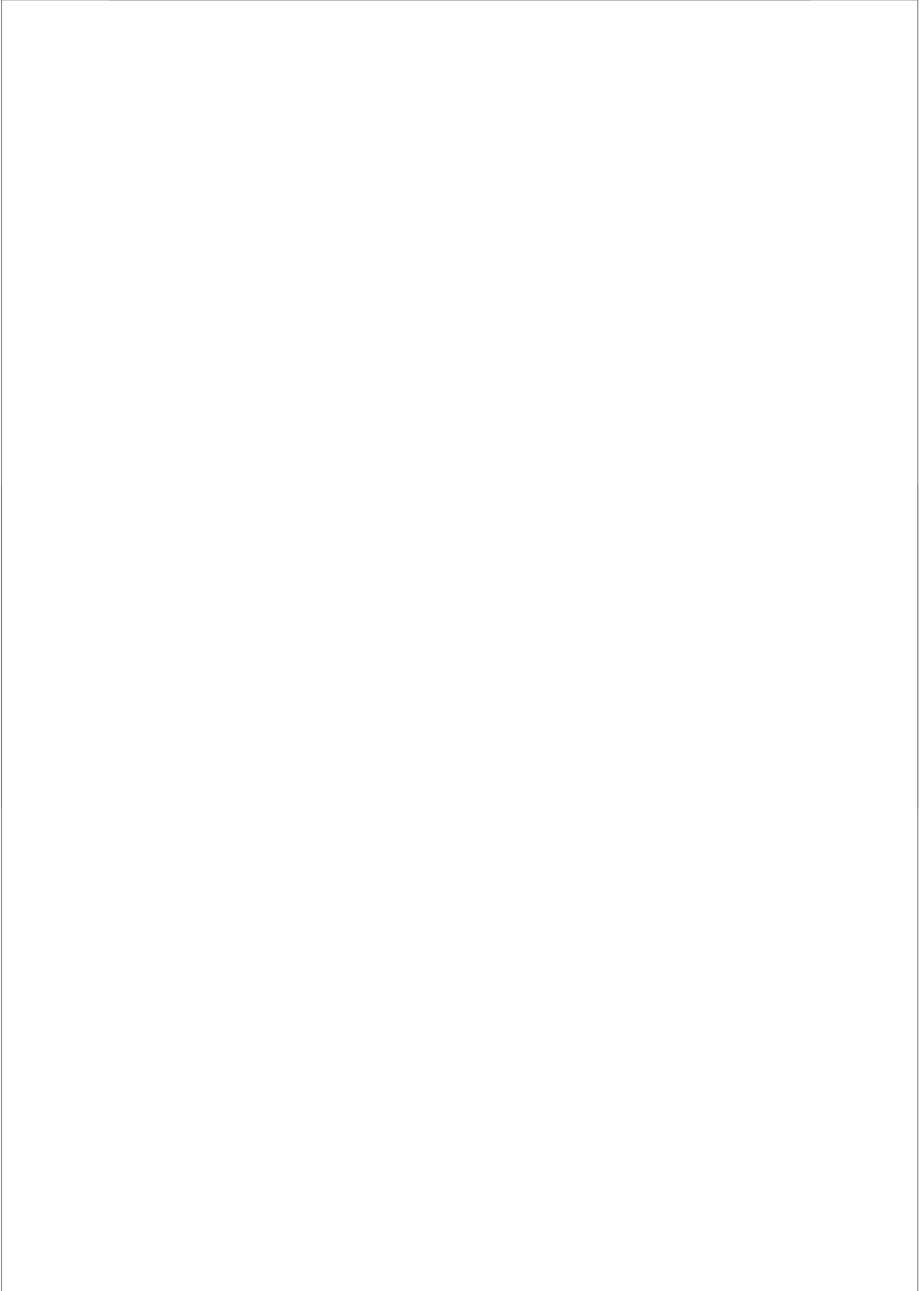
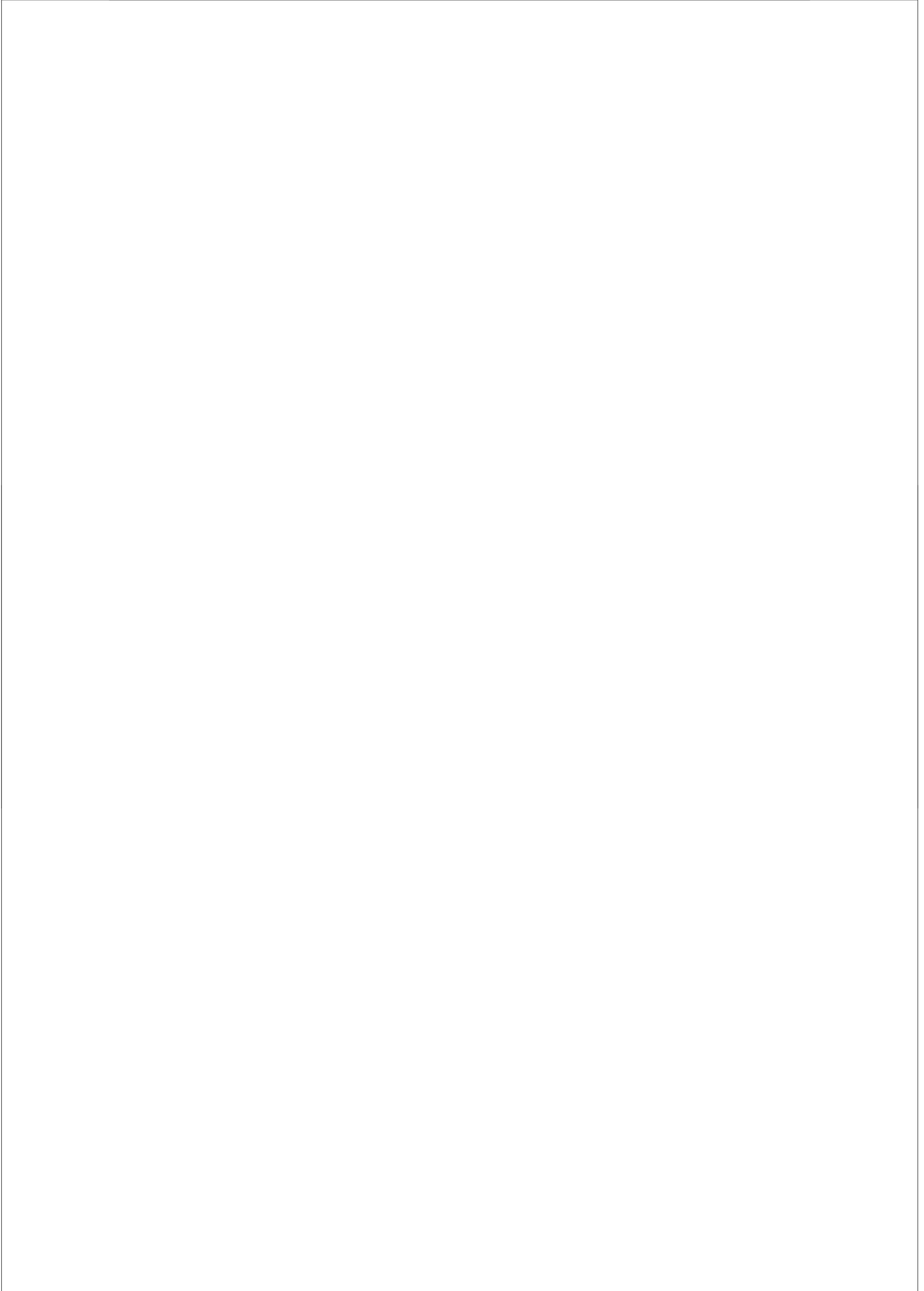
Voor Papa



Content

Chapter I	General introduction: scope and aim of the thesis	**9**
Chapter IIa	*Borrelia burgdorferi* infections with special references to horses. A review	**17**
Chapter IIb	Equine piroplasmosis: an overview	**33**
Chapter IIc	*Anaplasma phagocytophilum* in the horse: an overview	**41**
Chapter III	Development and evaluation of real-time PCR assays for the quantitative detection of *Babesia caballi* and *Theileria equi* infections in horses in South Africa	**45**
Chapter IV	*Anaplasma phagocytophilum* infection in horses in the Netherlands	**67**
Chapter V	Prevalence of the causative agents of equine piroplasmosis in the South-West of the Netherlands and the identification of two autochtonous clinical *Theileria equi* infections	**73**
Chapter VI	Classification of ticks collected from horses in the Netherlands and identification of the (zoonotic) agents they contain	**85**
Chapter VII	*Borrelia burgdorferi* and *Anaplasma phagocytophilum* in ticks and their equine hosts: A prospective clinical and diagnostic study of 47 horses following removal of a feeding tick	**95**
Chapter VIII	Repeated high dose imidocarb dipropionate treatment did not eliminate *Babesia caballi* from naturally infected horses as determined by PCR-reverse line blot hybridisation	**113**
Chapter IX	General discussion	**119**
Appendix I	Acute infection with *B. caballi* in a Dutch standardbred foal after visiting a stud in Normandy (France)	**127**
Appendix II	Teken bij paarden, niet zonder gevaar	**143**
	References	**147**
	Nederlandse samenvatting - English summary - Résumé Française - Deutsche Zusammenfassung	**169**
	Dankwoord	**189**
	Curriculum vitae	**195**
	Colored illustrations	**205**



Chapter 1

General introduction: scope and aim of the thesis

Background

Among the arthropod-borne diseases, organisms transmitted by ticks represent, at least from a geographical perspective, some of the world's most rapidly spreading infectious conditions with consequences for many vertebrate species, including man and horses (Jongejan and Uilenberg 2004). Four tick-borne agents are presently of importance to the horse industry in the Netherlands: *Borrelia burgdorferi*, *Anaplasma phagocytophilum* (formerly *Ehrlichia equi*), *Babesia caballi* and *Theileria equi* (formerly *Babesia equi*). The latter two are the causative agents of the disease piroplasmosis.

Ixodes ricinus is the most abundant tick species present in Europe (Stanek and Strle 2003) and is a known vector of both *B. burgdorferi* and *A. phagocytophilum*. *Dermacentor reticulatus* (the primary vector for *B. caballi* and *T. equi*) recently became established in the Netherlands, and permanent resident populations have now been documented (Nijhof, Bodaan et al. 2007), suggesting that the risk of equine piroplasmosis becoming endemic has increased substantially.
However, the limited data available on the competence of various tick species to act as vectors for the various equine agents, together with insufficient information on the effect of environmental factors, and changes therein, on the transmission of tick-borne organisms are of concern to both the veterinary and medical fields. In particular, the arrival of a novel agent in a virgin (i.e. previously unexposed) population may cause much more widespread morbidity, more severe disease and a higher risk of mortality than the agent causes in a population where it is endogenous (Maurer 1962).

Start of the study

The initial suspicion of a potential increase in the relevance of endemic tick-borne infections to horses in the Netherlands was prompted by the submission of a foal with severe anaemia as result of a *B. caballi* infection in 2003 (see **Appendix 1**).
In order to determine whether this was a sign of the establishment of a previously non-endemic tick-borne disease in the Netherlands, and how this related to the geographical distribution of potential tick hosts, studies were initiated into several aspects of equine tick-borne organisms in the Netherlands. Previously, horse owners in the Netherlands were primarily aware of *B. burgdorferi* as a potential tick-borne agent of equine disease. This was mainly due to the high level of public awareness of Lyme disease in general, but also because of the publication of a number of papers, or articles in the popular press, associating various clinical syndromes in horses with a *Borrelia* infection. In order to clarify the role of *B. burgdorferi*, the literature relating to borreliosis in horses available up to 2005 was reviewed. This review, including a subsequent

update, is presented in **Chapter IIa**. The scope of the thesis was subsequently extended to address other tick-borne infections that could potentially affect horses in the Netherlands; to introduce these studies on other tick-borne agents, overviews of equine piroplasmosis (**Chapter IIb**) and equine anaplasmosis (**Chapter IIc**) are also included in this thesis.

Presence of tick species and diagnostic procedures for detecting agents in ticks

Although *I. ricinus* was known to be the most abundant and geographically widespread tick species found on vertebrates and vegetation in the Netherlands, no information about tick infestation on horses or the prevalence of *I. ricinus* versus other tick species on this species was available. One of the aims of this thesis was, therefore, to investigate the occurrence and prevalence of various species of ticks found on horses in the Netherlands. Identification of ticks was performed using standard taxonomical descriptions (Estrada-Pena, Bouattour et al. 2004). In addition, to determine whether these ticks acted as (potential) vectors for the organisms known or suspected to infect horses, DNA was extracted from ticks collected from horses and used to search for the presence of agent-specific DNA using PCR-based assays such as the PCR-Reverse Line Blot (Nijhof, Bodaan et al. 2007).

Clinical and diagnostic aspects of tick-borne infections in horses

The majority of equine tick-borne infections lack pathognomonic clinical signs; definitive or presumptive diagnosis is therefore dependent on selecting accurate and adequate diagnostic tests. In this respect, acute *B. caballi* and *T. equi* infections are best diagnosed using PCR (Rampersad, Cesar et al. 2003; Nagore, Garcia-Sanmartin et al. 2004), because examination of stained blood smears is often insufficiently sensitive, i.e. the risk of a false negative is unacceptably high (Bruning 1996; Friedhoff and Soule 1996). After the acute phase, tick borne organisms often persist in the host to enhance the possibility of further transmission (carrier stage). In the case of an infection with *T. equi*, this carrier stage is life-long and the horse, therefore, effectively serves as a reservoir and potential source of infection for other horses (through a competent vector or as a result of iatrogenic transmission by, for example, blood or blood product transfusion). To avoid entry of these carriers into piroplasmosis-free countries, serological testing is often required prior to import; current serological tests include Indirect Fluorescent Antibody Test (IFAT) and cELISA, depending on the country involved (Unanimous 2012); **Chapter IIc**.

By contrast, acute *A. phagocytophilum* infection can be diagnosed with a reasonable degree of confidence by examination of a stained blood smear. Never-

theless, PCR is a more recent addition to the diagnostic armoury, and has been shown to be more sensitive in the sub-acute phase of the disease (Franzen, Aspan et al. 2005); **Chapter IIb**.
With regard to *B. burgdorferi,* infection with this spirochete has not (yet) been definitively proven to cause acute disease in horses. In addition, co-infection with *A. phagocytophilum*, a pathogen transmitted by the same vector, has been proposed to complicate or exacerbate the clinical picture during a *Borrelia* infection (Chang, McDonough et al. 2000). On the other hand, horses are often suspected by veterinary surgeons and owners to suffer from symptoms attributed to a chronic *Borrelia* infection, even though it is in actual fact rare for *B. burgdorferi* to be confirmed beyond reasonable doubt as the causative agent of disease. Serology is the most commonly used diagnostic tool in cases of suspected (chronic) borreliosis, but is of limited value in establishing a causal relationship between infection and clinical signs. This diagnostic challenge is illustrated in **Chapter VII** in which the question of whether co-infection with *Borrelia* and *Anaplasma* is responsible for (more severe) clinical disease under field circumstances is addressed; this study evaluated, in a prospective manner, (sub)clinical and diagnostic aspects of infection with these two organisms and related this data to symptoms of disease in horses after removal of feeding ticks.

A major difficulty with most of the tick-borne agents is that strain variations exist, with possible consequences for both pathogenicity and in terms of the likely accuracy of diagnostic tests, in particular, the sensitivity and specificity of the tests. For this reason, a real-time PCR assay for the quantitative detection of *B. caballi* and *T. equi* was developed (**Chapter III**) to determine whether strain variation influences test accuracy.

Horses with fever of unknown origin

Horses with fever of unknown origin can represent a considerable challenge to the equine veterinarian. To determine whether and how commonly tick-borne infections play a role in such patients, blood was collected from 61 horses with fever of unknown origin and tested for the tick-borne agents *B. burgdorferi, B. caballi*, *T. equi* and *A. phagocytophilum.* The results of this study are reported in **Chapter IV**

Does (autochthonous) equine piroplasmosis occur in the Netherlands?

In July 2009, a 12-year-old Dutch Warmblood gelding was presented to a referral clinic for treatment of a massive tick infestation accompanied by extensive skin swelling (figure 1), following a ride in a forest on Schouwen-Duiveland (an island in the Province of Zeeland). A blood sample from this horse tested posi-

tive for *B. caballi* DNA via PCR-RLB. Blood taken from the horse 6 weeks later was negative for *B. caballi* parasites in both the thin blood smear and the PCR-RLB, but was positive for *B. caballi* antibodies via IFAT. The clinical history suggested an autochthonous equine piroplasmosis infection and prompted the Dutch Ministry of Agriculture (subsequently renamed the Dutch Ministry of Economic Affairs, Agriculture and Innovation) to fund a study to evaluate the prevalence of *B. caballi* and *T. equi* in horses in the geographical area where the initial subclinical infection had occurred. The results of this study are described in **Chapter V**.

Tick species infesting horses in the Netherlands, and potential equine agents carried

Although there was increasing awareness of the risk of tick-borne infections in horses among practitioners, no structured information about tick infestations in horses and the possible consequences was available in the Netherlands. Questions posed by equine veterinarians and horse owners to the university equine clinic motivated the setting up of a research project to answer the following questions:

1. Which ticks (species and stage of development) infest horses in the Netherlands
2. What organisms are carried by these ticks?
3. Do infections with either *Borrelia* or *Anaplasma*, or both, lead to (sub)clinical disease in horses during either the acute phase or over a more extended time-course.

To obtain ticks and blood samples from the host-horses, equine practitioners were asked to submit ticks found on horses to Utrecht University (see **Appendix 2**) accompanied by a blood sample from the host horse. A second blood sample was supplied 6 to 12 weeks later to determine whether seroconversion had taken place. The results of this study are described in **Chapters VI** and **VII**.

Treatment versus chemo-sterilisation of tick-borne agents in horses.

At present, two issues are of relevance with regard to the treatment of tick-borne infections in horses:

1. Treatment versus chemo-sterilisation of *T. equi* infected horses
2. Treatment of horses with a *Borrelia* infection based on a positive antibody titre.

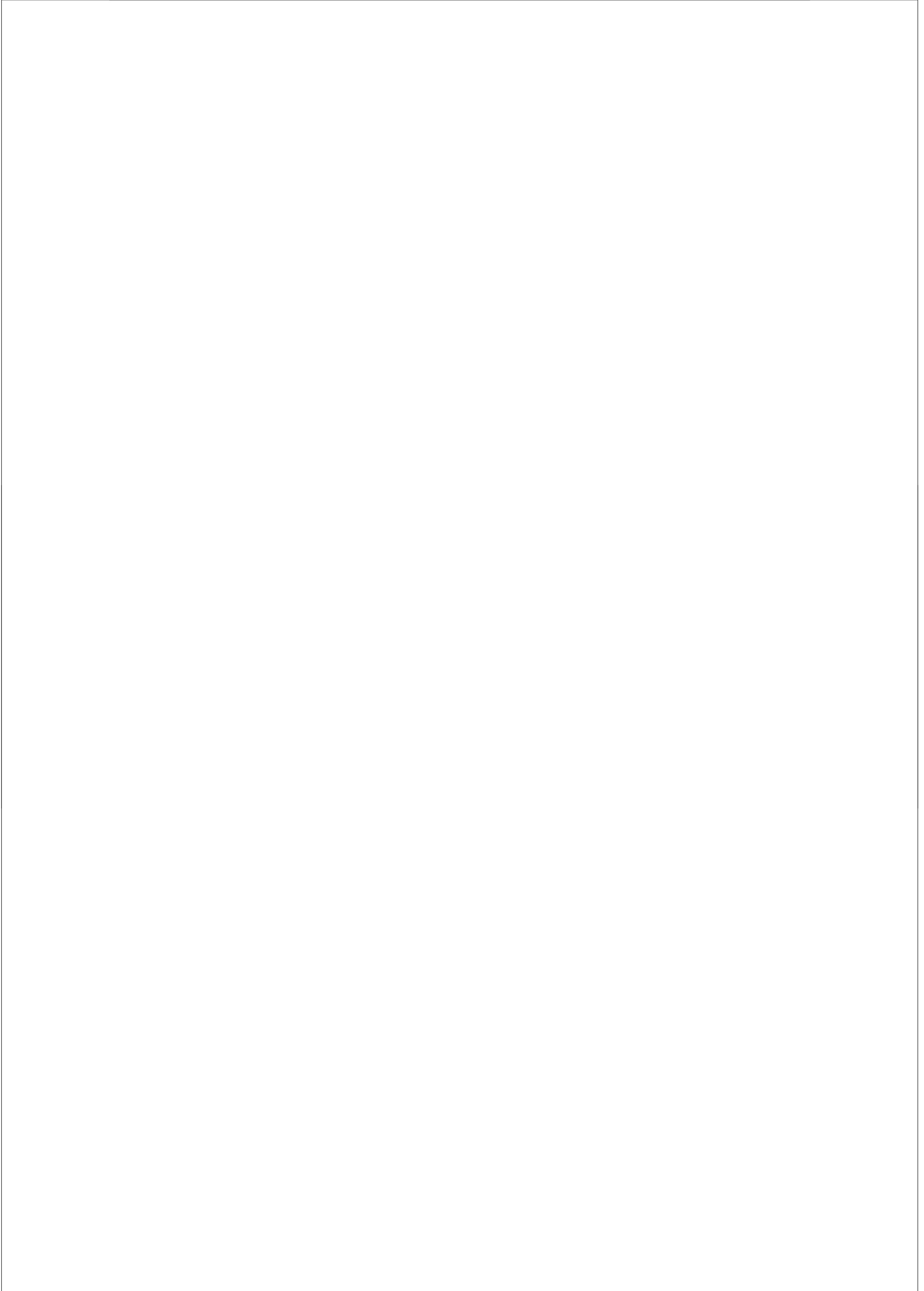
With regard to equine piroplasmosis (= clinical disease resulting from infection with *B. caballi* or *T. equi)*, treatment in the acute phase of disease is usually performed using imidocarb dipropionate as the drug of choice with a higher dose recommended for *T. equi* (4 mg/kg q72h, 3 times) than for *B. caballi* (2.2 mg/kg

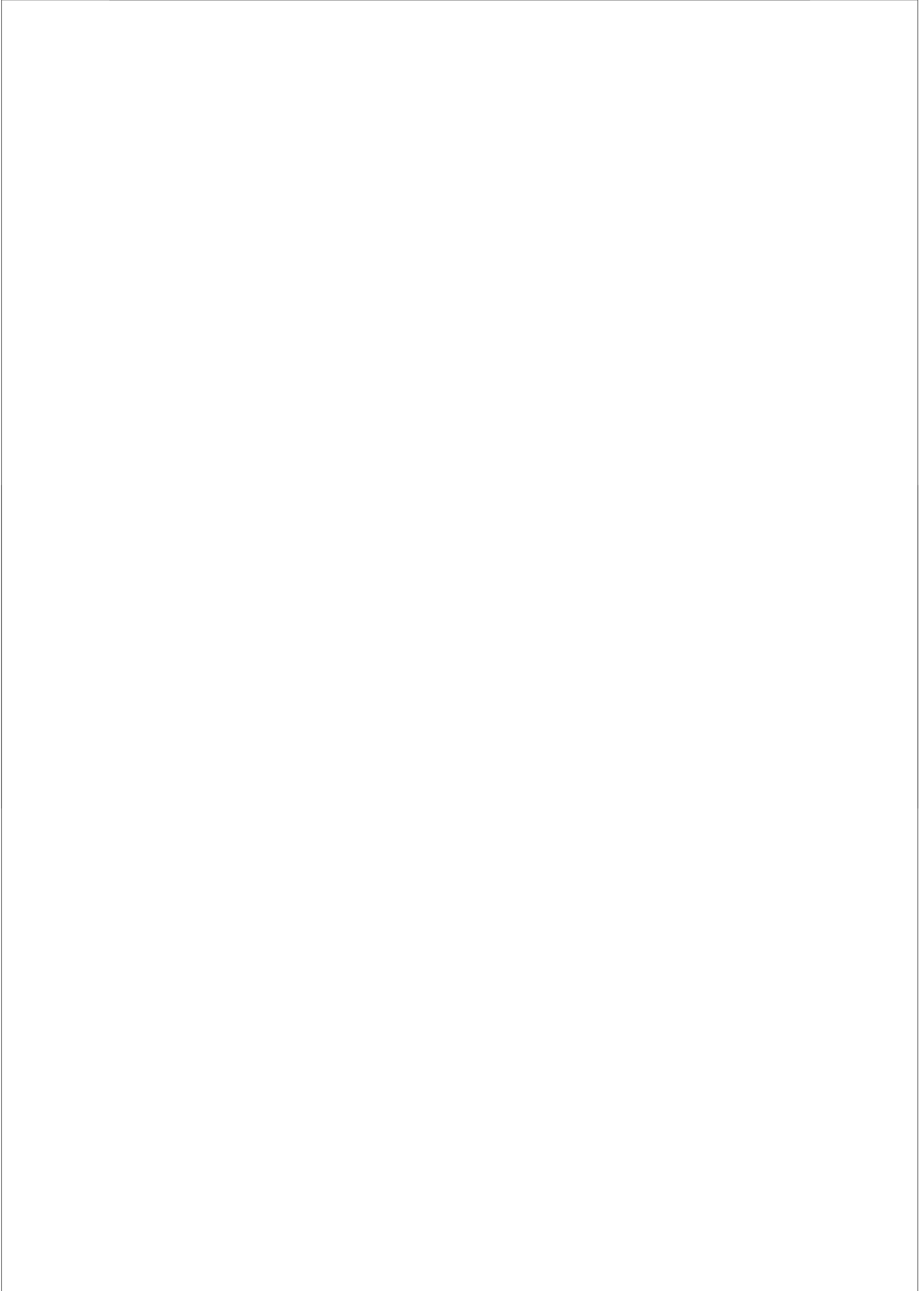
q24h, 2 times). Despite treatment, most *T. equi* infected horses will remain life-long subclinical carriers. There is, however, controversy about the efficacy of high doses of imidocarb dipropionate for clearing *T. equi* carriers (Frerichs and Holbrook 1974; Kuttler, Zaugg et al. 1987), a treatment that is mainly attempted for import and export purposes (Knowles 1996).
The efficacy of high dose imidocarb dipropionate treatment in clearing *T. equi* infected carrier horses was studied in a field situation and described in **Chapter VIII**.

With regard to the treatment of *Borrelia* infected horses, clinical signs associated with a *Borrelia* infection are non-specific and, for the most part, assumed or extrapolated from descriptions of chronic human borreliosis. Positive serology only indicates exposure to the organism (Egenvall, Franzen et al. 2001) and even after a *Borrelia* positive PCR result it remains difficult to determine whether *Borrelia* was the initiating cause or is "only" an incidental or complicating factor, since detection of *Borrelia* in affected tissue does not prove a causal relationship between infection and clinical signs (Chang, Novosol et al. 2000). Establishing the diagnosis borreliosis in horses is difficult. If treatment is warranted, the treatment of choice for *Borrelia* infected horses is oxytetracycline (6.6 mg/kg q12h iv), as only this antibiotic has been proven to clear tissues after an experimental *Borrelia* infection in ponies (Chang, Ku et al. 2005).

Aim

The aim of this thesis is to contribute to the knowledge of ticks and tick-borne infections in horses.





Chapter IIa

Borrelia burgdorferi infections with special references to horses. A review

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Summary

This review discusses the literature on *Borrelia Burgdorferi* infections in view of the rising incidence of this infection in general and the increasing concerns of horse owners and equine practitioners. Lyme disease, the clinical expression of *Borrelia* infections in man is an important health problem. The geographic distribution of *B. burgdorferi* infections in equidae should resemble that of human cases because the vector tick involved, *Ixodes ricinus*, feeds on both species and, indeed, the infection has been established many times in horses. However, a definite diagnosis of the disease "Lyme borreliosis" in human beings as well as in horses and other animals is often difficult to accomplish. Although a broad spectrum of clinical signs has been attributed to *B. burgdorferi* infections in horses, indisputable cases of equine Lyme borreliosis are extremely rare so far, if they exist at all.

Introduction

Lyme disease is a multisystemic tick-borne disease affecting humans in the Northern hemisphere. It is caused by the *B. burgdorferi* sensu lato (s.l.) complex which also infects a wide range of wild vertebrates, some of which act as infection reservoir. This tick-borne zoonosis occurs in particular habitats, named enzootic areas or natural foci, where both competent vectors and reservoirs are present (Humair and Gern 2000). In Europe, the main vector of *B. burgdorferi* s.l. is *I. ricinus*, and mammals such as small rodents and birds are known reservoirs (Gern, Estrada-Pena et al. 1998). Little is known about the clinical course and duration of *B. burgdorferi* infection in horses. At present, only wild small rodents were shown to remain infected outside of the tick activity period (Humair, Rais et al. 1999; Humair and Gern 2000).

Prevalence

Lyme disease was first recognised in 1975 in the city Old Lyme, Connecticut, USA, where it was found among children as an unusually frequent, juvenile rheumatoid-arthritic condition (Steere, Malawista et al. 1977). In 1982 the etiologic agent of the disease was discovered by Burgdorfer and co-workers (Burgdorfer, Barbour et al. 1982) and named *B. burgdorferi* in 1984. This was also the year in which Lyme borreliosis was first described in dogs with arthritis, arthralgia, and lameness (Lissman, Bosler et al. 1984). In 1985 antibodies to *B. burgdorferi* were reported in horses in New England, USA (Marcus, Patterson et al. 1985).

Seroprevalence in horses in some areas of the north-eastern USA is nearly 50% (Magnarelli, Ijdo et al. 2000) and might be expected to be higher than the seroprevalence in humans because of the increased risk of ticks attaching to horses for more than 24 hours (Divers, Chang et al. 2003). A study done by Carter et al. (1994) shows that infection with *B. burgdorferi* is common in the UK in horses, especially in areas that have a high incidence of human and canine borreliosis, however, clinical disease associated with a *B. burgdorferi* infection is uncommon in horses.

The reported prevalences of *I. ricinus* ticks infected with *B. burgdorferi* in Europe are 22-35% in Germany (Baumgarten, Rollinghoff et al. 1999; Rauter, Oehme et al. 2002), 10-35% in the Netherlands (Rijpkema, Molkenboer et al. 1995; Schouls, Van De Pol et al. 1999), 40% in Bulgaria (Christova, Van De Pol et al. 2003), 38% in Slovakia (Derdakova, Halanova et al. 2003) and 9% in Poland (Wodecka 2003). According to a study in Germany, a substantial rise in the average infection prevalence of *B. burgdorferi* s.l. spirochetes in questing ticks

has occurred over a 10-year period (Kampen, Rotzel et al. 2004). This increased infection prevalence might be the consequence of ecological changes, which favour the natural circulation of *B. burgdorferi* s.l.

Aetiology

The causative agents of borreliosis belong to the phylum of Spirochetes and are grouped in the *B. burgdorferi* s.l. species complex, which is divided into at least 11 different genospecies (*Borrelia afzelii*, *Borrelia garinii*, *Borrelia Burgdorferi* sensu stricto, *Borrelia andersoni*, *Borrelia japonica*, *Borrelia lusitaniae*, *Borrelia sinica*, *Borrelia tanuki*, *Borrelia turdii*, *Borrelia valaisiana*, *Borrelia bissettii*), which have been identified in different regions (Wang, van Dam et al. 1999; Stanek, Gray et al. 2004).

Borrelia are thin, elongated, motile bacteria with a fragile, fluid outer membrane surrounding the protoplasmic cylinder. In the periplasmic space there are 7 to 11 bipolar flagella (Barbour and Hayes 1986). These periplasmic flagella have a skeletal function and enable *B. burgdorferi* to stay motile in viscous media in which other bacteria would be immobilised (Motaleb, Corum et al. 2000). The outer cell membrane contains many polypeptides, of which the outer surface proteins (Osp's) A, B and C are the most extensively studied (Fikrig, Telford et al. 1992; Ma and Weis 1993; Lengl-Janssen, Strauss et al. 1994; Ma, Seiler et al. 1994; Weis, Ma et al. 1994; Wilske, Busch et al. 1996; Wooten, Modur et al. 1996; Chang, Novosol et al. 1999; Ohnishi, Piesman et al. 2001; Batsford, Dunn et al. 2004; Pal, Yang et al. 2004; Yang, Pal et al. 2004). *B. burgdorferi* has the largest number of plasmids known for any bacterium, and genes related with pathogenicity, including outer surface protein genes, are primarily located on these plasmids (Casjens, Palmer et al. 2000; Stewart, Byram et al. 2005). Spirochetes in the midgut of flat (unfed) ticks are metabolically active at a very low rate and express predominantly OspA on the cell membrane (De Silva and Fikrig 1995; Schwan, Piesman et al. 1995).

The transformation of *Borrelia* spirochetes is ignited as soon as blood begins to fill the ticks midgut: the spirochetes begin to divide, an increase in motility occurs and also morphological changes take place. Spirochetes in the midgut of fully engorged ticks express OspC on their surface instead of OspA (Tokarz, Anderton et al. 2004). OspC is produced by spirochetes at 32-37° C but not at 24° C and is therefore thought to be temperature dependant (Schwan, Piesman et al. 1995). In general, OspA and OspB seem to be tick-specific antigens, while OspC appears to be expressed only in feeding ticks and in the vertebrate host (de Silva and Fikrig 1997).

It has been demonstrated that the combined effect of blood influx and temperature shift on *B. burgdorferi* led to a differential expression of 154 genes (Tokarz, Anderton et al. 2004). Several chemotaxis and sensing genes were upregulated and these changes may be essential for the spirochete in its transmission and adaptation to the vertebrate host. It was also shown that the presence of *B. burgdorferi* in the ticks' salivary gland leads to an upregulation of a tick salivary protein named Salp15, which specifically interacts with *B. burgdorferi* outer surface protein C, and seems to cover the spirochete to facilitate its dissemination in the host (Ramamoorthi, Narasimhan et al. 2005). In North America, *B. burgdorferi* sensu stricto (s.s.) has been implicated as the only cause of *B. burgdorferi* borreliosis (Stanek, Gray et al. 2004; Steere, Coburn et al. 2004). The efficiency of transmission of *B. burgdorferi* s.s. appears to be better in North America compared to Europe, and although these genospecies are considered similar, their host adaptation appears to differ considerably between continents (Richter, Klug et al. 2004).
Clinical manifestations of *B. burgdorferi* infections in Europe are predominantly caused by only 3 of the described genospecies of the *B. burgdorferi* s.l. complex, namely *B. garinii*, *B. afzelii* and *B. burgdorferi* s.s. (Busch, Hizo-Teufel et al. 1996). The pathogenicity of two other *B. burgdorferi* species, *B. valaisiana* and *B. lusitaniae* is still questionable (Escudero, Barral et al. 2000). An Austrian study showed that *B. afzelii* was the predominant infectious agent in a group of clinically normal horses (Muller, Khanakah et al. 2002). The dissemination of tick-borne diseases requires the dispersal of tick vectors and in some cases the local presence of reservoir hosts.
A host species is considered a reservoir if co-existing with a pathogen for some time and transmitting this pathogen to competent vectors is possible (Humair and Gern 2000). At present 16 species of birds, seven medium-sized mammals as well as nine species of small mammals are considered able to transmit *B. burgdorferi* s.l. spirochetes to ticks, thus participating in the natural circulation of these pathogens (Gern, Estrada-Pena et al. 1998). Ticks of the *Ixodes* complex act as vectors to spread these spirochetes from one animal to another. In Europe, the main vector of *B. burgdorferi* s.l. is the *I. ricinus* tick, in North America the main vector is *I. scapularis*, and *I. persulcatus* is the vector in Asia (Steere, Coburn et al. 2004). *I. ricinus* ticks acquire the *B. burgdorferi* infection during an infective meal on a reservoir host. Recent studies provided evidence that specific associations exist in some areas between *B. burgdorferi* s.l. species and reservoir hosts, which implies that these species may be maintained through distinct maintenance cycles (Humair and Gern 1998; Pichon, Egan et al. 2003). This might be explained by the specific effect on each *B. burgdorferi* s.l. genospecies of the complement that is present in serum of the host (Bhide,

Travnicek et al. 2005). Patterns of resistance or sensitivity to specific complement differ among the different *B. burgdorferi* s.l. species, and survival of these spirochetes in the midgut of the tick depends on the source of blood and complement ingested by the tick (Kurtenbach, De Michelis et al. 2002). Also non-specific (not related to a specific host) maintenance cycles may occur since some small mammals were found to be infected by various *B. burgdorferi* genospecies (Humair and Gern 2000).

Migrating birds (with their great capacity of movement) might be responsible for pathogen dispersal along their migration routes, and the presence of *B. burgdorferi* s.l. in *I. uriae* ticks collected in various seabird colonies indicates that seabirds might play an important role in the dissemination of *Borrelia* across continents (Olsen, Duffy et al. 1995; Humair and Gern 2000). *Borrelia Burgdorferi* s.l. species have also been found in the digestive tract of horseflies, deerflies, and mosquitoes (Magnarelli and Anderson 1988; Kosik-Bogacka, Kuzna-Grygiel et al. 2004). These haematophagous insects might act as secondary vectors but probably do not really contribute to the dissemination of *B. burgdorferi* s.l.. In general, tick activity will begin in spring and early summer. In some areas a second less intense phase of questing tick activity occurs in the autumn (Wodecka 2003).

Epidemiology

The infection is usually acquired from larvae or nymphs feeding on small to medium sized wild animals which happen to be a *B. burgdorferi* reservoir. Adult ticks only engorge successfully on larger animals like deer, sheep, cows, and horses. *Borrelia burgdorferi* s.l. DNA was most frequently detected in female ticks, less frequently in nymphs and larvae, and least frequently in adult male ticks (Wodecka 2003). Adult *I. ricinus* males seldom feed and therefore the risk of getting infected with *B. burgdorferi* is highest due to contact with female ticks (De Meeus, Lorimier et al. 2004). Larvae may be carriers of the spirochete *B. burgdorferi* by transovarial acquisition which is however rare and inefficient in *I. ricinus* ticks (Humair and Gern 2000). Nymphs and adults may be *B. burgdorferi* carriers through transstadial passage, and all three stadia can get infected by so called co-feeding (Gern and Rais 1996).

When closely attached on the same host, *B. burgdorferi* spirochetes can be transmitted from one tick to another, without necessarily causing a generalised infection in this host (Gern and Rais 1996; Ogden, Nuttall et al. 1997). Once a tick attaches to a host and gets engorged, spirochetes which are present in the tick midgut migrate through the midgut wall and hemocoel, reach the salivary glands and are inoculated with the tick saliva into the host 2-3 days after attachment (Piesman, Mather et al. 1987). Sometimes inoculation occurs earlier if spirochetes are already present in the salivary glands of the infected tick (Alek-

seev, Arumova et al. 1995). The role of large mammals in the life cycle of *B. burgdorferi* is unknown. Even though these mammals are favourite hosts for adult ticks, the rare event of transovarial transmission of *B. burgdorferi* spirochetes makes it unlikely that large mammals play an important role in its maintenance cycle.

Pathogenesis

Borrelia burgdorferi is capable of evading the immune responses of vertebrate hosts and establishing chronic infection by residing in particular target tissues for long periods of time (Embers, Ramamoorthy et al. 2004; Liang, Brown et al. 2004; Singh and Girschick 2004). Once in the host skin, spirochetes may remain at the inoculation site for a few days before they start to migrate (Gern and Rais 1996). In most hosts, the organism seems to prefer to reside or move in skin, fascia, perineural tissue, and synovial membranes (Chang, Novosol et al. 1999; Hovius, Stark et al. 1999; Chang, Novosol et al. 2000; Steere, Coburn et al. 2004). *Borrelia burgdorferi* predominantly migrates within connective tissue which, as such, may protect the organism from humoral antibodies (31).
Infections with *Anaplasma phagocytophilum*, another common tick-borne infection, have been associated with immunosuppression, and coinfection with this pathogen could produce antagonistic immunologic responses or other immunologic effects that might affect the outcome of one or both infections (Persing 1997). It is generally accepted that an infected tick must be attached for at least 24 hours on the mammal for *B. burgdorferi* transmission to occur (Thanassi and Schoen 2000), but it has been demonstrated that *B. burgdorferi* can be transmitted to the host as early as 18 hours after attachment of an infected tick (Alekseev, Arumova et al. 1995). The increased risk of transmission with increased attachment time has been clearly demonstrated for *B. burgdorferi* in human beings (Piesman, Mather et al. 1987). *B. burgdorferi* has unique cellular and stimulatory activities that could be involved in the pathogenesis of infection.
A lot of research has been done recently concerning the outer surface lipoproteins (Osp's). Purified OspA and OspB stimulate polyclonal B-cell activation and cytokine production in macrophages and endothelial cells (Ma and Weis 1993; Wooten, Modur et al. 1996). This could explain how *B. burgdorferi* invades tissues through interaction with, and penetration through, vascular endothelium. Also neutrophils can be directly activated and primed by OspA similar to LPS (Morrison, Weis et al. 1997). Expression and up-regulation *in vivo* of two transcripts for IL-1a in synovial membranes of the dog in response to a *B. burgdorferi* infection was demonstrated (Straubinger, Viveiros et al. 1999). IL-1a is a pro-inflammatory cytokine reported to be involved in the regulation of cytokine expression in the synovium of joints (Niki, Yamada et al. 1998). No expression of transcripts for IL-1a, however, was seen in equine macrophages. *In vitro* research indicates that OspA and OspB are also potent stimulators of ni-

tric oxide (NO) production by bone marrow-derived macrophages (Ma, Seiler et al. 1994). If the production of NO results in control of the infection because of its antimicrobial properties, or promotes immune mediated injury, remains to be investigated. Lipid modification is essential for the immunologic properties of these lipoproteins (Weis, Ma et al. 1994) but not sufficient for inducing injury, as lipidated Osp B has not been associated with joint injury in humans, where lipidated OspA and OspC have been linked with severe arthritis (Batsford, Dunn et al. 2004).

Two *Borrelia* haemolysin genes (blyA and blyB) with erythrocyte-species specific haemolytic activity were discovered by genetic analysis (Williams and Austin 1992). The borrelial haemolysin, that is suggested to be oxygen-labile, is most active against horse erythrocytes compared to bovine, sheep and rabbit erythrocytes (Williams and Austin 1992). BlyA is a membrane interactive protein with haemolytic activity which functions as a pore forming toxin and its physiological effect can vary from a slight change in membrane polarisation and transport equilibrium to a more drastic and commonly observed effect, namely cell lysis by osmotic shock (Guina and Oliver 1997). This cytolytic activity could explain how the pathogen escapes destruction in the macrophage lysosomal compartment, and also their facilitated dispersal into surrounding cells or tissues in mammals as well as in ticks.

Research indicates that *B. burgdorferi* infection can also dramatically lower retinol levels (by an unknown mechanism), leading to transient or lasting vitamin A deficiency (Cantorna and Hayes 1996). As vitamin A down-regulates the synthesis of IL-12 and IFN-y *in vivo*, a deficiency of this vitamin predisposes an animal to produce an excessive inflammatory reaction, possibly leading to acute Lyme arthritis (Cantorna and Hayes 1996). The fact that immunodeficient mice also develop an inflammatory reaction that leads to chronic Lyme arthritis makes it plausible that non-specific immune responses are pathogenic as well in *B. burgdorferi* induced arthritic disease (Cantorna and Hayes 1996).

Persistent auto-immune responses against *B. burgdorferi* DNA in synovial membranes due to cross reactivity with self-components may play a role in the progressive reactive arthritis that can occur with Lyme disease (Raveche, Schutzer et al. 2005). Briefly, it is clear that *B. burgdorferi* has a strong potential for adaptation in both the invertebrate as well as the vertebrate host, and although much progress has been made in characterising the host immune reaction, spirochetal factors responsible for infectivity, immune evasion and disease pathogenesis are still largely obscure.

Borrelia burgdorferi infection in horses

To date, a broad spectrum of clinical manifestations has been attributed to *B. burgdorferi* infection in horses including arthritis (Burgess, Gillette et al. 1986; Hahn, Mayhew et al. 1996), lameness (Browning, Carter et al. 1993), muscle tenderness (Divers, Chang et al. 2003), anterior uveitis (Burgess, Gillette et al.

1986; Hahn, Mayhew et al. 1996), encephalitis (Burgess and Mattison 1987), abortion (Sorensen, Neely et al. 1990), foal mortality (Burgess, Genchon-Fitzpatrick et al. 1987), low grade fever and lethargy (Magnarelli, Anderson et al. 1988; Magnarelli, Flavell et al. 1997) without a conclusive diagnosis. A possible explanation for the described biological variation of the clinical manifestation in horses might be unapparent co-infection with other known or yet unknown pathogens (for example *A. phagocytophilum*) (Chang, McDonough et al. 2000; Magnarelli, Ijdo et al. 2000). The likelihood of co-infection increases if the agents involved are transmitted by the same arthropod vector, which is the case for *A. phagocytophilum* and *B. burgdorferi* (Chang, McDonough et al. 2000; Foley, Foley et al. 2004). Many of the horses "successfully" treated for presumed acute Lyme borreliosis may actually have an *A. phagocytophilum* infection, thus exhibiting similar symptoms (fever, leg oedema, stiffness) with an excellent response to tetracycline treatment.
In the past, some clinical cases suggesting a causal relationship between *B. burgdorferi* infection and disease in horses have been documented. Encephalitis for example was associated with a *B. burgdorferi* infection in a horse, and spirochetes isolated from the brain were identified as *B. burgdorferi* by direct immunofluorescence (Burgess and Mattison 1987). Unfortunately no microscopic examination of the brain was performed and no intrathecal antibody titre was determined to support the diagnosis. Arthritis and panuveitis in a Wisconsin pony were also attributed to an infection with *B. burgdorferi* and in this case spirochetes were detected microscopically in a section of the left eye stained with a Krajian silver stain (Burgess, Gillette et al. 1986). The final diagnosis was made on the basis of high IFA titres in serum and synovial fluid, detection of spirochetes in the anterior chamber of the left eye using silver stains and the absence of local and peripheral antibodies to other common causes of uveitis. However, silver stains are non-specific and difficult to interpret (Aberer 1992), culture for *B. burgdorferi* in blood, synovial- and anterior chamber fluid was negative and no *Borrelia* antibodies were detected in the anterior chamber fluid. Even early pregnancy failure in horses has been associated with *B. burgdorferi* but not proven by Sorensen et al. (1990), but was considered without significant correlation in a study by Eisner et al. (1994). Equine abortion however was associated with *B. parkeri* which belongs to the tick borne relapsing fever (TBRF) spirochete group and shows serological cross-reactivity with *B. burgdorferi* (Walker, Read et al. 2002). This emphasises the importance of appropriate tests to clearly distinguish between infections with *B. burgdorferi* and other spirochetes.
A causal relationship could not be established in a recent experimental *B. burgdorferi* infection study done by Chang et al. (2000). In this experiment seven specific pathogen free ponies were exposed to *B. burgdorferi* s.s infected adult ticks, while being treated with dexamethason over five consecutive days. None of the symptoms attributed to Lyme borreliosis in horses could be induced and only mild skin lesions were seen. Cultures of skin biopsies from

the site of tick exposure were positive for *B. burgdorferi*. Polymerase chain reaction (PCR) of various tissues was also positive and all of the infected ponies seroconverted. Despite the high antibody titres to *B. burgdorferi* in these ponies however, the spirochete could still be isolated from various post-mortem tissues, which indicates that persistent infection can be established in clinically healthy horses (Chang, Novosol et al. 2000).
In humans, different *B. burgdorferi* genospecies tend to cause distinct clinical manifestations affecting different organ systems (Humair and Gern 2000; Logar, Ruzic-Sabljic et al. 2004). In Europe *B. burgdorferi* s.s. infection has been associated with arthritis and found predominantly in joints, whereas *B. afzelii* prefers to reside in skin and causes erythema migrans. *Borrelia garinii* has been found in cerebrospinal fluid and appears to be the most neurotropic of the three (Balmelli and Piffaretti 1995; Steere, Coburn et al. 2004). Similar to human beings, variation in clinical signs of *B. burgdorferi* infected horses, might be due to infection with different *B. burgdorferi* genospecies.

Diagnosis in horses

The diagnosis of Lyme borreliosis in horses poses a number of challenges, similar to those challenges encountered in the diagnosis of Lyme borreliosis in humans and dogs. Erythema migrans is the only pathognomonic symptom, and is easily noticed in human beings, but useless as a clinical marker in dark-skinned dogs and horses. Isolation by culture (BSKII medium) or in situ detection of *B. burgdorferi* is feasible (Dumler 2001; Fritz and Kjemtrup 2003), but also possible in clinically healthy animals, which means that proving its detection does not allow etiological conclusions. Persistent *B. burgdorferi* infections without any clinical symptoms have been documented in horses (Chang, Novosol et al. 2000), and although *B. burgdorferi* is difficult to isolate from patients, culture of skin biopsies from early erythema migrans in horses has been successful (Chang, Novosol et al. 2000). Viable *B. burgdorferi* spirochetes have been found in urine of clinically normal horses in an endemic region (Manion, Khan et al. 1998), and this raised the question if non-tick transmission of *B. burgdorferi* may occur by direct urine/mucosal contact comparable with a known transmission mechanism of *Leptospira*.
Laboratory diagnosis of *B. burgdorferi* infection in horses has relied mainly on the detection of serum antibodies. Serological assays are widely available but their diagnostic value is open for discussion. The immunofluorescent antibody test (IFAT) and enzyme linked immunosorbent assay (ELISA) are two sensitive serologic tests to detect total or class-specific immunoglobulins to this organism in horses (Divers, Chang et al. 2003). More seropositive specimens were detected by ELISA compared to IFAT in horses in Berlin (Kasbohrer and Schonberg 1990). Tests on the specificity of the IFAT and ELISA method for *B. burgdorferi* revealed extensive cross-reactivity among *Borrelia* species and minimal cross-reactivity with heterologous *Leptospira* antigens (Marcus, Patterson et al.

1985; Magnarelli, Anderson et al. 1988; Carter, May et al. 1994; Magnarelli and Fikrig 2005). As the specificity of the IFAT and the ELISA is questionable, a two-step protocol has been recommended in the serologic diagnosis of early Lyme disease in humans (Trevejo, Krause et al. 1999) and has been recommended in horses as well (Magnarelli, Ijdo et al. 2000). Using an initial sensitive screening technique like ELISA and/or IFAT, after which a positive result is supplemented by a second method that generally uses protein immunoblotting (Western blot) to detect antibodies to specific *Borrelia* antigens of specific molecular sizes (Trevejo, Krause et al. 1999). However, a substantial heterogeneity exists in plasmid and protein profiles of different *B. burgdorferi* isolates in Europe (Bunikis, Garpmo et al. 2004; Casati, Bernasconi et al. 2004) hence, the use of native *B. burgdorferi* strains could improve the diagnostic performance of the immunoblot substantially (Hernandez-Novoa, Orduna et al. 2003).
An immunoblot with five *B. burgdorferi* strains was used to test horses from Austria and Vienna (Muller, Khanakah et al. 2002). None of the horses positive for *B. burgdorferi*, showed any clinical symptoms of disease, and *B. afzelii* seemed to be most prevalent. The laboratory diagnosis of active *B. burgdorferi* infection in horses has been based on a high ELISA titre and/or positive Western blot (Divers, Chang et al. 2003). One reason for not finding an elevated titre, despite active infection, is the slow multiplication of the spirochete within the body of the host, therefore titres may take 3-8 weeks to develop (Hollett 1989; Cohen 1996). Chang et al. (2000) discovered that ponies exposed to *B. burgdorferi* infected ticks developed detectable antibodies at 5-6 weeks, and the highest antibody levels were reached at 3 months after exposure. Given the fact that a high proportion of seropositive horses are clinically normal, a single high antibody titre is not sufficient for a presumptive diagnosis of active *B. burgdorferi* infection. A study done by Bernard et al. (1990) showed no increased frequency of positive serotest results for *B. burgdorferi* in horses with an open diagnosis of musculoskeletal or neurologic disease. Lack of significant correlation between antibodies to *B. burgdorferi* and signs of equine recurrent uveitis was shown in a study done by Gerhards et al. (1996).
Molecular tests, like PCR, could improve the sensitivity as well as the specificity of the detection of *B. burgdorferi* infection in horses, as it can detect both live and dead organisms, as well as intact and fragmented spirochete DNA (Chang, Novosol et al. 2000). However, in the differentiation between active and past infection, indirect tests alone are not enough to make a definitive diagnosis of equine Lyme borreliosis (Schonert, Grabner et al. 2002) with the exception perhaps of neuroborreliosis, where detection of intrathecal *Borrelia* antibody production is considered to be conclusive. Definite diagnosis of equine Lyme borreliosis however, is apparently problematic.

Differential diagnosis in horses

Lyme disease is a multi-systemic disorder that can affect a great number of organs and tissues in human beings and other mammals (Steere, Coburn et al. 2004). In horses, the associated clinical manifestations vary widely, ranging from asymptomatic or sub-clinical infection to signs and symptoms of systemic disease. As *B. burgdorferi* infection can affect many organ systems, the differential diagnosis is broad, and the list of differential diagnoses varies according to the predominant system affected. Lyme borreliosis in horses however is rare, and causes of arthritis (the primarily associated symptom) in horses are manifold and include infectious, metabolic, traumatic and immunologic aetiologies.

Pathology and histopathology in horses

Lesions in ponies infected with *B. burgdorferi* s.s. were restricted to skin (perivascular and perineural lymphohistiocytic aggregates in the superficial and deep dermis) near the attachment sites of *B. burgdorferi* infected ticks, similar to erythema chronicum migrans (the characteristic skin rash associated with human lyme disease) and prescapular lymph nodes with marked lymphoid hyperplasia (Chang, Novosol et al. 2000). In some ponies, perivascular and perineural lymphocytic reactions were observed especially in the skin, fascia, and perisynovial membranes (Chang, Novosol et al. 2000; Divers, Chang et al. 2003). Less commonly, but not infrequently, organisms were found in the heart, pericardium, kidney, bladder and rarely in the meninges (Chang, Novosol et al. 2000). Hyperplastic synovial membranes, focal meningoencephalitis, mild inflammation in the anterior chamber of the eye and local epicardial congestion and roughening were seen in a horse highly suspected of *B. burgdorferi* neuroborreliosis in the UK (Hahn, Mayhew et al. 1996).

Prevention and therapy in horses

Prevention is best achieved by avoiding tick infested areas, and by careful grooming of the horse to remove ticks as soon as possible. If present on the horse, ticks should be identified to determine whether they are *Ixodes* species, which may transmit *B. burgdorferi*. Various insecticidal sprays are used to prevent tick infestation, but most are not approved for use on horses and the efficacy of such use is unproven. No adverse effects have been noticed by use of the more common canine tick sprays on horses (Divers, Chang et al. 2001). Acaricids such as permethrin seem to be especially effective (Buschmich 1999).
Efforts have been made in the past several years to prevent *B. burgdorferi* infection in horses by vaccination, and a vaccine study done by Chang et al. (Chang, Novosol et al. 1999) suggests that OspA vaccination in horses would be effective in preventing infected ticks from transmitting *B. burgdorferi*. The

tick-gut is the primary site of *B. burgdorferi* growth and it seems that OspA antibody rich plasma inhibits *B. burgdorferi* growth within the tick while feeding on the host (de Silva and Fikrig 1997). Vaccination with recombinant OspA can only be used as a preventive measure for it can not eliminate the organisms from an already infected animal (Chang, Novosol et al. 1999). The matter of OspA heterogeneity was not addressed in the vaccine trial done by Chang et al. (1999), as one serotype of *B. burgdorferi* prevails in the United States. However, this is not the case in Europe (Dykhuizen, Polin et al. 1993), where extensive OspA heterogeneity makes the development of a commercial vaccine for equids much more complicated. Efficacy of the commercially available vaccine for dogs remains controversial. Low prevalence of equine Lyme borreliosis (if any at all) and the lack of complete knowledge of the natural history of *B. burgdorferi* in horses make development of equine vaccines difficult and commercially unattractive.

Treatment with appropriate antibiotics in early Lyme borreliosis is supposed to prevent the spreading of the organism. However, because of the variety in clinical symptoms ascribed to *B. burgdorferi* infection, only few horses are diagnosed in the early phase of a *B. burgdorferi* infection. Divers et al. (2003) reported that tetracycline (6,6 mg/kg IV, q 12 h), given for a three week period, is superior compared to orally administered doxycycline (10 mg/kg, q 12 h) or parenteral ceftiofur (2,2 mg/kg IM, q 12 h) in *B. burgdorferi* infected ponies. Only tetracycline treated ponies were negative for both culture and PCR, and the other two antibiotics showed inconsistent results in this study (Divers, Chang et al. 2003). Orally administered doxycycline has been shown to have a low bioavailability when administered to horses (Bryant, Brown et al. 2000), and this could explain its inferiority compared to tetracycline.

Prognosis in horses

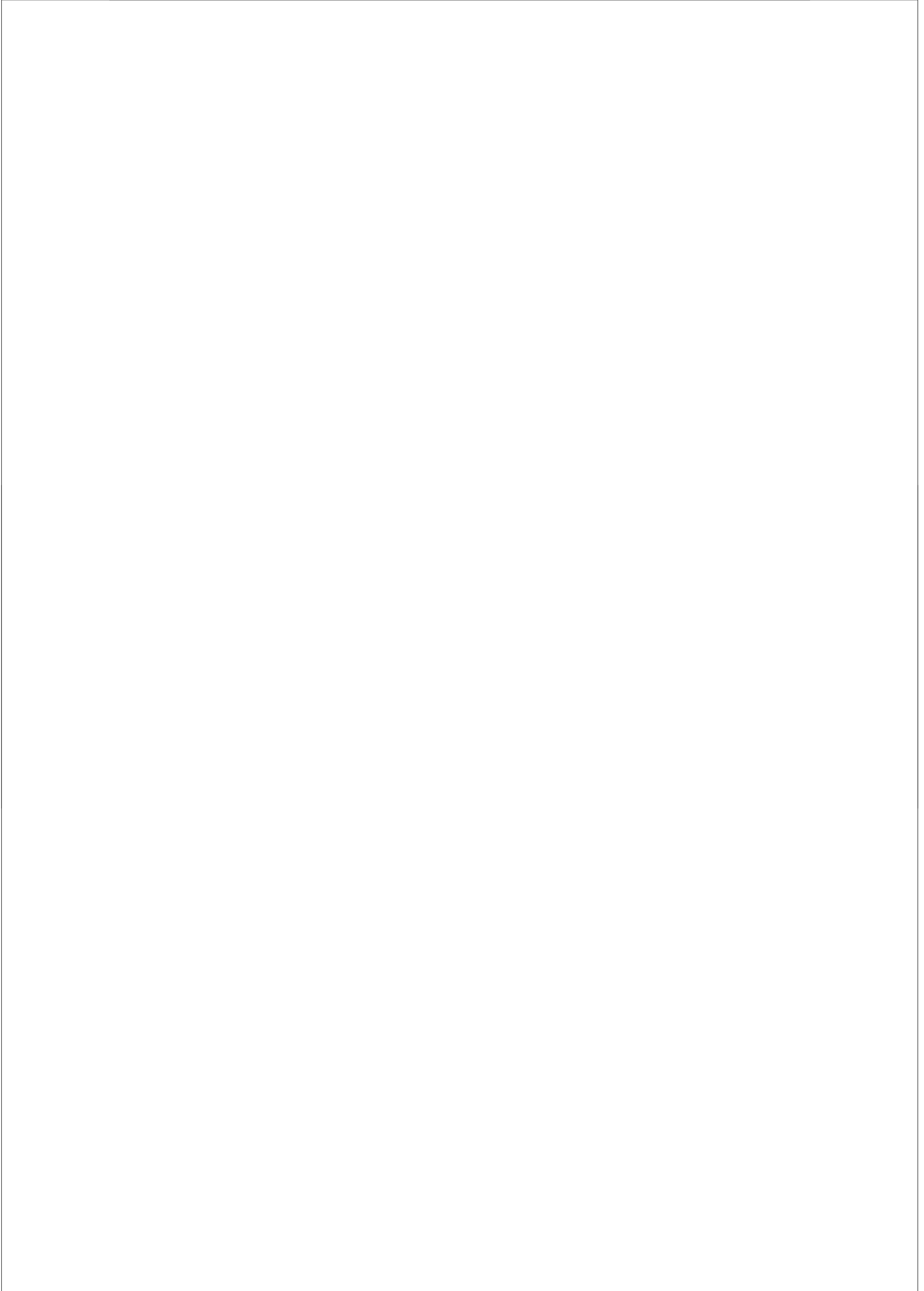
To the authors' knowledge, no indisputable confirmed case of Lyme borreliosis in horses has been reported. Therefore accurate prognostic data for horses with clinical signs related to a *B. burgdorferi* infection are not available. Prognosis for survival in general depends upon the organ system involved, severity and duration of infection and prompt initiation of treatment with the appropriate antibiotics whenever an acute infection with *B. burgdorferi* is highly suspected.

Addendum

Recent reports of the seroprevalence for *Borrelia* among horses in Europe confirm that *Borrelia* is endemic in most European countries even though clinical signs are seldom seen (Maurizi, Marie et al. 2009; Hansen, Christoffersen et al. 2010; Ebani, Bertelloni et al. 2012; Veronesi, Laus et al. 2012). While several cases of equine neurological disease (James, Engiles et al. 2010; Imai, Barr et al. 2011) and uveitis (Priest, Irby et al. 2012) have been associated with *B. burgdorferi* s.s. infection in recent years, the problem essentially remains the same: that is, how to relate the presence of the agent with an ongoing inflammatory process. In this respect, Chang et al.'s (2000) infection studies in ponies may prove to be pivotal. They infected ponies and then re-isolated the *Borrelia* from various tissues, despite the absence of obvious lesions, nine months after an initial experimental infection via a tick bite. In this study, all infected ponies showed a clear seroconversion. In fact, similar findings were previously reported for dogs following natural infection (Hovius, Rijpkema et al. 1999).
This encapsulates the essential difficulty of establishing the diagnosis of clinical borreliosis; neither the direct detection of the agent nor indirect demonstration of infection by antibody detection is conclusive evidence of clinical disease.
Chang et al.'s (2000) experimentally infected ponies did not show clinical signs during monitoring periods of up to nine months post-infection. Of course, this does not exclude the possibility that clinical signs would have developed at a later time point. The situation is complicated further by the difference between Europe, where at least four different genospecies of *B. burgdorferi* s.l. occur, and the USA where only one *B. burgdorferi* s.s. genospecies has been reported. It is, however, not entirely clear what effect this has on the pathogenesis or on the reliability of the serological tests commonly used. New 'diagnostic' tests for *Borrelia* in horses (C6 and a multiplex assay) have been developed in the USA but understandably focus on the detection of *B. burgdorferi* s.s. (Johnson, Divers et al. 2008; Wagner, Freer et al. 2011). With the aim of creating a diagnostic test capable of differentiating between the various stages of a *Borrelia* infection, a new quantitative test has been developed that focuses on simultaneous detection of OspA, OspC and OspF (Wagner, Freer et al. 2011). This test aims to differentiate vaccinated animals (OspA), from either animals with an acute infection (OspC) or those with a chronic infection (OspF) (Wagner, Freer et al. 2011).
The key issue is probably our lack of understanding of the pathogenic process. It is evidently complicated; there is a primary local inflammatory reaction in the skin of most, if not all, horses to which transmission occurs. In a few cases only, there is also a secondary inflammation in other tissues. Recent studies on *Leptospira interrogans*, another spirochete, have indicated that subclinical infections occur commonly in horses (Houwers, Goris et al. 2011) and that a leptospiral antigen induces auto-antibodies in the eyes of horses that suffer from

Equine Recurrrent Uveitis (Verma, Matsunaga et al. 2012) which is a comparatively rare disease in horses. The pathogenesis of borreliosis could involve similar features.

In summary, even though the recently documented *Borrelia* associated clinical cases (James, Engiles et al. 2010; Imai, Barr et al. 2011; Priest, Irby et al. 2012) emphasise the need to consider *B. burgdorferi* infection in the differential diagnosis of several equine diseases, incontrovertible cases of equine borreliosis are still extremely rare, if they exist at all.



Chapter IIb

Equine piroplasmosis: an overview

Introduction

Equine piroplasmosis (EP) is a non-contagious tick-borne disease caused by infection with either the intracellular haemoprotozoan parasites *Babesia caballi* or *Theileria equi* (previously known as *Babesia equi*). In the literature, EP is also frequently referred to as equine babesiosis (in the past this covered both *B. caballi* and *B. equi* but nowadays should strictly only refer to *B. caballi*), equine theileriosis (disease due to infection with *T. equi*), or biliary fever. Both parasites instigate haemolytic anaemia of varying severity with associated systemic illness, and can affect all equid species including horses, donkeys, mules and zebras (Schein 1988; Friedhoff, Tenter et al. 1990).

Epidemiology/ecology

Equine piroplasmosis occurs worldwide and is endemic in most (sub-) tropical countries. Infection is maintained within equine populations as long as competent vectors and suitable biotopes are present (Thompson 1969). Since transovarial transmission of *T. equi* does not occur in the known tick vectors, the persistently infected equid is considered the only reservoir (Ueti, Palmer et al. 2008). *Babesia caballi* is less dependent on availability of a suitable host for its maintenance, because transovarial transmission in the tick vectors does occur, such that the tick is considered the primary environmental reservoir (de Waal 1990). Multiple Ixodid tick species (*Dermacentor* spp., *Hyalomma* spp. and *Rhipicephalus* spp.) have been identified as either natural or experimental vectors of piroplasmosis (Stiller and Coan 1995; Uilenberg 2006). In southern Europe, *T. equi* is more prevalent than *B. caballi*; on the other hand, *B. caballi* has been identified further north than *T. equi* (Friedhoff, Tenter et al. 1990).
Given the available information on geographic distribution of infected horses, Central and South America, Africa, Asia, the Middle East and Southern Europe are all categorised as endemic regions (OIE July 2012). Northern Europe, including the Netherlands, is considered non-endemic. Competent ticks and iatrogenic transfer (e.g. via blood transfusion, contaminated syringes) are efficient modes of transmission (Short, Clark et al. 2012). Nevertheless, many factors need to be considered when estimating the risk of infection or disease; these include biotope, host availability, and aspects of a competent tick vector's life cycle.
The life cycle of most Ixodid ticks involves four developmental stages: egg, larva, nymph and adult. Each of the latter three stages requires a blood meal, although the different stages typically feed on different hosts (Sonenshine 1991). Pathogen transmission via ticks can occur in three ways: intrastadial (infection and transmission during the same stage), trans-stadial (transmission via a later developmental stage than parasite acquisition), or transovarial (pathogens enter the ovaries and are transmitted to offspring which subsequently in-

fect horses). While *T. equi* is generally transmitted in a trans-stadial fashion (Friedhoff and Soule 1996; Ueti, Palmer et al. 2008), *B. caballi* is transmitted both transstadially and transovarially by its vectors (de Waal 1990). Most studies on the prevalence of *T. equi* and *B. caballi* have been conducted in Southern Europe where the infection is endemic (Camacho, Guitian et al. 2005; Kouam, Kantzoura et al. 2010; Moretti, Mangili et al. 2010; Grandi, Molinari et al. 2011). Less information is available on the prevalence and possible emergence of EP in North Western Europe, with published data only available from Germany and Switzerland where seroprevalences of 5.6% and 4.4% for *T. equi* and 1.2% and 1.5% for *B. caballi*, respectively, have been reported (Boch 1985; Sigg, Gerber et al. 2010). Several clinical cases of EP have been recorded in Belgium (Mantran, Votion et al. 2004), but no information is available on the seroprevalence of EP in that country's horse population. Until 2007, none of the ticks known to act as natural vectors for *T. equi/B. caballi* in other parts of the world had been identified within the Netherlands (Nijhof, Bodaan et al. 2007).

Pathogenesis (transmission/life cycle of parasites)

While *B. caballi* is considered a classic 'babesia' species, the taxonomic classification of *B. equi* as a 'babesia' was controversial. *B. equi* was eventually reclassified as *T. equi* in 1998 based largely on the existence of an extra-erythrocytic stage within equine peripheral blood mononuclear cells (PBMCs) (Mehlhorn and Schein 1998).

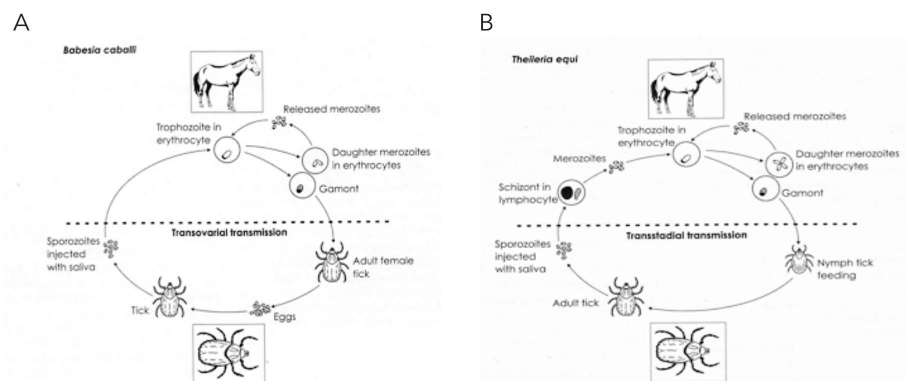


Fig. 1
The life cycle of Babesia caballi *(A) and the life cycle of* Theileria equi *(B) (Donellan and Marais 2009)*

The life cycle of both *B. caballi* and *T. equi* (figure 1) involve distinct stages that occur in either the host or in the tick vector (Schein, Rehbein et al. 1981; Moltmann, Mehlhorn et al. 1983). After pathogen transmission to a horse from a feed-

ing tick, the incubation time is 10-30 days for *B. caballi* and 12-19 days for *T. equi* (De Waal 1992).
While some details of the pathogenesis remain unknown, infection of horses with either *T. equi* or *B. caballi* causes erythrocyte lysis resulting in varying degrees of haemolytic anaemia. Clinical signs of disease will typically resolve in horses capable of mounting an appropriate immune response, or after effective treatment. However, recovered horses will become subclinical carriers, a condition that is life-long for *T. equi* and that lasts an estimated 2-4 years for *B. caballi* (de Waal and van Heerden 1994; Mehlhorn and Schein 1998; de Waal and Van Heerden 2004). Transplacental transmission from *T. equi* infected carrier mares to their foetuses has also been documented (Phipps and Otter 2004; Allsopp, Lewis et al. 2007; Chhabra, Ranjan et al. 2011; Georges, Ezeokoli et al. 2011), although the prevalence and significance of transplacental transmission are unknown.

Clinical disease

Clinical piroplasmosis can manifest as subacute, acute or chronic disease. Following initial infection with either parasite, horses can develop a variety of non-specific acute signs such as pyrexia, lethargy, anorexia, and weakness (Maurer 1962). Obvious signs of haemolytic anaemia may follow and include icterus or pallor of the mucous membranes, tachycardia, tachypnoea, and haemoglobinuria (Taylor, Bryant et al. 1969; de Waal 1992; Diana, Guglielmini et al. 2007). In more chronic cases of disease caused by either *T. equi* or *B. caballi*, the symptoms may be limited to non-specific signs such as mild anaemia, lethargy, partial anorexia, weight loss and poor performance. Not only can subclinical piroplasmosis negatively affect an animal's performance, it has been shown that strenuous exercise, such as that experienced during horse racing, can trigger subclinical infections to become acute (Hailat, Lafi et al. 1997). Importantly, however, most horses infected with either *T. equi* or *B. caballi* in both endemic and non-endemic regions become inapparent carriers with no obvious or appreciable signs of disease. There is thus a real need to diagnose both clinical and sub-clinical infections if attempts are to be made to prevent or limit transmission.

Diagnosis

There are a number of methods that can be used to aid the diagnosis of piroplasmosis, including stained blood smear examination, serological tests (ELISA, IFAT) and molecular techniques (PCR). Stained blood smear examination is the least sensitive technique because the parasites are even in the acute stage of infection often not detectable. The serological techniques, ELISA and IFAT, suf-

fer from low sensitivity in the acute phase of disease, when antibody production has yet to increase markedly, but are valuable diagnostic techniques in the sub-acute and chronic phases (Weiland, Aicher et al. 1984; Bruning 1996; Ogunremi, Georgiadis et al. 2007; Jaffer, Abdishakur et al. 2010). Molecular techniques like PCR have been shown to be sensitive and specific and are, therefore, useful diagnostic tools for detecting the presence of *Theileria* and *Babesia* species in both the acute and chronic phases of disease (Caccio, Camma et al. 2000; Rampersad, Cesar et al. 2003; Nagore, Garcia-Sanmartin et al. 2004).

Sensitive diagnostic tests are essential to reduce the risk of transmission and spread of EP by, for example, the importation of subclinical carriers into non-endemic countries. This is especially important in the case of *T. equi* because infected horses are considered both to be life-long carries and to be the only environmental reservoir for the pathogen (Ueti, Palmer et al. 2008). In suspected acute and chronic EP cases, the ranking of EP among the differential diagnoses should be based upon whether the horse resides in, or has visited, an EP endemic region. In general, clinical signs similar to those encountered during EP could also be caused by equine infectious anaemia (EIA), equine viral arteritis (EVA), equine anaplasmosis, purpura haemorrhagica, leptospirosis, and immune mediated haemolytic anaemia (Rothschild and Knowles 2006).

Treatment

The goals of EP treatment may, in part, depend on the disease status of the country in which the affected horse is located. In endemic regions, treatment of piroplasmosis-infected horses is usually aimed simply at resolving clinical signs, whereas attempts to eliminate the organism are generally only considered when future export to an EP-free country is anticipated. Complete clearance of the organisms is not the goal of treatment in endemic areas because prolonged low-level infection is thought to confer life-long immunity, which is desirable as a means of resistance to clinical disease. Nevertheless, imidocarb diproprionate is the most commonly used pharmaceutical for both alleviation of clinical signs and attempted ‘chemo-sterilisation’ (Kuttler, Zaugg et al. 1987; Schwint, Ueti et al. 2009; Grause 2011). While chemotherapeutic clearance of *T. equi* in the horse has been reported, the initial studies were conducted prior to the development of sensitive tests for the detection of carriers with very low grades of infection (Frerichs, Allen et al. 1973; Frerichs and Holbrook 1974). Until recently, therefore, it was widely accepted that true chemo-sterilisation of a horse infected with *T. equi* or *B. caballi* using any agent was unachievable (Kuttler, Zaugg et al. 1987). However, recently imidocarb dipropionate was shown to eliminate *T. equi* infection in experimentally infected horses (Grause 2011). Whether treatment will be as effective in eliminating *T. equi* from naturally infected carriers is yet to be definitively demonstrated.

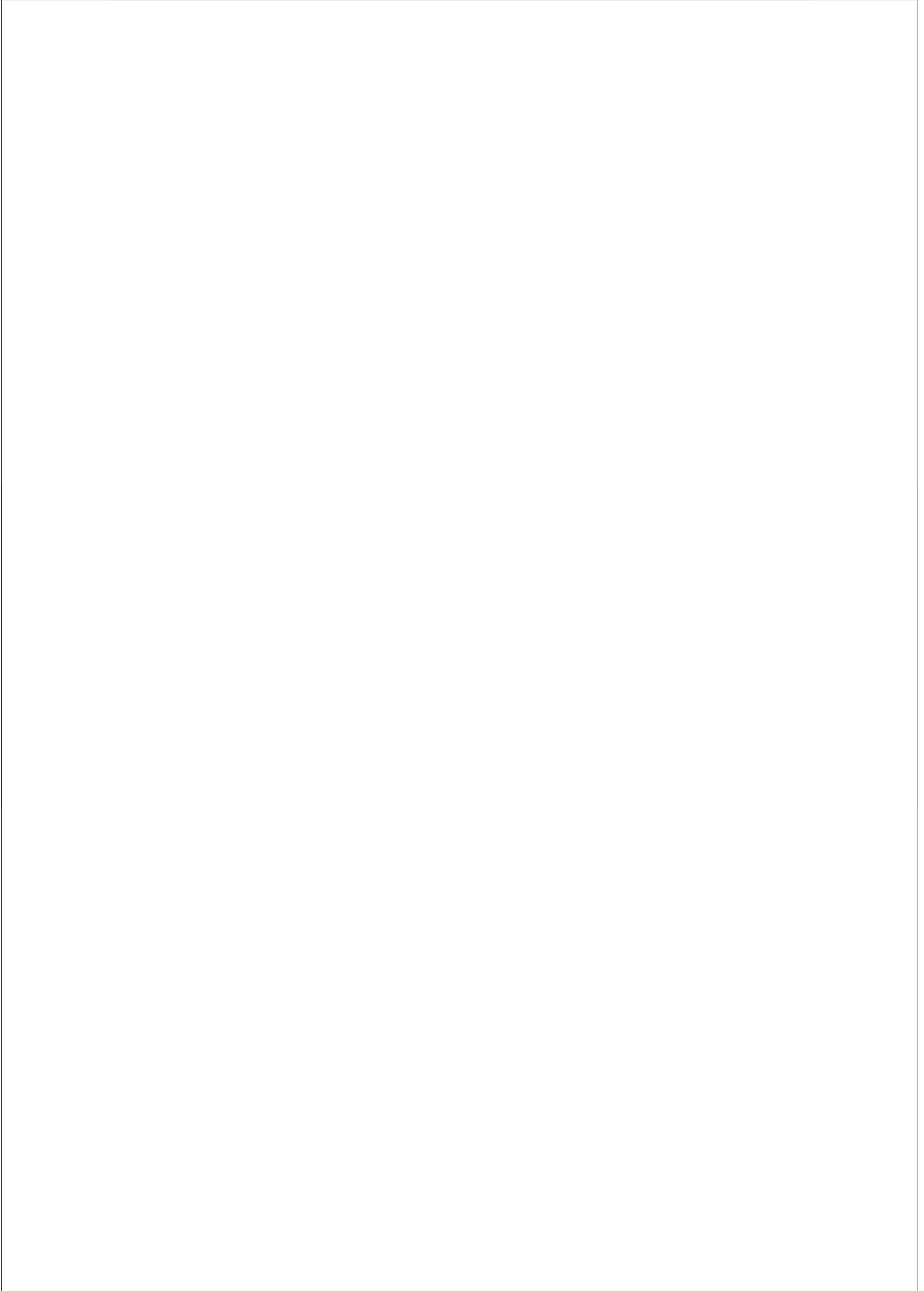
Prevention/vaccination

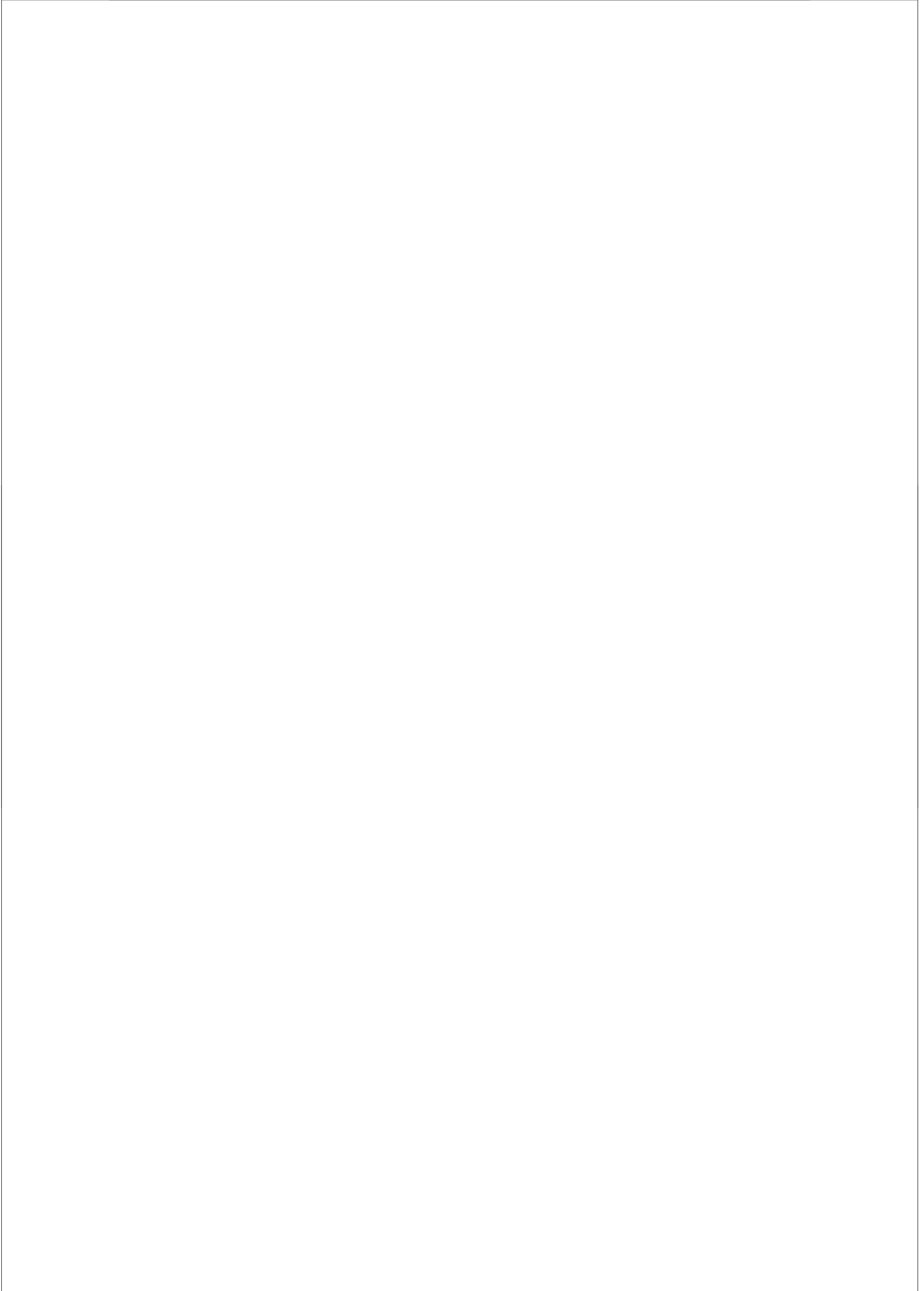
Prevention of EP in endemic regions is virtually impossible and it is therefore neither sensible nor desirable to attempt complete prevention. Rather, it is considered important for foals and young horses to be exposed to the pathogens and to recover from disease early in life, primarily because it is presumed that the generation of antibodies will protect the horse from recurrent disease following subsequent exposures. In non-endemic regions where competent vector ticks are present, prevention is based around the regulation of horse movement (i.e. import) from endemic regions or countries; this generally revolves around serological testing prior to travel (Friedhoff, Tenter et al. 1990; Knowles 1996). On the other hand, it may be impossible to prevent the introduction of infected ticks into non-endemic regions, because migrating birds appear to play a role in tick dispersal (Dietrich, Gomez-Diaz et al. 2011; Palomar, Santibanez et al. 2012). Diligent measures must therefore be taken to reduce or minimise the contact between horses and ticks. This includes avoiding high risk areas (where ticks are common), surveillance of horses and surrounding environment for the presence of ticks, clearing certain types of vegetation and application of acaricides. For a non-endemic country to remain infection-free, necessary tools include accurate screening tests and thorough knowledge of the tick populations and their ability to transmit and/or act as a reservoir for *B. caballi* and/or *T. equi*.

The potential use of vaccines to induce immunity to *T. equi* infection has been studied in donkeys, but there is to date no commercially available vaccine (Kumar, Malhotra et al. 2002a; Kumar, Malhotra et al. 2002b). Indeed, even though immunisation using *T. equi* whole merozoites appeared to be protective in donkeys (Kumar, Malhotra et al. 2002a), it is not known how effective a similar vaccination would be in horses.

Conclusion

Equine piroplasmosis follows the distribution of its vector ticks, which is a multifactorial dynamic process. Even though the risk of infection to horses in Northern European countries where piroplasmosis is non-endemic is still low, monitoring of the resident tick and horse populations is essential to avoid unexpected outbreaks of this disease in immunologically naïve horses.





Chapter IIc

Anaplasma phagocytophilum: an overview

Introduction

Anaplasma phagocytophilum is a tick borne pathogen of emerging veterinary and medical importance, for it can cause disease on its own in several mammals and potentially complicate disease processes caused by other pathogens (Belongia 2002). First described as the cause of equine granulocytic ehrlichiosis in horses in 1969 (Gribble 1969), the same pathogen was also identified in humans causing disease in 1994 (Dumler, Barbet et al. 2001).

Etiology

Anaplasma phagocytophilum is an obligate intracellular, gram-negative coccoid with a tropism for granulocytes and most commonly found in neutrophils as cytoplasmic inclusions (morulae). To reflect the close genome homology and similarity in pathophysiology of three species of granulocytic bacteria causing disease in humans (human granulocytic ehrlichiosis agent), ruminants (*Ehrlichia phagocytophilum*) and in horses (*E. equi*), these were designated as variants of the same species *Anaplasma phagocytophilum* in 2001 (Dumler, Barbet et al. 2001).

Epidemiology

Anaplasma phagocytophilum has been detected in animals and *Ixodes* ticks in nearly all European countries but the strains or variants and the resulting disease vary with the geographic location (Woldehiwet 2006; Silaghi, Liebisch et al. 2011) pointing towards different natural cycles with different reservoir hosts.
Transmission of *A. phagocytophilum* from ticks to mammals occurs transstadial, in which infection is acquired by the feeding of larvae or nymphs on infected hosts and transmission occurring by the next tick stage, nymphs or adults (Hodzic, Fish et al. 1998). The tick is an essential biological vector in which *A. phagocytophilum* replicates and persists through molting between sequential blood meals. As no transovarial transmission has been shown, a reservoir animal is necessary for the maintenance in nature (Woldehiwet 2006). Wildlife and birds are implicated as reservoir hosts for *A. phagocytophilum* (Hildebrandt, Franke et al. 2010; Palomar, Santibanez et al. 2012), with migratory birds dispersing ticks and consequently expanding the range of tick populations and associated pathogens into new locations.
Prevalences found in previous surveys conducted in other European countries such as Italy and France show a prevalence of around 25% (Leblond, Pradier et al. 2005; Passamonti, Veronesi et al. 2010). *Anaplasma* DNA, however, is rarely detected in healthy seropositive horses or ticks in Northern Europe and reaches prevalences in ticks of 0.5-3 % (Hartelt, Oehme et al. 2004; Passa-

monti, Veronesi et al. 2010; Lommano, Bertaiola et al. 2012; Palomar, Santibanez et al. 2012). The low percentage of *Anaplasma* DNA positive horses contrary to the high seroprevalence imply that this animal is not a reservoir.

Diagnosis

Clinical signs in infected horses vary in severity from subclinical to lethal and include high fever, anorexia, lethargy, limb oedema, reluctance to move, and ataxia (Reubel, Kimsey et al. 1998; Bermann, Davoust et al. 2002; Franzen, Aspan et al. 2005). Characteristic haematological abnormalities in experimentally infected horses include mild anaemia, leucopenia, and thrombocytopenia (Franzen, Aspan et al. 2005). A diagnosis of equine anaplasmosis can be made when characteristic intra-granulocytic morulae are seen on a blood smear. Blood smear examination, seems a sensitive tool in the diagnosis of this disease if applied in the acute stadium when fever is still present. PCR assays are also available and allow rapid and sensitive, early diagnosis, especially in the sub-acute stage of infection when observable morulae are absent (Pusterla, Huder et al. 1999; Franzen, Aspan et al. 2009).

Treatment and prognosis

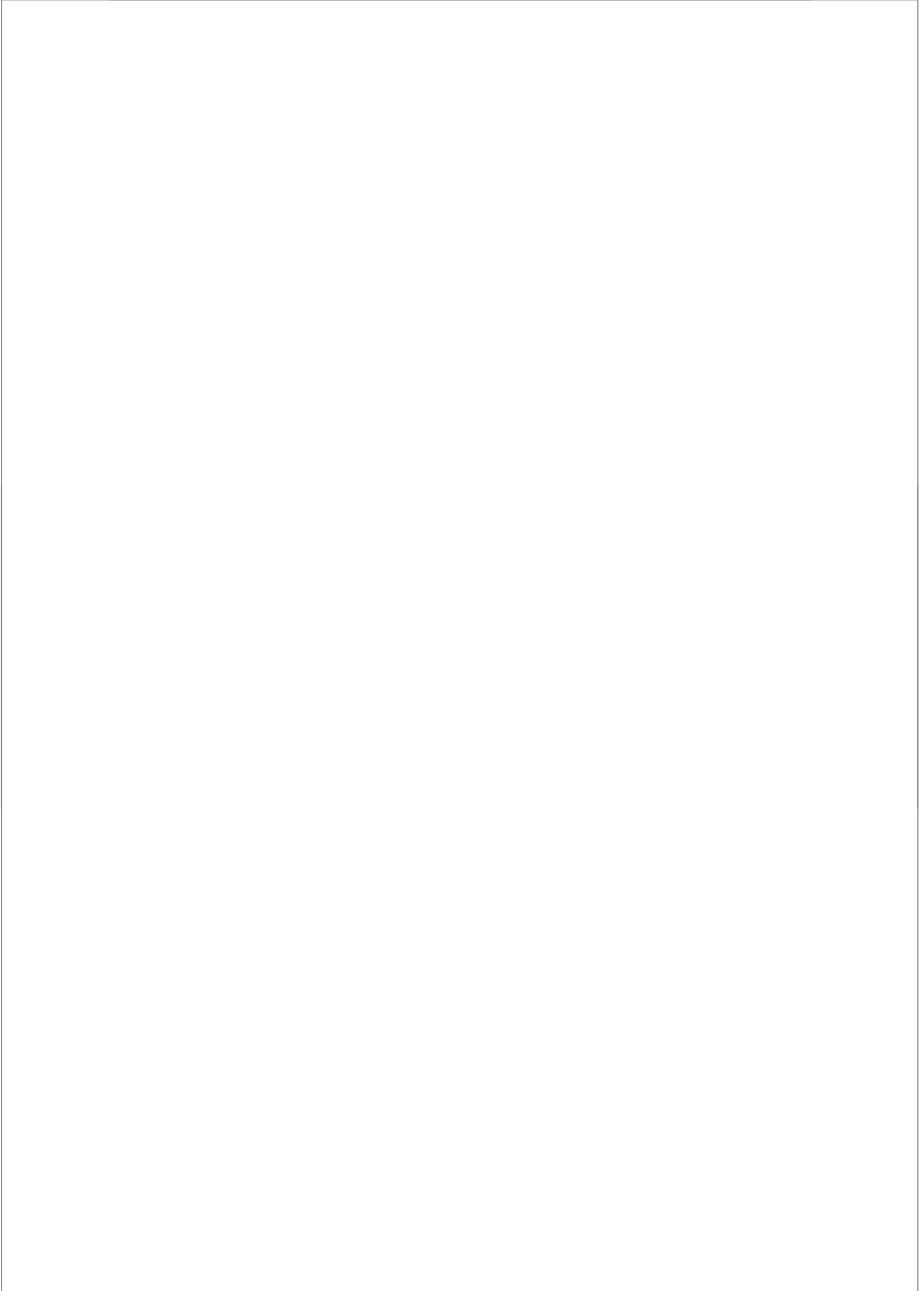
While most *A. phagocytophilum* infections in horses develop subclinical or seem only moderate in severity and are usually self-limiting in 2 to 3 weeks (Franzen, Aspan et al. 2009), treatment with oxytetracycline (6.6 mg/kg iv q24h for 5 days) is occasionally indicated when clinical signs are severe. It has been suggested that horses younger than three to four years of age generally experience less severe clinical disease (Gribble 1969; Madigan and Gribble 1987) when infected with *A. phagocytophilum*. Protective immunity lasting 2 years develops after natural and experimental infection and a carrier state beyond 4 months does not seem to develop (Franzen, Aspan et al. 2009).

Prevention and control

Development of vaccines is complicated by the intracellular nature and strain diversity of this bacterium. Therefore, prevention is currently primarily focused on tick control and avoiding high-risk areas.

Conclusion

Equine anaplasmosis is quite common in Europe, but often not diagnosed because of its a-specific clinical signs and mainly subclinical course of disease.



Chapter III

Development and evaluation of real-time PCR assays for the quantitative detection of *Babesia caballi* and *Theileria equi* infections in horses from South Africa

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Abstract

A quantitative real-time polymerase chain reaction (qPCR) assay using a TaqMan minor groove binder (MGBTM) probe was developed for the detection of *Babesia caballi* infection in equids from South Africa. Nine previously published sequences of the V4 hypervariable region of the *B. caballi* 18S rRNA gene were used to design primers and probes to target unique, conserved regions. The *B. caballi* TaqMan MGBTM qPCR assay was shown to be efficient and specific. The detection limit, defined as the concentration at which 95% of positive samples can be detected, was determined to be 0.000114% parasitised erythrocytes (PE). We further evaluated a previously reported *Theileria equi*-specific qPCR assay and showed that it was able to detect the 12 *T. equi* 18S rRNA sequence variants previously identified in South Africa. Both qPCR assays were tested on samples from two ponies experimentally infected with either *T. equi* or *B. caballi*. The qPCR assays were more sensitive than the indirect fluorescent antibody test (IFAT) and the reverse-line blot (RLB) during the early onset of the disease. The assays were subsequently tested on field samples collected from 41 horses, resident on three stud farms in the Northern Cape Province, South Africa.
The IFAT detected circulating *T. equi* and *B. caballi* antibody in, respectively, 83% and 70% of the samples. The RLB detected *T. equi* parasite DNA in 73% of the samples, but none of the samples were positive for *B. caballi*, although 19 *T. equi*-positive samples also hybridised to the Babesia genus-specific probe. This could indicate a mixed *T. equi* and *B. caballi* infection in these samples, with either the *B. caballi* parasitaemia at a level below the detection limit of the *B. caballi* RLB probe, or the occurrence of a novel Babesia genotype or species. In contrast, the qPCR assays correlated fairly well with the IFAT. The *B. caballi* TaqMan MGBTM qPCR assay was able to detect *B. caballi* parasite DNA in 78% of the samples. The *T. equi*-specific qPCR assay could positively detect *T. equi* DNA in 80% of the samples. These results suggest that the qPCR assays are more sensitive than the RLB assay for the detection of *T. equi* and *B. caballi* infections in field samples.

Introduction

Babesia caballi and *T. equi* are haemoprotozoan parasites that cause equine piroplasmosis (Mehlhorn and Schein 1998). The disease is of worldwide importance and occurs throughout the tropical and subtropical parts of the world with its prevalence being related to the distribution of its tick vectors (de Waal 1992).

Fourteen species of ixodid ticks of the genera *Dermacentor*, *Hyalomma* and *Rhipicephalus* have been identified worldwide as vectors of either *T. equi* or *B. caballi* (de Waal 1992). Both parasites cause disease, which may be either acute or chronic with mortalities ranging from less than 10% up to 50%. The disease is generally characterised by fever and anaemia. The clinical signs are often variable and nonspecific, making it easy to confuse the disease with other conditions, therefore complicating diagnosis. It is also not possible to differentiate between *B. caballi* and *T. equi* infections based on clinical signs alone. Once infected, horses may remain life-long carriers of *T. equi* infections whereas with *B. caballi* infections, which are self-limiting, horses remain carriers for up to four years (de Waal and van Heerden 1994).

The global transport of horses has led to the spread of equine piroplasmosis from its endemic tropical and subtropical zones to more temperate regions. Stringent regulatory import restrictions are in place in some countries to prevent the entrance of horses that are carriers of *B. caballi* and *T. equi* as they may act as reservoirs of infection (Friedhoff, Tenter et al. 1990; Sluyter 2001). Regulations often require the serological testing of horses in order to confirm seronegativity and to identify seropositive animals whose movement is restricted (Bose, Jorgensen et al. 1995; Bruning 1996). A variety of serological methods, which include the complement fixation (CF) test, the indirect fluorescent antibody test (IFAT) and the competitive-inhibition ELISA (cELISA), have been developed for the detection of specific antibodies (Donnelly, Phipps et al. 1982; Weiland, Aicher et al. 1984; Knowles, Perryman et al. 1991; Bose, Jorgensen et al. 1995; Bruning, Phipps et al. 1997; Kappmeyer, Perryman et al. 1999). The reliability of these serological assays is, however, restricted by antibody detection limits and cross-reactivity (Bruning, Phipps et al. 1997).

Molecular-based diagnostic tests, which have higher sensitivities and specificities than serological tests, and which can detect samples with very low parasitaemias, have been developed. *Babesia caballi*- and *T. equi*-specific oligonucleotide probes based on sequence differences in the small subunit (18S) ribosomal RNA (rRNA) genes (Allsopp, Cavalier-Smith et al. 1994) were used in a preliminary study to demonstrate transplacental transmission of *T. equi* (Lewis, Penzhorn et al. 1999). Further developments using 18S rRNA genes as target sequences include species-specific nested polymerase chain reaction (PCR) assays (Bashiruddin, Camma et al. 1999; Rampersad, Cesar et al. 2003) and the reverse-line blot (RLB) assay, which allows for the identification of novel genotypes or species and also allows for the detection of mixed infections

(Gubbels, de Vos et al. 1999; Nagore, Garcia-Sanmartin et al. 2004; Bhoora, Franssen et al. 2009). Several parasite outer membrane protein gene sequences have also been targeted in the development of molecular diagnostic assays for equine piroplasmosis (Nicolaiewsky, Richter et al. 2001; Ueti, Palmer et al. 2003; Alhassan, Pumidonming et al. 2005; Alhassan, Thekisoe et al. 2007; Heim, Passos et al. 2007). A quantitative real-time polymerase chain reaction (qPCR) assay targeting the gene ema-1 encoding the equi merozoite antigen-1 (EMA-1) was developed to determine the number of *T. equi* parasites in the mammalian host and in *Rhipicephalus* (*Boophilus*) *microplus* ticks (Ueti, Palmer et al. 2003). A multiplex assay, using the ema-1 qPCR assay and a qPCR based on the rhoptry associated protein (BC 48) gene of *B. caballi*, was subsequently developed and used to determine the prevalence of both *T. equi* and *B. caballi* parasites in horses in Brazil (Heim, Passos et al. 2007).
A qPCR assay, based on the 18S rRNA gene, was recently developed for the detection of *T. equi* infections in horses (Kim, Blanco et al. 2008). This assay proved to be highly sensitive and specific for *T. equi*, and it allowed for the simultaneous detection and quantification of *T. equi* DNA in infected equine samples. In the present study, we describe the development and application of a TaqMan minor groove binder (MGBTM) qPCR assay, also based on the 18S rRNA gene, for detection of *B. caballi* in equine field blood samples. Furthermore, we evaluate the ability of the *T. equi*-specific qPCR assay to detect all *T. equi* 18S rRNA variants that have been shown to occur in South Africa (Bhoora, Franssen et al. 2009).

Materials and methods

In vitro culture of South African *B. caballi* and *T. equi* isolates

The South African *B. caballi* 502 culture was initiated from blood samples collected from an infected horse at the South African National Yearling Sale in March 2000 (Zweygarth, Lopez-Rebollar et al. 2002). In February 2006, blood collected from a *T. equi*-infected horse resident on a farm in Fastfontein, Onderstepoort was used to culture the *T. equi* WL isolate used in this study. The *B. caballi* 502 and *T. equi* WL isolates were propagated in purified equine red blood cells using established culture systems previously described (Zweygarth, Just et al. 1995; Zweygarth, Lopez-Rebollar et al. 2002).

Infection trial

Experimental infections were approved by the Committee of Animal Welfare of the Faculty of Veterinary Medicine, Utrecht University. Blood stabilates of *T. equi* (strain Zaria) and the USDA strain of *B. caballi* (Kappmeyer, Perryman et al. 1999) were used in the infection trial. *Theileria equi* (strain Zaria) was isolated in Nigeria in 1973. A blood stabilate was prepared from an infected horse, frozen

and transported to the Faculty of Veterinary Medicine at Utrecht University in the Netherlands, where pony "Nico" was infected subcutaneously with the stabilate. Blood was collected and frozen 18 days later. Five years later (1978) Pony 187 was infected both subcutaneously and intramuscularly with the blood stabilate from pony "Nico". Blood was collected and frozen ten days post-infection (p.i.) and stored in liquid nitrogen. This stabilate was used in the current investigation. A blood stabilate of the USDA strain of *B. caballi* was kindly supplied by Prof. Friedhoff from the Faculty of Veterinary Medicine, Hannover, Germany. Two three-year-old, male Shetland ponies were housed together in the large animal experimental facility, Utrecht University, where they were kept under tick-free conditions. Prior to infection both ponies were tested negative for both *T. equi* and *B. caballi* by PCR/RLB hybridisation and IFAT. Before infection the ponies were immunosuppressed by the administration of dexamethasone (Dexadresone, Intervet®, Boxmeer, the Netherlands) at a dose of 0.04 mg/kg body weight (BW) by intramuscular injection, three times: four and two days before infection and on the day of infection. Pony A, a stallion weighing 150 kg, was infected with *T. equi* (Zaria) blood stabilate by 2ml intravenous and 2ml subcutaneous inoculation. Pony B, a gelding weighing 104 kg, was infected with 1ml of *B. caballi* (USDA) blood stabilate administered subcutaneously and 1ml intravenously. The horses were monitored daily for clinical responses. General physical appearance, early morning rectal temperatures, pulse and respiratory rates were monitored, mucosal membranes were inspected and lymph nodes were checked for possible enlargement. Serum and EDTA blood samples were taken daily from the jugular vein of each pony from day 3 until day 52 p.i. Haematocrit readings were taken daily, and between 3000 and 5000 erythrocytes were examined in Giemsa-stained blood smears for the presence of parasites. 200 ml of blood was spotted in duplicate on FTA filter paper (Whatman®). Filter papers were sent to South Africa where DNA was extracted. On day 60 p.i. both ponies were euthanised using 20 ml pentobarbital (30%) intravenously.

Field samples

Serum and EDTA blood samples were collected from 41 horses of unknown piroplasm status, resident on three stud farms in the Northern Cape Province, South Africa, where tick control measures were only implemented when high tick burdens were observed.

Indirect fluorescent antibody test (IFAT)

A standard IFAT protocol, as described in the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2004 (Anonymous 2012) was used; antigens were locally produced in the Netherlands or South Africa. Bound equine antibodies were detected with fluorescein isothiocyanate-conjugated rabbit

anti-horse immunoglobulin (RAHo/IgG(H + L)FITC, Nordic Immunology, Tilburg, the Netherlands) and examined in a wet mount by fluorescence microscopy.

DNA extraction

DNA was extracted from 200ml of *in vitro* culture material or EDTA-anticoagulated blood using the QIAamp DNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, except that DNA was eluted in 100 ml. Six round holes, 3mm in diameter, were punched out of blood samples stored on FTA paper (Whatman®), and DNA was extracted using the dried blood spot protocol from the QIAamp DNA mini kit (Qiagen, Hilden, Germany).

PCR amplification and reverse-line blot (RLB) hybridization

Primers RLB-F2 (50-GAC ACA GGG AGG TAG TGA CAA G-30) and RLB-R2 (50-biotin-CTA AGA ATT TCA CCT CTG ACA GT-30), which are specific for *Theileria* and *Babesia* species, were used to amplify the V4 hypervariable region of the parasite 18S rRNA gene, as described by Nijhof et al. (2005). Samples were subjected to RLB hybridisation as described previously (Bhoora, Franssen et al. 2009).

Design of a *B. caballi*-specific TaqMan® MGBTM qPCR assay

Six 18S rRNA gene sequences (GenBank Accession numbers: EU642512, EU642513, EU642514, EU888900, EU888901, EU888904), obtained from *B. caballi* positive field samples in a previous study (Bhoora, Franssen et al. 2009), along with three other previously published *B. caballi* 18S rRNA sequences, Z15104 (Allsopp, Cavalier-Smith et al. 1994), AY309955 (Criado-Fornelio, Gonzalez-del-Rio et al. 2004) and AY534883 (Nagore, Garcia-Sanmartin et al. 2004), were used to develop the assay. A TaqMan minor groove binder (MGB™) probe qPCR assay was designed using the Primer express software v2.0 (Applied Biosystems). A primer pair, [Bc_18SF402: 5'-GTA ATT GGA ATG ATG GCG ACT TAA-3' and Bc_18SR496: 5'-CGC TAT TGG AGC TGG AAT TAC C-3' (IDT)], and a TaqMan® MGB™ probe [Bc_18SP: 5'-6-FAM-CCT CGC CAG AGT AA-MGB-3' (Applied Biosystems)], were designed to amplify and detect a 95 bp fragment in the V4 hypervariable region of the 18S rRNA gene.
The TaqMan MGB™ probe was labeled with the fluorescent dye 6-carboxyfluorescein at the 5' end and a non-fluorescent quencher at the 3' end (Applied Biosystems). The forward primer and the probe are specific for *B. caballi*, but the reverse primer is not. Real-time quantitative PCR was performed in Micro-Amp optical 96-well reaction plates using the StepOnePlus™ Real-time PCR instrument (v. 2.0, Applied Biosystems). All qPCR assays were

run in a total reaction volume of 20 ml comprising 1x TaqMan Fast Universal PCR Master Mix (Applied Biosystems), 0.9 mM of each forward and reverse primer, 0.25 mM TaqMan MGB™ probe and 5 ml of target DNA. The qPCR cycling conditions were as follows: activation of the FastStart DNA polymerase at 95 ℃ for 20 s, then 40 cycles of 1 s at 95 ℃ and 20 s at 60 ℃.

Theileria equi-specific TaqMan qPCR assay

Primers Be18SF (5'-GCG GTG TTT CGG TGA TTC ATA-3') and Be18SR (5'-TGA TAG GTC AGA AAC TTG AAT GAT ACA TC-3') and the TaqMan probe, Be18SP (5'-6-VIC-AAA TTA GCG AAT CGC ATG GCT T-3'), previously designed for a *T. equi*-specific qPCR assay (Kim, Blanco et al. 2008) were used. The qPCR was performed as described above.

Efficiency, sensitivity and specificity of the qPCR assays

The in vitro cultured South African *B. caballi* 502 and *T. equi* WL isolates with percentage parasitised erythrocytes (PE) of approximately 8.1 (~7.29×10^5 parasites/ml) and 13.8 (~1.24×10^6 parasites/ml), respectively, were used in the generation of standard curves from which the efficiencies of the qPCR assays were determined. A tenfold dilution series (10^0 to 10^7) from each of the *in vitro* cultured equine parasites was prepared in duplicate using a suspension of uninfected equine red blood cells. DNA was extracted from all diluted samples and qPCR amplifications of both standard dilution series were repeated in triplicate and on ten separate occasions. The data generated from each of the 30 runs were used to calculate linear regression equations of quantification cycle (Cq) (Bustin, Benes et al. 2009) against log copy number. The efficiency of each assay was determined from the regression equations. SigmaPlot1 (ver. 11) was used to plot a graph from which the sensitivity by concentration, for each assay, could be determined. The estimated sensitivity and 95% confidence intervals for the true sensitivity for each group of dilutions prepared were calculated using the standard error of the estimated sensitivity of each dilution group (Sibeko, Oosthuizen et al. 2008).
The analytical specificity of each assay was evaluated using DNA extracted from other protozoal parasites expected to occur in equids, including *Trypanosoma brucei evansi*, *Trypanosoma brucei equiperdum*, *Trypanosoma vivax* and either *T. equi* for the *B. caballi* qPCR assay or *B. caballi* for the *T. equi* assay. DNA extracted from a piroplasm-free horse was included in each assay as a negative control.

<u>Comparison of the qPCR assays with other tests for the detection of *B. caballi* and *T. equi*</u>

The qPCR assays were used to detect parasites in daily blood samples taken from two experimentally infected ponies as well as from 41 field samples of unknown piroplasm status. The results were compared with serological detection by IFAT and detection of parasites by RLB hybridisation.

Results

<u>Specific detection of *B. caballi* and *T. equi* using the qPCR assays</u>

The *B. caballi* TaqMan MGB™ qPCR assay proved to be efficient in the amplification of a 95 bp fragment of the V4 hypervariable region of the 18S rRNA gene from an in vitro cultured *B. caballi* isolate (*figure 1*). No amplification signal was detected from negative control DNA extracted from a horse free from piroplasms or from DNA extracted from an in vitro cultured *T. equi* isolate (*figure 1*). Furthermore, no amplification signals were observed from DNA extracted from *T. b. evansi*, *T. b. equiperdum* and *T. vivax*, other protozoal parasites expected to occur in horses (*figure 1*).

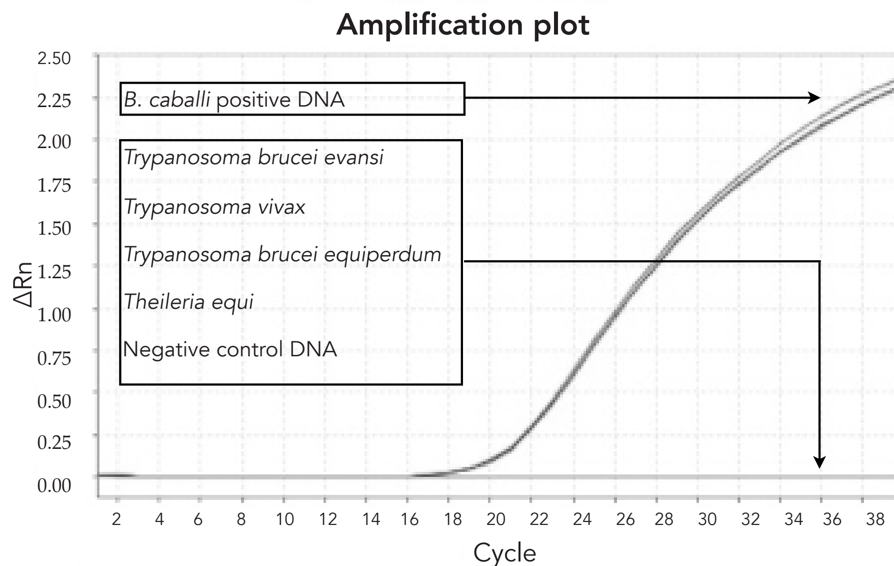

Fig. 1
Detection of Babesia caballi *positive control DNA using the* Babesia caballi *TagMan MGB™ qPCR assay, indicated by an increase in the fluorescence signal. No increase in fluorescence was observed in the negative control sample (DNA extracted from blood from a piroplasm free horse), or from* Theileria equi *control DNA or DNA from other protozoal parasites expected to occur in equids*

Similarly, the *T. equi*-specific qPCR assay recently developed by Kim et al. (2008) was shown to be successful in the amplification of a fragment of the *T. equi* 18S rRNA gene from DNA extracted from an *in vitro* cultured *T. equi* isolate (results not shown). No amplification signal was obtained from negative control DNA, DNA extracted from an *in vitro* cultured *B. caballi* isolate, or from *T. b. evansi*, *T. b. equiperdum* and *T. vivax* DNA (results not shown). The latter result agrees with the findings of Kim et al. (2008) who showed that the *T. equi* qPCR assay did not detect *B. caballi* and *Trypanosoma evansi* DNA.

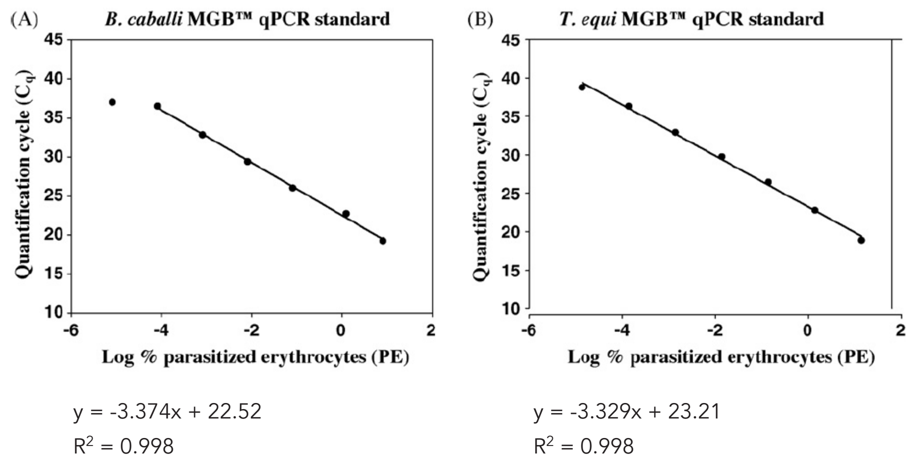

Fig. 2
Standard curve for the quantification of (A) the Babesia caballi *18S rRNA gene and (B) the* Theileria equi *18S rRNA gene. C_q values were plotted against the log % parasitised erythrocytes (PE) of the initial tenfold dilution series of parasite DNA equivalent to 8.1% PE to 8.1×10^{-7} % PE for* Babesia caballi *and 13.8%PE to 1.38×10^{-6}% PE for* Theileria equi.

The efficiencies of the qPCR assays were determined from linear regression equations, generated from tenfold se-rial dilutions of genomic DNA extracted from *B. caballi* and *T. equi*-infected equine erythrocytes (*Figure 2*). For *B. caballi*, the efficiency of the TaqMan MGB™ qPCR assay was determined to be 97.8% and for the *T. equi*-specific qPCR assay, an efficiency of 99.7% was calculated.

Table 1
Ability of the qPCR assays to detect previously identified South African Babesia caballi *and* Theileria equi *18S rRNA genotypes in field samples containing single and mixed infections*

Sample name	Species and 18S rRNA genotype[a]	*B. caballi*-specific B. qPCR result (Cq)[b]	*T. equi*-specific qPCR result (Cq)
LFEQ23	*T. equi* group A	Negative	26.42
RBEQ32[c]	*T. equi* group A	35.85	20.88
RBEQ63[c]	*T. equi* group A	34.24	21.9
RBEQ178[c]	*T. equi* group A	39.62	28.65
RBEQ96	*T. equi* group B	Negative	23.33
RBEQ101	*T. equi* group B	Negative	24.81
EQ08	*T. equi* group C	Negative	28.73
EQ10	*T. equi* group C	Negative	29.79
EQ70	*T. equi* group C	Negative	32.38
EQ75	*T. equi* group C	Negative	32.81
LFEQ45	*T. equi* group C	Negative	35.43
LFEQ47	*T. equi* group C	Negative	36.09
LFEQ177	*T. equi* group C	Negative	33.33
RBEQ105	*T. equi* group C	Negative	27.41
RBEQ122	*T. equi* group C	Negative	31.69
CABEQ30[c]	*B. caballi* group A	18.31	34.99
CABEQ31[c]	*B. caballi* group B1	18.38	34.76
CABEQ33[c]	*B. caballi* group B1	17.37	35.11
CABEQ50	*B. caballi* group B1	17.7	Negative
CABEQ51[c]	*B. caballi* group B1	19.91	29.53
CABEQ52[c]	*B. caballi* group B1	18.52	36.78
CABEQ107[c]	*B. caballi* group B1	35.83	38.07
CABRBEQ164	*B. caballi* group B1	32.72	Negative
CABRBEQ179	*B. caballi* group B1	33.91	Negative
CABRBEQ73	*B. caballi* group B2	21.09	Negative
CABRBEQ74	*B. caballi* group B2	19.66	Negative
CABRBEQ115	*B. caballi* group B2	19.46	Negative

[a] The sequence of the 18S rRNA genotype in each sample was obtained in a previous study in which phylogenetic analyses indicated that the *T. equi* 18S rRNA sequences could be grouped into three main clades (A, B, C) and the *B. caballi* sequences could be divided into two clades (A, B1 and B2) (Bhoora, Franssen et al. 2009).
[b] Quantification cycle value. A high Cq value indicates a low concentration of parasite DNA and therefore a low parasitaemia in the field sample.
[c] Field samples with dual *T. equi* and *B. caballi* infections as indicated by the qPCR results. Only one sequence was obtained from these samples in our previous study (Bhoora, Franssen et al. 2009), because in each case, one of the parasites was present at a higher parasitaemia than the other (as indicated by the Cq value), and the 18S rRNA gene would have been amplified preferentially from the parasite present at higher parasitaemia.

The qPCR assays were further tested for their ability to detect parasite DNA in field samples representative of the *T. equi* and *B. caballi* 18S rRNA genotypes

previously identified in South Africa (Bhoora, Franssen et al. 2009). The *B. caballi* TaqMan MGB™ qPCR assay successfully detected *B. caballi* parasite DNA in field samples representative of each of the groups of *B. caballi* 18S rRNA genotypes (groups A, B1 and B2) (*table 1*). Similarly, the *T. equi*-specific qPCR assay could detect parasite DNA in field samples representative of each of the previously identified groups of *T. equi* 18S rRNA genotypes (groups A, B and C) (*table 1*). Mixed infections are likely to occur in field samples and the use of the qPCR assays allowed for the identification of samples with dual *T. equi* and *B. caballi* infections.

Analytical sensitivity of the qPCR assays

Babesia caballi DNA could be detected in all 30 replicates of the dilutions of the cultured *B. caballi* 502 isolate ranging from undiluted (8.1% PE) to 8.1 x 10^{-4} % PE. The detection limit of the assay, defined as the concentration at which 95% of positive samples are detected (Bustin, Benes et al. 2009), was determined from the sensitivity curve (*figure 3A*) to be 1.14 x 10^{-4} % PE, equating to a quantification cycle (C_q) of 35.82.

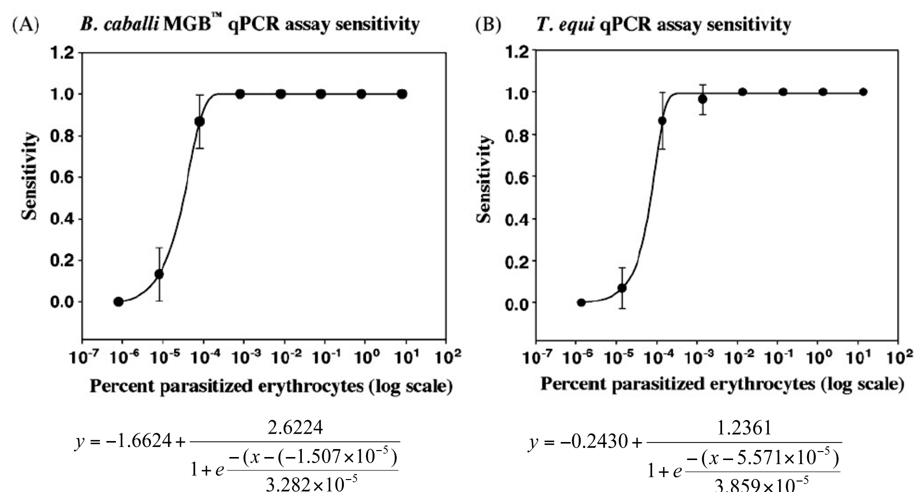

Fig. 3
The sensitivity and 95% confidence intervals for the qPCR assays. (A) Babesia caballi *TaqMan MGB™ qPCR sensitivity assay determined using a tenfold dilution series from 10^0 to 10^{-7} prepared from a* Babesia caballi *in vitro culture with approximately 8.1 % PE. (B)* Theileria equi *qPCR sensitivity assay determined using a tenfold dilution series from 10^0 to 10^{-7} prepared from a* Theileria equi *in vitro culture with approximately 13.8 % PE.*

The sensitivity of the assay decreased at higher dilutions. At 8.1 x 10^{-5} % PE, the sensitivity decreased to 86.7% with a 95% confidence interval (CI) of 74.5–98.8%, and at 8.1 x 10^{-6} % PE, the sensitivity was 13.3% with a 95% CI of 1.16–

25.4% (*figure 3A*). Similarly, *T. equi* DNA was detected in all 30 replicates of the dilutions ranging from 13.8% PE to 1.38 x 10^{-2} % PE. The detection limit (95% sensitivity) of the *T. equi* qPCR assay was determined to be 1.9 x 10^{-4} % PE at a C_q value of 35.89. The sensitivity decreased from 96.5% (95% CI 89.9–100%) at 1.38 x 10^{-3} % PE to 6.8% (95% CI 0–16.1%) at 1.38 x 10^{-5} % PE (*figure 3B*).

Comparison of the qPCR assays with other tests for the detection of *B. caballi* and *T. equi*

The newly developed *B. caballi*-specific TaqMan MGB™ qPCR assay and the previously reported *T. equi* specific qPCR assay were compared with IFAT and RLB by testing the ability of the different tests to detect parasites or antibody in the blood of two experimentally infected ponies.
Pony A, which was infected with *T. equi*, had a temperature rise (40 ℃), that was observed on day 8 p.i. and remained high until day 10 p.i.. During this period of increased temperature the pony was lethargic, but showed no other clinical signs. On day 12 p.i. parasites were seen in blood smears but the parasitaemia never became high enough to calculate reliably (table 2). From day 14 p.i. no further parasites were found in any of the blood smears. The haematocrit started to decline after infection and was less than 36% from day 6 p.i., with the lowest haematocrit value of 25% recorded on day 15 p.i. The haematocrit remained under the normal level of 36–40% until day 52 p.i.when it reached 37%. The first sample that was IFAT positive was taken on day 9 p.i. (table 2) and Pony A remained IFAT positive until the end of the experiment. IFAT was not performed on all samples, since antibody titres were not expected to vary much from day to day. The first positive result of the RLB test was on day 8 p.i. and Pony A samples remained positive until the last test was performed on day 52 p.i., with five exceptions (days 16–18, 34 and 45) (table 2). The qPCR test appeared to be the most sensitive of the three tests as it was able to detect *T. equi* in Pony A from day 7 p.i. to day 52 p.i.. Pony B, infected with *B. caballi*, had an increased temperature (40 °C) from day 6 p.i. until day 15 p.i..
During this time the animal suffered from general malaise; it moved slowly, lay down frequently and had a decreased appetite. Parasites were seen in the blood smear from day 9 p.i. (table 2). From day 19 p.i. no parasites were found, except for day 22 p.i. when a single parasite was seen.

Table 2
Results of microscopic examination of blood smears, IFAT, RLB and qPCR tests for Pony A infected with Theileria equi *and Pony B infected with* Babesia caballi

Days p.i.	Pony A (infected with *T. equi*)				Pony B (infected with *B. caballi*)			
	Blood smear	IFAT (reciprocal titre)	RLB	*T. equi*-specific qPCR (C_q)	Blood smear	IFAT (reciprocal titre)	RLB	*B. caballi*-specific qPCR (C_q)
3	–	0	–	–	–	0	–	–
4	–		–	–	–		–	–
5	–		–	–	–		–	–
6	–		–	–	–		–	–
7	–	0	–	35.11	–	0	–	36.3
8	–	0	+	34.12	–	0	–	34.43
9	–	160	+	31.89	+	0	+	31.79
10	–	640	+	30.85	+	160	+	29.63
11	–	1280	+	30.72	+	320	+	28.48
12	–		+	30.27	+		+	26.47
13	–		+	30.98	+		+	26.53
14	–		+	35.18	+		+	31.67
15	–	>2560	+	32.93	+	320	+	27.34
16	–		–	35.35	+		+	29.93
17	–	5120	–	37.77	+	2560	+	29.24
18	–		–	38.3	+		+	31.08
19	–	2560	+	35.57	–	640	+	31.17
20	–		+	32.99	–		+	31.17
21	–		+	33.04	–		+	31.16
22	–	2560	+	31.65	+	1280	+	32.85
24	–		+	31.4	–		+	31.08
26	–	>2560	+	33.62	–	640	+	30.5
28	–		+	37.32	–		+	30.5
31	–	2560	+	31.54	–	640	+	30.44
34	–		–	34.31	–		+	32.81
38	–	>2560	+	33.05	–	1280	+	32.5
45	–	>2560	–	36.17	–	1280	+	32.95
48	–	5120	+	32.39	–		+	32.76
52	–	>1280	+	33.40	–	640	–	32.75

After infection, the haematocrit value declined to a low of 20% on day 19 p.i. and then increased again, reaching a normal haematocrit value (36%) on day 46 p.i.. Pony B tested IFAT positive on day 10 p.i. and remained IFAT positive until the last test was performed on day 52 p.i. (table 2). According to the RLB test, the first positive sample was on day 9 p.i., and samples remained positive until day 51 p.i. The RLB was negative on day 52 p.i. when the last test was performed (Table 2). The TaqMan MGB™ qPCR test was able to detect *B. caballi* from day 7 until day 52 p.i..

The PCR-based DNA detection tests used in this study were more sensitive than IFAT during the early onset of the disease, since they could detect *T. equi* and *B. caballi* DNA in the circulation prior to antibody production. After day 9 p.i. for Pony A and day 10 p.i. for Pony B all three tests were in full agreement, with the exception of five days when the RLB was negative for *T. equi* (Pony A) and one day when it was negative for *B. caballi* (Pony B). On most days when the RLB was negative, the qPCR Cq values were very high (table 2), indicating that there were fluctuations in the circulating parasitaemia over time. The negative RLB results were thus probably as a result of periods when the parasitaemia dropped below the detection limit of the RLB. The qPCR assays were more sensitive than the RLB as they were able to detect parasite DNA in both ponies earlier than the RLB hybridisation assay, and qPCR results remained positive until the last day.

In order to further evaluate the *T. equi* and *B. caballi* qPCR assays, we tested 41 field samples of unknown piroplasm status (table 3). Due to the low prevalence of *B. caballi* in field blood samples in South Africa, we targeted three stud farms in the Northern Cape Province, where tick vectors for both *T. equi* and *B. caballi*, namely, *Hyalomma truncatum* and *Rhipicephalus evertsi evertsi* occur (de Waal and van Heerden 1994). Tick control strategies were only implemented on the selected stud farms when high tick burdens were observed and therefore horses were almost certainly exposed several times to the tick vectors and parasites, making them an ideal target population for detecting natural *T. equi* and *B. caballi* infections. At a cutoff quantification cycle (Cq) value of 35.82, the *B. caballi* TaqMan MGB™ qPCR assay could detect *B. caballi* parasite DNA in 23 of the 41 field samples with Cq values that ranged between 23.62 and 34.85. A further nine samples tested positive for *B. caballi* with Cq values greater than 35.82. The *T. equi* qPCR assay detected *T. equi* parasite DNA (cut-off Cq value of 35.89) in 33 of the 41 samples with Cq values between 22.37 and 34.49. The qPCR results were compared to those obtained by IFAT and RLB (table 3). The IFAT detected circulating *T. equi* and *B. caballi* antibody in, respectively, 83% and 70% of the samples tested (table 3).

The RLB detected *T. equi* parasite DNA in 73% of samples tested, while none of the samples were positive for *B. caballi*. However, 19 of the *T. equi* positive samples also hybridised to a *Babesia* genus specific probe. This could indicate a mixed *T. equi* and *B. caballi* infection with either the *B. caballi* parasitaemia at a level below the detection limit of the *B. caballi* RLB probe, or the occurrence of a novel *Babesia* genotype or species. *B. caballi* infections have been reported to occur at very low parasitaemias that rarely exceed 1% (Hanafusa, Cho et al. 1998). Previous reports indicate that *B. caballi* is extremely difficult to detect in blood at any stage of the disease except the early acute phase, and once a carrier status is established, there may be complete absence of circulating parasites (Frerichs, Holbrook et al. 1969; Holman, Chieves et al. 1994).

The occurrence of such low parasitaemias could possibly explain the inability of the RLB to detect all positive *B. caballi* infections. Alternatively, the presence of

sequence variation, which has previously been reported to occur in the region of the 18S rRNA gene where the RLB primers and probes were designed (Bhoora, Franssen et al. 2009), could explain the discrepant results. Despite the apparently low parasitaemias, or the possible presence of sequence variants, the *B. caballi* TaqMan MGB™ qPCR assay proved to be efficient in detecting infected animals. The assay detected *B. caballi* parasite DNA in 78% of the samples tested, 34% (17) of which were from samples co-infected with *T. equi* as shown by the *T. equi*-specific qPCR assay. The *T. equi*-specific qPCR assay was also shown to be more sensitive than the RLB, and could detect parasite DNA in 80% of the samples tested.

Table 3
IFAT, RLB and qPCR results for 41 equine field blood samples of unknown piroplasm status, obtained from three stud farms in the Northern Cape Province, South Africa, where limited tick control measures were implemented

Sample no.	*T. equi* IFAT	*B. caballi* IFAT	RLB	*T. equi*-specific qPCR (Cq)	*B. caballi*-specific qPCR (Cq)
1	Positive	Positive	*T. equi*, B. catch all[a]	25.06	32.76
2	Positive	Positive	*T. equi*, B. catch all	25.14	32.43
3	Positive	Positive	*T. equi*	34.49	37.41
4	Positive	Positive	*T. equi*, B. catch all	24.18	32.49
5	Positive	Positive	*T. equi*	29.99	34.85
6	Positive	Positive	*T. equi*, B. catch all	25.53	32.83
7	Positive	Positive	T/B. catch all[b]	25.07	32.72
8	Positive	Positive	Negative	34.13	30.72
9	Positive	Positive	*T. equi*, B. catch all	23.86	28.43
10	Positive	Positive	*T. equi*, B. catch all	24.19	29.36
11	Positive	Positive	*T. equi*, B. catch all	Negative	Negative
12	Positive	Positive	*T. equi*, B. catch all	22.37	29.76
13	Positive	Negative	*T. equi*, B. catch all	25.09	36.69
14	Positive	Positive	*T. equi*, B. catch all	24.09	28.29
15	Positive	Positive	*T. equi*, B. catch all	24.72	31.58
16	Positive	Positive	*T. equi*, B. catch all	26.04	36.43
17	Positive	Positive	*T. equi*, B. catch all	26.18	31.7
18	Negative	Positive	Negative	30.13	37.29
19	Positive	Positive	*T. equi*, B. catch all	25.39	29.95
20	Positive	Positive	*T. equi*, B. catch all	Negative	26.41
21	Positive	Positive	*T. equi*, B. catch all	23.24	29.7
22	Positive	Positive	*T. equi*, B. catch all	25.02	36.39
23	Positive	Positive	*T. equi*	30.0	33.49
24	Positive	Positive	*T. equi*	22.87	34.84
25	Positive	Positive	*T. equi*	24.5	29.64
26	Positive	Positive	T/B. catch all[b]	32.16	36.51
27	Positive	Negative	*T. equi*	22.48	33.9
28	Positive	Positive	*T. equi*, B. catch all	22.86	23.62
29	Positive	Positive	*T. equi*, B. catch all	31.06	Negative
30	Positive	Positive	*T. equi*	23.17	36.18
31	Negative	Negative	Negative	Negative	Negative
32	Positive	Negative	*T. equi*	29.08	Negative
33	Negative	Negative	Negative	Negative	36.53
34	Negative	Negative	Negative	Negative	Negative
35	Negative	Negative	Negative	Negative	Negative
36	Negative	Positive	Negative	Negative	Negative
37	Positive	Negative	*T. equi*	30.13	36.83
38	Positive	Negative	*T. equi*	30.43	Negative
39	Positive	Negative	*T. equi*	28.64	36.4
40	Negative	Negative	Negative	Negative	Negative
41	Positive	Negative	Negative	34.12	32.98

[a] B. catch all - *Babesia* genus-specific probe.
[b] T/B. catch all - *Theileria/Babesia* genus-specific probe

Discussion

In the present study we describe the development and application of a TaqManMGB™ qPCR assay targeting the *B. caballi* 18S rRNA gene, for the quantitative detection of the parasite from equine blood samples, as well as the evaluation of a qPCR assay for the detection of *T. equi* infections in South Africa. Molecular tests previously developed for the detection and differentiation of equine parasite species were based on conventional PCR and probe-based assays, which are relatively sensitive, but involve complex procedures which are time-consuming (Allsopp, Baylis et al. 1993; Bashiruddin, Camma et al. 1999; Nicolaiewsky, Richter et al. 2001; Rampersad, Cesar et al. 2003; Alhassan, Pumidonming et al. 2005). Quantitative PCR technology has recently been applied to the diagnosis of many organisms of veterinary and medical importance (Jeong, Kweon et al. 2003; Lindh, Gorander et al. 2007; J, Sedano-Balbas et al. 2008; Wengi, Willi et al. 2008).
This technology provides several advantages over the use of conventional PCR assays. Detection and quantification of a PCR product takes place in a single tube during the cycling process, thus eliminating the need for post-PCR manipulation and reducing the risk of contamination. Quantitative PCR tests have been developed for a number of haemoparasitic diseases including *Theileria sergenti* (Jeong, Kweon et al. 2003), *Babesia bovis* and *Babesia bigemina* (Jeong, Kweon et al. 2003; Buling, Criado-Fornelio et al. 2007; Kim, Blanco et al. 2008; Sibeko, Oosthuizen et al. 2008), *Anaplasma marginale* (Carelli, Decaro et al. 2007) and *Theileria parva* (Sibeko, Oosthuizen et al. 2008). These qPCR assays have significantly improved the sensitivity and specificity of parasite detection. While a qPCR assay for the detection of *T. equi* (Kim, Blanco et al. 2008), based on the amplification of the 18S rRNA gene has been developed, there is no report on the application of a qPCR test for the quantitative diagnosis of equine *Babesia* parasites using this gene.
Although sequence heterogeneity has been demonstrated in the 18S rRNA gene of *B. caballi* in South Africa (Bhoora, Franssen et al. 2009), we chose this gene as the target for development of a *B. caballi*-specific qPCR assay. Other genes previously targeted for the development of molecular diagnostic assays encode outer membrane proteins (Nicolaiewsky, Richter et al. 2001; Ueti, Palmer et al. 2003; Alhassan, Pumidonming et al. 2005; Alhassan, Thekisoe et al. 2007; Heim, Passos et al. 2007) which are under immune selection pressure, and sequence heterogeneity in the genes encoding these proteins is therefore likely to be even greater than in the 18S rRNA gene. Prior to the development of the primers and probes, the *B. caballi* 18S rRNA sequence variants were carefully examined for conserved regions. TaqMan MGB™ probes have previously been demonstrated to be helpful in the case of variable nucleotide sequences, since they allow for the use of smaller probes that are capable of detecting shorter conserved regions and with lower fluorescent background signals, as the 30 end of the probe is labelled with a non-fluorescent quencher

(Kutyavin, Afonina et al. 2000). Therefore, for a robust and quantitative assay, a TaqMan MGB™ probe was designed in a conserved target sequence of the V4 hypervariable region of the *B. caballi* 18S rRNA gene.

The *T. equi*-specific qPCR assay designed by Kim et al. (Kim, Blanco et al. 2008) targets a region of the 18S rRNA gene that occurs outside of the V4 hypervariable region. Inspection of the 18S rRNA sequences obtained from the twelve distinct South African *T. equi* variants (Bhoora, Franssen et al. 2009), indicated the occurrence of a single nucleotide difference in the forward primer target sequence and no differences in the regions where the reverse primer and probe had been designed. These differences did not appear to affect the sensitivity of the qPCR assay developed by Kim et al. (Bhoora, Franssen et al. 2009) as it was able to detect all *T. equi* variants thus far identified. The *B. caballi*-specific TaqMan MGB™ qPCR assay was highly sensitive proving to be able to detect as few as 0.000114% PE. The sensitivity of the *B. caballi* TaqMan MGB™ qPCR assay is comparable to that reported for the qPCR assay developed for the detection of *T. parva* (8.79×10^{-4} % parasitaemia) in cattle and buffalo in South Africa (Sibeko, Oosthuizen et al. 2008). Kim et al. (2008) determined the detection limit of their *T. equi* qPCR to be 1.5 parasites per ml per sample, which equates to 1.5×10^{-5} % PE. In our hands, the detection limit (95% sensitivity) of the *T. equi* qPCR assay was 1.9×10^{-4} % PE. Both assays were shown to be specific for the target organism and no amplification signals were observed from DNA of other protozoal parasites expected to occur in equids. *Babesia caballi* infections generally tend to occur at extremely low parasitaemias, often due to the early elimination of the parasite after a short period of infection, making diagnosis almost impossible (Frerichs, Holbrook et al. 1969). The development of a highly sensitive and specific qPCR assay provides a major advantage in the detection of *B. caballi* infections in field blood samples.

The high seroprevalence of *T. equi* and *B. caballi* on three stud farms in the Northern Cape Province where limited tick control measures were implemented confirmed the occurrence of both *T. equi* and *B. caballi* infections on the selected farms. Although there was significant correlation between the qPCR and IFAT results in the detection of *B. caballi* and *T. equi* in infected horses, minor differences between the results were observed. Two samples were IFAT-positive for *B. caballi* but the qPCR results were negative. In addition, *B. caballi* DNA could be detected in six samples that were reported to be *B. caballi* IFAT negative. Similarly for *T. equi*, two IFAT-positive samples were negative when tested using the qPCR, while *T. equi* parasite DNA could be detected in one sample that was reported to be IFAT-negative. Quantitative PCR tests detect the presence of parasite DNA, whereas IFAT detects antibodies, which can be present in the absence of parasites (Holman, Chieves et al. 1994). The sampling time thus plays a critical role in the detection of circulating parasites. *B. caballi* infections are self-limiting, usually lasting one to three years, and horses are generally able to eliminate the infection naturally or drugs can

be used to sterilise the infection (Friedhoff and Soule 1996; Bruning, Phipps et al. 1997), although a recent study has shown that even high dose treatment with imidocarb may not be capable of eliminating *B. caballi* infections from healthy carriers (Butler, Nijhof et al. 2008). It is thus possible that *B. caballi* antibody titres remain at detectable levels for some period after the parasite has been cleared, resulting in animals testing IFAT-positive but negative using PCR-based methods. *T. equi* infections are not self-limiting and once infected, horses remain life-long carriers of the parasite.
Samples that test positive for *T. equi* by IFAT should therefore be detectable by qPCR, unless the parasitaemia is below the detection limit of the assay. However, IFAT positive and qPCR-negative results could also be explained by the existence of parasite 18S rRNA gene sequence variants that have not yet been identified and can therefore not be detected by the qPCR tests. The occurrence of IFAT-negative but qPCR positive results maybe explained by the observation that parasites may be detected in newly infected animals prior to the development of antibodies to the parasite. To our knowledge, this is the first report on the development of a quantitative TaqMan MGB™ qPCR assay, based on the 18S rRNA gene, for the detection of *B. caballi* infections in equine blood samples. Accurate diagnosis of equine piroplasmosis is essential for effective control measures. Previous assays proved to be limited in their ability to detect *B. caballi* infections in field blood samples due to the extremely low or undetectable parasitaemias observed. We have demonstrated rapid and accurate quantification of *B. caballi* from sub-clinically infected or carrier animals, using the TaqMan MGB™ qPCR assay on a StepOnePlus™ real-time PCR instrument. We envisage that application of this assay, along with the *T. equi*-specific qPCR assay developed by Kim et al. (2008), will provide better confirmation of diagnosis of equine piroplasmosis, particularly in cases where symptoms are non-specific. However, while we were able to show that the *B. caballi* and *T. equi* qPCR assays were able to detect all known 18S rRNA sequence variants that have previously been identified in South Africa, we do not know whether other variants exist in the field. It would therefore be prudent to develop a multiplex qPCR assay, including a "catch-all" TaqMan probe, similar to the *Theileria/Babesia* genus-specific probe used in the RLB, to ensure that if a piroplasm parasite is present in a sample, it will be detected by the qPCR test. Once validated, the tests could be incorporated as required tests by the OIE for the import and export of horses and for checking whether attempts at sterilising equine piroplasmosis infections have been successful.

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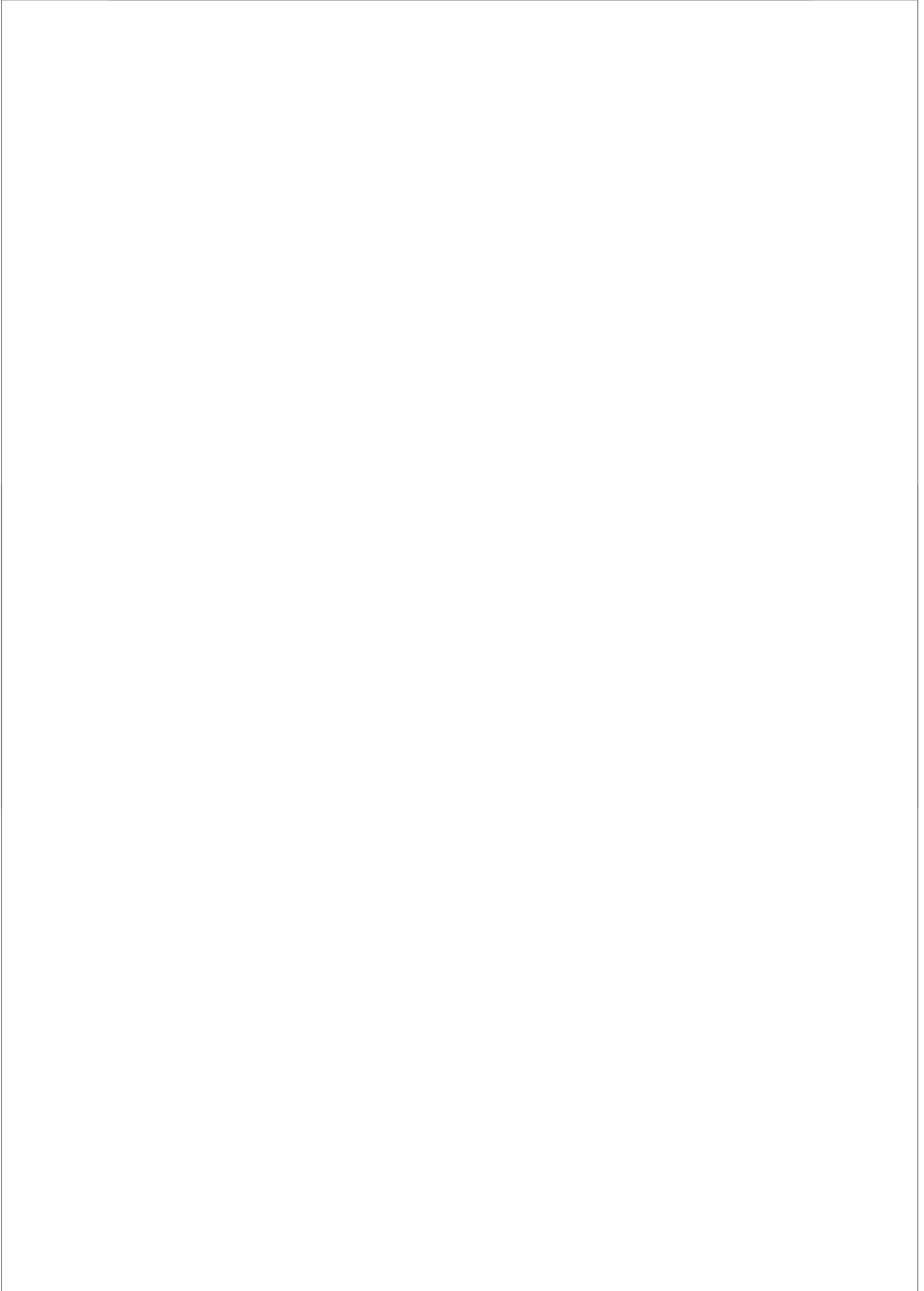

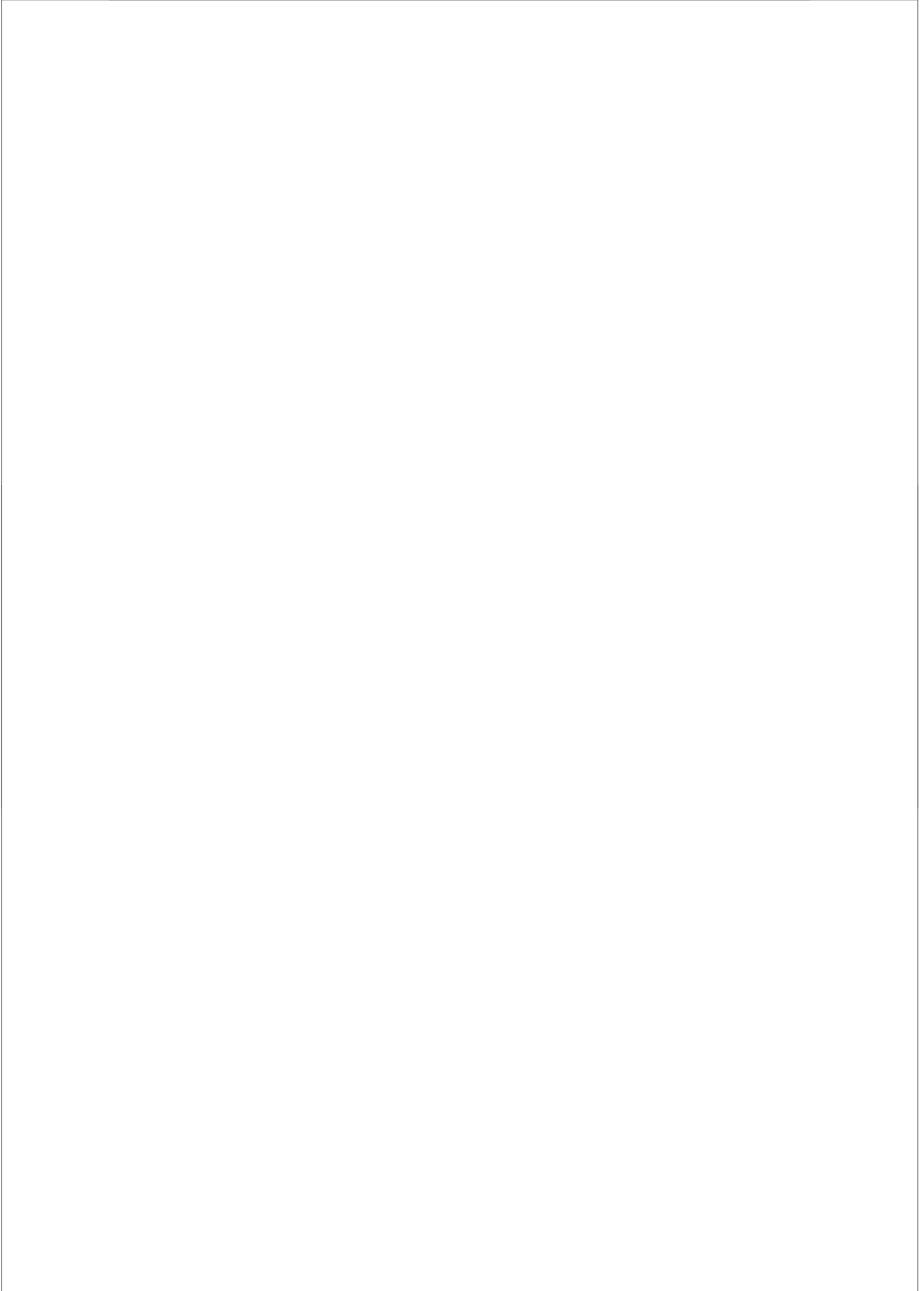

Chapter IV

Anaplasma phagocytophilum infection in horses in the Netherlands

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Introduction

Equine granulocytic anaplasmosis is a tick-borne disease caused by the obligate intracellular bacterium *Anaplasma phagocytophilum* (previously *Ehrlichia equi*), which can elicit febrile disease in animals and human beings (Dumler, Barbet et al. 2001). The disease has previously been referred to as equine granulocytic ehrlichiosis, and is transmitted in Europe by *Ixodes ricinus* ticks. Ticks of the *I. ricinus* complex also act as vectors in the spread of *Borrelia burgdorferi* from one animal to another, and co-infections of *A. phagocytophilum* and *B. burgdorferi* have been confirmed in horses (Chang, McDonough et al. 2000; Magnarelli, Ijdo et al. 2000). Equine granulocytic anaplasmosis was first described in the USA in 1969 (Gribble 1969), and has since been reported in other countries, including Switzerland, Sweden, France, Germany, Italy and the UK (Korbutiak and Schneiders 1994; Artursson, Gunnarsson et al. 1999; Bermann, Davoust et al. 2002; Von Loewenich, Stumpf et al. 2003; Alberti, Zobba et al. 2005).
Following an incubation period of approximately 10 days (Gribble 1969; Pusterla, Leutenegger et al. 1999; Pusterla, Chae et al. 2002), infected horses may experience subclinical disease or develop overt signs that include fever, depression, inappetence, a reluctance to move and distal limb oedema. The disease can be self-limiting when untreated, and clinical signs usually last from seven to 14 days (Gribble 1969). These signs, however, are not pathognomonic for the disease, and demonstration of granulocytic inclusions, either morulae or initial bodies, in Wright-Giemsa- or haematoxylin and eosin-stained blood smears can confirm a clinical diagnosis (Gribble 1969; Engvall and Egenvall 2002). These organisms can be found microscopically in peripheral blood only for a few days in the acute stage of the disease (Gribble 1969; Stannard, Gribble et al. 1969; Rikihisa 1991), and molecular techniques like PCR (Engvall, Pettersson et al. 1996; Pusterla, Huder et al. 1999) or serology (Van Andel, Magnarelli et al. 1998; Artursson, Gunnarsson et al. 1999) can be valuable tools in the confirmation of infection. This short communication describes the analysis of 61 blood samples obtained from horses with fever of unknown origin, for the presence of *A. phagocytophilum*, *B. burgdorferi*, *Babesia caballi* and *Theileria equi*.

Material and Methods

Blood samples from 61 horses, which had been admitted to the authors' clinic with fever of unknown origin between 2002 and 2005, were collected in EDTA tubes. Quick stain haematoxylin and eosin blood smears were made of buffy coat and analysed microscopically for granulocytic and erythrocytic inclusions. In addition, Wright-Giemsa stained blood smears were made and analysed at the veterinary diagnostic laboratory, Faculty of Veterinary Medicine, Utrecht

University. The remaining blood was stored at –20 °C, until DNA was extracted using a DNA extraction kit (Qiagen), according to the manufacturer's instructions. PCR products for the detection of *Anaplasma*, *Borrelia*, *Babesia* and *Theileria* species by reverse line blot (RLB) hybridisation were amplified in an automated thermocycler (Bio-Rad Laboratories) as described by Schouls and others (1999), Bekker and others (2002), Nijhof and others (2005). RLB hybridisation was performed as described by Nijhof and others (2005). Two additional and previously undescribed species-specific oligonucleotide probes for the detection of *B. caballi* and *T. equi* were incorporated in the RLB. They were deduced from gene sequences available from GenBank and synthesised by Isogen Life Science (table 1).

Table 1
Oligonucleotide probes used for the detection of Babesia caballi *and* Theileria equi

Oligonucleotide probe specificity	18S rRNA sequence (5' to 3')	GenBank accession number	Melting point (℃)
B. caballi	GTGTTTATCGCA-GACTTTTGT	AY309955, AY534883 and Z15104	52.4
T. equi	TTCGTTGACTGCG(CT)TTGG	AY150062, AY150063, AY1510064 and Z15105	56.9

Results and discussion

There were 10 stallions, 26 geldings and 25 mares of Dutch warmblood, Quarter horse, Friesian, Icelandic, Shetland pony, Belgian draft horse breeds in the study, ranging in age from one to 16 years. Equine granulocytic anaplasmosis was diagnosed in six horses of different breeds, aged between four and 15 years. Clinical signs consisted of lethargy, pyrexia (38.7 to 41.1 °C), oedema of the hind legs or all four legs, and partial or total anorexia (table 2). Haematology revealed a relatively low packed-cell volume (23 to 29 l/l, reference range

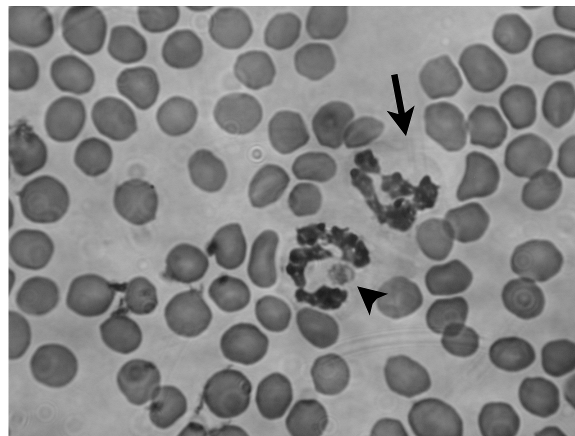

Fig. 1
Blood smear from horse 4 showing a neutrophil with cytoplasmic inclusions (arrowhead) of Anaplasma phagocytophilum *and one normal neutrophil (arrow)*

30 to 42 l/l), and in four of these horses a marked thrombocytopenia (22 to 26 x 10^9/l, reference range >100 x 10^9/l) was present (table 2). Blood smears revealed neutrophils with cytoplasmic inclusions (figure 1) consistent with *A. phagocytophilum* infection in five of the six horses, and PCR-RLB confirmed the diagnosis by showing positive results for *A. phagocytophilum* in these five horses plus one other. The clinical signs were most severe in a four-year-old Friesian gelding (horse 2), which showed ataxia, became recumbent and was euthanised on humane grounds due to the deterioration in its condition despite intravenous treatment with 7 mg/kg oxytetracycline every 24 hours. The other five horses showed mild clinical signs comprising pyrexia, lethargy, partial anorexia and oedema of the hind limbs. Clinical disease in these horses resolved without treatment.

Table 2
Clinical signs, haematology and PCR-reverse line blot (RLB) results for horses infected with Anaplasma phagocytophilum

Horse	Age (years)	Breed	PCV (l/l)	PLT (x 10^9/l)	Lethargy	T (℃)	Oedema	Ataxia	Anorexia	Blood smear result	PCR-RLB for *A. phagocytophilum*
1	10	Dutch warmblood	29	100	Yes	40.4	None	No	Partial	-	+
2	4	Friesian	28	344	Yes	41.1	All legs	Yes	Total	+	+
3	14	Dutch warmblood	23	22	Yes	38.7	Hindlegs	No	Partial	+	+
4	15	Dutch warmblood	24	26	Yes	40.6	Hindlegs	No	Partial	+	+
5	15	Dutch warmblood	25	23	Yes	41.0	Hindlegs	No	Partial	+	+
6	10	Dutch warmblood	29	22	Yes	41.0	Hindlegs	No	Partial	+	+

PCR-RLB and stained blood smears for all the horses were negative for all other tick-borne pathogens. It has been suggested that horses younger than three to four years of age generally experience less severe clinical disease (Gribble 1969; Madigan and Gribble 1987) when infected with *A. phagocytophilum*. However, in the present study, the youngest positive horse, which was four years of age, showed severe clinical signs and did not survive, whereas the other positive horses were aged between nine and 15 years and their infections resolved without treatment.
To confirm an infection with tick-borne pathogens in horses, molecular tests such as PCR in combination with RLB could improve the sensitivity as well as the specificity because, compared with stained blood smears, both live and

dead organisms, as well as intact and fragmented DNA of multiple parasites can be detected in one test (Chang, Novosol et al. 2000). In the present study, however, five of the 61 horses were diagnosed as being positive for *A. phagocytophilum* using a haematoxylin and eosin-stained as well as a Wright-Giemsa stained blood smear, and only one further positive horse was identified using the more sensitive and specific, but also expensive and time consuming PCR-RLB. Stained blood smears and PCR-RLB were also used to detect *B. caballi* and *T. equi*, but for the detection of *B. burgdorferi* only PCR-RLB was used. No other tick-borne pathogens (except *A. phagocytophilum*) were found in any of the blood samples from the 61 horses.

Conclusions

Clinical anaplasmosis in horses is probably still underdiagnosed in the Netherlands as most horses recover spontaneously and clinical signs are similar to those caused by infections with other pathogens such as *B. burgdorferi*, *B. caballi*, *T. equi*, equine herpesvirus, equine infectious anaemia virus, equine arteritis virus and Leptospiraceae. In the authors' experience, microscopic interpretation of a buffy coat haematoxylin and eosin-stained blood smear is a sensitive and practical diagnostic tool for veterinarians considering possible infection with *A. phagocytophilum* in horses with pyrexia, but some cases may require PCR testing for diagnosis.

Acknowledgements

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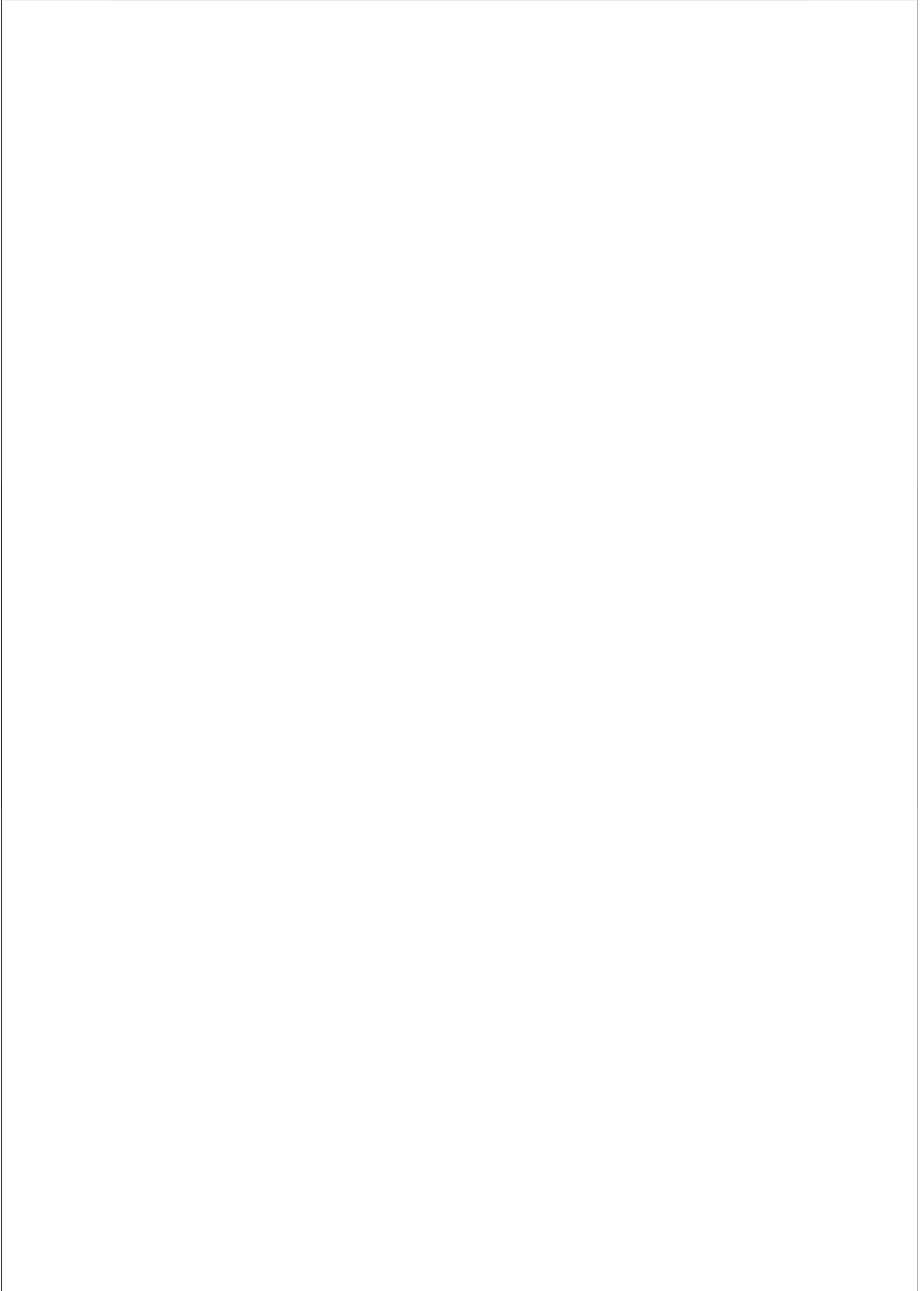

Chapter V

Prevalence of the causative agents of equine piroplasmosis in the South West of the Netherlands and the identification of two autochtonous clinical *Theileria equi* infections

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Abstract

Equine piroplasmosis (EP) has not been considered indigenous in the Netherlands. However, following the detection of an apparently indigenous subclinical *Babesia caballi* infection in a horse on Schouwen- Duiveland (an island in the Zeeland Province), a survey was undertaken between May and September 2010 to assess the prevalence of the causative agents of EP in the South-West of the Netherlands. Blood samples from 300 randomly selected horses were tested for specific antibodies against *Theileria equi* and *B. caballi* using an indirect fluorescence antibody test (IFAT), and for parasite DNA using a specific polymerase chain reaction combined with reverse line blotting (PCR-RLB). Twelve of the horses (4%) were seropositive for EP. Of these, nine (75%) were positive (titre ≧ 1:160) for *B. caballi* alone and three (25%) were also positive for *T. equi*. PCR-RLB detected *T. equi* DNA in five horses (1.6%), two of which were seronegative. Four (1.3%) of the positive horses (three positive for *T. equi* and one for both *B. caballi* and *T. equi*) were considered truly indigenous. During the study, two indigenous ponies from a farm situated outside the sampling area were diagnosed with acute clinical piroplasmosis characterised by severe anaemia and pyrexia. Blood smears showed *T. equi*–like inclusions in red blood cells, and *T. equi* infection was confirmed in both ponies by PCR-RLB. The initial subclinical *B. caballi* infection, the survey results and the two acute clinical EP cases confirmed the autochthonous transmission of *B. caballi* and *T. equi* infections in the Netherlands.

Introduction

Equine piroplasmosis (EP) is an important tick-borne protozoan disease that poses a serious threat to the horse industry and has important implications for the international movement of horses (Friedhoff, Tenter et al. 1990). The disease is caused by *B. caballi* and/or *T. equi* and is endemic in many tropical and subtropical areas (de Waal 1992). Since EP can occur in any region or environment where horses are exposed to vector ticks, horses in countries with a moderate climate can also be affected (Mehlhorn and Schein 1998). Relocation of carrier horses and infected ticks by international transport is a potential means of spreading the infection. The expansion of permissive tick vectors to hitherto non-endemic countries and the changes in habitat for both ticks and wildlife species as a result of climate change are other promoting factors (Sreter, Szell et al. 2005). Ticks capable of acting as vectors for *B. caballi* and *T. equi* include those belonging to the genera *Dermacentor*, *Hyalomma* and *Rhipicephalus* (Uilenberg 2006). According to the World Organisation for Animal Health (OIE) Manual (WAHID 2012), the Netherlands is considered free of autochthonous EP. However, the recent discovery of resident *Dermacentor reticulatus* populations (Nijhof, Bodaan et al. 2007), combined with a subclinical *B. caballi* infection in a horse that had never left the Netherlands, prompted investigation of the actual prevalence of *B. caballi* and *T. equi*. The aims of this study were to determine the prevalence of *T. equi* and *B. caballi* infections in horses in the area where the initial subclinical *B. caballi* infection occurred. In addition, we report the first confirmed acute autochthonous *T. equi* infections in indigenous ponies in the Netherlands.

Materials and methods

Case that prompted the study

The subclinical case that prompted the present study involved a 12-year-old Dutch Warmblood gelding presented to a referral clinic (DAP Bodegraven) for treatment of a massive tick infestation with extensive skin swelling following a ride in a forest on Schouwen-Duiveland (an island in the Province of Zeeland) in July 2009. The skin swelling was treated with a combination of a non-steroidal anti-inflammatory drug (flunixin meglumine, 1 mg/kg body weight [BW] intravenously [IV]) and an antibiotic (oxytetracycline, 6.6 mg/kg BW, every 12 h IV for 7 days). The infestation of the limbs and ventral abdomen was so extensive that an acaricide (fipronil, Frontline, Merial) was sprayed on the affected areas. Most ticks were identified as engorged *Ixodes ricinus* nymphs, but there were simply too many ticks to identify all of them. Thus, species other than *I. ricinus*, which is not considered a vector of EP, may have been present. On presentation, a Giemsa-stained blood smear was negative for inclusions, and an indirect

fluorescence antibody test (IFAT) was negative for *B. caballi* IgG. However, a polymerase chain reaction combined with reverse line blotting (PCR-RLB) was positive for *B. caballi* DNA. Blood taken from the horse 6 weeks later was negative for *B. caballi* parasites in both the thin blood smear and PCR-RLB but was positive for *B. caballi* antibodies in the IFAT (table 1). In view of this subclinical case, the Dutch Ministry of Economic Affairs, Agriculture and Innovation) supported a seroprevalence survey in Zeeland to evaluate the situation with regard to EP. During the course of the investigation, one of the authors was consulted about two indigenous ponies in the south of the country that appeared to be suffering from acute clinical EP. At present, little information is available on the epidemiology of EP in North Western Europe.

Sample collection

To detect a 1% incidence of infection with a certainty of 95% in a population estimated at 5000–10,000 horses living in the 'high risk area' where the subclinical EP had been diagnosed, it was calculated that a minimum of 300 animals needed to be tested (Win Episcope, Risk-based sampling) (figure 1). For this we used the formula:

$n = [1 - (\alpha)^{1/D}][N - (D - 1)/2]$

where n = required sample size; α = 1 - confidence level (0.05); D = estimated minimum number of diseased animals in population; N = population size) (Dohoo, Martin et al. 2010).
Between May and August 2010, jugular vein blood samples were collected into EDTA and serum tubes from 300 horses. Apparently healthy horses that were either privately owned or owned by a riding school were selected and sampled based on (1) the owner's willingness to cooperate, (2) the horse having lived in the area for at least 1 year and (3) the horse having access to pasture and/or being used for outdoor recreation. Blood sampling of client-owned horses for diagnostic purposes is not considered animal experimentation under Dutch law; this was confirmed by Utrecht University's Animal Ethical Committee for this specific study.
Samples were placed in and kept on ice immediately after collection and subsequently stored at 4 C for a maximum of 18 h before processing. After haematological screening, the remaining EDTA blood was stored at -20 ℃ until DNA extraction was undertaken. Serum was stored at -20 ℃ until the serological tests were performed. Seven stallions, 147 geldings and 146 mares ranging in age from 12 weeks to 25 years and representing various breeds (Dutch Warmblood, Quarter horse, Paint horse, Merens, Friesian, Icelandic, Fjord, Shetland pony, Welsh pony, Falabella, Standardbred, Thoroughbred, Belgian Draft, Irish Cob, Arab) were included in the study. Since it is often difficult to establish whether horses are truly autochthonous and have not travelled to another

country at some point in their lives, it was decided to only attempt to trace the full background and movements of horses that were EP positive.

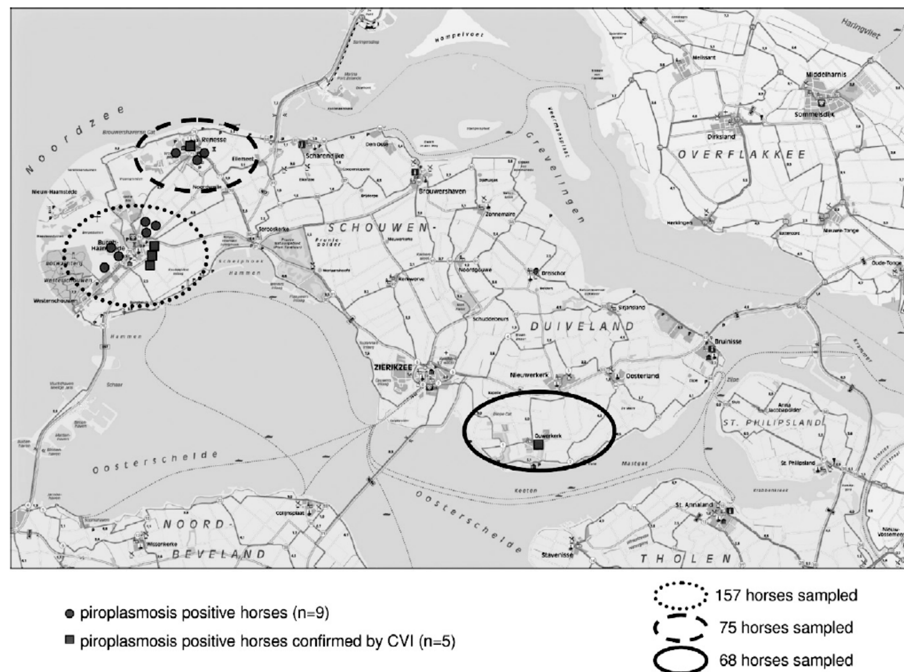

Fig. 1
A map of Schouwen-Duivenland (Zeeland, the Netherlands) including the locations of both sampled and piroplasma positive horses

Haematology

Haematology was used to determine whether (in the group of 300 apparently healthy horses included in the survey) positivity for piroplasmosis had an influence on packed cell volume, white blood cell count, or thrombocyte count. All 300 EDTA samples were therefore analysed on the day of sampling using a Medonic Haematology Analyzer.

IFAT

Samples were screened for antibodies against *T. equi* and *B. caballi* using a standard IFAT protocol, as described by Madden and Holbrook (1968). Bound equine IgG antibodies were detected using a fluorescein isothiocyanate conjugated rabbit anti-horse secondary antibody (RAHo/IgG(H + L)FITC; Nordic Immunology). All samples were screened at a starting dilution of 1:80 in phosphate-buffered saline (pH 7.2), as described in the manufacturer's protocol. Positive samples were subsequently analysed to determine the titre. Antibody titres were categorised into three groups: 1:80, 1:160 and >1:320. Any sample

showing fluorescence at a dilution of 1:160 was considered positive. Positive and negative controls were included in each run.

DNA extraction

DNA was extracted from EDTA blood using the QIAamp DNA mini kit (Qiagen) as described in the manufacturer's protocol. PCR amplification and reverse line blot (RLB) hybridisation. The primers RLB-F2 (5'-GAC ACA GGG AGG TAG TGA CAA G-3') and RLB-R2 (5'-biotin-CTA AGA ATT TCA CCT CTG ACA GT-3') were used to amplify the V4 hypervariable region of the piroplasmid 18S rRNA gene, and the PCR products were subsequently subjected to RLB hybridisation as described previously (Nijhof, Pillay et al. 2005). Positive and negative controls were included in each run.

Clinical EP cases

Two pregnant ponies (A and B; aged 18 and 20 years, respectively), housed together and acquired 2 and 5 months previously (from different parts of the Netherlands) for the purpose of commercial plasma collection, were presented to a local practitioner with signs of lethargy, anorexia and pyrexia. Blood was collected in EDTA and serum tubes. Because the acute signs suggested EP, the samples were analysed for the presence of *T. equi* and *B. caballi* by microscopic examination of stained thin blood smears and by serology (IFAT) and molecular testing (PCRRLB).
Blood collected (and stored with EDTA at 4 ℃) from pony B approximately 7 days prior to clinical signs was also tested. Once piroplasmosis was confirmed, the ponies received a blood transfusion, and treatment with imidocarb dipropionate (4 mg/kg BW every 72 h intramuscularly) was recommended.

Statistical evaluation

All data are given as absolute numbers, as percentages or as means ± standard deviations. Statistical evaluation of the blood parameters was performed using a two sample t test (R, A Language and Environment for Statistical Computing, R Development Core Team, R Foundation for Statistical Computing, 2010). $P < 0.05$ was considered to be statistically significant.

Results

Survey

Of the 300 horses included in the survey, 12 (4%) tested seropositive for EP (table 2). Of these, nine (3%) were seropositive (titre ≧ 1:160) for *B. caballi* and three (1%) were positive for both *B. caballi* and *T. equi*. In addition five horses (1.6%) tested positive for *T. equi* DNA, although only three had *T. equi* antibody titres (table 2).

Table 2
Results of an indirect fluorescence antibody test (IFAT) for Theileria equi *and* Babesia caballi *(positive if titre ≧ 1:160) and/or polymerase chain reaction combined with reverse line blotting (PCR-RLB;* – negative; + positive) *for* Theileria equi *or* Babesia caballi *for the 14 horses that tested positive for piroplasmosis in a survey of 300 randomly selected horses in the South-West of the Netherlands*

Horse number	IFAT *T. equi*	IFAT *B. caballi*	PCR-RLB	Age (years)	Breed	Origin of the horse according information of the owner
5[a]	1:640	1:160	+ (*T. equi*)	11	Merens	Visited France
7	–	–	+ (T. equi)	13	Merens	Visited France
10[a]	1:5120	1:160	+ (T. equi)	20	Thoroughbred	Imported from Russia 16 years ago
12[a]	–	1:160	–	16	Hackney	the Netherlands, no travel last 9 years
17[a]	1:640	1:160	+ (T. equi)	8	Pony unknown	the Netherlands, no travel
24	–	1:160	–	18	Dutch Warmblood	the Netherlands, no clear history
37	–	1:160	–	16	Dutch Warmblood	the Netherlands, no clear history
61	–	1:160	–	25	Dutch Warmblood	the Netherlands, no clear history
92	–	1:160	–	16	Dutch Warmblood	the Netherlands, no clear history
111	–	1:160	–	15	Shetland pony	the Netherlands, no travel last 9 years
114	–	–	+ (T. equi)	25	Dutch Warmblood	the Netherlands, no travel last 19 years
142[a]	–	1:160	–	2	Shetland pony	the Netherlands, no travel
210	–	1:160	–	11	Belgian draft horse	the Netherlands, no travel
236	–	1:160-320	–	5	Belgian draft horse	the Netherlands, no travel

[a] *Also tested by the Central Veterinary Institute Lelystad, the National Reference Laboratory, and reported to the OIE by the Chief Veterinary Officer of the Netherlands on 16th February 2011, together with the two clinical pony cases (Anonymous 2011).*

B. caballi DNA was not detected by PCR-RLB, but one horse (horse 123) was positive for the *Babesia* catch-all genus probe used in conjunction with a *B. bovis* probe (not included in table 2). In samples that were positive for EP, the platelet count was lower than in the negative samples (P = 0.025; table 3).

Table 3
Haematological parameters for 300 randomly selected horses divided by piroplasma status (positive or negative horses). Values are shown as means (± standard deviation). PCV, packed cell volume; WBC, white blood cell count, PLT, thrombocyte count

	All horses in survey n = 300	Piroplasmosis positive (IFAT or PCR-RLB)[a]; n = 14	Piroplasmosis negative (IFAT and PCR-RLB); n = 286	Reference values[b]
PCV (l/l)	0.33 ± 0.05	0.36 ± 0.02	0.33 ± 0.00	0.32 - 0.50
WBC (G/l)	8.2 ± 2.1	8.2 ± 0.6	8.2 ± 0.1	7 - 10
PLT (G/l)	94 ± 33	79 ± 8[c]	95 ± 2	75 - 300

[a] *IFAT, indirect fluorescence antibody test; PCR-RLB, polymerase chain reaction combined with reverse line blotting.*
[b] *Reference values from (Sellon and Wise 2010).*
[c] *A statistically significant reduction in thrombocyte counts in piroplasmosis-positive horses compared to piroplasmosis-negative horses (P = 0.025)*

Clinical cases

Haematological assessment of the two ponies (A and B) that presented with signs suggesting acute piroplasmosis revealed marked anaemia in both cases and a marked thrombocytopenia in pony A (table 1). Microscopic examination of thin blood smears revealed round to pear-shaped bodies and an occasional 'Maltese cross' inside the red blood cells, indicating *T. equi* organisms (fig-ure 2). No antibodies to *T. equi* were detected and PCR-RLB was negative for *T. equi* in blood collected from pony B 1 week before clinical signs were apparent. However, during the period in which the ponies showed clinical signs, both *T. equi* antibodies and DNA (via PCR-RLB) were detected in blood from both animals (table 1).

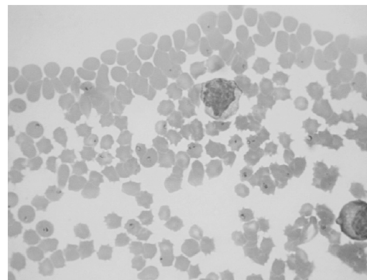

Figure 2
Theileria equi *parasites in red blood cells as visualised by Giemsa staining of a thin blood smear from pony A*

Table 1
Haematological results (reference values in column heading); packed cell volume (PCV), white blood cell count (WBC) and thrombocyte count (PLT). Blood smear results; Theileria equi *or* Babesia caballi *inclusions visible in red blood cells. Serological results; indirect fluorescence antibody test (IFAT) for* Theileria equi *and* Babesia caballi *(positive if titre ≧ 1:160), and molecular (polymerase chain reaction combined with reverse line blotting [PCR-RLB]: - negative; + positive) results of blood samples collected from a subclinical* Babesia caballi *- infected horse (KWPN) and two* Theileria equi *- infected ponies (pony A and pony B) with clinical symptoms of equine piroplasmosis. All three horses were indigenous to the Netherlands*

Horse	Date	PCV (0.32-0.50 l/l)	WBC (7-12 G/l)	PLT (75-300 G/l)	Blood smear	IFAT	PCR-RLB
KWPN	June 2009	0.39	6.8	74	–	–	+ (*B. caballi*)
	July 2009	0.39	5.5	50	–	1:160	–
Pony A	July 2010	0.11	24.5	39	+ (*T. equi*)	1:640	+ (*T. equi*)
Pony B	July 2010	0.27	6.6	222	–	–	–
	July 2010	0.15	11.7	108	+ (*T. equi*)	1:320	+ (*T. equi*)

Discussion

Blood analysis and serological and molecular testing demonstrated the presence of *T. equi* in two indigenous ponies and *B. caballi* in one indigenous Dutch Warmblood horse. The subclinical course of infection in the latter animal may have been due to the immediate prophylactic administration of oxytetracycline in an attempt to curb the potential sequelae of the severe tick infestation; oxytetracycline has been shown to exhibit an inhibitory effect on *T. equi* sporozoites cultured in vitro and on *Babesia divergens* replication in cattle (Taylor, Elliott et al. 1986) if administered early during infection (Zweygarth, Ahmed et al. 1984).
With respect to the acute *T. equi* cases described in this study, the owner stated that the two ponies had been on the premises for only 2 months (pony B) and 5 months (pony A), respectively, having been purchased from different areas of the Netherlands (Provinces of Gelderland and Friesland). Both ponies showed clinical signs suggestive of acute piroplasmosis. Importantly, although specific antibodies were not detected in pony B at first presentation, a *T. equi* titre of 1:320 was detected by IFAT 7 days later, indicating that pony B had acquired the infection at its new location in the Netherlands. Pony A showed clinical signs indicative of piroplasmosis 1 week before pony B. Unfortunately, no initial blood sample was available from pony A. Both ponies were pregnant and used for commercial plasma collection so it is possible that the immunosuppressant effects of pregnancy or the stress of pregnancy and/or plasma collection

activated a dormant infection leading to clinical disease. In this respect, it cannot be excluded that pony A was a *T. equi* carrier and experienced reactivation, after which it became a reservoir for transmission to pony B, either via a tick vector or iatrogenically (in this case as a result of blood collection procedures). The latter mode of transmission is unlikely, however, because new disposable catheters were used for each horse as a standard procedure. It is therefore possible that both ponies were infected via *D. reticulatus* ticks present in the surrounding environment. *Dermacentor reticulatus* ticks are distributed in Europe by animals travelling to and from endemic areas, with the majority originating from Southern Europe, where *D. reticulatus* is well established (Estrada-Pena, Quiez et al. 2004). In theory, several wildlife species including migrating birds could also play a role in tick distribution.

The results of our survey show that although piroplasma infections are found in horses imported into the Netherlands, the prevalence of seropositive or DNA positive horses in both the indigenous and imported horse populations is still relatively low. Most studies on the prevalence of *T. equi* and *B. caballi* have been conducted in Southern Europe where the infection is endemic (Camacho, Guitian et al. 2005; Leblond, Pradier et al. 2005; Kouam, Kantzoura et al. 2010; Moretti, Mangili et al. 2010). Little information is available on the prevalence and possible emergence of EP in North Western Europe, with published data only available from Germany and Switzerland where seroprevalences of 5.6% and 4.4% *T. equi* and 1.2% and 1.5% *B. caballi*, respectively, have been reported (Boch 1985; Sigg, Gerber et al. 2010). Several clinical cases of EP have been recorded in Belgium (Mantran, Votion et al. 2004) but no information is available on the seroprevalence of EP in that country's horse population. The overall seroprevalence in the present study is of a similar magnitude to data reported in Switzerland and Germany. Furthermore, the seroprevalence for *B. caballi* in the current study was higher than for *T. equi*, which is also in line with both other reports.

While antibody titres indicate past infections of unknown age, positive PCR-RLB results demonstrate current active parasite infestation. Positive PCR-RLB results for *T. equi* in the peripheral blood of two indigenous and three imported horses are reasons for concern with regard to the spread of this pathogen in the Netherlands; horses are considered to be the primary reservoir for *T. equi* (Allsopp, Lewis et al. 2007; Ueti, Palmer et al. 2008) and established populations of *D. reticulatus* are now present in the Netherlands (Nijhof, Bodaan et al. 2007). Two of the five horses that tested positive for *T. equi* in the PCR-RLB did not have an antibody titre when examined by IFAT, and it is therefore likely that they had been infected relatively recently. An interesting finding in this survey was the detection of *B. bovis* DNA via PCR-RLB performed on blood collected from horse 123. This particular horse was apparently healthy, and its haematological parameters were within the normal range. Even though it has been reported once previously (Criado, Martinez et al. 2006), the significance of *B. bovis* DNA in a horse is unclear.

Clinicopathological abnormalities in horses with a *T. equi* and/or a *B. caballi* infection may include a reduced red blood cell count, platelet count and haemoglobin concentration (de Waal 1992). The significantly lower platelet count in the piroplasmosis positive horses in the present study is therefore consistent with (sub)clinical piroplasmosis.

Conclusions

This study confirmed that autochthonous EP occurs in the Netherlands, albeit sporadically. With the apparent establishment of indigenous *D. reticulatus* populations (Nijhof, Bodaan et al. 2007) and unrestricted importation of horses from piroplasmosis endemic areas, the chance of encountering clinical EP cases in the Netherlands is likely to increase and veterinarians should therefore consider this disease in the differential diagnosis for anaemic horses, even if the animals have never left the country.

Acknowledgements

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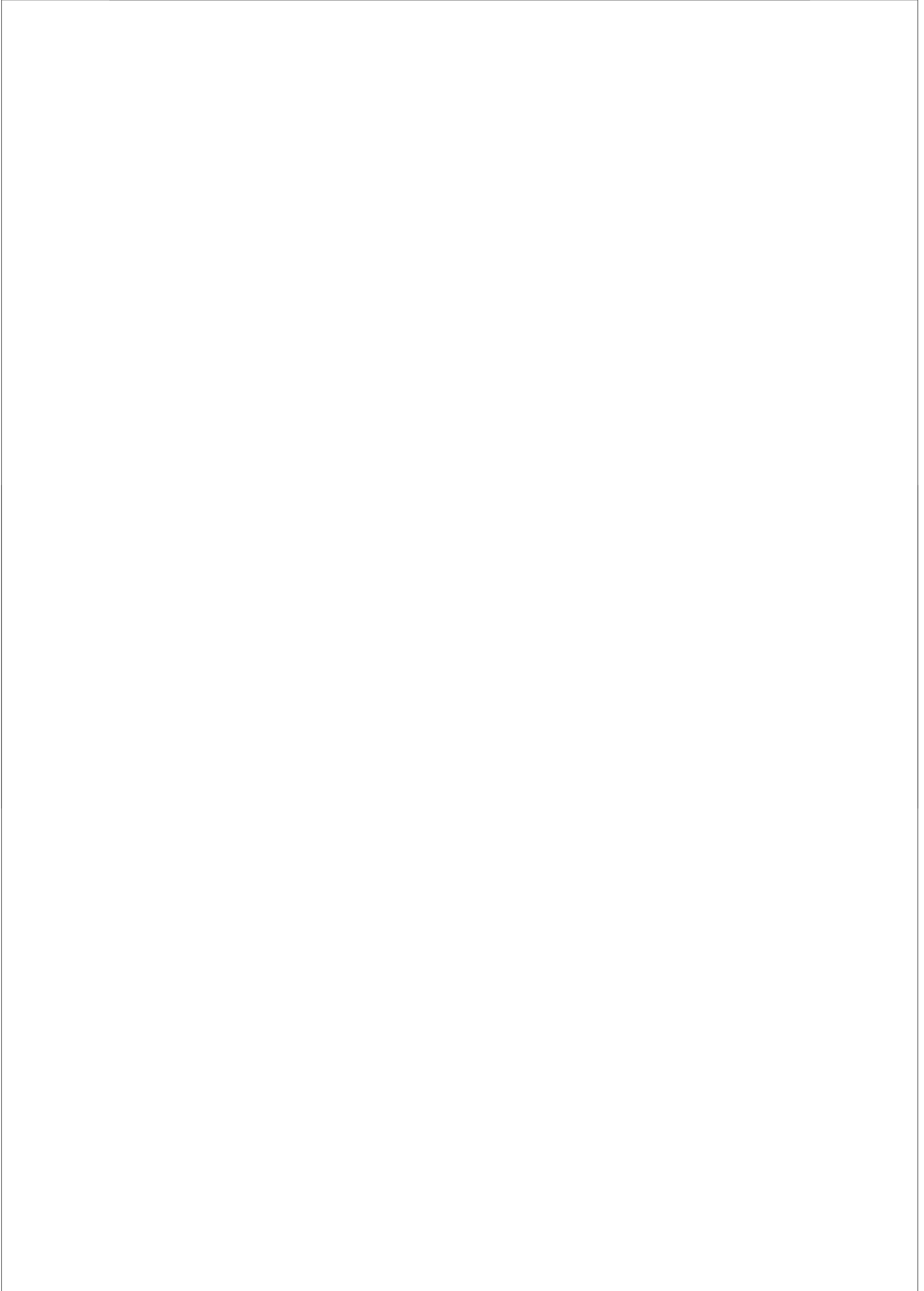

Chapter VI

Classification of ticks collected from horses in the Netherlands and identification of the (zoonotic) agents they contain

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Submitted

Abstract

This study determined which hard tick species (*Ixodidae*) are presently found on domestic horses in the Netherlands and which potential pathogens they carried. In the period 2008-2009, 130 ticks were collected, classified and screened for the presence of DNA from specific tick-borne pathogens using PCR-RLB. The numbers of ticks of the various species found were: 68 *Ixodes ricinus*, 58 *Ixodes* spp. (57 nymphs and 1 larva), 2 *Dermacentor reticulatus* and 2 *Hyalomma marginatum*. DNA from *Borrelia valaisiana* was detected in 49% of these ticks, *Borrelia afzelii* in 22%, *Borrelia burgdorferi* sensu stricto (s.s.) and *Borrelia garinii* in 3% and 2%, respectively. *Rickettsia helvetica* was detected in 9% of examined ticks, *Anaplasma phagocytophilum* in 1.5%, *Babesia venatorum* in 4%, and *Babesia caballi* and *Theileria equi* in 1.5 and 3%, respectively. There were considerable regional differences suggesting focal distribution of these potential pathogens.

Introduction

Ticks play a significant role as vectors of several bacterial, protozoal and viral pathogens of worldwide medical, veterinary and economic importance (Jongejan and Uilenberg 2004; de la Fuente, Estrada-Pena et al. 2008). Among the various species of hard ticks endemic to Western Europe, *I. ricinus* is the most frequently found (Stanek, Gray et al. 2004). In the Netherlands, *I. ricinus* is the predominant species recovered from domestic animals (Nijhof, Bodaan et al. 2007) and is the most important vector of a number of emerging pathogens of veterinary and medical significance, including *Borrelia burgdorferi* senso lato (s.l.), *Babesia* spp., and *Ehrlichia-Anaplasma* spp. (Estrada-Pena and Jongejan 1999). Like other Ixodid ticks, *I. ricinus*' developmental cycle includes four stages (egg, larva, nymph, adult) and takes 2-3 years to complete (Sonenshine 1991). At each stage, the tick needs a blood meal in order to develop to the next stage and therefore has to find a new host. Consequently, ticks are an important means of transmitting blood or skin borne pathogens between hosts. Adult ticks feed mainly on large mammals such as cattle, sheep and deer, whereas larvae and nymphs usually feed on small- and medium-sized vertebrates (Sonenshine 1991).

Although studies on the prevalence of tick-borne pathogens in tick vectors (Ferquel, Garnier et al. 2006; Wielinga, Gaasenbeek et al. 2006; Hartelt, Pluta et al. 2008; Wielinga, Fonville et al. 2009; Reye, Hubschen et al. 2010; Smith, Ballantyne et al. 2012), and vertebrate hosts (Nijhof, Bodaan et al. 2007; Passamonti, Veronesi et al. 2010; Lommano, Bertaiola et al. 2012) in the Netherlands and other North-western European countries have been performed, there is little data on the classification and the frequency of the various tick species that infest horses or of the prevalence of significant pathogens in these ticks.

Horse numbers have increased in the Netherlands over the last 2 decades, with the horse becoming an ever-more popular recreational- or "companion animal". In this respect, horses are considered to share their owners' biotope, at least with respect to tick exposure, even more than dogs which tend to have more intense contact with low growing shrubs. In this respect, ticks from horses may be regarded as excellent sentinels for tick-borne infections of zoonotic significance. In recent years, there has been evidence of the spread of tick species into areas where they were previously unable to survive year-round, probably as a result of climate change; this spread of tick species into new areas has been accompanied by a parallel spread in tick-borne pathogens across areas of Western Europe previously considered free of endemic disease (Nijhof, Bodaan et al. 2007; Estrada-Pena, Ayllon et al. 2012).

This study aimed to investigate the distribution and types of ticks specifically collected from horses in the Netherlands and to determine the proportion of these ticks that carried not only the known equine pathogens *B. burgdorferi* s.l.

spp., *T. equi*, *B. caballi*, and *A. phagocytophilum*, but also other agents of potential medical/veterinary importance that have been identified previously in Europe, including *Babesia divergens*, *Babesia bovis*, *Babesia microti*, *Babesia venatorum*, *Babesia canis*, *Rickettsia helvetica*, *Ehrlichia schotti*, and *Anaplasma marginale*.

Materials and methods

Study design and tick collection

Equine practitioners in the Netherlands were contacted early in 2008 by the Utrecht Centre for Tick borne Diseases (UCTD) with a request to submit ticks recovered from horses. Of the ticks submitted between May 2008 and September 2009, a total of 130 from 56 horses belonging to 50 different owners were identified to species level, together with developmental stage and gender, using standard keys (Estrada-Pena, Bouattour et al. 2004). The ticks were then fixed and stored in 70% ethanol prior to subsequent screening for specific pathogens using polymerase chain reaction-reverse line blot (PCR-RLB). Forty-seven of the 56 horses were also included in a prospective study examining clinical and diagnostic aspects of *B. burgdorferi* and *A. phagocytophilum* infections following a known tick-bite (Chapter VII).

Classification and determination of sex and developmental stage

Ticks were classified using standard keys (Estrada-Pena, Bouattour et al. 2004) and categorised as larvae, nymphs, adult females or adult males.

DNA extraction and PCR-RLB for specific pathogen detection

DNA was extracted from the ticks using the Nucleospin Tissue kit (Macherey-Nagel, Düren, Germany) following the manufacturer's instructions for the extraction and purification of genomic DNA from insects. Previously validated PCR primer pairs for the detection of *Borrelia*, *Anaplasma*, *Ehrlichia*, *Rickettsia*, *Theileria* and *Babesia* DNA were used to detect the major groups of pathogens via a PCR-assay using an automated thermocycler (Bio-Rad Laboratories, Veenendaal, the Netherlands) as described previously (Schouls, Van De Pol et al. 1999; Bekker, de Vos et al. 2002; Nijhof, Pillay et al. 2005). Subsequently, the PCR products generated were applied to an RLB hybridisation blot prepared with specific probes for the agents *B. burgdorferi* s.l. (four genospecies), *T. equi*, *B. caballi*, *A. phagocytophilum*, *B. divergens*, *B. bovis*, *B. microti*, *B. venatorum*, *B. canis*, *R. helvetica*, *E. schotti* and *A. marginale*, as described by Nijhof et al. (2005). Positive control DNA for each specified agent was included in each test run.

Results

Tick species, developmental stages and gender

Of the 130 ticks examined, 68 were classified as adult *I. ricinus* (63 female and 5 male), 58 were immature *Ixodes* spp (57 nymphs and 1 larva), 2 were classified as adult female *D. reticulatus*, and 2 as adult female *H. marginatum*. All ticks were further processed for detection of DNA for the pathogens of interest.

Table 1
The identity and incidence of specific pathogens for which DNA was detected in 130 ticks (adults or nymphs) by PCR-RLB analysis

	Nymph	Adult female	Adult male
Babesia caballi	1	1	0
Babesia canis	0	0	0
Babesia divergens	4	2	0
Babesia microti	0	1	0
Babesia venatorum	3	2	0
Anaplasma phagocytophilum	0	2	0
Ehrlichia schotti	3	10	1
Borrelia afzelii	15	13	1
Borrelia burgdorferi sensu stricto	2	2	0
Borrelia garinii	0	3	0
Borrelia valaisiana	31	33	0
Rickettsia helvetica	4	8	0
Theileria equi	2	2	0
Theileria equi like	5	1	0
Total	70	80	2

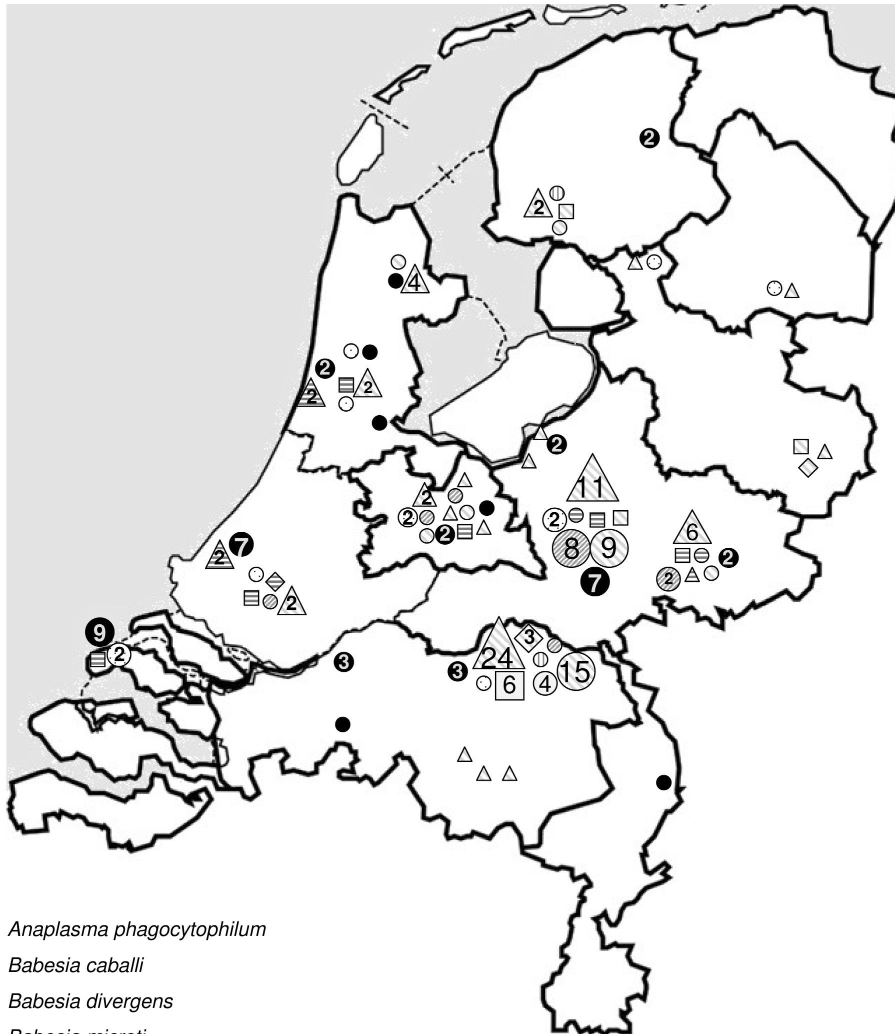
A total of 45 of the 130 ticks were negative for DNA of the above agents and 28 ticks had co-infections with 2 or more *Borrelia* species:
B. valaisiana and *B. afzelii (n = 23)*
B. burgdorferi s.s. and *B. garinii* (n = 1)
B. valaisiana, *B. afzelii* and *B. garinii* (n = 1)
B.*valaisiana*, B. afzelii and *B. burgdorferi* s.s. (n = 3)

Detection of DNA for tick-borne pathogens using PCR-RLB

The results of the PCR-RLB are summarised in table 1. DNA from the pathogens of interest was detected in 95 (73%) of the 130 ticks examined. *Borrelia* sp. were the most commonly detected pathogens and were recovered form 72 ticks of which 28 were co-infected with 2 or more species (table 1). The incidence of pathogen DNA was similar between adult ticks and nymphs. The geographical distribution of the various pathogens is depicted in figure 1. The distribution of pathogens differed between regions, most strikingly for *T. equi* (and the *T. equi*-like pathogens), which were detected in only one area in the south of the country.

Discussion

Following a request to Dutch equine practitioners early in 2008 to submit ticks collected from horses, a total of 130 ticks were submitted by the end of 2009; these were classified and subjected to PCR-RLB for detection of potential pathogens. The majority of ticks were classified as *Ixodes* spp, as was the case for previous studies of ticks collected from other (non-equine) host species in this part of Europe (Nijhof, Bodaan et al. 2007; Pavlidou, Gerou et al. 2008; Jaenson, Jaenson et al. 2012). That 40% of collected ticks were nymphs was surprising, given that nymphs (and larvae) are generally considered to feed primarily on small and medium sized mammalian hosts (Sonenshine 1991). This suggests either that horses are actually a preferred host for *Ixodes* nymphs, or that suitable small mammal hosts were insufficiently available in the habitat where these horses acquired the ticks. The finding of two adult *D. reticulatus* is in line with recent reports that this tick species has become established in the Netherlands (Nijhof, Bodaan et al. 2007). The two adult specimens of *H. marginatum* are also noteworthy; they were recovered from horses living more than 100 Km apart from each other along the eastern border of the Netherlands. *Hyalomma* spp are regarded as non-indigenous to Western Europe, so whether they were incidental drop-offs from migrating birds or early signs of establishment of a permanent population remains to be seen. Only the larval and nymph stages of *Hyalomma* feed on birds, therefore the fact that the *Hyalomma* ticks recovered were adults suggests that the weather conditions along the eastern border of the Netherlands allowed the moulting of *Hyalomma*. Establishment of this tick species in virgin areas could potentially have serious consequences given that it is a vector of Crimean-Congo haemorrhagic fever virus (Zeller, Cornet et al. 1994), an important pathogen in man, and probably also of West Nile virus, a pathogen of clinical importance in both man and horses (Chevalier, de la Rocque et al. 2004; Formosinho and Santos-Silva 2006).



Anaplasma phagocytophilum
Babesia caballi
Babesia divergens
Babesia microti
Babesia venatorum
Borrelia afzelii
Borrelia garinii
Borrelia sensu-stricto
Borrelia valaisiana
Ehrlichia schotti
Ricketsia helvetica
Theileria equi
Theileria equi-like
no specific DNA detected

Fig. 1
Geographic distribution of the number of ticks with DNA from specific agents per region or location of collection (if no number is indicated it is 1)

The geographic distribution of the submitted ticks is depicted in figure 1. The clustering, however, is most likely in part a consequence of sampling bias: as a result of the voluntary study design, it is likely that some equine practitioners were simply more active in collecting and submitting ticks than others. The distribution of DNA from specific pathogens in the ticks also indicates regional differences in the distribution of these agents. This underlines the complex dynamics of ticks and the complicated dynamics and epidemiology of tick-borne infections.
The results for the incidence of *Borrelia*-DNA detection for 120 of the 130 ticks reported in the current article are discussed in Chapter VII (horse and tick infection with *Borrelia* and *A. phagocytophilum*); the 10 extra ticks do not alter the general picture that *B. valaisiana* and *B. afzelii* were the most prevalent species and *B. burgdorferi* s.s. much less common. The potential consequences in terms of clinical borreliosis are discussed in Chapter VII, as are the implications of detecting *A. phagocytophilum* in two *I. ricinus* ticks (1.5%).
The recovery from the ticks of DNA from several *Babesia* spp (*B. venatorum* (B EU1), *B divergens*, *B. microti* and *B. caballi*) and *R. helvetica* is interesting because all have been reported previously in the Netherlands in ticks collected from the vegetation (Wielinga, Fonville et al. 2009) and dogs (Nijhof, Bodaan et al. 2007). Moreover, all are reported to be pathogenic to man. In particular, *B.venatorum*, *B. divergens* and *B. microti* are capable of causing human babesiosis (Herwaldt, Caccio et al. 2003; Gray 2006). Indeed, most documented human cases of babesiosis are caused by *B. divergens* (Vannier and Krause 2009), a pathogen that was present in 6/130 (4.6%) of the ticks removed from horse, and which is a higher percentage than previously reported in the Netherlands or other Western European countries (Duh, Petrovec et al. 2001; Nijhof, Bodaan et al. 2007; Wielinga, Fonville et al. 2009; Capelli, Ravagnan et al. 2012). This suggests that the risk of human babesiosis may be increasing. With regard to another zoonotic pathogen, *R. helvetica*, DNA was detected in 12 (9%) of the collected ticks. *R. helvetica* infection in people is associated with a mild flu-like disease (Fournier, Allombert et al. 2004), although chronic infections have been proposed to play a role in sarcoidosis and chronic peri-myocarditis (Nilsson, Lindquist et al. 1999; Nilsson, Pahlson et al. 2002). The prevalence of *R. helvetica* DNA in the ticks in this study (9%) was lower than usually found in Europe (Christova, Van De Pol et al. 2003; Hartelt, Oehme et al. 2004; Nijhof, Bodaan et al. 2007), although this may be a result of seasonal fluctuations or, more likely, the high percentage of nymphs recovered in the present study.
The presence of *B. caballi* DNA (2/130: 1.5%) in one *I. ricinus* female and one *Ixodes* nymph is a novel finding for the Netherlands which was considered free of this pathogen; nevertheless, the finding of *B. caballi* in ticks explains the recent description of an autochthonous case of equine babesiosis in the Netherlands (Butler, Sloet van Oldruitenborgh-Oosterbaan et al. 2012). The two other

pathogens that cause equine piroplasmosis, namely *T. equi* and *T. equi*-like, were also detected for the first time in *Ixodes* ticks in the Netherlands (table 1). Although the vector capacity of *I. ricinus* for these equine pathogens has not been established, the fact that *D. reticulatus* is a capable vector allied to the emergence of resident *Dermacentor* populations, indicates that there is now appreciable risk of equine piroplasmosis in horses that have never left the Netherlands, a fact that equine practitioners should take into account when confronted with a horse suffering from pyrexia of unknown origin. Strikingly, the ticks containing *Theileria*-DNA were all from one geographical collection area (equine practice); the first autochthonous piroplasmosis cases caused by this agent also occurred in this area (Butler, Sloet van Oldruitenborgh-Oosterbaan et al. 2012).
E. schotti DNA was detected in 14 ticks (11%), and therefore at a slightly higher rate than the 3.4-6.8% reported by Wielinga et al. in 2006 (2006). In addition, the distribution of ticks containing this agent appeared to be rather focal (figure 1). It was recently reported that *E. schotti* is identical to *Neoehrlichia mikurensis* (Jahfari, Fonville et al. 2012) and probably establishes a reservoir in small rodents; however, its pathogenic potential is not yet known. All of the pathogens detailed were detected in both nymphs and adult ticks, with a high incidence of co-infections (table 1). The high frequency of co-infection may be significant because it has been suggested that co-infection enhances pathogenicity of a number of tick-borne pathogens in both man and animals (Thomas, Anguita et al. 2001; Belongia 2002).

In conclusion: This study demonstrated that *I. ricinus* is the predominant tick species infesting horses in the Netherlands and, moreover, that it carries a variety of emerging (zoonotic) tick-borne pathogens; the most frequently encountered pathogen was *B. valaisiana*. The detection of a surprisingly large number of *Ixodes* nymphs on domestic horses is an indication that, at least in some regions of the Netherlands, tick density is relatively high; both the relatively high incidence of ticks and the range of pathogens carried have important possible implications for the risks of tick-borne infections in man, since horses appear to be excellent sentinels.

Acknowledgements

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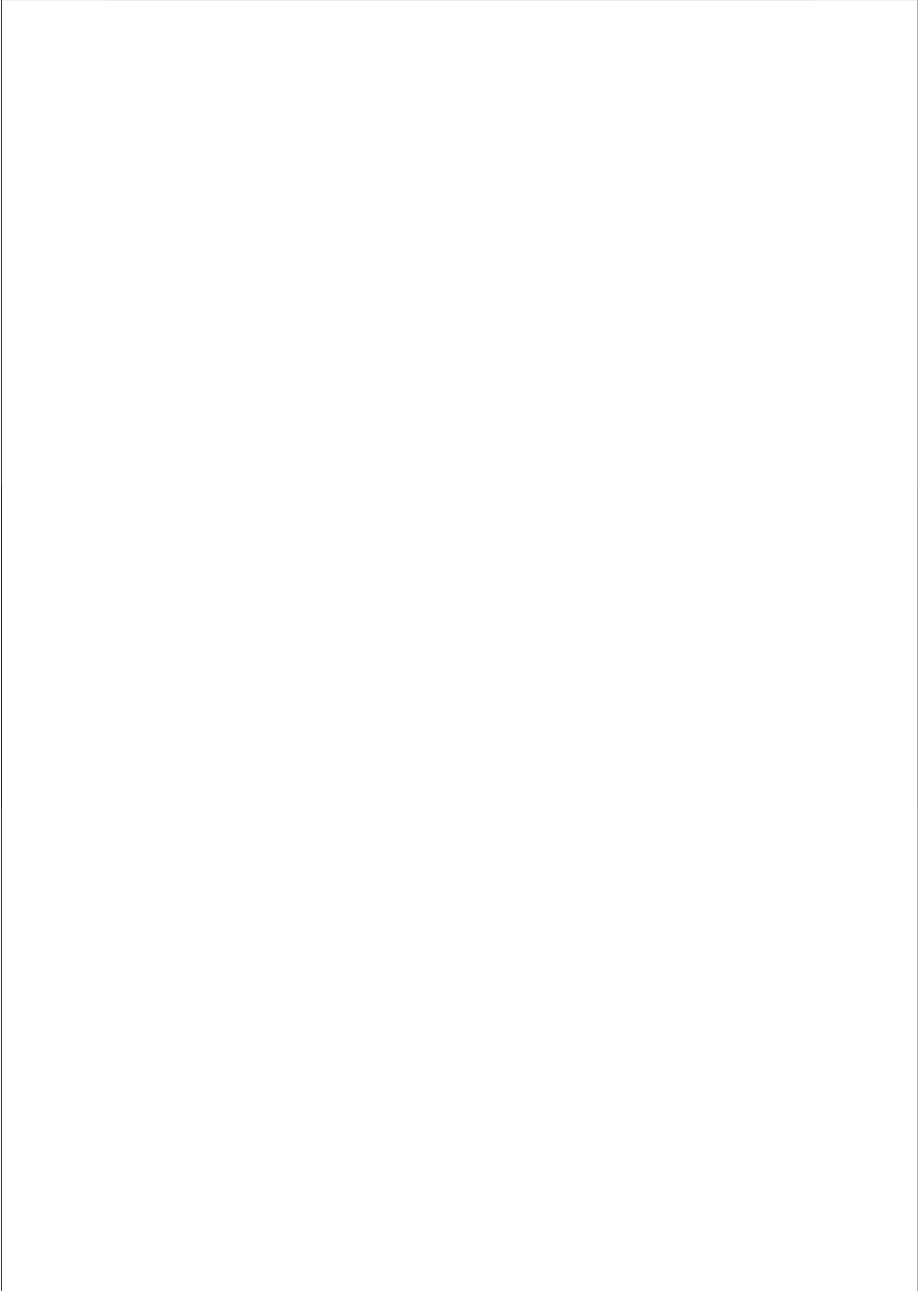

Chapter VII

Borrelia burgdorferi and *Anaplasma phagocytophilum* in ticks and their equine hosts: A prospective clinical and diagnostic study of 47 horses following removal of a feeding tick

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Submitted

Abstract

A total of 47 horses, and 120 ticks that had been removed from them, were tested for infections with the zoonotic pathogens *Borrelia burgdorferi* and *Anaplasma phagocytophilum*. All horses were examined at the time of tick removal and 6-12 weeks later; thirteen horses were examined again 9-23 months after tick removal. Serology was performed using an IFAT, an ELISA and a commercially available rapid test. Initially, 45% of horses were positive for *B. burgdorferi* antibodies and 23% tested positive for *A. phagocytophilum*; 15% of horses were seropositive for both pathogens. Although seven horses showed evidence of seroconversion to *Borrelia*, only 1/7 showed potentially associated clinical signs. On the other hand, *Anaplasma* seropositivity was correlated with low thrombocyte counts. *Borrelia* DNA was detected in 43% of the removed ticks, with a predominance of *B. valaisiana*. By contrast, *A. phagocytophilum* DNA was detected in only 1 tick.

Introduction

Public awareness of tick-borne infections has increased steadily in Europe over the last two decades. *Borrelia* species are the best-known pathogens transmitted by *Ixodes ricinus* (Stanek and Strle 2003), which is the predominant tick species in Western Europe. Whether there is a direct causal relationship between *Borrelia* infection and clinical disease is, however, still subject to debate. In horses, a broad spectrum of clinical manifestations has been attributed to *Borrelia burgdorferi* sensu lato (s.l.) infections including arthritis (Burgess, Gillette et al. 1986; Hahn, Mayhew et al. 1996), lameness (Browning, Carter et al. 1993), anterior uveitis (Burgess, Gillette et al. 1986; Priest, Irby et al. 2012), neuroborreliosis (Burgess and Mattison 1987; James, Engiles et al. 2010; Imai, Barr et al. 2011), and low grade fever and lethargy (Magnarelli, Anderson et al. 1988; Magnarelli, Flavell et al. 1997). However, establishing the cause of disease conclusively is difficult because detection of the agent, either directly by PCR or indirectly using serological tests, does not prove causality (Chang, Novosol et al. 2000). Moreover, seroconversion to the agent is also seen in healthy subjects. In this respect, some investigators have suggested that an overlooked co-infection with *A. phagocytophilum* might explain the biological variation in the clinical manifestations described, and the difficulty of establishing a conclusive causal link between *Borrelia* and associated disease in horses (Chang, McDonough et al. 2000; Magnarelli, Ijdo et al. 2000). In general, the likelihood of co-infection with different vector-borne pathogens increases if the agents concerned are transmitted by the same arthropod vector; this is the case for *A. phagocytophilum* and *B. burgdorferi* (Chang, McDonough et al. 2000; Foley, Foley et al. 2004).

Little is known about the clinical course of natural *B. burgdorferi* infections in horses, let alone the effects of concurrent infections. Laboratory diagnosis of *B. burgdorferi* infection in horses has relied primarily on serology and, although serological assays are widely applied, their diagnostic value is also the subject of debate. As a result, even when the assays are capable of distinguishing past and present infections, the above outlined difficulty of proving causality remains (Marcus, Patterson et al. 1985; Carter, May et al. 1994; Manion, Buschmich et al. 1998; Magnarelli and Fikrig 2005). In the U.S.A., testing for *B. burgdorferi* antibodies has recently focused on the so-called C6 antigen, which is reported to be highly specific for *B. burgdorferi* sensu stricto (s.s.) (Liang, Jacobson et al. 2000; Bacon, Biggerstaff et al. 2003; Johnson, Divers et al. 2008); moreover, it is thought that C6 antibodies indicate active infection (Philipp, Wormser et al. 2005). In Europe, however, there are several other *Borrelia* species besides *B. burgdorferi* s.s., and it is as yet unclear whether these are capable of inducing C6 antibodies in horses.

In contrast to *B. burgdorferi*, diagnosis of clinical *A. phagocytophilum* infections in horses is straightforward and based on a combination of clinical signs

and the detection of the agent in the blood, either microscopically in smears or via molecular tests (Franzen, Aspan et al. 2005; Butler, Nijhof et al. 2008; Franzen, Aspan et al. 2009). While serological assays are commonly used for prevalence studies, there is as yet no established gold standard test.
Concern among owners and veterinarians with regard to the prevalence and possible severity of tick-borne infections, particularly 'Lyme borreliosis', in horses has grown considerably in recent years. Moreover, it remains difficult to reach a conclusive diagnosis of clinical borreliosis while very little is known about the possible significance of concurrent *A. phagocytophilum* infection to the appearance or severity of clinical disease. For these reasons, a prospective field study was designed to investigate clinical progression and compare diagnostic tests in horses with a confirmed recent tick-bite, during a follow-up period of 6 weeks to 23 months. Enrolment of individual horses into the study was permitted following removal of an attached tick by an attending veterinarian. The ticks were subsequently tested for the presence of DNA for the two pathogens of interest in order to give some indication of the likely risk of transmission of these agents to horses following a tick-bite. To investigate whether one or both agents were transmitted, the horses' blood was tested on two or three occasions for pathogen-specific DNA and for specific antibodies. Since there are no established gold standard tests for either pathogen in horses, in-house tests (ELISA/ IFAT) and a commercially available test kit (4Dx SNAP®; IDEXX, Hoofddorp, the Netherlands) were performed in parallel and the results compared. The health status of the horses was evaluated over time using clinical follow ups and haematological parameters. The ticks were also examined for other pathogens of veterinary and medical importance; the latter results are described in Chapter VI.

Materials and methods

Study design and sampling

During 2008, a total of 47 domestic horses were included in the study and screened for *B. burgdorferi* and *A. phagocytophilum* infection using various methods, namely; light microscopic examination of blood smears, polymerase chain reaction-reverse line blot (PCR-RLB) and serology (indirect ELISA, IFAT and 4Dx SNAP® test). The horses enrolled included one stallion, twenty geldings and twenty-six mares of various breeds (twenty-one Dutch Warmblood, ten Friesian, five Icelandic, three Arabian, one Quarter horse, one New Forest pony, one Welsh pony, one Standardbred, one Haflinger, one Hanoverian, one Andalusian and one Fjord horse), ranging in age from six weeks to 19 years.
Horses were eligible for the study if their owner found one or more ticks attached to them, and the local veterinarian was called to attend to the horse because the owner was worried about tick-borne infections. After a general

clinical examination, a blood sample was collected from the jugular vein, before any tick(s) was (were) removed by the veterinarian. After removal, ticks were stored in a tube with 70% ethanol and sent with the blood samples to the Utrecht Centre for Tick-borne Diseases (UCTD). A second clinical examination, including a blood sample, was performed on all horses 6-12 weeks later.
Finally, in order to extend the observation period in case of more chronic presentation of disease, 13 of the horses (mainly those with persistent high titres for antibodies against *B. burgdorferi*) were also examined and blood sampled a third time 9-23 months after the first sample. All owners were asked to monitor their horse(s) carefully for signs of ill-health, and to keep records of any potential symptoms (lethargy, fever, anorexia or lameness of unidentified origin) during the study period. Any symptoms of ill-health were reported immediately to the first author.

Blood collection

Ten millilitres (ml) of jugular venous blood was collected into both an EDTA tube and a serum tube and these tubes were immediately posted to UCTD. Upon arrival at the laboratory, two blood smears were made from the EDTA tube and a sample was used for haematological examination. The remaining blood was centrifuged at 11000xg for 10 min, after which the cellular fraction (pellet) from the EDTA tube and the serum from the clotted sample were separated and stored at -20 °C.

Haematology

Packed Cell Volume (PVC), White Blood Cell (WBC) and thrombocyte counts (PLT) were determined using a Haematology Analyser CA530, Vet 16 Parameter System (Medonic, Stockholm, Sweden).

Blood smears

Blood smears were stained with Hemacolor (Diff Quick, Merck, Germany) and examined by light microscopy at 1000x magnification for the presence of inclusion bodies suggestive of tick-related pathogens.

Serology

Anaplasma phagocytophilum Indirect Fluorescent Antibody Test (IFAT)
Samples were screened for IgG against *A. phagocytophilum* using commercially available IFA slides (VMRD, Pullman, WA, U.S.A.). Bound equine IgG was detected using a fluorescein isothiocyanate-conjugated rabbit anti-horse IgG secondary antibody (Nordic Immunology, Tilburg, the Netherlands). All sera

were tested at a 1:80 dilution in phosphate-buffered saline solution (PBS) at pH 7.2, following the manufacturer's instructions. Slides were examined using a fluorescence microscope at 400x magnification. Samples that showed a bright green fluorescence for *A. phagocytophilum* morulae (inclusion bodies) were subsequently diluted serially to ascribe a titre of 1:80, 1:160 or >1:320. Positive control serum from a previously confirmed clinical case (Butler, Nijhof et al. 2008) and a negative control were included in each run. At a dilution of 1:80 it was sometimes difficult to distinguish between specific and non-specific fluorescence, therefore only titres ≥160 were considered true positives.

Borrelia burgdorferi whole cell indirect ELISA

Commercially available antigen (*B. burgdorferi* strain B31) coated wells (Virion/Serion, Wurzburg, Germany) and horse radish peroxidase labelled rabbit anti-horse IgG (MP Biomedicals, Eindhoven, the Netherlands) were used to establish an ELISA, with hydrogen peroxide serving as substrate and 2,2'-azino-bis 3-ethylbenzothiazoline-6-sulphonic acid (ABTS) as the chromogenic agent. Three serum dilutions (1:80, 1:320 and 1:1280) were tested alongside positive and negative controls. Results were read by eye and scored relative to the control samples. All samples with a titre of ≥1:320 were considered positive. This procedure was based on a test previously developed for dogs (Hovius, Rijpkema et al. 1999). The validity of this test has been evaluated in ten annual proficiency tests organised by Dr. K. Bergstrom of the Swedish National State Veterinary Institute (SVA), Uppsala, Sweden; two other laboratories involved performed an in house IFAT. Concordance over the years between the two IFATs and the indirect ELISA used in the current study was excellent for both horses and dogs.

In-office rapid immunoassay

A commercially available test was used to detect antibodies to the IR6 region of the *Borrelia* membrane protein VlsE, the so-called C6 antigen (4Dx SNAP®; IDEXX, Hoofddorp, the Netherlands). Although marketed for use in dogs, the assay should detect antibodies in a range of mammalian species because it employs a non species-specific anti-IgG conjugate. An earlier version of this test (3Dx SNAP®) has been validated for the detection of *B. burgdorferi* antibodies in horses in the USA (Johnson, Divers et al. 2008). The 4Dx SNAP® test can also detect antibodies to *A. phagocytophilum* in horses (Maurizi, Marie et al. 2009), although it had not yet been validated for this purpose. The test was performed according to the manufacturer's instructions; results were scored as either positive or negative.

DNA extraction from blood cell fractions and ticks

DNA was extracted from blood cell fractions using the QIAamp DNA mini kit (Qiagen, Venlo, the Netherlands), as described in the manufacturer's protocol. DNA from the ticks was extracted using the Nucleospin Tissue kit (Macherey-Nagel, Düren, Germany) following the manufacturer's protocol for the purification of genomic DNA from insects.

PCR amplification with Reverse Line Blot (RLB) hybridisation (PCR-RLB)

PCR for the detection of *Anaplasma* and *Borrelia* DNA was performed in an automated thermocycler (Bio-Rad Laboratories, Veenendaal, the Netherlands) as described previously (Schouls, Van De Pol et al. 1999; Bekker, de Vos et al. 2002). Subsequently, RLB hybridisation was performed with the PCR products generated, as described by Nijhof et al. (2005). DNA-extracted from blood and skin of previous equine cases of *A. phagocytophilum* and *Borrelia garinii* infection, respectively, were used as positive controls.

Statistical analysis

For both pathogens, logistic regression models were run to investigate whether gender, age or tick-load were related to 1) seropositivity at the 1st sampling, or 2) seroconversion by the 2nd sampling, for animals that were initially seronegative only. Haematology data for all horses are presented as mean ± SD. Data were analysed using a linear mixed model with horse as a random effect and group, time and the interaction between group and time as fixed effects. If the interaction was not significant, a model with only main effects was fitted to the data. Residuals were checked for normality using normal probability plots. Computations were performed using R, A Language and Environment for Statistical Computing, R Development Core Team, R Foundation for Statistical Computing, 2010. $P < 0.05$ was considered to indicate statistically significant differences. The apparent seroprevalences for *B. burgdorferi* and *A. phagocytophilum* were compared between the IFAT, ELISA and 4Dx SNAP® tests; correlations between the various testing methods were assessed using kappa statistics.

Results

<u>Sequential detection of antibodies to *B. burgdorferi* using ELISA and 4Dx SNAP®</u>

The results of the ELISA and the 4Dx SNAP® tests are presented on a per horse basis in table 1. The initial proportion of animals recorded as seropositive in the whole-cell ELISA ranged from 44.7% (21/47 at a titre ≥1:320) to 19.1% (9/47 at a titre ≥1:1280). At the second sampling 6-12 weeks later, the proportions were similar, although five horses had changed from seropositive to negative and five showed a conversion from negative to positive. Using the 4Dx SNAP® (C6 antibodies), the proportion of positive serum samples was 44.7% (21/47) at the time of tick removal and 53.2% (25/47) in the second sample; two horses subsequently became seronegative while 6 showed evidence of seroconversion. Thirteen horses that were sampled on a third occasion showed proportions of ELISA positive samples ranging from 11/13 (≥1:320) to 8/13 (≥1:1280). The C6 status remained unchanged between the second and third samples; 12/13 positive.

Tables 2a,b
Two-by-two comparisons of the indirect whole cell ELISA and 4Dx SNAP® tests for antibodies against Borrelia burgdorferi sensu lato in 47 horse serum samples (a: first sample, b: paired sample recovered 6-12 weeks later).

Table a

		4Dx SNAP®	
		Positive test result	Negative test result
iELISA	Positive test result	21	5
	Negative test result	5	16
Kappa value: 0.57			

Table b

		4Dx SNAP®	
		Positive test result	Negative test result
iELISA	Positive test result	19	7
	Negative test result	3	18
Kappa value 0.58			

Only one horse (no. 72) showed a clear seroconversion over time in the ELISA test; from negative at the first sample to a titre of 1:1280 in the third; this horse remained negative for C6 antibodies. Only one horse (no. 65) showed the opposite change (i.e. ELISA positive to negative), although it remained positive for C6 antibodies.

Table 1
Borrelia serology results in sequential serum samples recovered from 47 horses after a tick bite.

Horse	Time point 1		Time point 2		Time point 3	
	ELISA	SNAP C6	ELISA	SNAP C6	ELISA	SNAP C6
1	0	-	0	-	nd	nd
2	320	+	0	+	nd	nd
3	0	+	0	+	nd	nd
4	0	+	0	+	nd	nd
5	320	+	0	+	nd	nd
6	320	+	0	+	nd	nd
7	0	+	0	-	nd	nd
8	0	-	0	-	nd	nd
11	0	-	0	-	nd	nd
14	1280	+	1280	+	1280	+
15	0	+	0	+	nd	nd
16	0	-	0	-	nd	nd
17	1280	+	1280	+	640	+
18	0	_	320	-	nd	nd
19	1280	+	1280	+	1280	+
21	0	-	0	-	nd	nd
22	0	-	0	-	nd	nd
23	0	-	0	-	nd	nd
24	320	-	0	-	nd	nd
26	320	+	320	+	nd	nd
27	0	-	0	-	nd	nd
28	0	-	0	-	nd	nd
30	1280	+	1280	+	1280	+
33	320	+	1280	+	1280	+
35	1280	+	1280	+	1280	+
36	0	-	0	-	nd	nd
37	320	+	320	+	nd	nd
42	0	-	0	-	nd	nd
44	0	+	320	+	nd	nd
46	0	-	0	-	nd	nd
48	0	-	0	+	nd	nd
49	0	-	0	-	nd	nd
51	0	-	320	+	nd	nd
52	0	-	0	-	nd	nd
53	320	+	320	+	nd	nd
56	0	-	320	+	nd	nd
60	320	-	1280	+	nd	nd
61	1280	-	320	+	320	+
64	320	-	0	-	nd	nd
65	1280	-	320	+	0	+
66	0	-	0	-	0	-
12	320	+	320	+	320	_
70	0	-	0	-	nd	nd
71	1280	+	1280	+	1280	+
72	0	-	320	-	1280	-
73	320	+	320	-	nd	nd
74	1280	+	1280	+	1280	+

Tables 2a and b show the two-by-two comparisons of the serological results for the first two sampling time-points. The kappa values were calculated for each sampling time-point (47 sera), subsequently the mean of both values was calcu-

lated as 0.575 (i.e. a reasonable correlation). The major difference was that the 4Dx SNAP® appeared to be more sensitive than the ELISA.

Sequential detection of antibodies to *A. phagocytophilum* using IFAT and 4Dx SNAP®

Table 3 shows the results of the tests on the paired samples collected from every horse (i.e. initial and after 6-12 weeks). The proportion of positive serum samples in the IFAT ranged from 23.4% (11/47) for titres ≥1:160 to 17% (8/47) for titres ≥1:320 at the time of tick removal. Samples taken 6-12 weeks later tested positive in 34.0% (16/47) of horses using a titre of ≥1:160 as the cut-off and 19.1% (9/47) for titres ≥1:320. Using the 4Dx SNAP® test, 23.4% (11/47) of horses tested positive at the time of tick removal and 29.8% (14/47) tested positive 6-12 weeks later.
Five horses tested positive by IFAT in the second sample and four were also classified as seropositive using the 4Dx SNAP®. Horse no. 51 was negative on both occasions by IFAT, whereas the 4Dx SNAP® indicated a positive. Horse no. 65 showed a clear positive titre using the IFAT but was classed as negative via 4Dx SNAP®.
Tables 4a and b show the two-by-two comparisons for the serological results at the two different time-points; the mean kappa value was 0.815 (i.e. a substantial correlation).

Table 4a,b
Two-by-two comparison of the results of IFAT and 4Dx SNAP® tests for the detection of antibodies against Anaplasma phagocytophilum *in 47 horse serum samples from the field (a: first sample, b: paired sample recovered 6-12 weeks later)*

Table a

		4Dx SNAP®	
		Positive test result	Negative test result
IFAT	Positive test result	10	1
	Negative test result	1	35
Kappa value: 0.88			

Table b

		4Dx SNAP®	
		Positive test result	Negative test result
IFAT	Positive test result	12	3
	Negative test result	2	30
Kappa value: 0.75			

Table 3
Anaplasma *serology results for 47 horses after a tick bite (- = negative test result; + = positive test result; numbers indicate antibody titre)*

		Test time point 1		Test time point 2	
Horse	Age	IFAT 1	4Dx SNAP®	IFAT 2	4Dx SNAP®
1	13 years	0	–	0	–
2	1 year	0	–	0	–
3	9 years	0	–	0	–
4	1 year	0	–	0	–
5	4 years	0	–	0	–
6	10 years	0	–	0	–
7	1 year	0	–	0	–
8	5 years	0	–	0	+
11	7 years	0	–	160	+
12	6 weeks	0	–	0	–
14	16 years	>320	+	>320	+
15	6 years	0	–	0	–
16	2,5 years	0	–	0	–
17	4 years	>320	+	160	+
18	11 months	0	–	0	–
19	8 years	0	–	160	–
21	10 years	160	+	0	–
22	8 years	0	–	>320	+
23	8 years	0	–	>320	+
24	4 years	>320	+	160	+
26	19 years	0	–	0	–
27	7 years	0	–	0	–
28	18 years	0	–	0	–
30	12 years	0	–	0	–
33	15 years	0	–	0	–
35	10 years	0	–	0	–
36	8 years	0	–	160	–
37	7 years	0	–	0	–
42	19 years	0	–	0	–
44	6 years	160	+	>320	+
46	6 years	0	–	0	–
48	14 years	0	–	0	–
49	16 years	0	–	160	–
51	11 years	0	+	0	+
52	15 years	0	–	0	–
53	5 years	0	–	0	–
56	7 years	0	–	0	–
60	5 years	>320	+	>320	+
61	3 years	>320	+	>320	+
64	16 years	>320	+	320	+
65	18 years	>320	–	320	–
66	3 years	>320	+	320	+
70	15 years	0	–	0	–
71	12 years	0	–	0	–
72	19 years	160	+	160	+
73	15 years	0	–	0	–
74	15 years	0	–	0	–

Coexistence of antibodies to *B. burgdorferi* and *A. phagocytophilum*

Of all the horses tested, 19.1% (9/47) had antibodies against both *A. phagocytophilum* and *B. burgdorferi* in the whole cell ELISA and the IFAT at the first

sampling point; this had decreased to 12.7% (6/47) 6-12 weeks later. Using the 4Dx SNAP® assay, only 6.4% (3/47) of the horses showed an initial double positive result, and this had dropped to 4.2% (2/47) by the second sample.

Statistical analysis

No relationship between seropositivity in the 1st sample and the risk factors gender, age or tick-load (taken as the fraction of ticks per horse that carried the pathogen in question), was found by logistic regression analysis. There was also no clear relationship between these risk factors and seroconversion by the 2nd sampling in initially seronegative horses.

Blood smear interpretation and PCR-RLB results of EDTA blood samples

All blood smears were negative for *Anaplasma* inclusion bodies and all blood cell fractions were negative via PCR-RLB for *Borrelia* and *Anaplasma* DNA.

Clinical progression and haematology of the 47 horses after tick removal

Only one horse (no. 72) showed symptoms consistent with disease caused by *Borrelia* or *Anaplasma*, namely distal limb oedema of variable intensity during the first two weeks after tick removal, in this case removal of several *Ixodes* nymphs. None of the other horses showed any signs of disease during the study period. Furthermore, no abnormalities, other than an initial moderate to severe local skin reaction to the tick bite in some horses, were detected during the clinical examinations performed by the attending veterinary surgeons. The longer observation period for the horses that were examined and sampled for a third time did not affect the results.
Mean results of haematological examination are given in table 5. Statistical analysis showed that the thrombocyte count was significantly lower ($P = 0.0273$) in the group *Borrelia* negative and *Anaplasma* positive animals compared to the group animals negative for both agents at both time points.

Classification of the ticks removed from the horses

Of the 120 ticks (63 adult females, 7 adult males, 49 nymphs and 1 larva) removed from the 47 horses, 67 (56%) were identified as *I. ricinus*, 50 (41%) as *Ixodes* spp., 2 (2%) as *Hyalomma marginatum*, and 1 (1%) as *Dermacentor reticulatus*.

Table 5

*Haematological data categorised per time point and test result (*Borrelia *and* Anaplasma *serology) for 47 horses sampled at the time of tick removal and 6-12 weeks later*

	Bor 01 pos.	Bor 01 neg.	Ana01 pos	Ana01 neg	Bor02 pos.	Bor02 neg.	Ana02 pos.	Ana02 neg.
	n = 21	n = 26	n = 11	n = 36	n = 21	n = 26	n = 16	n = 31
PCV	0.36 ± 0.05	0.36 ± 0.05	0.36 ± 0.05	0.36 ± 0.05	0.37 ± 0.06	0.36 ± 0.04	0.37 ± 0.04	0.36 ± 0.05
PLT	129 ± 36	118 ± 42	116 ± 55	125 ± 34	128 ± 57	102 ± 42	111 ± 62	114 ± 44
WBC	7.1 ± 1.8	6.2 ± 2.2	7.9 ± 1.6	6.3 ± 2.1	7.0 ± 2.2	6.3 ± 2.3	6.7 ± 2.2	6.4 ± 2.4

(PCV - packed cell volume in l/l; PLT - thrombocytes x 10^9/l; WBC - white blood cell count x 10^9/l; Bor01 = Borrelia *titre test 1; Bor02 =* Borrelia *titre test 2; Ana01 =* Anaplasma *titre test 1; Ana 02 =* Anaplasma *titre test 2).*

PCR-RLB results for the 120 ticks

In the PCR-RLB, 52/120 ticks (43.3%) were positive for *Borrelia* DNA but only 1 was positive for *A. phagocytophilum*; these pathogen containing ticks were harvested from 27 horses. The other ticks tested negative for both agents. table 6 presents the proportions of *Borrelia*-DNA containing ticks that were male, female or at the various developmental stages.

Various *Borrelia* spp. were detected; *B. valaisiana* (51/120: 42.5%) was the most prevalent, followed by *B. afzelii* (19/120: 15.8%), *B. garinii* (3/120: 2.5%) and *B. burgdorferi* s.s. (2/120: 1.6%). A total of 18/120 (15%) ticks contained 2 or more *Borrelia* spp.; 11/63 (17%) of the adult female ticks and 7/49 (14%) of nymphs. Only one adult male (1/7: 14%) was positive for *B. afzelii*.

Table 6

Distribution of Borrelia*-DNA over the sexes and developmental stages of 68 ticks removed from 27 horses with one or more positive ticks. All ticks were* Ixodes ricinus *or* Ixodes *spp. except for one female* Hyalomma marginatum.

	Total ticks on 27 horses	*Borrelia*-DNA positive ticks (%)
Nymph	26	20 (76.9)
Female	39	31 (70.5)
Male	2	1 (50)
Larva	1	0
Total	68	52 (76.5)

Discussion

Overall, the results show that roughly half of the horses in this study had previously been exposed to *Borrelia* sp. and a quarter to *A. phagocytophilum*. Whereas almost half of the ticks examined contained *Borrelia* spp DNA, only one contained *A. phagocytophilum* DNA. Although these results are biased by

the sampling method, they indicate that the average horse in the Netherlands runs a reasonable risk of being bitten by a tick carrying *Borrelia* spp., but a much lower risk of being exposed to *A. phagocytophilum* via a tick bite. At the same time, it is clear that infections resulting after a tick-bite rarely lead to clinical symptoms in the horse; in this respect, none of the horses had a clinical history consistent with either disease, and none of the horses that seroconverted during the study showed contemporaneous or delayed associated clinical symptoms.

The only possible exception may be horse no. 72, which showed mild, but potentially related clinical symptoms in the period after the tick bite, and was also one of the seven horses to show a clear seroconversion to *Borrelia*; there was, however, no definitive proof of a causal relationship. By contrast, haematology results showed a somewhat different picture in that lower thrombocyte counts were associated with seropositivity to *A. phagocytophilum*. A recent experimental infection study in horses with *A. phagocytophilum* indicated a long-lasting thrombocytopenia as a typical feature (Davies, Madigan et al. 2011). Taken together, this indicates that *A. phagocytophilum* infections may cause subclinical pathology in horses, which may be a reason for concern in animals used for performance sports.

The finding that five horses with an initial titre of 1:320 in the ELISA tested negative at the second sampling 6-12 weeks later, is probably explained by the absence of active infection and consequently declining antibody titres (Chang, Novosol et al. 2000). Although a limited number of horses had antibodies to both pathogens, since none of the blood samples were positive for DNA for either pathogen there was no proof of concurrent infection. In short, the present results neither support nor disprove the possibility of a pathogenic interaction between concurrent *A. phagocytophilum* and *Borrelia* infections in horses.

Most studies on *Borrelia* infections in horses have been performed in the U.S.A. where *B. burgdorferi* s.s. is the only species present (Bunikis, Garpmo et al. 2004; Piesman and Gern 2004; Steere, Coburn et al. 2004). In contrast, there are at least four *Borrelia* species present in Europe, and they are associated with different disease manifestations in man (Balmelli and Piffaretti 1995; Logar, Ruzic-Sabljic et al. 2004). Moreover, the report that only *B. burgdorferi* s.s. is relatively resistant to the bactericidal effects of horse serum suggests that equine hosts may react differently to infections with different genospecies (Kurtenbach, Sewell et al. 1998). It is therefore likely that the differences between the genospecies complicate studies of *Borrelia* infections in horses; this probably explains some of the variation in results of experimental or natural infections with *Borrelia* in the literature.

In the ticks recovered from the horses in this study, *B. valaisiana* and *B. afzelii* were the most prevalent *Borrelia* species detected, at 42.5% and 15.8%, respectively; *B. burgdorferi* s.s. was the least common *Borrelia* found in the ticks, at 1.6%; it is therefore assumed that the majority of detected anti-*Borrelia* anti-

bodies were not induced by *B. burgdorferi* s.s.. This may have affected the clinical outcome of this study, but it did not appear to affect the results of the serological assays. It has been reported that antibody specificity to the C6 antigen may vary according to the infecting *Borrelia* genospecies (Sillanpaa, Lahdenne et al. 2007; Krupka, Knauer et al. 2009). Nevertheless, given the reasonable correlation between the whole cell ELISA and the 4Dx SNAP®, and the low prevalence of *B. burgdorferi* s.s. in the ticks, it is concluded that the genospecies prevalent in the Netherlands (*B. valaisiana* and *B. afzelii*) almost certainly induce the formation of antibodies against the C6 antigen in horses.
The results also suggest that the 4Dx SNAP® is more sensitive than the whole cell ELISA, which employs a 'broad' antigen. It is, however, unlikely that hosts produce only antibodies against the C6 antigen; nevertheless, horses. 3, 4, 7 and 65 tested positive for C6 alone. Moreover, three of the horses that became negative in the whole cell ELISA by the second sampling time-point remained C6 positive. These results raise questions about the specificity of the 4Dx SNAP® with respect to *Borrelia* antibodies in horses. Indeed, since C6 antibodies are thought to indicate active infection, a lower sensitivity than that of the whole cell ELISA would be logical. Nevertheless, the two tests agreed sufficiently to suggest that either or both can be used at a serological survey level.
At a diagnostic level, the results presented here demonstrate that seropositivity does not correlate with clinical disease. Therefore, in clinically suspect equine patients seropositivity, in terms of a high titre in the whole-cell ELISA and/or for the C6 antigen, indicates only that borreliosis cannot be ruled out; it does not confirm it as the cause of disease. The results of this study broadly agree with a study performed in Austria, where samples were collected before and at the end of the tick season 8-9 months later; the seroprevalence was high but there were no clinical signs, and the horses were infected mainly with *B. afzelii* (Muller, Khanakah et al. 2002).
Despite the relatively high seroprevalence, no *Borrelia*-DNA was detected in the blood cell fractions by PCR. This is in line with the report that the bacterium preferentially disseminates into various connective tissues in which it may persist for long periods despite its DNA being very difficult to detect in the blood (Chang, Novosol et al. 2000). Overall, the results presented here support previous reports that most *Borrelia*-infections in horses, even those involving *B. burgdorferi* s.s., remain sub-clinical (Magnarelli, Anderson et al. 1988; Egenvall, Franzen et al. 2001; Muller, Khanakah et al. 2002).
The two tests used in this study for the detection of antibodies against *A. phagocytophilum* showed a substantial kappa-value, i.e. a good correlation with regard to horses classified as seropositive or negative. Since IFAT has 'the morphological specificity' of displaying the in situ detected pathogen on the slide, it can be concluded that the 4Dx SNAP® also reliably detects *A. phagocytophilum* antibodies in horses. Although four to five horses seroconverted during the examination period, none came up positive in either of the infec-

tious agent detection tests (blood smear; PCR-RLB). This is most probably explained by the relatively long intervals between sampling; infected horses have been reported to clear the parasite within weeks, although Franzen et al. (2009) did find *A. phagocytophilum* DNA intermittently in blood, but not in tissues, months after an experimental infection. In short, even if horses do become persistently infected, their role as a reservoir for transmitting infection should be comparatively minor.
Interestingly, the seroprevalence of *A. phagocytophilum* (23.4%) in sampled horses was of a different order to the incidence of its DNA (0.8%) in the recovered ticks. This low incidence of *A. phagocytophilum* DNA corroborates earlier studies of ticks in other North-European countries (Lommano, Bertaiola et al. 2012; Schicht, Schnieder et al. 2012) suggesting that the result is not primarily an artefact of poor test sensitivity. Moreover, the low incidence in the removed, partly engorged ticks suggests that horses do not present an important reservoir. Indeed, the discrepancy between the seroprevalence in horses and incidence of DNA in attached ticks suggests that other vectors may play a role in transmission, e.g. mosquitos (*Culicoides* spp.) or biting flies. Alternatively, there could be an extremely strong seasonal variation in the incidence of *A. phagocytophilum* carriage among ticks.
With respect to the tick species identified, *I. ricinus* was predominant, as expected, while finding one *D. reticulatus* was not surprising given that this tick species has recently become indigenous to the Netherlands (Nijhof, Bodaan et al. 2007). On the other hand, the two specimens of adult *H. marginatum* on two unrelated horses were interesting because this tick is not considered to be indigenous to North-Western Europe.

In conclusion: the high seroprevalences of *B. burgdorferi* and *A. phagocytophilum* among horses in this prospective study, and the high incidence of *Borrelia*-DNA in the ticks removed from them, indicates that horses living in the Netherlands are at appreciable risk of tick-borne infections. On the other hand, only one horse showed clinical signs consistent with tick-borne disease during the study period, even though a number clearly seroconverted. This suggests that the infections do not cause disease or that symptoms are subclinical; the relative thrombocytopenia associated with *Anaplasma* seropositivity is a probable example of subclinical pathology. The low incidence of clinical disease may have been influenced by the predominance of *B. valaisiana* and *B. afzelii* over *B. burgdorferi* s.s., and by the fact that the studied population consisted of recreational horses, rather than high level sport horses. The discrepancy between a 23.4% seroprevalence of *A. phagocytophilum* in horses and a very low occurrence in ticks suggests that other vectors may exist for this parasite. Finally, this study confirms that seropositivity for *Borrelia* in horses with suggestive clinical symptoms justifies considering borreliosis among the differential diagnoses, but is not diagnostic per se.

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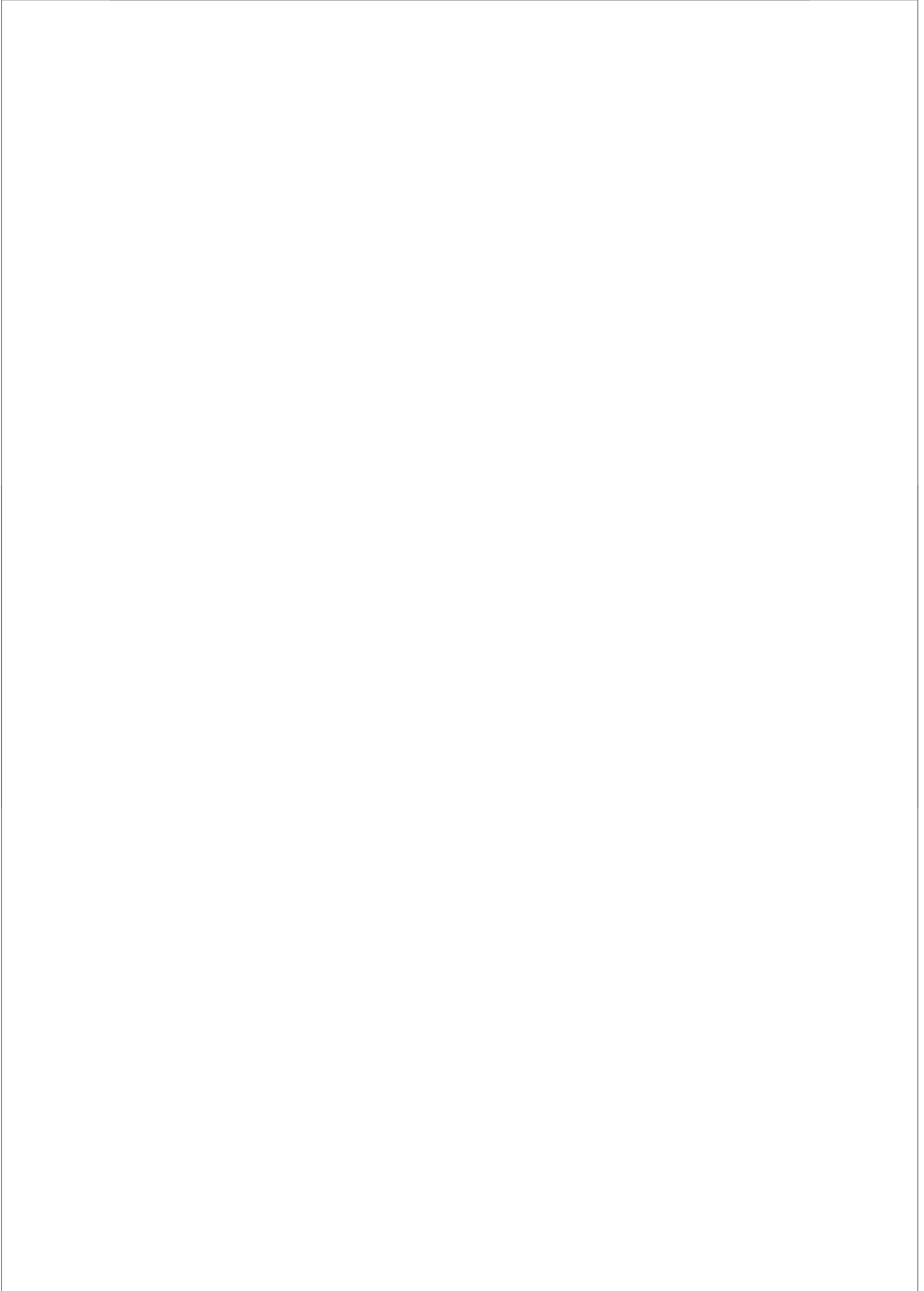

Chapter VIII

Repeated high dose imidocarb dipropionate treatment did not eliminate *Babesia caballi* from naturally infected horses as determined by PCR-reverse line blot hydridisation

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Abstract

Imidocarb dipropionate treatment of horses infected with *Babesia caballi* is supposed to eliminate the infection, but data on the efficacy of this treatment is scarce. The study presented here concerns four Paso Fino horses, which were imported into the island of Curacao on the basis of a piroplasmosis negative complement fixation test (CFT). Upon retesting with an indirect fluorescent antibody test immediately after arrival in Curacao, two horses appeared to have antibodies to *B. caballi* and all horses had antibodies to *Theileria equi*. Subsequent testing with polymerase chain reaction combined with a reverse line blot yielded positive results for both agents in all four horses. Treatment with five consecutive doses of imidocarb dipropionate (4.7 mg/kg BW im q 72 h), temporarily resulted in negative results, but *B. caballi* and *T. equi* were detected again in the samples taken at 6 and 18 weeks after completion of the treatment. These results confirm that the CFT is not a suitable test for pre-import testing and that even high dose treatment with imidocarb dipropionate may not be capable of eliminating *B. caballi* and *T. equi* infections from healthy carriers.

Introduction

Equine piroplasmosis, caused by *B. caballi* and *T. equi*, is an important tick-borne protozoan disease (Friedhoff, Tenter et al. 1990). The agents are endemic throughout the (sub-) tropics and stringent regulatory import restrictions are in place in some countries to prevent their spread among both resident and transient equid populations (Friedhoff, Tenter et al. 1990). Equine piroplasmosis occurs in acute, sub-acute and chronic forms. Carriers of the infection are mostly asymptomatic.
Identification of parasites in blood smears is the diagnostic mainstay, but this bears certain limitations, particularly when parasitaemia is low (Krause, Telford et al. 1996). Serodiagnosis by the use of complement fixation test (CFT) alone may give false negative test results, especially in horses that are parasite carriers, and has been shown to be less sensitive than the indirect fluorescent antibody test (IFAT) (Frerichs, Holbrook et al. 1969; Donnelly, Joyner et al. 1980; Tenter and Friedhoff 1986; Weiland 1986). Polymerase chain reaction (PCR) proved very useful for the detection of haemoparasites (Caccio, Camma et al. 2000), and combined with reverse line blot (RLB) offers the possibility of simultaneous detection and identification of different species infecting horses (Nagore, Garcia-Sanmartin et al. 2004). Imidocarb-dipropionate treatment of *B. caballi* infected horses has been shown to eliminate the infection (Frerichs and Holbrook 1974; Bruning 1996), but data on the efficacy of this treatment is scarce and more sensitive diagnostic tools have developed over the years. In an attempt to eliminate the infection from the horses in this study, treatment with a high dose of imidocarb dipropionate (4.7 mg/kg BW im q 72 h 5 times) was initiated and blood was tested using PCR-RLB to monitor the effect of treatment.

Materials and methods

A group of four Paso Fino horses, naturally infected with *B. caballi* and *T. equi*, ranging in age from 6 to 8 years, with a weight between 325 and 350 kg were treated five consecutive times with a high dose of imidocarb dipropionate (Imizol® 4.7 mg/kg BW im q 72 h; Shering-Plough S.A., Friesoythe, Germany). Following treatment, blood samples were collected sequentially. The blood was stored at 20 °C, until DNA was extracted using a DNA extraction kit (Qiagen, Hilden, Germany), following the manufacturer's protocol. The procedures for obtaining PCR products used for the detection of *Theileria* and *Babesia* species by RLB and the RLB hybridisation itself have previously been described (Nijhof, Pillay et al. 2005). Negative and positive control samples were included in every run. A standard IFAT protocol was used as previously described (Madden

and Holbrook 1968), using locally produced antigens. Bound equine antibodies were detected with fluorescein isothiocyanate conjugated rabbit anti-horse immunoglobulin (RAHo/ IgG(H + L)FITC, Nordic Immunology, Tilburg, the Netherlands) and examined in a wet mount by fluorescence microscopy.

Results and discussion

The study presented here concerns four Paso Fino horses, which were imported into the island of Curacao on the basis of a negative piroplasmosis CFT. Upon retesting with IFAT immediately after arrival in Curacao, two horses appeared to have anti-bodies to *B. caballi* and all horses had antibodies to *T. equi*. Subsequent testing with PCR-RLB yielded positive results for both agents in all four horses. Travel-related stress might have affected them immunologically (Hailat, Lafi et al. 1997), causing a flare up of the infection resulting in the positive PCR-RLB after arrival in Curacao. None of the horses showed clinical signs of disease during their stay in regulatory quarantine. Treatment with a high dose of imidocarb dipropionate (4.7 mg/kg BW im q 72 h 5 times) was initiated, and blood samples (EDTA, Vacuette®) were collected sequentially between 4 September 2002 and 31 January 2003. PCR-RLB results are given in table 1.

Table 1
Results of PCR-RLB for Babesia caballi *and* Theileria equi *in the blood of four horses during and after five consecutive imidocarb dipropionate treatments (4.7 mg/kg BW im q 72h) commencing on 4 September 2002*

Sample date	Horse 1		Horse 2		Horse 3		Horse 4	
	B. caballi	*T. equi*	*B. caballi*	*T. equi*	*B. caballi*	*T. equi*	*B. caballi*	*T. equi*
4 September 2002	+	+	+	+	+	+	+	+
7 September 2002	+	+	+	+	+	+	+	+
10 September 2002	–	+	–	+	–	+	–	+
13 September 2002	–	+	–	–	–	–	–	+
16 September 2002	–	+	–	–	–	–	–	+
30 October 2002	+	+	+	+	–	+	+	+
31 January 2003	+	+	+	+	+	+	+	+

+: postive result; –: negative result

It is clear that *B. caballi*, and to a lesser degree *T. equi*, detection fails after two doses of the five-dose course but that they are detected again in the samples taken at 6 and 18 weeks after completion of the treatment. The lack of efficacy was surprising since a much lower dose of imidocarb 2 mg/kg q24 h twice) was previously reported to be capable of eliminating *B. caballi* (Frerichs and Holbrook 1974). Detectable DNA actually disappeared from the circulation during

the treatment and re-appeared later. Moreover, since dead agents/organisms are rapidly degraded and removed by warm blooded hosts and since long time survival of DNA in the circulation seems incompatible with life, it is highly unlikely that the DNA of dead *B. caballi* organisms was detected at 6 and 18 weeks post-treatment. In addition, re-infection is also an unlikely explanation for our findings since the tick species required for transmission did and do not occur in Curacao.
In conclusion, the results reported here confirm that the CFT is not a suitable test for pre-import testing and suggest that even high dose treatment with imidocarb dipropionate may not be capable of eliminating *B. caballi* and *T. equi* infections from healthy carriers.

Acknowledgement

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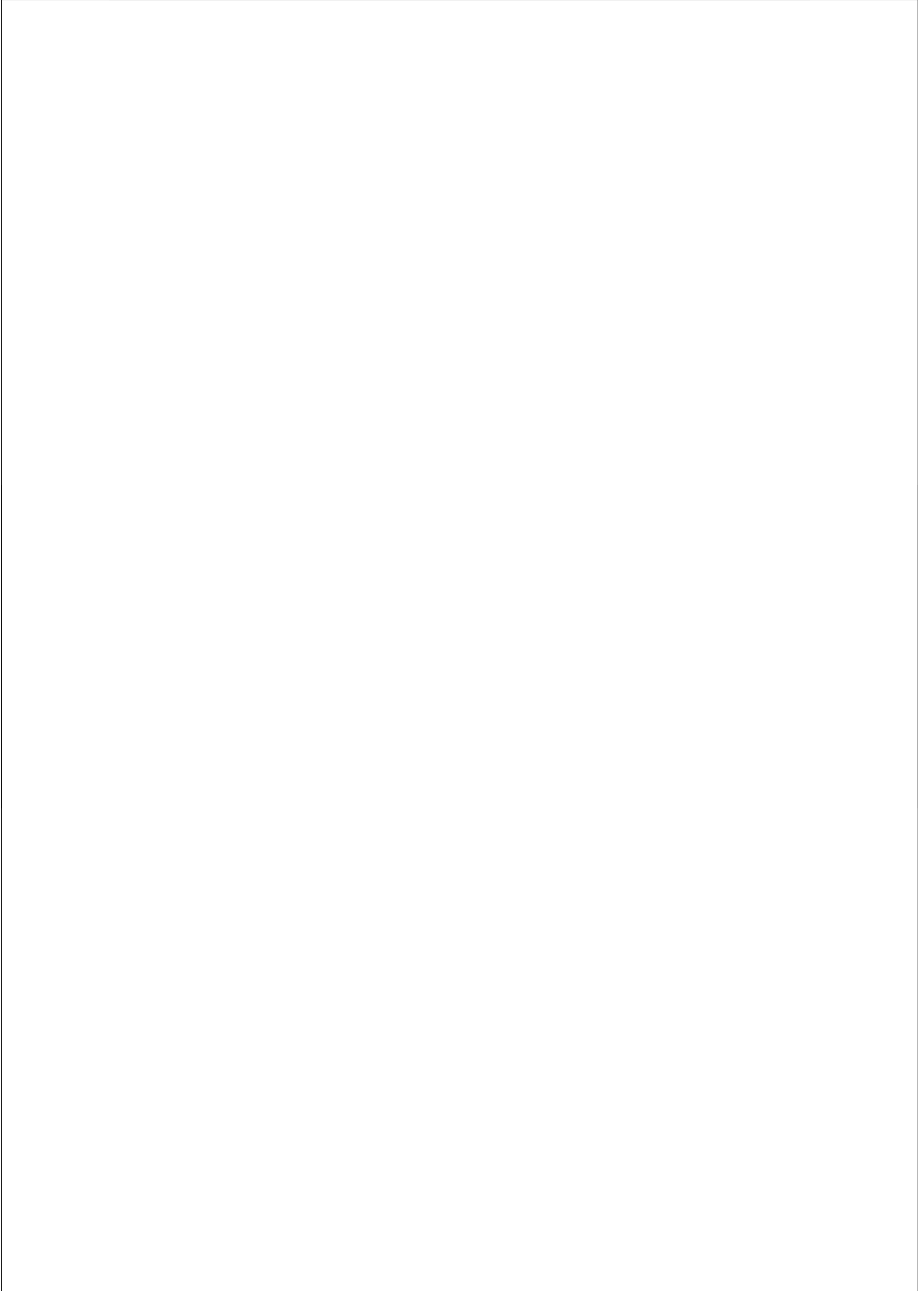


Chapter IX

Summarising discussion

Summarising discussion

Ticks and tick-borne infections

Tick-borne infections are common in (sub-) tropical areas, and their incidence is rising in more temperate climate zones (de la Fuente, Estrada-Pena et al. 2008; Hartelt, Pluta et al. 2008; Estrada-Pena, Ayllon et al. 2012). Many aspects of the major tick-borne infections are poorly understood, despite the fact that several appear to have zoonotic potential such that there is a compelling extra reason for concern about their increasing distribution and incidence.
The epidemiology of tick-borne infections is complicated, primarily because it involves the availability of a competent vector, the tick, and an animal species that can act simultaneously as a reservoir for the pathogen and as a host for the tick, allowing it to obtain the blood meal essential for progression to the next stage of its lifecycle and for transferring the pathogen. An animal acting as a reservoir of infection basically permits an infection to persist in its body for a reasonable period of time; the agent and its reservoir host are adapted to one another to allow co-existence in a state of relative equilibrium. Evidently, individuals of a species vary to some extent in their capacity to reach and maintain that equilibrium, meaning that some will develop disease and may die, while others will show no clinical signs at all. Likewise, the length of time that an animal host acts as reservoir varies per individual. Consequently, animal populations that have not yet adapted to a given agent, i.e. a naive population, may suffer severe disease and mortality when they are exposed for the first time.
Tick-borne agents themselves have varying host specificities; some may infect a wide range of animal species including humans while other only infect one species (Sonenshine 1991). Tick-borne agents also have tick specificity; every species of agent has specific tick species that are capable of its transmission from one host to another; in many cases the tick actively contributes to the agent's transmission in several ways, not the least of which is active propagation of the agent (de la Fuente, Blouin et al. 2007; Kocan, de la Fuente et al. 2008). Given their capacity to adapt to different hosts, there may be considerable variation among strains of a single species of tick-borne agent. Essentially, tick-borne agents must be highly dynamic to survive.
Ticks have complicated life cycles, and the details vary between species (Sonenshine 1991). Tick survival is linked to many environmental factors, including the availability of suitable hosts to feed on. Together these factors determine the geographical distribution of a given tick species. Ticks have relatively long generation intervals, sometimes years, which means that as a species they have limited capacity to adapt to lasting changes in their environment; on the other hand, they may be transported by their feeding hosts to new biotopes with a suitable environment.
In conclusion, tick-borne agents are in many ways 'terra incognita', we are only just starting to unravel their intricate relationships with the ticks that transmit

them and the animal hosts that maintain them. The intricate relationship of ticks with their environment and the 'mobility' provided by their hosts, are factors that add to a highly dynamic, challenging and fascinating subject to study. Moreover, incipient changes in the global climate may necessitate considerable changes in the way Western Europe views tick-borne infections (Estrada-Pena, Ayllon et al. 2012).

Detection of equine tick-borne infections

In recent years and largely as a result of the emergence and development of molecular diagnostic techniques, substantial improvements have been made in the sensitivity and specificity of detection of many agents of importance to human and veterinary medicine. Even very low burdens of multiple tick-borne agents can nowadays be detected using polymerase chain reaction (PCR) based techniques. These techniques, and in particular PCR-RLB, have become indispensable in the diagnosis and monitoring of (equine) tick-borne infections. PCR-RLB and real time PCR are the dominant techniques used in the studies described in this thesis; while PCR-RLB has the advantage of detecting multiple agents in a single test, it is time-consuming, appears to be less sensitive than real time PCR (Chapter III), and gives only a qualitative result (yes/no). Real-time PCR on the other hand, has proven to be a rapid quantitative test for identifying equine piroplasmosis carriers (Chapter III). As with many PCR assays, the primers used need to be specifically designed, and validated to ensure optimal performance in the face of strain variations and emergence of new pathogens.
Despite the fact that PCR detects the DNA of an agent rather than the viable agent itself, quantitative PCR examination of carrier horses and vector ticks enables studies to determine the minimum pathogen load which allows a host to act as reservoir. This could lead to tests to discriminate between potential reservoir hosts and infected non-reservoir hosts.
As many tick-borne agents are propagated, and in some cases even 'activated', in a competent vector tick while available vector tick species may differ per geographical location, it would be of interest to investigate whether differences in pathogenicity of a specific agent are associated with its vector. A recent article by Villar et al. (2012) describes the potential of a quantitative proteomic approach to characterise the pathogen-tick interaction. Such an approach could unravel differences in the development of *Piroplasmata*, *Borrelia* and/or *Anaplasma* spp. in different *Ixodes* spp., as well as shedding light on the proteins involved in the interaction between the ticks and the agents they transmit, and between the ticks and their hosts. One of the potential benefits of unravelling the differences in pathogen development in different tick species is the identification of specific targets for the development of vaccines or novel therapeutic modalities.

Equine tick-borne infections in the Netherlands

Until recently, *Borrelia burgdorferi* was the only tick-borne agent that horse owners in the Netherlands were concerned about, primarily because of its zoonotic potential and media attention. The recent detection of *A. phagocytophilum* (Chapter IV), *T. equi* and *B. caballi* (Chapter V) in indigenous horses together with the reports that a previously "exotic" tick (*D. reticulatus*) known to be a competent vector for equine piroplasmosis and canine babesiosis (Nijhof, Bodaan et al. 2007) has become established in parts of the Netherlands has changed things considerably and should help increase the awareness of tick-borne diseases. The studies described in this thesis show that horses in the Netherlands are actually at appreciable risk of acquiring tick-borne infections. Since some of these agents have zoonotic potential, humans sharing the same biotype as affected horses must also be considered 'at risk'. In this respect horses kept for recreational purposes have become popular in the Netherlands over the last decade. While keeping and riding horses outdoors, especially in forests and undergrowth, the rider/owners are equally exposed to tick infestation and thus to tick-borne infections. Although dogs are regarded as good sentinels for the likely tick exposure of their owners, the typical behaviour of dogs may lead to a different, and less representative, exposure to tick habitats than that of horses under saddle. Further research using horses as sentinels is necessary to objectively identify, and subsequently monitor, the high tick-borne disease risk areas in the Netherlands.

Clinical disease caused by equine tick-borne infections

Clinical signs observed after infection with *Borrelia* and/or *Anaplasma* in recreational horses appear to be minimal (Chapter VII). However, the association of *A. phagocytophilum* antibodies with low thrombocyte counts suggests that changes did occur, but were subclinical in the horses studied. It remains to be seen if such changes would be similarly subclinical in top level sport horses. The controversy surrounding the clinical relevance of *Borrelia* infections in various mammals has yet to be resolved. The key issue is the problem of establishing the diagnosis, which is essentially the question of how to indisputably link the presence of the agent with an ongoing pathological process. On the other hand, the details of the pathogenic process itself are also poorly understood, but evidently complicated: there is a primary local inflammatory reaction in the skin in most, if not all, horses to which the spirochaete is transmitted; however, only relatively few cases appear to suffer a secondary process in other tissues. These secondary inflammatory processes, when they occur, may well be of an auto-immune nature similar to what has been suggested for *Leptospira*; infections with the latter are also common in horses (Houwers, Goris et al. 2011) and the agent probably persists in certain niches where it may elicit antibodies that

react with tissue elements thereby triggering a local chronic inflammatory reaction (Verma, Matsunaga et al. 2012). If this concept were also to apply to *Borrelia* infections it would place the immune capacity and response of the individual host, and any related feedback mechanisms, at the heart of the pathogenic mechanism, which might help explain the enormous variety in the apparent outcomes of this relatively widespread infection.

A fairly similar issue applies to horses persistently infected with *T. equi* and *B. caballi* (Chapter VIII). In order to enhance their chances of survival, most tick-borne agents rely on establishing a long-lasting equilibrium with their host, which appears to be mediated through evasion and dysregulation of the hosts' immune response following infection. Disturbance of this delicate equilibrium between an agent and its host may precipitate clinical disease in a previously asymptomatic carrier, as has been documented for persistent *T. equi* infections in racehorses after intensive exercise (Hailat, Lafi et al. 1997).

The carrier-status equilibrium is the result of agent-host interaction, which is a dynamic process, and its disturbance may be particularly likely in, and most likely to have serious health repercussions in, horses that have to perform at their physical limits. Thus, poor performance in high-level competition horses could be associated with subclinical persistent, tick-borne infections. In view of the special relationship between the tick-borne pathogen and its host, absence of fever and a normal white blood cell count may not a priori exclude involvement of these agents in disease. Although the study presented in Chapter VII neither supported nor refuted the relevance of co-infection of *Anaplasma* and *Borrelia*, the implications of co-infections in general require further attention.

Spread of tick vectors into Northern/Western Europe

The importance of migratory birds as vehicle for the distribution of ticks into new biotopes has been substantiated by recent research (Dietrich, Gomez-Diaz et al. 2011; Palomar, Santibanez et al. 2012). Subtle changes in climate, biotope and availability of reservoir hosts can result in the survival and establishment of previously exotic tick species. The detection of two partly engorged, adult *Hyalomma* spp on two different horses in the Netherlands (Chapter VI) is a signal that the biotope in this part of Europe may (have) become suitable for the development and, eventually, establishment of this tick (Estrada-Pena, Ayllon et al. 2012). This would obviously increase the risk of equine piroplasmosis becoming endemic in the Netherlands. In this respect, because of the introduction and establishment of *D. reticulatus* in the Netherlands (Nijhof, Bodaan et al. 2007), indigenous horses are already at increased risk of infection with *T. equi* and *B. caballi*. While the equine piroplasma seroprevalence and piroplasma DNA incidence was still low among horses in the parts of the Netherlands examined (Chapter V), this does not prove that the situation is similar in other parts of the country. Results of the current study showed that a certain

population of previously imported, apparently healthy *T. equi* carrier horses are present, and may even serve as reservoir; such animals were DNA positive in the PCR-RLB (Chapter V). To be able to estimate the risk associated with these carriers and the 'import' of exotic ticks, a survey targeting specific breeds (originating from endemic countries) in other parts of the country, combined with monitoring of tick populations to establish their dispersal and infectious status, would be indicated.

Prevention of equine tick-born infections

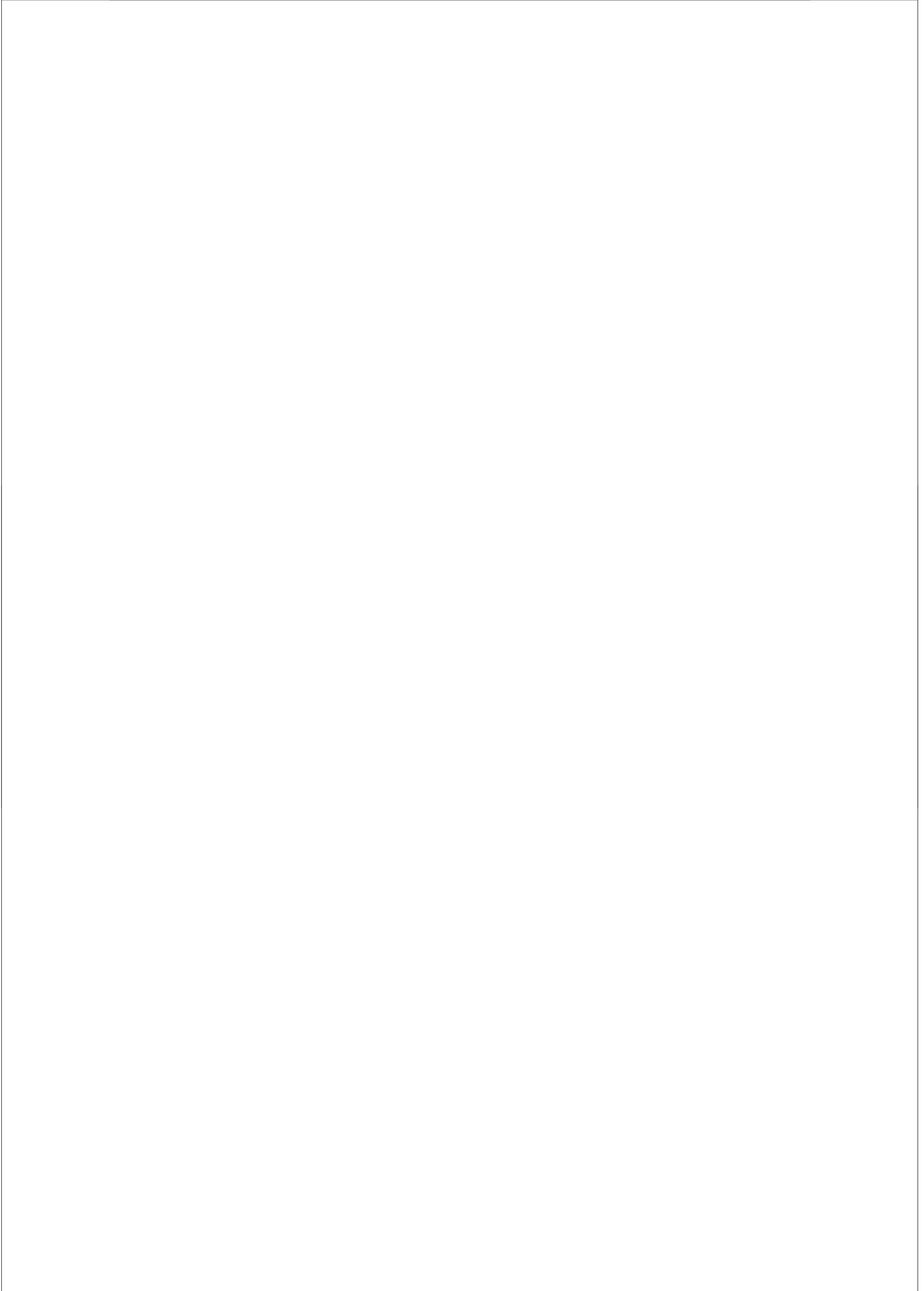
Without any meaningful disease control at the borders between neighbouring EU countries to prevent the entrance of carrier horses and/or ticks from endemic areas into the Netherlands, exotic ticks and their associated agents will continue to pose a threat to the equine population. Moreover, preventing ticks from crossing the border on wild-life and migrating birds is impossible. Even though the transmission of tick-borne infections is basically possible by preventing the tick-bite, no acaricides have been licensed for horses in the Netherlands. Those for dogs and cats may be safe and effective for horses, but they are relatively expensive. In many cases, removing attached ticks as early as possible, preferably before engorgement, also strongly reduces the risk of transmission. The development of vaccines against tick-borne infections is fundamentally hampered by the persistent nature of these agents in their host and the often geographically dependent strain variations they exhibit. Moreover, if vaccines can be developed, they will probably not be designed specifically for equids because the equine 'niche' is a fairly small market. Another option for a practical and sustainable approach lies in the development of "anti-tick" vaccines. However, developing vaccines against parasites is extremely difficult for several reasons. On the other hand, the fact that a vaccine for cattle against *Boophilus microplus* is actually on the market demonstrates that it is possible (de la Fuente, Almazan et al. 2007). Since the tick population in North-Western Europe mainly consists of *Ixodes ricinus*, a vaccine against this tick could strongly reduce the risk of tick-borne infections in the future.
In the mean time, an important weapon against tick-borne infections is gathering specific information and distributing it to the public. Standardised measurement of the agent in tick populations or vertebrate reservoirs has yet to be established. This is of crucial importance as the identification of foci of infection, or of parasite presence in the environment, enables specified notification; a carefully targeted flow of specific information to those it most concerns may therefore be the best medicine available.

Conclusions

The results of this thesis contribute to a better understanding of the occurrence and relevance of ticks (endemic and exotic) and equine tick-borne infections in

the Netherlands. Hopefully, the studies published in scientific, professional and lay journals will increase the awareness of tick-borne disease among veterinary surgeons and horse owners. The initial steps in establishing the current status of equine tick-borne infections in the Netherlands and increasing awareness among practitioners/owners have been made and include:

- The development of a real-time PCR assay for the detection of *B. caballi*, providing a better diagnostic tool for equine piroplasmosis, particularly in cases with very low parasitaemia and non-specific clinical signs (Chapter III).
- Detection of equine *A. phagocytophilum* infections, confirming that this tick-borne infection does occur in horses in the Netherlands and, should therefore, be included in the differential diagnosis for horses with pyrexia of unknown origin (Chapter IV).
- Detection of autochthonous acute *T. equi* and *B. caballi* infections in horses in the Netherlands (Chapter V). Such cases are expected to become more common as a result of the presence of subclinical *T. equi* carriers and the establishment of competent vector ticks.
- Examination of ticks collected from horses resident in the Netherlands has revealed that an unexpectedly high percentage of attached ticks were *Ixodes* nymphs carrying multiple agents of zoonotic potential (Chapter VI).
- Distribution of specific tick-borne agents seems to be geographically focussed (Chapter VI).
- Adult *Hyalomma marginatum* ticks were detected on 2 horses (Chapters VI and VII) and this may be a sign of climatic and/or biotope changes allowing this tick to moult and move on to the next host, in the Netherlands.
- Confirmation that *B. caballi* and *T. equi* are not always eliminated from carrier horses after high dose imidocarb dipropionate treatment (Chapter VIII). Further research is necessary to investigate whether this lack of efficacy is influenced by strain-variations or is the result of drug resistance.



Appendix I

Een literatuuroverzicht van equine piroplasmose naar aanleiding van acute babesiose bij een Nederlands draver veulen na verblijf in Normandië

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Samenvatting

Piroplasmose is een aandoening die voornamelijk voorkomt in tropische en subtropische gebieden maar ook landen met een gematigd klimaat lijkt te veroveren. Dit artikel geeft een literatuuroverzicht van equine piroplasmose en beschrijft een geval van een acute infectie met *Babesia caballi* bij een Nederlands draver veulen na een kort verblijf op een dekstation in Normandië.

Summary

This article gives a review of equine piroplasmosis and describes an acute case of infection with *Babesia caballi* in a Dutch Standardbred foal after a short stay at a stud in Normandy (France). A 3-month-old stallion foal was presented with lethargy, fever of 41°C, and pale mucosal membranes. Haematology revealed a low packed cell volume (14 l/l) leucytosis (25 G/l) and a high blood urea nitrogen concentration (20.1 mmol/l). Infection with *B. caballi* was diagnosed on the basis of Giemsa stained blood smears and was confirmed by polymerase chain reaction in combination with RLB. Treatment with imidocarb dipropionate and a blood transfusion resolved the haemolytic crisis.

Inleiding

De piroplasmata, die voornamelijk de genera *Babesia* en *Theileria* omvatten, zijn teek overdraagbare parasieten die zeer pathogeen kunnen zijn voor herkauwers, paarden, varkens, honden, katten en, in sommige gevallen, voor de mens. Equine piroplasmose, tegenwoordig opgesplitst in equine babesiose en equine theileriose, is een door teken overgedragen aandoening van paarden, muildieren, ezels en zebra's die veroorzaakt wordt door de hemoprotozoa *Theileria equi* en *Babesia caballi*. De infectie is endemisch in de meeste tropische en subtropische landen (Pfeifer Barbosa, Bose et al. 1995; Gummow, de Wet et al. 1996; Avarzed, De Waal et al. 1997; Kerber, Ferreira et al. 1999; Xuan, Chahan et al. 2002) waarbij *T. equi* vaker voorkomt dan *B. caballi* en de laatste verder noordwaarts reikt (Friedhoff and Soule 1996). In Europa is de infectie onder andere in Portugal, Spanje, Frankrijk en Italië endemisch. In Nederland, Ierland, Engeland en Duitsland is de ziekte niet endemisch, terwijl er in België, Zwitserland, Oostenrijk en Polen gebieden zijn waar autochtone infecties kunnen voorkomen. Australië is het enige continent waar equine babesiose en equine theileriose zich niet hebben kunnen vestigen, ook niet na introductie via geïnfecteerde geïmporteerde paarden in 1976, waarschijnlijk omdat de benodigde vector teek daar niet aanwezig is (Martin 1999).

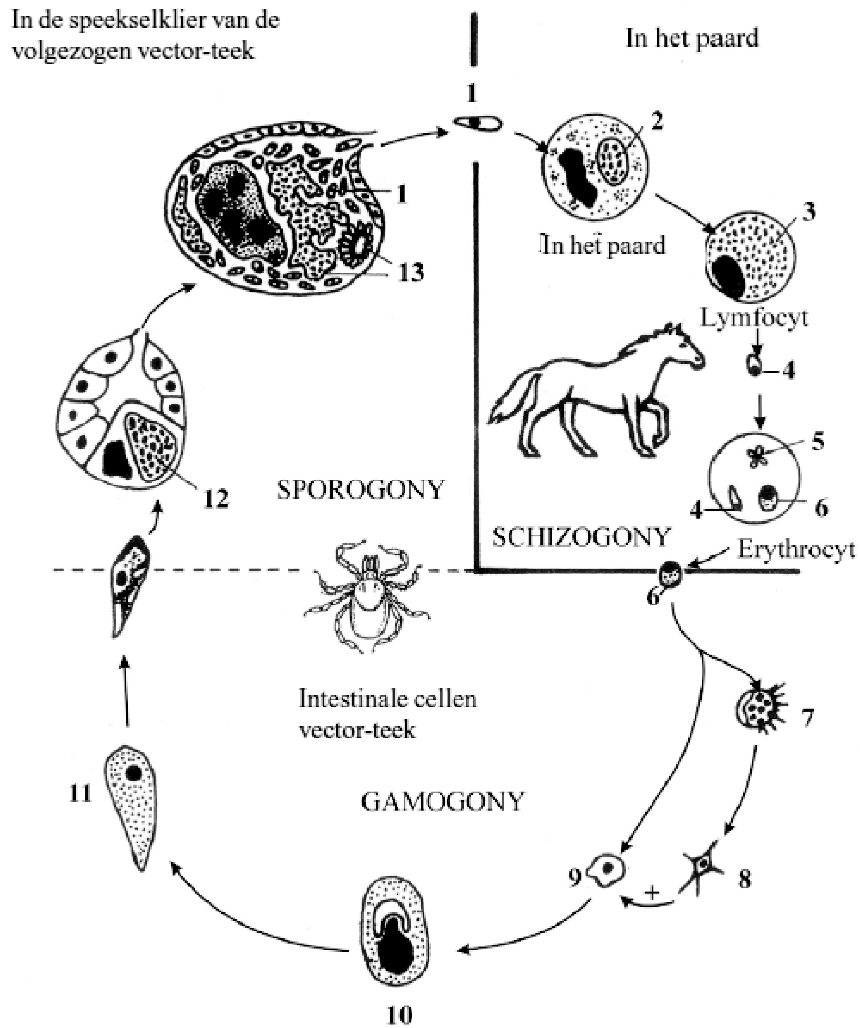
De parasieten

Equine babesiose en equine theileriose worden overgedragen door teken van de familie *Ixodidae* (harde teken), waaronder de *Dermacentor reticulatus*, *Dermacentor nuttalli* (Battsetseg, Lucero et al. 2002a), *Boophilus microplus* (Battsetseg, Lucero et al. 2002b) en de *Ixodes ricinus*. Deze teken hebben een typische 3-gastheercyclus waarbij elk stadium van de teek (larve, nimf en volwassen) op een andere gastheer parasiteert.

Teken zijn hematofage arthropoden die wereldwijd op bijna elke klasse van vertebraten parasiteren (Parola and Raoult 2001). Eenmaal volgezogen valt de teek van de gastheer af en zoekt een rustplaats om het bloed te verteren en zich om te vormen tot het volgende stadium. Teken zijn erg afhankelijk van omgevingsfactoren, zoals temperatuur, vochtigheidsgraad en de hoeveelheid zonlicht. Door hun grote gevoeligheid voor uitdrogen komen ze voornamelijk voor in grasland en bos. Elke tekensoort heeft zijn eigen optimale omgevingscondities wat voor een groot deel de geografische distributie van de teek bepaalt.

Teken kunnen besmet raken bij het voeden op een geïnfecteerde gastheer en deze besmetting -transstadiaal- behouden tijdens de overgang van het larvale stadium via nimf naar volwassen teek. Met *T. equi* geïnfecteerde paarden zijn een mogelijke bron (reservoir) van verspreiding aangezien de infectie van *T. equi* bij de teek alleen transstadiaal en niet transovarieel wordt overgedragen.

Levenscyclus van *T. equi*



1. sporozoïet, 2. macroschizont, 3. microschizont, 4. merozoïet,
5. maltees kruis, 6. gamont, 7. microgamont, 8. microgameet,
9. macrogameet, 10. zygote, 11. kineet, 12. sporont, 13. sporoblast

Fig. 1
Levenscyclus van T. equi *aangepast naar Zapf en Schein (1994)*

Daarentegen kan een *B. caballi* infectie door middel van transstadiale en transovariële overdracht in meerdere tekengeneraties persisteren. Dit heeft tot gevolg dat *B. caballi* gemakkelijker migreert (Friedhoff and Soule 1996).

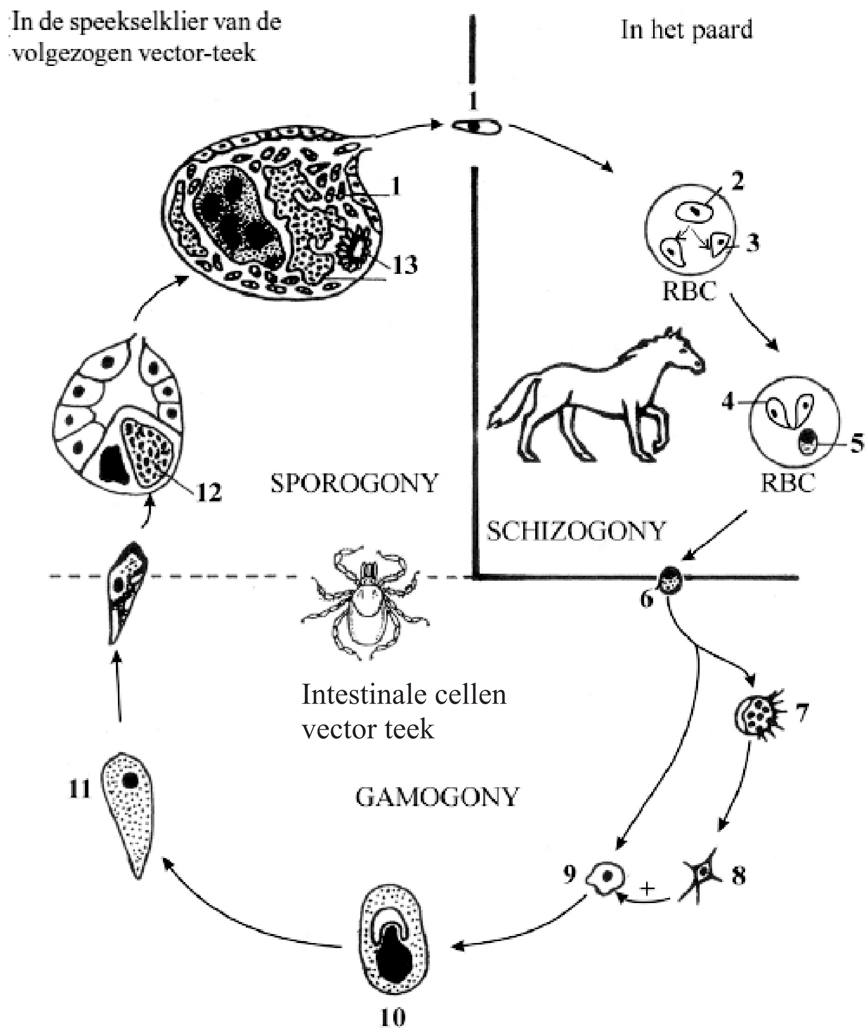


Fig. 2
Levenscyclus van B. caballi *aangepast naar Zapf en Schein (1994)*

Gedurende de levenscyclus van *B. caballi* en *T. equi* (figuur 1 en 2) worden sporozoïeten geïnoculeerd in paarden door vector-teken (Friedhoff and Soule 1996). Vector teken zijn teken waarin de betreffende parasiet kan overleven. *B.*

caballi sporozoïeten infecteren uitsluitend erytrocyten, waarin ze transformeren tot trophozoïeten. Elke trophozoïet groeit en deelt zich dan in twee peervormige merozoïeten van 2-5 μm lang bij 1.3-3 μm in diameter (Friedhoff and Soule 1996). Deze grote gepaarde merozoïeten komen tegen elkaar te liggen en geven zo het karakteristieke beeld van een *B. caballi* infectie in erytrocyten (figuur 3).

Theileria equi, vroeger bekend als *Babesia equi* (Mehlhorn and Schein 1998), heeft een pre-erytrocytaire ontwikkeling in lymfoïde cellen voordat de erytrocyten geïnvadeerd worden (Zapf and Schein 1994; Zweygarth, Lopez-Rebollar et al. 2002b).

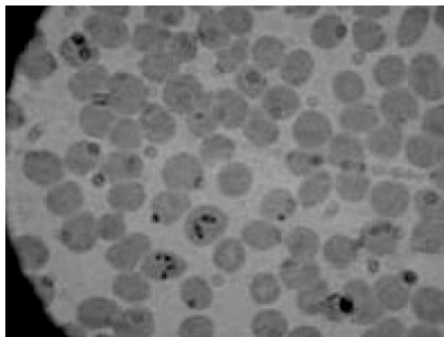


Fig. 3
Microscopisch beeld, 1000x vergroot, van een Giemsa-gekleurd bloeduitstrijkje met B. caballi *parasiet zichtbaar in de erythrocyten*

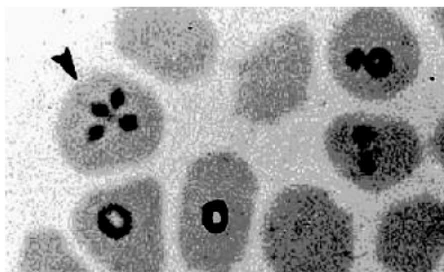


Fig. 4
Microscopisch beeld, 2000x vergroot, van een Giemsa-gekleurd bloeduitstrijkje met een Maltees kruis van T. equi *zichtbaar in een erythrocyt (Mehlhorn and Schein 1998)*

Theileria equi- sporozoïeten dringen eerst lymfocyten binnen en beginnen hier een schizogene fase die resulteert in het ontstaan van macroschizonten (fase van nucleaire reproductie) en microschizonten (fase van merozoïetformatie). Er worden ongeveer tweehonderd motiele merozoïeten per geïnfecteerde cel gevormd die na destructie van de lymfocyt vrijkomen en rode bloedcellen infecteren. Deze schizogene fase ontbreekt bij *Babesia*-species. De merozoïeten vermeerderen door middel van de simultane vorming van een merozoïetviertal oftewel een Maltees kruis dat karakteristiek is voor een *T. equi* infectie (figuur 4). De prepatent periode omvat twee tot vijf dagen en pas twaalf tot veertien dagen na infectie kunnen erytrocytaire merozoïeten in bloeduitstrijkjes worden waargenomen (Mehlhorn and Schein 1998).
De parasitaemie bij *T. equi* varieert van 1- 80 procent. Bij een infectie met *B. caballi* is de parasitaemie zelden hoger dan één procent. Dieren die herstellen van een primo-infectie met *T. equi* blijven vaak levenslang geïnfecteerd en kunnen zo als infectiereservoir dienen (Bruning 1996). In deze fase is de parasitaemie vaak te laag om microscopisch gedetecteerd te worden en zijn er sensitieve diagnostische testen nodig om symptoomloze dragers op te sporen. Zo is de gevoeligheid van een PCR voor *T. equi* ongeveer duizend keer

groter dan die van het microscopisch onderzoek (Bashiruddin, Camma et al. 1999).

Symptomen

De incubatietijd van een *T. equi* infectie bij de gastheer varieert van twaalf tot negentien dagen en voor *B. caballi* van tien tot dertig dagen. Theileriose en babesiose kunnen voorkomen in een acute, subacute en chronische vorm. In de acute gevallen wordt hoge koorts (>40 °C), dyspneu, anemie, icterus, hemoglobinurie, congestie van de muceuze membranen en soms lichte koliek gezien. Icterus, anemie en hemoglobinurie zijn vaak erger bij een infectie met *T. equi*. In subacute gevallen zijn de symptomen gelijk aan de acute gevallen, maar er is dan vaak gewichtsverlies, intermitterende koorts en de slijmvliezen kunnen variëren van lichtroze tot helgeel. In de chronische fase zijn er geen specifieke symptomen, maar hebben de paarden vage klachten zoals gebrekkige eetlust, slecht presteren en verlies van spiermassa (Friedhoff and Soule 1996).

Diagnostiek

Detectie van de parasieten

Microscopie

Microscopisch onderzoek van een bloeduitstrijkje kan de parasieten aantonen in de rode bloedcellen gedurende de acute fase van de infectie. De betrouwbaarheid van een uitstrijkje bij visualisatie van de parasiet in de erytrocyten is honderd procent, maar de gevoeligheid is laag aangezien de parasitaemie vaak niet groter is dan één procent. Bij een negatief bloeduitstrijkje zijn aanvullend serologische en/of moleculaire testen nodig om de diagnose te stellen.

In vitro cultuur

In vitro cultuur is moeilijk en kan een aantal weken duren (Holman, Frerichs et al. 1993). In Nederland wordt geen gebruikgemaakt van *in vitro* cultuur in de diagnostiek van theileriose en babesiose. Voortschrijdend onderzoek en betere kennis van de optimale groeicondities zorgen ervoor dat *in vitro* cultuur steeds makkelijker wordt en het infecteren van proefdieren (in verband met antigeen productie) overbodig maakt (Avarzed, Igarashi et al. 1997; Holman, Becu et al. 1998). Het zou een aanvulling kunnen zijn op serologische testen en geeft accuraat aan of de levende parasiet nog aanwezig is bij het paard (Holman, Hietala et al. 1997; Zweygarth, Lopez-Rebollar et al. 2002b) en paard-achtigen (Zweygarth, Lopez-Rebollar et al. 2002a).

Aantonen van DNA met behulp van de polymerase chain reaction (PCR)

PCR is een techniek waarbij het DNA van de parasiet vele malen vermenigvuldigd wordt waardoor de gevoeligheid enorm toeneemt (Bose, Jorgensen et al. 1995; Rampersad, Cesar et al. 2003). De sensitiviteit en de specificiteit zijn afhankelijk van de gebruikte primers. Als aanvulling op de PCR kan voor speciëring een reverse line blot (RLB) worden gedaan waarmee een hogere specificiteit bereikt wordt.
Bij een RLB wordt het PCR-product gehybridiseerd op specifieke probes die vastzitten op een membraan (Nagore, Garcia-Sanmartin et al. 2004). De combinatie PCR-RLB maakt het mogelijk tegelijkertijd te testen op verschillende parasieten, waarbij zowel de specificiteit als de sensitiviteit van deze test erg hoog zijn (Gubbels, de Vos et al. 1999).

Detectie van specifieke antilichamen tegen de parasiet

Complement fixatie test (CFT)
De CFT is een oudere serologische techniek die gebruikt wordt in Amerika, Australië en Japan (Ikadai, Nagai et al. 2002) met de bedoeling de invoer van paarden besmet met *Babesia/Theileria* te voorkomen. De CFT heeft echter een lage sensitiviteit bij chronische infecties met veel vals-negatieve (subklinische dragers) uitslagen (Knowles, Perryman et al. 1991b; Knowles 1996). In Amerika was men sinds 1 november 2004 overgestapt op het gebruik van een cELISA maar heeft men zeer recent de CFT weer in gebruik genomen.

Indirecte fluorescentie antilichaam test (IFAT)
Bij de IFAT wordt serum van een te testen paard toegevoegd aan gefixeerde parasieten en worden de eventueel gebonden antilichamen met behulp van een fluorescerend conjugaat aangetoond. Deze test is gemakkelijk en gevoelig maar wel arbeidsintensief. Een vergelijkende studie wijst uit dat de IFAT ten opzichte van de CFT eerder positief is, langer positief blijft, hogere titers geeft en niet beïnvloed wordt door behandeling met imidocarb dipropionaat (Kuttler, Zaugg et al. 1987).

Ezyme-linked immunosorbent assay (ELISA)
De kwaliteit van een ELISA is afhankelijk van de kwaliteit van de antigenen waar de test op gebaseerd wordt (Kumar, Kumar et al. 2003). Paarden geïnfecteerd met *T. equi* produceren antilichamen tegen tenminste elf merozoïet eiwitten (Knowles, Perryman et al. 1991a). Knowles et al. (1992) toonden aan dat het equi merozoïet antigeen 1 (EMA- 1) een epitoop bezit dat immunodominant en wereldwijd aanwezig is. Dit recombinant EMA-1 kan betrouwbaar gebruikt worden om *T. equi* specifieke antistoffen aan te tonen, ondanks lichte variatie in het EMA-1-gen (Knowles, Kappmeyer et al. 1992; Nicolaiewsky, Richter et al. 2001; Xuan, Igarashi et al. 2001; Cunha, Kappmeyer et al. 2002; Kumar, Malhotra et al. 2002b). Paarden geïnfecteerd met *B. caballi* binden het EMA-1 epitoop niet. Xuan et al. (2001) toonden aan dat het EMA-1 reagens

relatief simpel geproduceerd kan worden door een celcultuur te infecteren met een recombinant virus dat EMA-1 tot expressie brengt. Voor de detectie van *T. equi* en *B. caballi* zijn er sinds kort twee commerciële cELISA-testen op de markt (VMRD, Inc Pullman, WA 99163 USA) die gebaseerd zijn op twee verschillende antigenen.

Sneltest (Point of care test)
Deze recent ontwikkelde (nog niet commercieel verkrijgbare) test is gebaseerd op de detectie van antilichamen van *T. equi* met behulp van nitrocellulose membraan teststrips. Volgens Huang et al. (2004) is dit een snelle, simpele en betrouwbare test die gemakkelijk door de practicus in het veld gedaan kan worden. Het diagnostisch traject bij een babesiose/theileriose verdacht dier zou dus moeten beginnen met microscopie van een gekleurd bloeduitstrijkje. Bij een negatief resultaat is PCR de volgende stap aangezien er in de acute fase nog geen antilichaam respons is die met serologie kan worden aangetoond. In de subacute en chronische fase zijn serologie en PCR de aangewezen diagnostische middelen om een infectie met *babesia/theileria* vast te stellen.

Bestrijding

Behandeling

De behandeling van equine babesiose/theileriose is gericht op eliminatie van de parasiet uit zieke paarden of uit paarden die van een endemisch gebied naar een niet-endemisch gebied reizen. Imidocarb dipropionaat (een carbanilide derivaat) is nog steeds het middel van eerste keus. *Babesia caballi* kan geëlimineerd worden met een dosis van 2 mg/kg lichaamsgewicht intramusculair 1dd gedurende twee dagen. *Theileria equi* kan bestreden worden, maar niet geëlimineerd, met een dosis van 4 mg/kg lichaamsgewicht intramusculair vier doses met 72 uur tussen elke injectie (Belloli, Crescenzo et al. 2002). Imidocarb dipropionaat is twaalf uur na injectie niet meer aan te tonen in plasma en wordt voornamelijk opgeslagen in de lever en uitgescheiden door de nier (Belloli, Crescenzo et al. 2002). Een geringe overdosering kan dodelijk zijn voor het paard. Er zijn veelbelovende alternatieve behandelmethoden die nu echter nog in de experimentele fase zijn (Bork, Yokoyama et al. 2003a; Bork, Yokoyama et al. 2003b; Kumar, Gupta et al. 2003; Bork, Yokoyama et al. 2004).

Preventie

De mogelijkheid tot preventie van equine babesiose/theileriose is afhankelijk van de epidemiologische situatie. In *theileria/babesia* vrije gebieden zijn de enige bronnen van infectie de introductie van geïnfecteerde paarden en/of teken (Friedhoff, Tenter et al. 1990). *T. equi* kan ook doorgegeven worden met bloed gecontamineerde instrumenten en ook intra-uteriene infecties van de

foetus tijdens de dracht kunnen voorkomen (Phipps and Otter 2004). Transmissie via sperma is nog niet aangetoond, maar er bestaat wel bezorgdheid over mogelijke transmissie bij contaminatie van sperma met bloed (Metcalf 2001). Subklinische infecties zijn van groot belang voor de sportpaarden industrie vanwege de veelvuldige verplaatsing van paarden (Coffman 1997). Zware training kan een subklinische infectie acuut maken, zoals aangetoond werd bij race-paarden in Jordanië door Hailat et al. (1997). De preventie is dus voornamelijk gericht op het voorkomen van de import van subklinisch geïnfecteerde paarden oftewel dragers in onder andere Amerika, Canada, Australië, Japan en enkele andere landen (Friedhoff, Tenter et al. 1990). De insleep van besmette teken via andere dieren (onder andere klein wild en vogels) is bijna niet te voorkomen. Het gebruik van acaricide middelen en een goede controle van individuele paarden op teken zijn uiteraard van groot belang (Kerber, Ferreira et al. 1999). De meeste *Babesia* parasieten induceren een langdurige infectie bij de gastheer en er wordt gedacht dat zowel *Theileria* als *Babesia* spp. meerdere mechanismen kunnen aanwenden om de immuunrespons van de gastheer te ontwijken (Allred 2003). Er zijn nog geen effectieve vaccins tegen equine babesiose/theileriose beschikbaar (Friedhoff, Tenter et al. 1990).

Fig. 5
Draver veulen op de leeftijd van drie maanden

Casuistiek

Ziektegeschiedenis

Op 22 april 2003 werd één van ons (Drs. J.van Gils) geroepen bij een draver hengstveulen van drie maanden oud (figuur 5) met de klachten algehele malaise, sloom en hoge koorts (41 °C). Het veulen was in de periode van 23-02-2003 tot 08-04-2003 met de merrie op een dekstation in Normandië geweest. Bij terugkomst in Nederland kregen merrie en veulen weidegang in de koppel. Het veulen werd veertien dagen na terugkomst in Nederland ziek.

Klinisch onderzoek

Bij klinisch onderzoek had het veulen een rectale temperatuur van 41 °C, een ademfrequentie van 40-50 per minuut en een pols van 60 slagen per minuut. De slijmvliezen waren op dat moment bleekroze, de lymfeknopen waren niet

afwijkend, maar bij longauscultatie werd verscherpt vesiculair ademen gehoord. Omdat gedacht werd aan een virusinfectie van de luchtwegen werd het veulen behandeld met flunixine meglumine[1] (1 mg/kg lg IV 1dd) gedurende vier dagen en ter voorkoming van secundaire bacteriële infecties werd ceftiofur[2] (10 mg/kg lg IM 1dd) gedurende elf dagen toegediend. Aangezien geen verbetering optrad werd het veulen drie dagen later op 25 april 2003 nog een keer onderzocht. Het veulen vertoonde op dat moment een 'dronkemansgang', had nog steeds koorts en nu werden bleekgele slijmvliezen vastgesteld. Bij bloedonderzoek werden een verlaagde hematocriet en hemoglobine concentratie gevonden alsook een sterk verhoogde ureum concentratie en een leucocytose (tabel 1).

Tabel 1

Datum/Bloeduitslag	25/04/2003	05/05/2003	20/05/2003	Normaal waarden
Hematocriet (l/l)	14	28	22	30-42
Hemoglobine (mmol/l)	3,6	6,4	5,4	7,5-9,0
Leukocyten (G/l)	25	54,3	12,3	7-10
Totaal eiwit (g/l)	50	65	–	60-85
Ureum (mmol/l)	20,1	4,3	–	< 8

Diagnostiek

Microscopisch onderzoek van een opgestuurd bloeduitstrijkje (bekeken door het Brabants Veterinair Laboratorium te Diessen) bleek in tweede instantie positief te zijn op *B. caballi*, met de *B. caballi* parasiet zichtbaar in de rode bloedcellen (figuur 3). De IFAT, bij de afdeling Parasitologie en Tropische Diergeneeskunde, Faculteit der Diergeneeskunde te Utrecht, was positief voor *B. caballi* (1:640/1280 op 5 mei 2003 en 1:320 op 10 mei 2003) en negatief voor *T. equi*. PCR gecombineerd met een RLB, uitgevoerd volgens de methode beschreven door Gubbels et al. (1999), gaf met beide monsters een positief resultaat voor B. caballi (figuur 6).

[1] Finadyne®, Schering-Plough Santé Animale

[2] Excenel®, Pharmacia B.V., Woerden

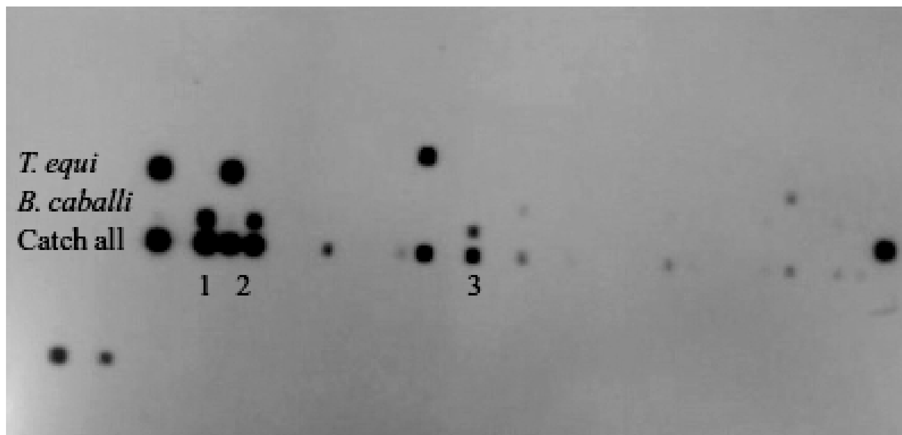


Fig. 6
Reverse line blot uitslag van het veulen positief voor B. caballi *op de data 05-05-2003 en 21-05-2003. Nummer 1: positieve controle* B. caballi*; nummer 2: uitslag op 05-05-2003; nummer 3: uitslag op 21-05-2003*

Differentiële diagnose

Anemie kan veroorzaakt worden door bloedverlies, verhoogde afbraak van erytrocyten (hemolyse) en verminderde aanmaak van erytrocyten. Bij anemie door bloedverlies moet gedacht worden aan bloedverlies na trauma, operatie of ruptuur van een groot bloedvat, maar ook chronisch verlies van kleine hoeveelheden bloed kan anemie veroorzaken. In het laatste geval kan gedacht worden aan ulceraties (onder andere door NSAID-intoxicatie), parasitaire infecties (strongylose) en neoplasiën (plaveiselcelcarcinoom van de maag) in het maagdarmkanaal. Hemolyse (intravasculaire of extravasculaire afbraak van erytrocyten) kan voorkomen bij meerdere aandoeningen, onder andere bij immuungemedieerde aandoeningen (vaak secundair aan virale, bacteriële of protozoaire infecties, blootstelling aan bepaalde medicamenten of neoplasie), infectieuze aandoeningen (babesiose, theileriose en equine infectieuze anemie), oxidant geïnduceerde schade (phenotiazines, uien, rode esdoornbladeren) en iatrogene inductie (toedienen van hypertone vloeistoffen). Anemie ten gevolge van verminderde aanmaak van erytrocyten kan het gevolg zijn van chronische ontstekingsprocessen, zoals pneumonie, pleuritis, peritonitis en abcessen, tumoren (onder andere lymfosarcoma), ijzertekort, beenmergaandoeningen, chronische lever- (verminderde aanmaak van stollingsfactoren) en/of nierproblemen (verlaagde erytropoëtine productie).

Behandeling en vervolg

Het veulen werd opgenomen en behandeld met een bloedtransfusie (500 ml vol bloed) afgenomen van een donorruin. Omdat eerst gedacht werd aan een autoimmune aandoening werd dexamethason[3] (0,4 mg/kg lg IM 1dd) gegeven. Vanaf het moment dat de diagnose babesiose definitief gesteld was werd het veulen behandeld met imidocarb dipropionaat[4] (1,7mg/kg lg IM) tweemaal met een interval van 48 uur. Na behandeling op 28 en 30 april was het veulen koortsvrij en vertoonde bij klinisch onderzoek geen afwijkingen meer. Op 5 mei werd opnieuw bloedonderzoek gedaan waarbij de hematocriet 28 l/l en het ureum 4,3 mmol/l waren, waarop het veulen als klinisch genezen naar huis is gegaan. Op 20 mei werd een klinische terugval vastgesteld met opnieuw koorts (39.3 °C) en een haemolytische crisis (Ht: 22 l/l). Er bleek sprake te zijn van een recidief aangezien de PCR-RLB van monster-datum 21 mei 2003 positief was op *B. caballi* (figuur 6). Het veulen werd opnieuw behandeld met imidocarb dipropionaat (1,7mg/kg IM) en in verband met een aangetroffen teek op de voorborst tevens met fipronil[5] (niet geregistreerd voor het paard). Het veulen herstelde vlot na behandeling en heeft geen klachten meer gehad (figuur 7).

Fig. 7
Draverveulen op de leeftijd van acht maanden

Discussie

Bovenstaande casus wijst op de mogelijkheid van babesiose bij het paard in Nederland (in dit geval na verblijf in het buitenland). Recente uitbraken van autochtone babesiose bij honden zonder buitenland anamnese zijn een aanwijzing dat babesiose endemisch zou kunnen worden in Nederland (Matjila, Nijhof et al. 2005). De verantwoordelijke vector-teek *Dermacentor reticulatus* die ook equine babesiose/theileriose overdraagt is op enkele plaatsen in Nederland aangetroffen en kan zich mogelijk permanent vestigen. Dit zou aanleiding kunnen gaan geven tot autochtone infecties bij paarden en paardachtigen in Nederland. Om die reden zal men bij een paard met anemie ook aan babesiose/ theileriose moeten denken. Recent onderzoek toont aan dat er een be-

[3] Dexadreson®, Intervet International B.V., Boxmeer

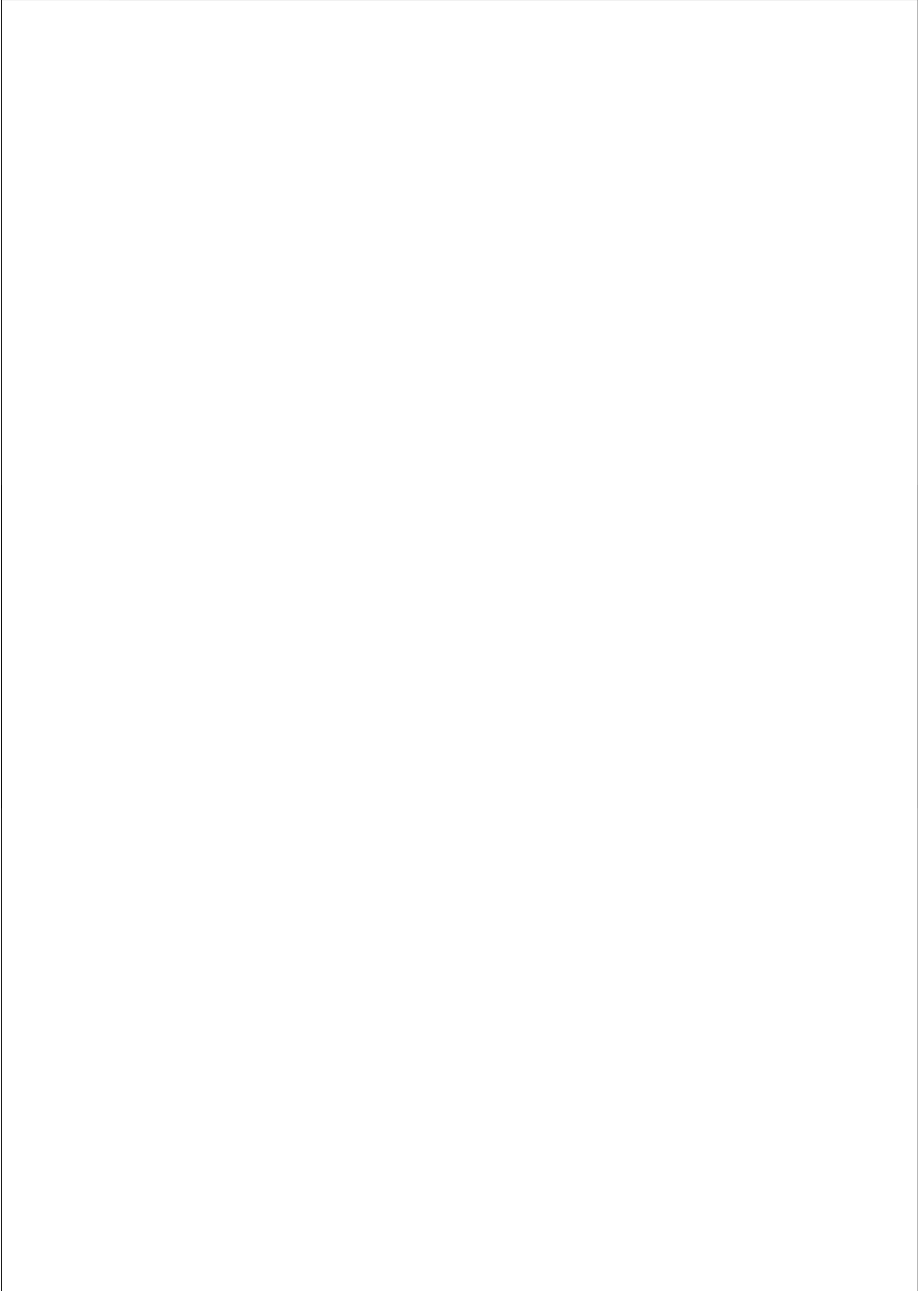
[4] Carbesia®, Schering-Plough Santé Animale

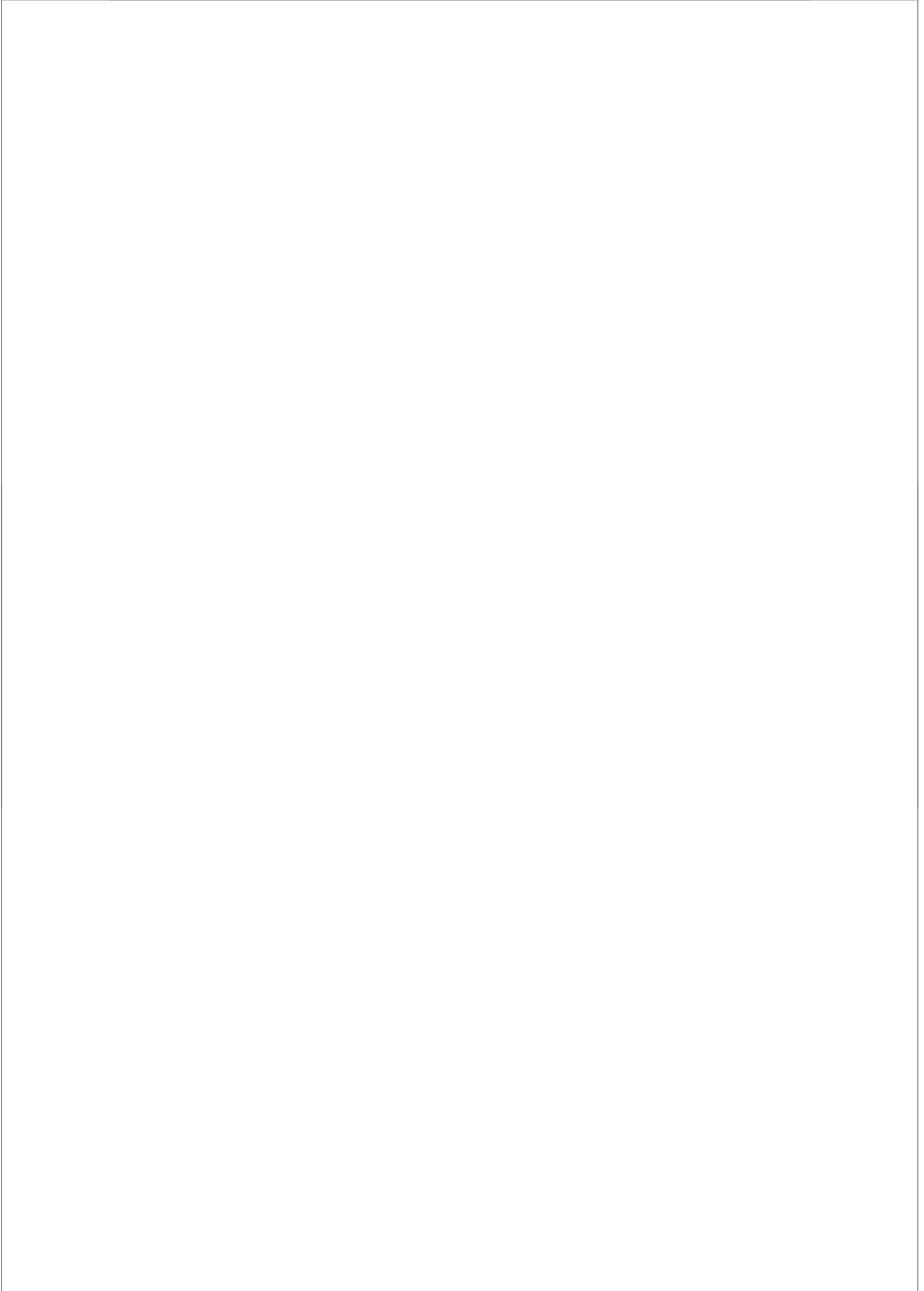
[5] Frontline®, Merial, Amstelveen

hoorlijk hoge graad van genetische variatie bestaat binnen de groep van de babesiae en de theileriae (Criado-Fornelio, Martinez-Marcos et al. 2003b; Criado-Fornelio, Martinez-Marcos et al. 2003a; Criado-Fornelio, Gonzalez-del-Rio et al. 2004).
Deze variatie is onder andere van belang bij de ontwikkeling van nieuwe diagnostische testen en vaccins (Katzer, McKellar et al. 1998). Kumar et al. (2002a) toonden aan dat ezels (ook de gesplenectomeerde) beschermd waren tegen infectie met *T. equi* na vaccinatie (gecombineerd met een booster na 21 dagen) met geïnactiveerde *T. equi* merozoïeten. Dit wijst op de mogelijkheid van effectieve vaccinatie. Veel protozoa hebben echter ontsnappingsmechanismen ontwikkeld om het immuunsysteem van de gastheer te ontwijken, en vaccinatie tegen één soort geeft geen garantie voor bescherming tegen andere soorten (Jenkins 2001). Oplettendheid bij dierenartsen, goede diagnostiek, een beter begrip van de gastheer-vectorteek interacties en kennis over het immuunmechanisme, zodat veilige en effectieve vaccins gemaakt kunnen worden zijn van eminent belang.

Dankbetuiging

De auteurs willen het Departement Infectieziekten en Immunologie, afdeling Parasitologie en Tropische Diergeneeskunde, Faculteit der Diergeneeskunde te Utrecht bedanken voor het uitvoeren van de IFAT en de hulp bij de PCR-RLB.





Appendix II

Niet zonder gevaar....

Teken bij paarden

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Inleiding

Er wordt de laatste jaren veel gesproken over de toename van het aantal teken en tekensoorten en de risico's die daar uit voortvloeien voor mens en dier.
Teken zijn geleedpotige organismen die behoren tot de spinachtigen. De twee- tot driejarige levenscyclus van teken bestaat uit meerdere stadia. Elk stadium (larve, nimf en volwassen teek) parasiteert op een andere gastheer en kan op die manier ziektekiemen overbrengen. Een teek loopt vaak meerdere uren op een gastheer rond voordat een plek uitgekozen wordt om bloed te zuigen. Eenmaal volgezogen laat de teek los en zoekt hij een rustplaats om het bloed te verteren en zich om te vormen tot het volgende stadium. Door hun grote gevoeligheid voor uitdrogen komen teken voornamelijk voor in ruw grasland, bos en duinen.

Schapenteek

In Nederland zijn slechts enkele tekensoorten van belang waarvan de *Ixodes ricinus* (schapenteek) de meest voorkomende is. *Ixodes ricinus* heeft een groot gastheerbereik en kan de ziekte van Lyme (veroorzaakt door de bacterie *Borrelia burgdorferi*) en anaplasmose (veroorzaakt door de bacterie *Anaplasma phagocytophilum*) overdragen op de mens, het paard en andere diersoorten. Teken kunnen ook andere ziekteverwekkers bevatten waarvan de klinische betekenis voor onder andere het paard nog onbekend is.
De ziekte van Lyme wordt bij het paard vaak geassocieerd met koorts, kreupelheid, gewrichtsontstekingen en spierpijn. Een duidelijk verband tussen een infectie met *Borrelia burgdorferi* en de ziekteverschijnselen is moeilijk met zekerheid vast te stellen. Dit geldt overigens voor alle diersoorten (inclusief de mens). Anaplasmose bij het paard is recent vastgesteld in Nederland. De bacterie kan koorts, verminderde eetlust, lichte bloedarmoede, sloomheid, spierpijn en dikke achterbenen veroorzaken. De aandoening komt vooral voor in de lente en de herfst. De diagnose kan gesteld worden door middel van bloedonderzoek.
Er is nog maar weinig bekend over het voorkomen van teek gerelateerde aandoeningen bij het paard in Nederland. De klimaatverandering en het transport van mens en dier over de hele wereld zouden nieuwe tekensoorten en daarmee onbekende ziekten kunnen introduceren in de Nederlandse populatie. Sommige van deze teek gerelateerde infecties komen al veelvuldig voor in het buitenland, onder andere België, Frankrijk en Spanje. In die landen vertonen geïnfecteerde paarden vaak geen of nog slechts geringe ziektesymptomen. Ze hebben specifieke afweerstoffen in het bloed ontwikkeld. Als er wel symptomen optreden, zijn die bij veel van deze teek gerelateerde infecties gelijksoortig. Dat maakt de juiste diagnose moeilijk.

Onderzoek

Vanwege het toenemende belang om de stand van zaken met betrekking tot teek-gebonden aandoeningen bij onder andere het paard goed in kaart te brengen, hebben het Departement Gezondheidszorg Paard en het Utrecht Centre for Tickborne Diseases (UCTD) een inventariserend onderzoek gestart. Voor dit onderzoek is elke teek die op een paard wordt aangetroffen van belang.

Wat te doen?

- Verwijder de teek/teken met een pincet of tekentang. Gebruik geen alcohol of andere vloeistoffen om de teek te bedwelmen. Bewaar de teek in een droog afgesloten buisje of potje.
- Vraag uw dierenarts om twee buisjes EDTA-bloed van het paard af te nemen. Het bloed en de teek/teken kunnen opgestuurd worden naar het UCTD. Om te kunnen onderzoeken of de teek een infectie op uw paard heeft overgebracht hebben de onderzoekers nog een bloedmonster (twee buisjes EDTA-bloed) nodig dat vier tot zes weken later is afgenomen. De resultaten worden via de dierenarts naar de deelnemende paardeneigenaren teruggekoppeld. Dit kan enkele maanden duren. De teken en de bloedmonsters worden kosteloos onderzocht. Bloedafname door de dierenarts is niet kosteloos.
- Informatie die voor het onderzoeksteam van belang is met betrekking tot het paard: ras, leeftijd, geslacht, waar de teek is gevonden op het paard, of eerder teken op het paard zijn aangetroffen, of een bestrijdingsmiddel is gebruikt (wat, hoe, waar), of het paard in het buitenland is geweest (zo ja: waar, wanneer) en de postcode van het stalling adres.
- Voor specifieke informatie over teek gebonden aandoeningen bij paarden kan gemaild worden naar Catherine Werners-Butler: c.m.butler@uu.nl, Specialist Inwendige Ziekten Paard bij de Faculteit Diergeneeskunde en werkzaam bij het UCTD.

De op deze wijze verkregen informatie over teken bij paarden is van belang om inzicht te krijgen in de infecties die thans in teken voorkomen en welke daarvan paarden kunnen infecteren en eventueel ziek maken. Kennis die voor alle paardenhouders van belang is.

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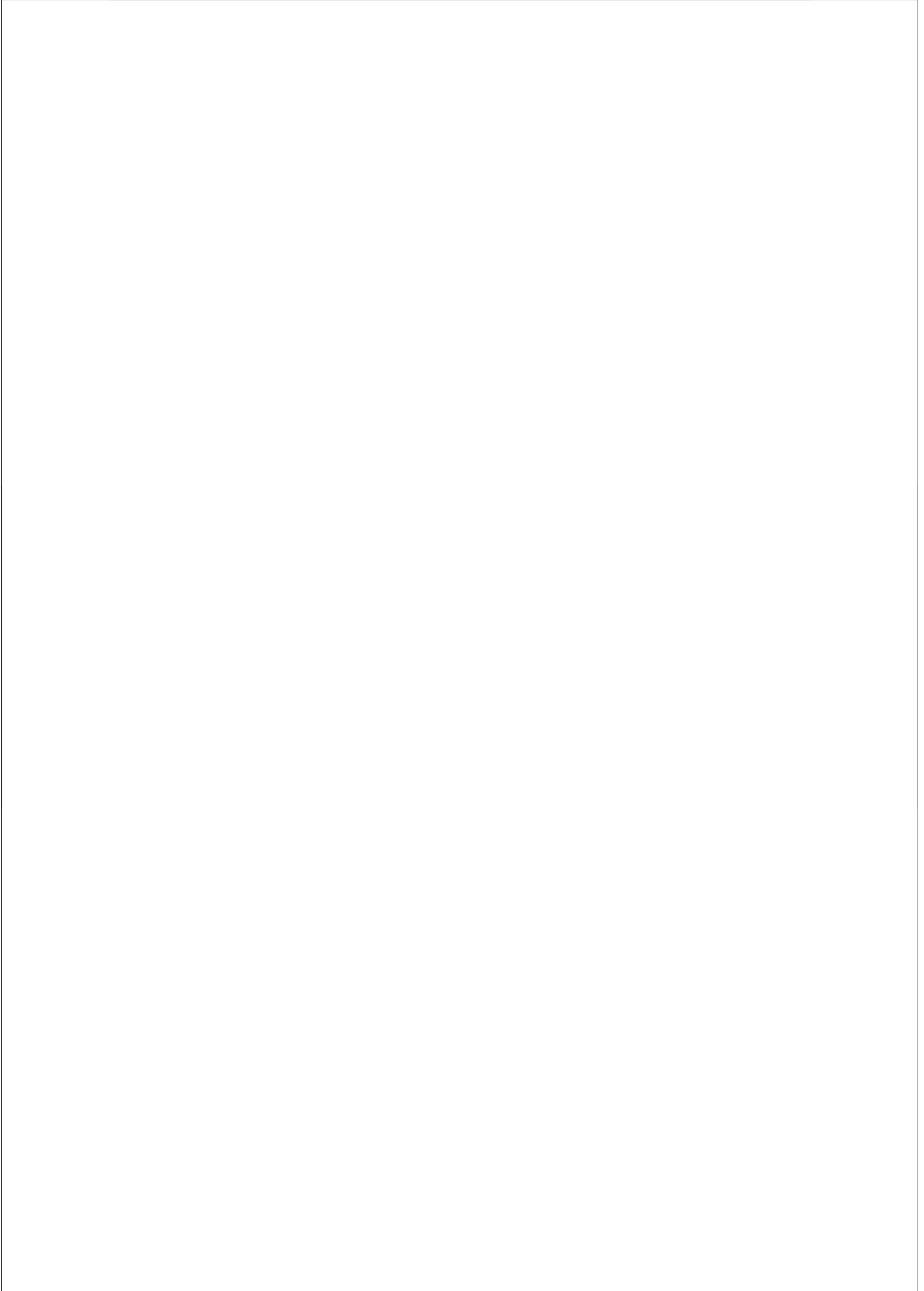
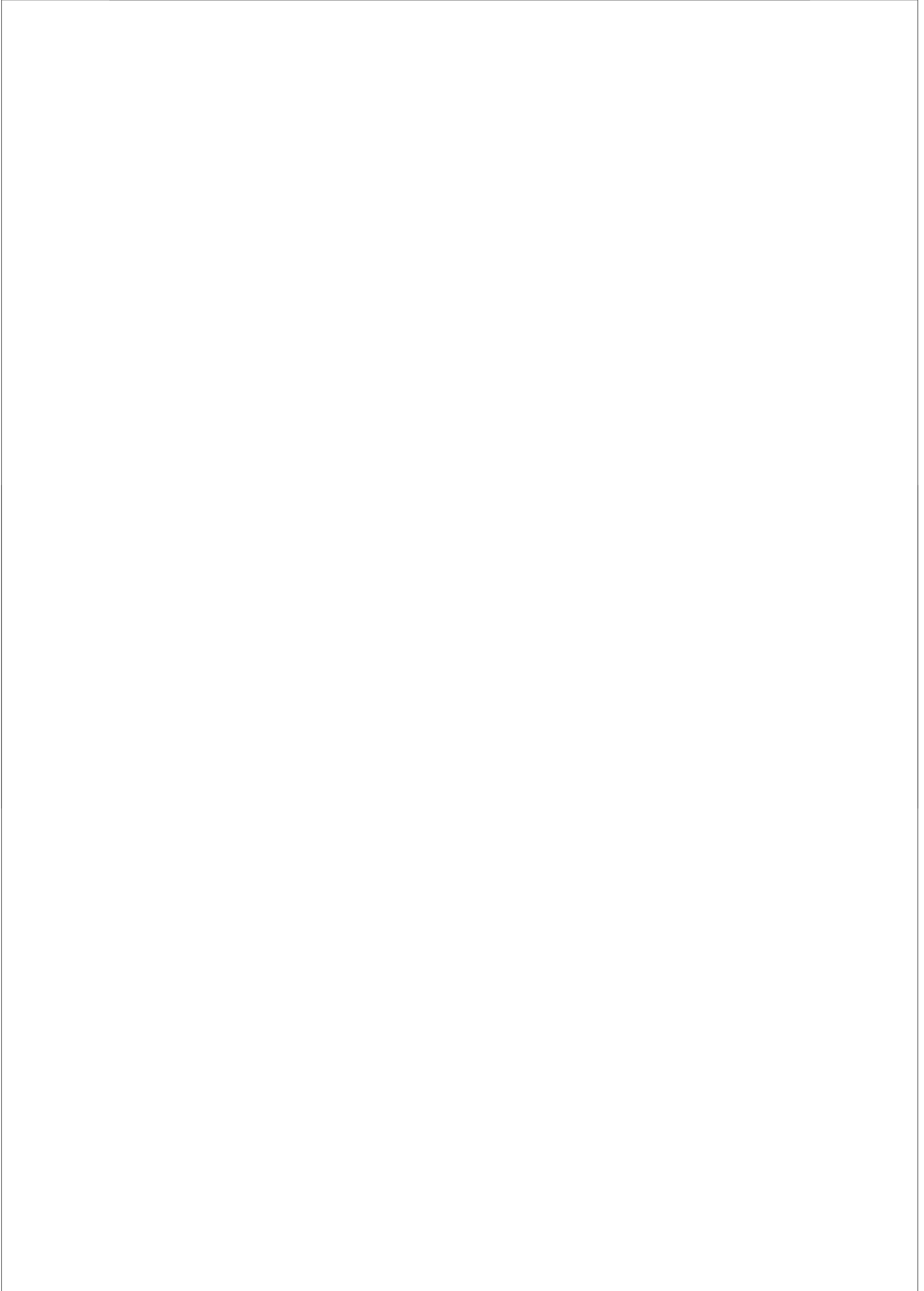




Nederlandse samenvatting

Dit proefschrift richt zich op het voorkomen en het belang van teek gebonden infecties bij paarden in Nederland. Speciale aandacht wordt gegeven aan de diagnose, klinische relevantie en behandeling van deze infecties.

In **hoofdstuk 2** wordt een literatuuroverzicht gegeven van de 4 agentia die bij paarden in Nederland voorkomen; *Borrelia burgdorferi*: oorzakelijk agens van (equine) borreliose. *Theileria equi* en *Babesia caballi*: oorzakelijke agentia van equine piroplasmose (EP). *Anaplasma phagocytophilum*: oorzakelijk agens van (equine) anaplasmose. In deze literatuuroverzichten worden de hedendaagse opvattingen met betrekking tot de diagnose, behandeling en preventie van de genoemde teek gebonden infecties beschreven.

In **hoofdstuk 3** worden de moeilijkheden toegelicht die zich voor kunnen doen bij het identificeren van zogenaamde piroplasmose-dragers; paarden met een chronische/persisterende infectie met *T. equi* en/of *B. caballi*. Ondanks het feit dat PCR een onmisbare techniek is geworden in de detectie van *T. equi* en *B. caballi*, kunnen kleine variaties in het DNA ervoor zorgen dat een "te" specifieke PCR de betreffende agentia niet meer herkent. Naast het probleem van de specificiteit bestond er ook een probleem met betrekking tot het detecteren van *B. caballi* dragers. Dit bleek te worden veroorzaakt door een te lage sensitiviteit van de bestaande testen. De hier beschreven nieuwe, kwantitatieve test bleek zodanig specifiek en gevoelig dat genoemde problemen opgelost lijken te zijn.

Het doel van **hoofdstuk 4** was het aantonen van de aanwezigheid van teek gebonden agentia bij paarden met onverklaarde koorts. Van 61 paarden met koorts van onbekende oorsprong werd, na uitsluiting van veel voorkomende oorzaken, het bloed getest op de aanwezigheid van teek gebonden agentia door middel van microscopisch onderzoek van een gekleurd bloeduitstrijkje en een PCR-RLB. Zes (10%) van de paarden bleken *A. phagocytophilum* positief te zijn in de PCR-RLB, en 5 van deze 6 paarden werden ook positief bevonden bij onderzoek van het bloeduitstrijkje. Deze bevindingen toonden enerzijds aan dat *A. phagocytophilum* voorkomt bij paarden in Nederland en anderzijds dat het bekijken van een gekleurd bloeduitstrijkje een gevoelige en praktische test is om *A. phagocytophilum* aan te tonen bij paarden in de acute fase van de infectie. Bij de overige 55 paarden werden geen teek gebonden infecties gevonden.

In **hoofdstuk 5** wordt de prevalentie beschreven van *B. caballi* en *T. equi*; deze agentia kunnen samen of alleen equine piroplasmose (EP) veroorzaken. De studie werd uitgevoerd in Schouwen-Duiveland waar eerder een diagnose van subklinische equine babesiose werd gesteld bij een paard die bij een bosrit een zware teken-infestatie had opgelopen. Nederland is officieel nog steeds een piroplasmose-vrij land hoewel het risico op insleep van deze "exotische"

aandoening is toegenomen. Dit verhoogde risico is het gevolg van de aanwezigheid van een van de vector-teken - *Dermacentor reticulatus* - en het ontbreken van specifieke maatregelen met betrekking tot de import van paarden uit omringende, EP endemische, landen. Uit dit inventariserend onderzoek kwam naar voren dat de prevalentie van antistoffen en/of *piroplasma*-DNA bij autochtone en geïmporteerde paarden nog zeer laag is.

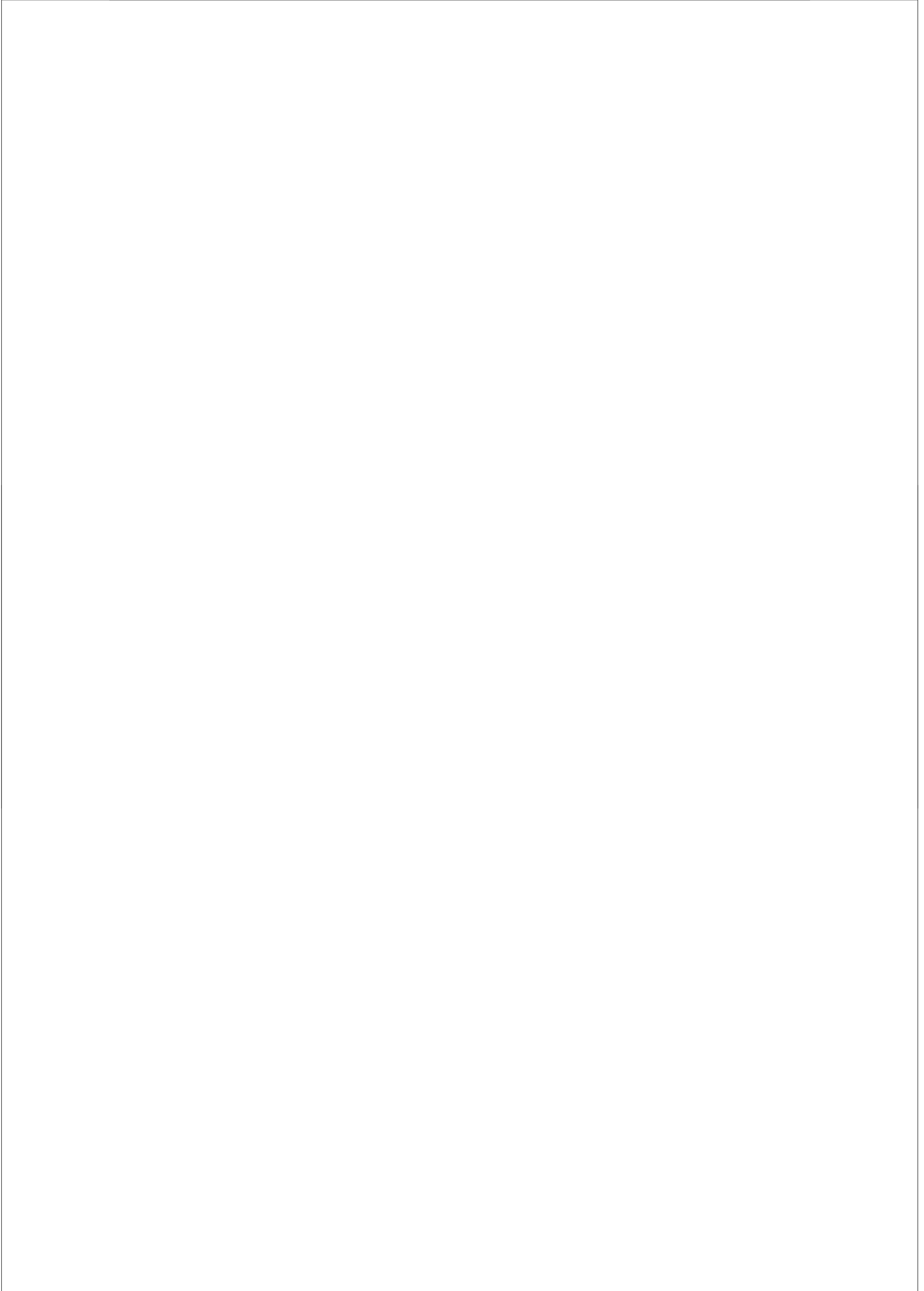
Hoofdstuk 6 beschrijft een studie naar de tekensoorten die op gezonde paarden in Nederland worden aangetroffen. *Ixodes ricinus*, -larve, nimf en volwassen-, bleek de meest op paarden voorkomende teek te zijn. Ook werden enkele volwassen exemplaren van *Dermacentor reticulatus* en *Hyalomma marginatum* aangetroffen. Het vinden van *H. marginatum* was een onverwachte bevinding aangezien deze teek normaal gezien niet kan overleven in ons klimaat. Een deel van de teken werd onderzocht op het dragen van een aantal specifieke teek gebonden agentia. Er werd een grote verscheidenheid aan DNA van (zoönotische) agentia in de teken gevonden waarbij *Borrelia valaisiana* de meest voorkomende was. Het grote aantal *Ixodes* nimfen dat op de paarden werd aangetroffen geeft aan dat de densiteit aan teken in bepaalde gebieden in Nederland relatief hoog is. Omdat paarden, anders dan de hond, op min of meer dezelfde wijze met de natuurlijke omgeving in contact komen als de mens vormen ze een uitstekende 'snuffelpaal' voor het tekenbeet-risico voor de mens in een bepaald gebied. Tegelijkertijd betekent dat dan dat de mens daarmee ook blootstaat aan de hier gevonden, veelal potentieel zoonotische, agentia.

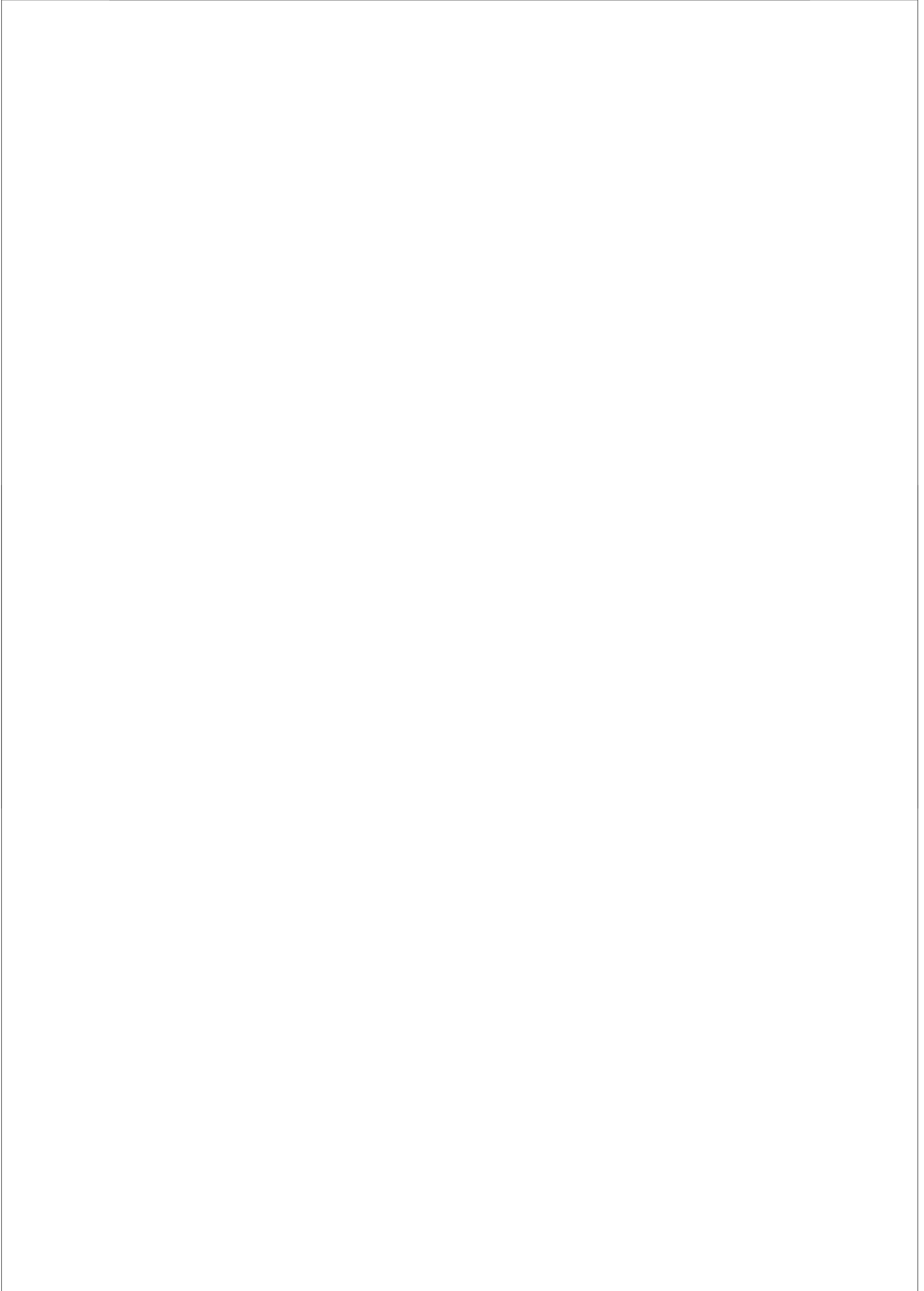
In verband met de vraag of er, en zo ja welke, klinische verschijnselen bij paarden worden veroorzaakt door teek gebonden infecties werd een prospectieve studie uitgevoerd bij 47 individuele recreatie-paarden nadat er bij hen een vastzittende teek was verwijderd. Hiermee zou hopelijk ook de onduidelijkheid rond de relevantie van *Borrelia burgdorferi* sensu lato infecties bij paarden kunnen worden opgeheven (**hoofdstuk 7**). De verwijderde teek (teken) en een op dat moment afgenomen bloedmonster van het paard werden onderzocht op de aanwezigheid van een aantal teek gebonden agentia. Zes tot twaalf weken hierna werd bij alle 47 paarden opnieuw bloed afgenomen. De betrokken locale practicus deed een algemeen klinisch onderzoek en de eigenaar werd verzocht elke aanwijzing voor ziekte te melden. Bij 13 paarden werd na 9-23 maanden nog een keer bloed afgenomen en ze werden tegelijkertijd klinisch geevalueerd. Een hoog percentage van de paarden bleek vanaf het begin antistoffen tegen *Borrelia* en *Anaplasma* te hebben. In 43% van de 120 teken die van de paarden waren afgehaald bleek *Borrelia* DNA aanwezig te zijn en in 0.8% van de 120 teken werd *Anaplasma* DNA gevonden. Deze studie toont aan dat ondanks het feit dat de kans op overdracht van een infectie bij een tekenbeet groot lijkt, en er ook veel paarden antistoffen tegen *Borrelia* en *Anaplasma* blijken te hebben, er toch vrijwel geen klinische symptomen zijn

waargenomen, ook niet bij die dieren die tijdens de studie een seroconversie lieten zien. Bij de paarden die antistoffen tegen *Anaplasma* hadden werd echter wel een significant lager aantal thrombocyten in het bloed gevonden. Dit alles leidt tot de conclusie dat veel paarden subklinische infecties doormaken met verschillende teek gebonden agentia. Het blijft vooralsnog de vraag of dit ook zo is bij sportpaarden die grote fysieke inspanningen moeten leveren en/of langdurig blootstaan aan stress.

Het doel van **hoofdstuk 8** was om de effectiviteit van meerdere injecties met de hoge dosering imidocarb dipropionaat op de eliminatie van *B. caballi* en *T. equi* bij chronisch/persisterend geïnfecteerde paarden te onderzoeken. Vier piroplasmose positieve paarden zonder klinische klachten werden behandeld met vier hoge doseringen imidocarb dipropionaat intramusculair. Moleculair onderzoek toonde aan dat kort na deze behandeling de paarden weer positief waren voor beide agentia. Het is onduidelijk of deze resultaten het gevolg zijn van resistentie-ontwikkeling tegen imidocarb dipropionaat; het is niet onwaarschijnlijk dat de dieren ook al eerder waren behandeld met dit middel. Eliminatie van met name *T. equi* is van groot belang bij paarden die positief worden bevonden voor transport naar een piroplasmose negatief land en/of positief worden bevonden na aankomst in een piroplasmose-negatief land aangezien het paard het reservoir vormt van dit agens.

In **hoofdstuk 9** worden de belangrijkste resultaten en conclusies besproken. Conclusie: dit proefschrift draagt bij aan de kennis omtrent de teek gebonden aandoeningen bij paarden in Nederland. Teek gebonden infecties blijken meer voor te komen dan eerder gedacht en het is dan ook van belang dat deze infecties verder worden onderzocht, enerzijds ter bevordering van de gezondheid van het paard en anderzijds in het kader van de (potentiële) zoönotische implicaties.





English summary

This thesis focuses on the emergence and establishment of equine tick-borne infections in the Netherlands, with particular attention to their diagnosis, clinical relevance and treatment.

Chapter 2 includes overviews of four tick-borne agents (*Borrelia burgdorferi, Theileria equi, Babesia caballi and Anaplasma phagocytophilum)* that appear to be present in the Dutch horse population. The current state-of-the-art of their diagnosis, treatment and prevention are outlined.

Chapter 3 describes the difficulties encountered in identifying equine carriers of piroplasmosis as a result of strain differences which may frustrate the PCR-based tests that have become indispensable in the detection of (equine) tick borne infections in recent years. Besides the specificity issue, there was also a problem with the sensitivity for detection of *B. caballi* carriers due to the extremely low level of parasitaemia. A specific, sensitive and quantitative test for *B. caballi* was developed, which appears to resolve these problems.

The objective of **Chapter 4** was to evaluate the presence of tick-borne agents in horses with fever of unknown origin (FUO). A total of 61 horses were tested for the presence of tick-borne agents by stained blood smear examination and PCR-RLB, after attempting to rule out other causes of FUO. It transpired that 6 horses (10%) were suffering from acute *A. phagocytophilum* infection based on positive PCR-RLB in all 6 horses and a positive stained blood smear in 5 of the 6 horses. *A. phagocytophilum* had not previously been documented in horses in the Netherlands. The cause of fever in the remaining 55 horses was not determined. The study also confirmed that stained blood smear examination is a sensitive and practical tool for diagnosing *A. phagocytophilum* infection in pyrexic horses.

In **Chapter 5,** the prevalence of *B. caballi* and *T. equi* infections was assessed in an area of the Netherlands where a horse with subclinical *B. caballi* infection had been detected. It is shown that the risk of this so called "exotic disease" is increasing in the Netherlands, probably in association with the recent establishment of its vector -*Dermacentor reticulatus*- as an indigenous tick in this geographical area along with the unrestricted import of horses from surrounding piroplasmosis endemic areas. However, even though piroplasma infections were found in indigenous horses as well as horses imported into the Netherlands, this study showed that the prevalence of seropositive or DNA positive horses in both populations is still very low. It was also shown that if serology alone is used, some infected horses would go undetected.

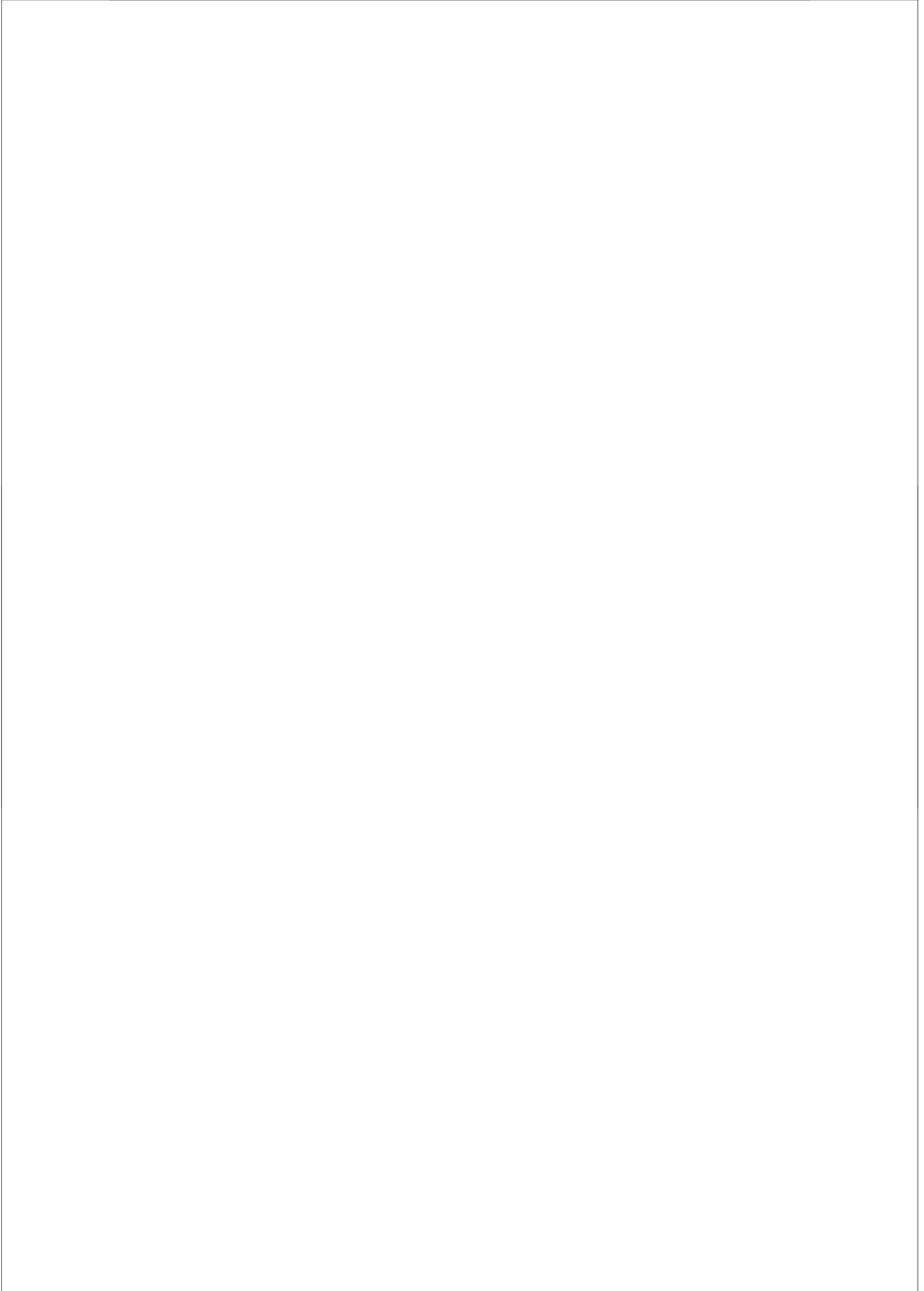
Chapter 6 describes the classification of ticks collected from healthy horses in the Netherlands and the identification of the potential pathogens that they

contained. *Ixodes ricinus* appeared to be by far the most common tick species. Among a variety of emerging (zoonotic) tick-borne pathogens, *Borrelia valaisiana* was the most frequently encountered agent in these ticks. A surprisingly large number of *Ixodes* nymphs were found, which indicates that tick density is relatively high in at least some areas of the Netherlands. As horses appear to be excellent sentinels, the combined relatively high incidence of ticks and the frequency and range of the potential zoonotic agents carried by these ticks suggest that people in these areas are at considerable risk of acquiring tick-borne infections.

The objective of **Chapter 7** was to prospectively investigate whether horses in the Netherlands are at risk of developing tick-borne disease after a tick-bite. Forty-seven recreational horses were examined using serological and molecular tests for the presence of a tick-borne infection, shortly after removing a feeding tick. Follow- ups were conducted in 47 horses 6-12 weeks after tick removal and in 13 horses a further 9-23 months later. In addition, the 120 ticks removed from these 47 horses were tested for the presence of a number of specific tick-borne agents. The sero-prevalences among the horses were high for *B. burgdorferi* and *A. phagocytophilum*, and a high incidence of *Borrelia*-DNA was detected in the ticks removed from these horses. This study showed that even though there was a high risk of infection the incidence of clinical disease was very low, with the exception of low thrombocyte counts associated with *Anaplasma* seropositivity. In other words, most tick-borne infections in recreational horses in the Netherlands appear to run a subclinical course.

Chapter 8 describes a study of four naturally infected piroplasmosis carrier horses in which clearance of the agent (*B. caballi* and or *T. equi*) was attempted by repeated administration of high doses of imidocarb dipropionate. Post-treatment follow-up (using PCR-RLB) showed that, despite treatment, neither *B. caballi* nor *T. equi* were eliminated from these natural infected carriers. Elimination of these protozoa is especially important for *T. equi* carriers imported and/ or resident in countries considered free of infection, because the horse is a known reservoir. This study also showed that the detection of carriers is considerably improved by molecular techniques like PCR-RLB.

In **Chapter 9** the main results and conclusions of this thesis are integrated and discussed. The principal conclusion is that equine tick-borne infections are prevalent in the Netherlands and deserve ongoing scientific attention both in their own right, and because of their potential zoonotic implications.



Résumé Français

Cette thèse porte sur l'émergence et l'établissement aux Pays-Bas d'infections équines transmises par les tiques, avec une attention particulière à leur diagnostic, pertinence clinique et traitement.

Le **chapitre 2** présente une revue de quatre agents transmis par les tiques (*Borrelia burgdorferi, Theileria equi, Babesia caballi* et *Anaplasma phagocytophilum*) identifiés dans la population équine néerlandaise, incluant l'état de l'art de leur diagnostic, traitement et prévention.

Le **chapitre 3** décrit les difficultés rencontrées dans l'identification des chevaux porteurs de la piroplasmose, dues aux différences entre les souches qui perturbent les tests PCR, devenus ces dernières années indispensables pour détecter les infections équines transmises par les tiques. Outre la question de la spécifi-cité, il existe aussi un problème de sensibilité pour détecter les porteurs de *B. caballi* en raison d'une parasitémie extrêmement faible. Un test quantitatif sensible et spécifique pour *B. caballi* a été développé, qui semble résoudre ces problèmes.

L'objectif du **chapitre 4** était d'évaluer la présence d'agents transmis par les tiques chez des chevaux présentant une fièvre d'origine indéterminée (FOI). La présence d'agents transmis par les tiques a été recherchée chez 61 chevaux par l'utilisation de frottis sanguins avec coloration et PCR-RLB, après avoir tenté d'écarter d'autres causes de FOI. Il est apparu que 6 chevaux (10%) souffraient d'une infection aiguë à *Anaplasma phagocytophilum*, diagnostiquée sur la base de résultats positifs au test PCR-RLB (6 chevaux) et au frottis sanguin (5 chevaux sur 6). La présence d'*Anaplasma phagocytophilum* chez les chevaux n'avait pas encore été documentée aux Pays-Bas. La cause de la fièvre chez les 55 autres chevaux n'a pas pu être déterminée. L'étude a également confirmé que l'examen des frottis sanguins avec coloration était un outil sensible et pratique pour diagnostiquer l'infection à *Anaplasma phagocytophilum* chez les chevaux présentant une hyperthermie.

Le **chapitre 5** porte sur la prévalence des infections à *Babesia caballi* et *Theileria equi* dans une région des Pays-Bas où un cheval présentant une infection subclinique à *B. caballi* a été détecté. Il y est démontré que le risque de cette soi-disant "maladie exotique" est en augmentation aux Pays-Bas, probablement en association avec l'établissement récent de son vecteur -*Dermacentor reticulatus*- comme une tique indigène dans cette zone géo-graphique, ainsi qu'avec la libre importation de chevaux de zones environnantes endémiques pour la piroplasmose. Cependant, même si des infections à *Piroplasma* ont été identifiées chez des chevaux indigènes ainsi que chez des chevaux importés aux Pays-Bas, cette étude a montré que la prévalence dans les deux populations considérées de chevaux séropositifs ou chez lesquels on a détecté l'ADN

des parasites est encore très faible. Il a également été démontré que des chevaux infectés ne seraient pas détectés par utilisation de la sérologie seule.

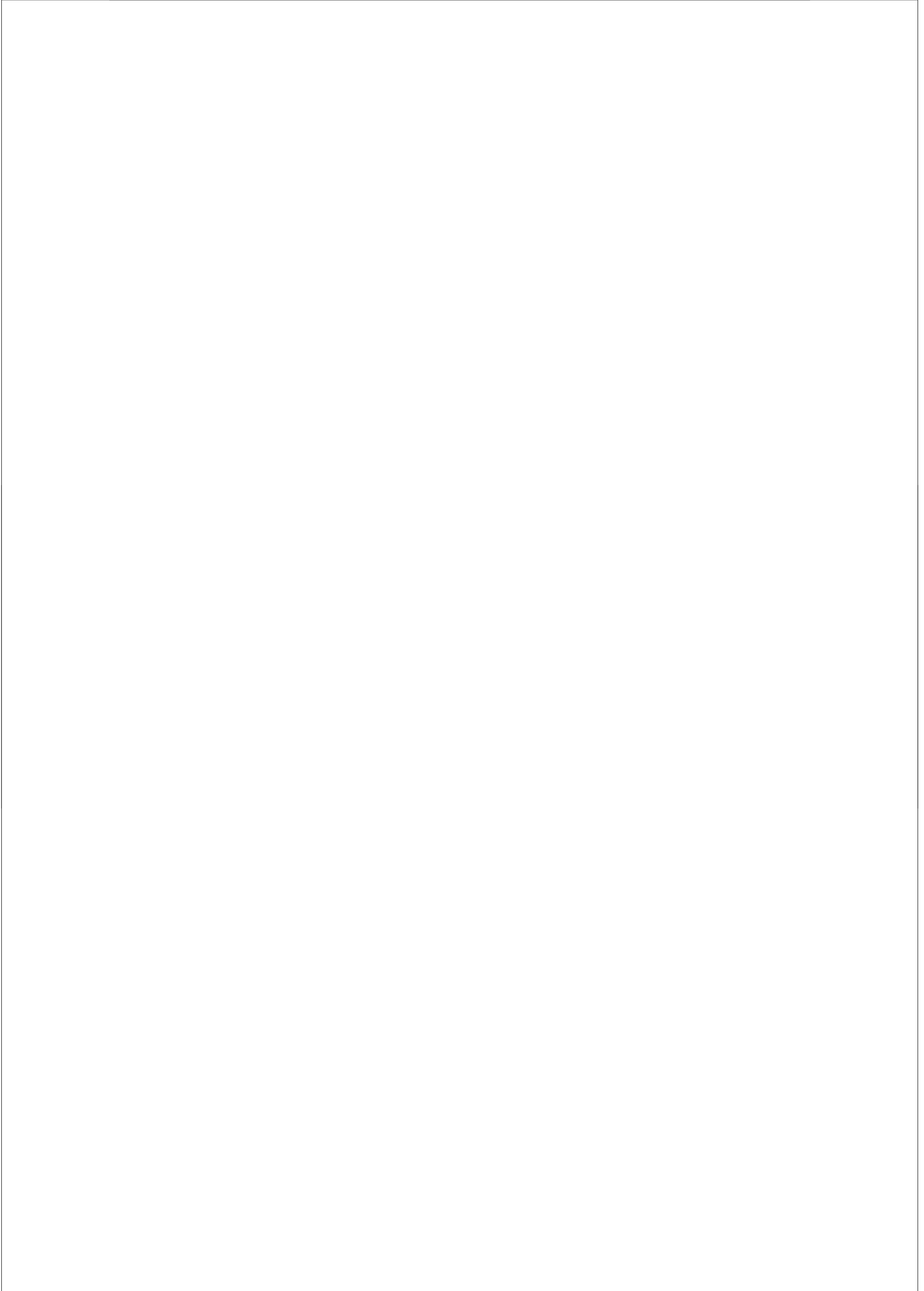
Le **chapitre 6** décrit la classification des tiques prélevées sur des chevaux sains aux Pays-Bas et l'identification des agents pathogènes potentiels qu'elles portaient. *Ixodes ricinus* semble être de loin l'espèce de tiques la plus courante. Parmi une variété d'agents pathogènes émergents (zoonotiques) transmis par les tiques, *Borrelia valaisiana* était l'agent le plus fréquemment rencontré. On a identifié un nombre de nymphes d'*Ixodes* étonnamment élevé, ce qui indique une densité de tiques relativement importante, au moins dans certaines régions des Pays-Bas. Etant donné que les chevaux semblent être d'excellentes sentinelles, l'association d'une incidence relativement élevée de tiques avec la fréquence et l'extension géographique d'agents zoonotiques potentiels transmis par ces tiques suggère que les habitants de ces régions sont exposés à un fort risque d'infections transmises par les tiques.

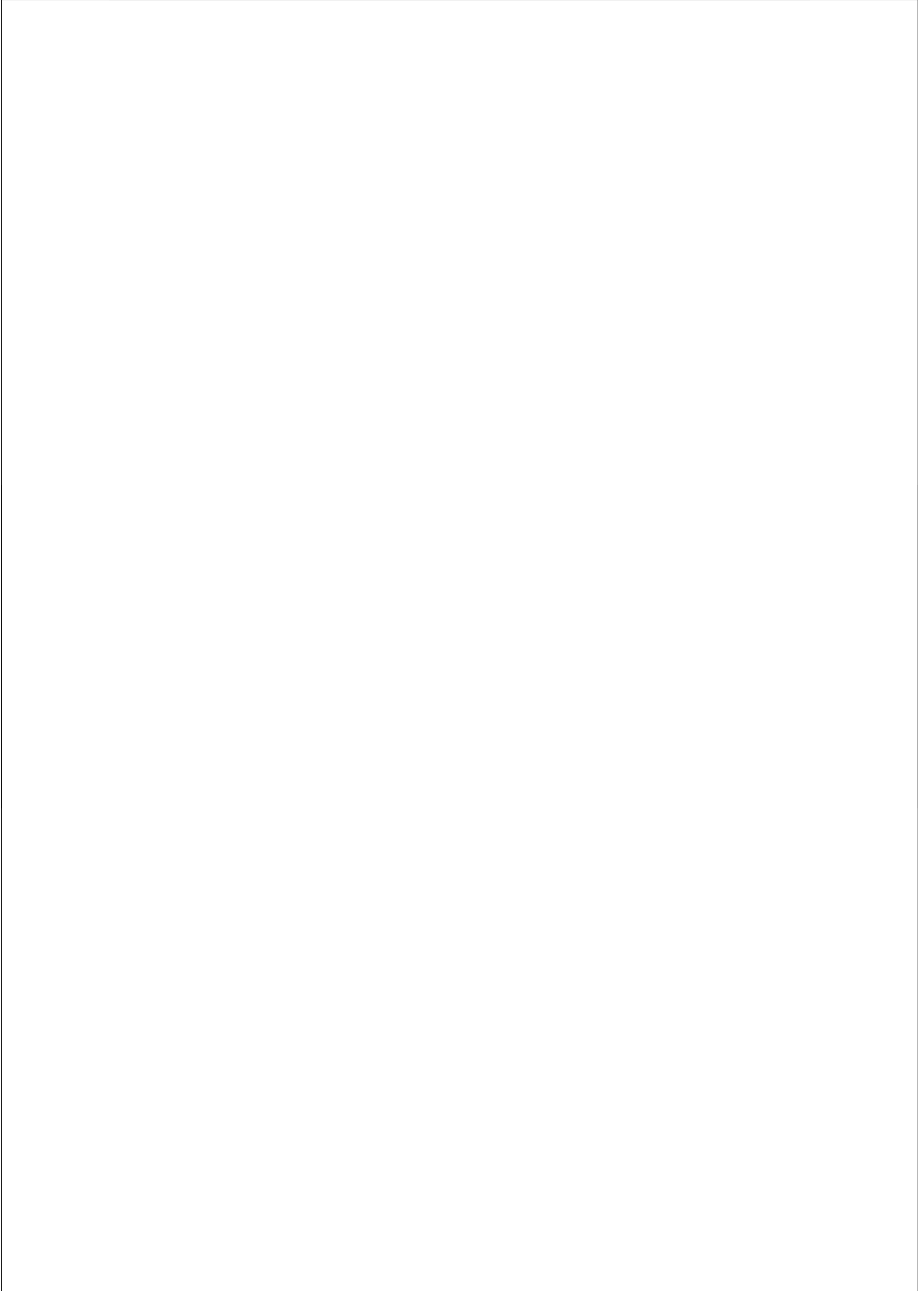
L'objectif du **chapitre 7** était d'évaluer de façon prospective si les chevaux aux Pays-Bas sont à risque de développer une maladie transmise par les tiques après une piqûre de tique. Quarante-sept chevaux de loisir ont été examinés par des tests sérologiques et moléculaires afin de détecter la présence d'une infection transmise par les tiques, peu de temps après avoir retiré une tique gorgée. Un suivi a été effectué sur les 47 chevaux 6 à 12 semaines après le retrait des tiques et, pour 13 chevaux, une nouvelle fois 9 à 23 mois plus tard. De plus, on a recherché la présence d'un certain nombre d'agents transmis par les tiques dans les 120 tiques prélevées sur ces 47 chevaux. On a détecté une forte séro-prévalence de *B. burgdorferi* et *A. phagocytophilum* chez ces animaux, ainsi qu'une forte incidence d'ADN de *Borrelia* dans les tiques prélevées sur eux. Cette étude a démontré que, bien que le risque d'infection soit élevé, l'incidence de maladie clinique est très faible, à l'exception de numérations thrombocytaires basses associées à une séropositivité à *Anaplasma*. En d'autres termes, la plupart des infections transmises par les tiques chez les chevaux de loisirs aux Pays-Bas semblent avoir une évolution subclinique.

Le **chapitre 8** décrit une étude sur quatre chevaux présentant une piroplasmose avec infection naturelle, chez lesquels on a tenté l'éradication de l'agent responsable (*B. caballi* et/ou *T. equi*) par administration répétée de fortes doses de dipropionate d'imidocarbe. Le suivi post-traitement (en utilisant la PCR-RLB) a montré que, malgré ni *B. caballi* ni *T. equi* ont été éradiqués chez ces chevaux porteurs infectés naturellement. L'élimination de ces protozoaires est particulièrement important pour les porteurs de *T. equi* qui sont importés et/ou résident dans des pays jugés indemnes de l'infection, car le cheval est un réservoir connu. Cette étude a également montré que la détection des porteurs

est considérablement améliorée par l'utilisation de techniques moléculaires comme la PCR-RLB.

Au **chapitre 9**, les principaux résultats et conclusions de cette thèse sont synthétisés et discutés. La principale conclusion est que les infections équines transmises par les tiques sont fréquentes aux Pays-Bas, ce qui justifie l'étude scientifique des agents et de leurs éventuelles implications zoonotiques.





Deutsche zusammen-fassung

Diese Arbeit bezieht sich auf die Entstehung und Etablierung von durch Zecken übertragene Infektionskrankheiten beim Pferd in den Niederlanden, mit besonderem Augenmerk auf Diagnose, klinische Relevanz und Behandlung.

Kapitel 2 gibt einen Überblick über vier durch Zecken übertragene Agenzien (*Borrelia burgdorferi*, *Theileria equi*, *Babesia caballi* und *Anaplasma phagocytophilum*), die in der niederländischen Pferdepopulation eine Rolle spielen. Diagnose, Behandlung und Prävention nach neusten Erkenntnissen werden hier beschrieben.

In **Kapitel 3** werden die Schwierigkeiten beschrieben, die aufgrund von Stammunterschieden bei der Identifizierung von Trägern von Piroplasmose auftreten. Diese Schwierigkeiten vereiteln die PCR-basierten Tests, die bei der Auffindung von (equinen) Zecke übertragenen Infektionen in den letzten Jahren unverzichtbar geworden sind. Neben dem Problem der Spezifität gab es auch Probleme bezüglich der Sensitivität beim Nachweis von Trägern von *B. caballi*, aufgrund der extrem niedrigen Parasitämielevel. Ein spezifischer, sensitiver und quantitativer Test für *B. caballi* würde diese Probleme lösen.

Das Ziel in **Kapitel 4** war es, das Vorhandensein von durch Zecken übertragene Agenzien bei Pferden mit Fieber unklarer Genese (FUO) zu evaluieren. Insgesamt 61 Pferde wurden auf das Vorhandensein von durch Zecken übertragene Agenzien im gefärbten Blutausstrich untersucht und mittels PCR-RLB getestet, nachdem andere Ursachen von FUO ausgeschlossen worden waren. Es stellte sich heraus, dass 6 Pferde (10%) von einer akuten *A. phagocytophilum* Infektion betroffen waren, basiert auf positivem PCR-RLB bei allen 6 Pferden und positivem Blutausstrich bei 5 von 6 Pferden. *A. phagocytophilum* ist bisher nicht dokumentiert worden bei Pferden in den Niederlanden. Bei den verbleibenden 55 Pferden konnte die Ursache des Fiebers nicht evaluiert werden. Diese Studie bestätigte auch, dass gefärbte Blutausstriche ein sensibles und taugliches Mittel sind für die Diagnose von *A. phagocytophilum* beim Pferd mit Fieber.

In **Kapitel 5** wurde die Prävalenz von *B. caballi* und *T. equi*-Infektionen untersucht in einem Gebiet der Niederlanden, wo ein Fall mit subklinischer *B. caballi* Infektion aufgetreten war. Es wurde gezeigt, dass das Risiko dieser sogenannten "exotischen Krankheit" in den Niederlanden erhöht ist, wahrscheinlich im Zusammenhang mit der jüngsten Etablierung des betreffenden Vektors -*Dermacentor reticulatus* - als indigene Zecke in diesem geografischen Gebiet zusammen mit der unbeschränkten Einfuhr von Pferden aus Gebieten, in denen die Piroplasmose endemisch ist. Doch obwohl *Piroplasma*-Infektionen bei einheimischen Pferden sowie bei importierten Pferde gefunden wurden, zeigte diese Studie, dass die Prävalenz von seropositiven oder DNA-positiven Pferde in beiden Populationen noch sehr gering ist. Es wurde auch gezeigt, dass, bei

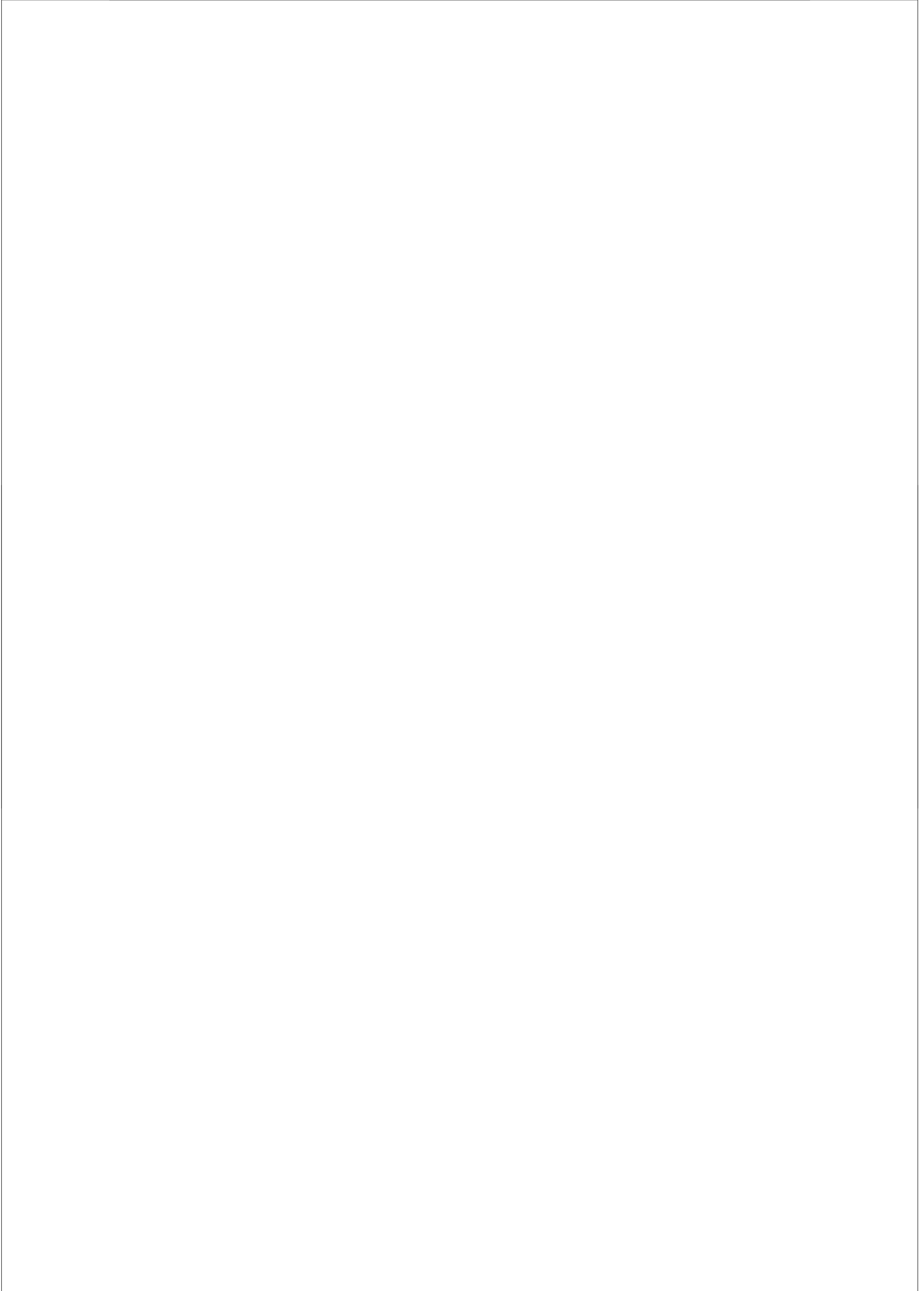
alleiniger Verwendung der Serologie einige infizierte Pferden unentdeckt bleiben.

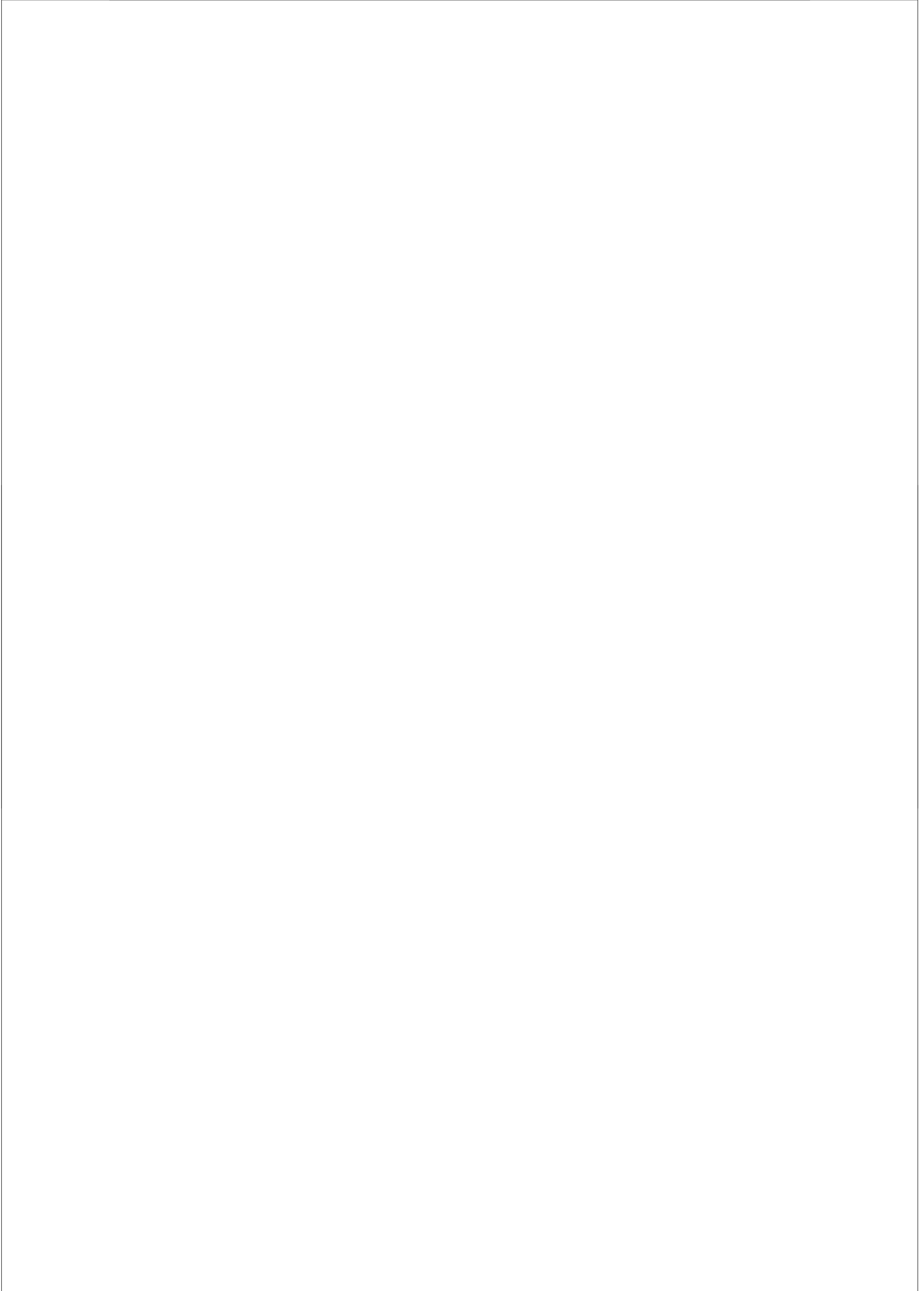
Kapitel 6 beschreibt die Klassifizierung von Zecken die bei gesunden Pferden in den Niederlanden eingesammelt wurden, und die Identifizierung der potentiellen Krankheitserreger die in diesen Zecken enthalten waren. *Ixodes ricinus* schien bei weitem die häufigste Zeckenart. Unter einer Vielzahl von aufkommenden (zoonotischen) von Zecken übertragenen Krankheiterregern war *Borrelia valaisiana* das am häufigsten vorkommende Agens bei diesen Zecken. Eine überraschend große Anzahl von *Ixodes*-Nymphen wurde gefunden, was bedeutet, dass die Zeckendichte relativ hoch ist, zumindest in einigen Gebieten der Niederlande. Die Kombination der relativ hohen Zecken-Inzidenz zusammen mit der Häufigkeit und dem Umfang der von diesen Zecken möglicherweise übertragenen Zoonoseerregern lässt vermuten, dass Menschen in diesen Gebieten einem erheblichem Risiko für Infektionen mit diesen durch Zecken übertragenen Infektionen unterstehen.

Das Ziel von **Kapitel 7** war die prospektive Evaluation der Frage, ob Pferde in den Niederlanden nach einem Zeckenbiss dem Risiko der Entwicklung von durch Zecken übertragenen Krankheiten unterstehen. Siebenundvierzig Freizeitpferde wurden mittels serologischer und molekularer Tests auf das Vorkommen von durch Zecken übertragenen Infektionen untersucht, kurz nach dem Entfernen einer saugenden Zecke. Nachfolgeuntersuchungen wurden bei 47 Pferden nach 6-12 Wochen und bei 13 Pferden nach weiteren 9-23 Monaten durchgeführt. Darüber hinaus wurden die 120 Zecken, die von diesen 47 Pferden entfernt wurden, auf das Vorhandensein einer Reihe von spezifischen Zecken übertragenen Substanzen untersucht. Die Seroprävalenz für *B. burgdorferi* und *A. phagocytophilum* war hoch, und bei den untersuchten Zecken ergab sich eine hohe Inzidenz für *Borrelia*-DNA. Diese Studie zeigte, dass, obwohl ein hohes Risiko für das Auftreten einer Infektion vorlag, die Inzidenz einer klinischen Erkrankung sehr gering war, mit Ausnahme der niedrigen Thrombozytenzahlen die mit *Anaplasma* Seropositivität korrelierten. In anderen Worten, die meisten durch Zecken übertragene Infektionen bei Freizeitpferde in den Niederlanden weisen eine subklinischen Verlauf auf.

Kapitel 8 beschreibt eine Studie von vier natürlich mit Piroplasmose infizierten Pferden, bei denen versucht wurde, mittels wiederholter Verabreichung von hohen Dosen Imidocarb dipropionat das Agens zu eliminieren. Verlaufsuntersuchungen (mit PCR-RLB) nach Therapie zeigten, dass trotz Behandlung weder *B. caballi* noch *T. equi* aus diesen Träger eliminiert wurden. Die Beseitigung dieser Protozoen ist besonders wichtig für importierte Träger von *T. equi* und solche die in Ländern leben, die als frei von der Infektion gelten, weil das Pferd ein bekanntes Reservoir ist. Diese Studie zeigte, dass das Auffinden von Trägern durch molekulare Methoden wie PCR-RLB beträchtlich verbessert wird.

In **Kapitel 9** werden die wichtigsten Ergebnisse und Schlussfolgerungen dieser Arbeit zusammengefasst und diskutiert.
Die Hauptschlussfolgerung ist, dass durch Zecken übertragenen Infektionen bei Equiden in den Niederlanden weit verbreitet sind, und dass weitere Untersuchungen dazu nötig sind, vor allem auch wegen des bestehenden Zoonosepotentials.





Dankwoord

Dankwoord

Het schrijven van een proefschrift is al een soort van bevalling en zeker als een mens van de directe communicatie en weinig woorden, zijn het samenstellen van discussies bij tijd en wijle een hele beproeving. Zo ook dit dankwoord; daarom een soort chronologische volgorde zonder daarmee mensen te kort te willen doen en het vooral niet te wollig of te langdradig te maken.

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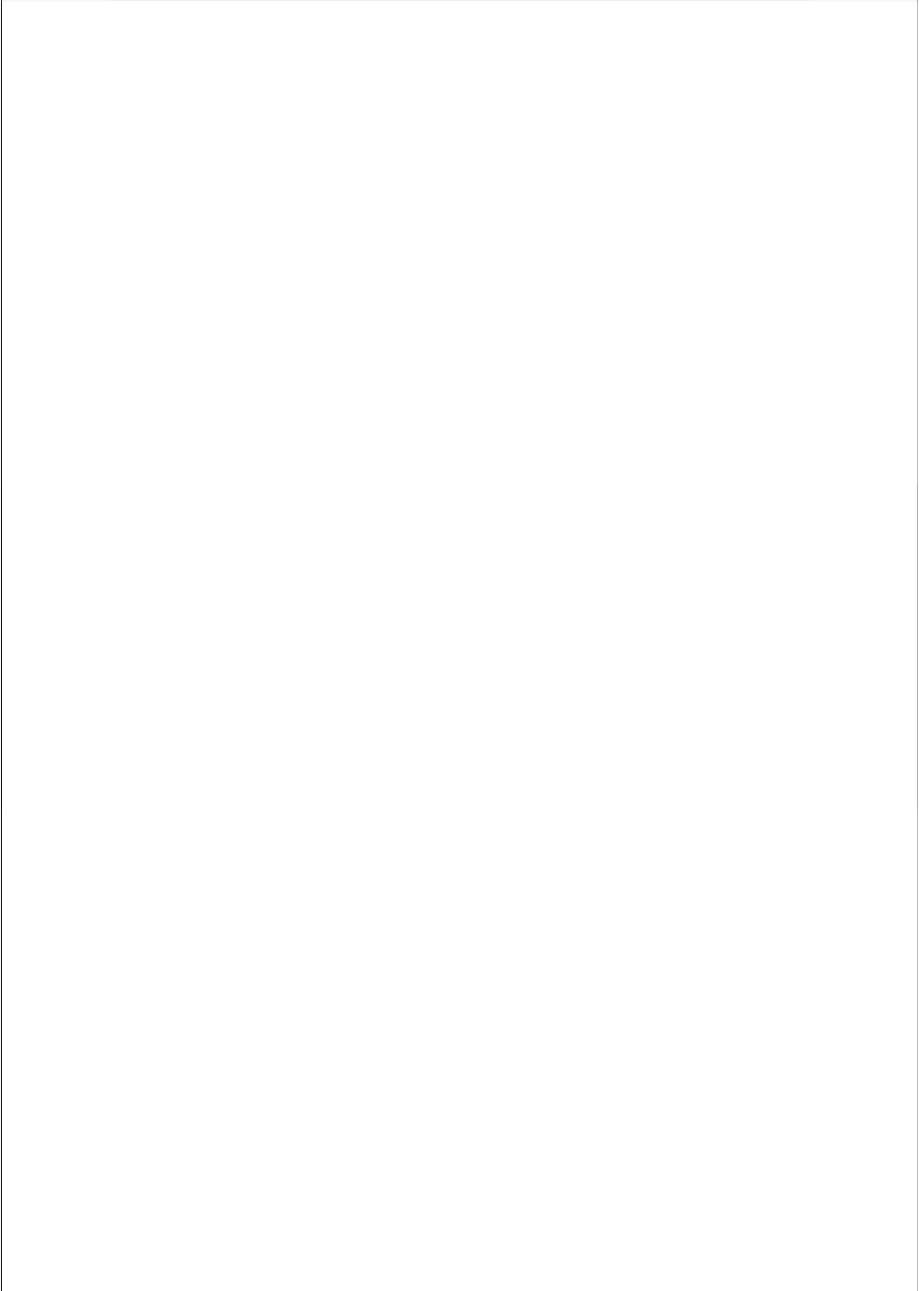
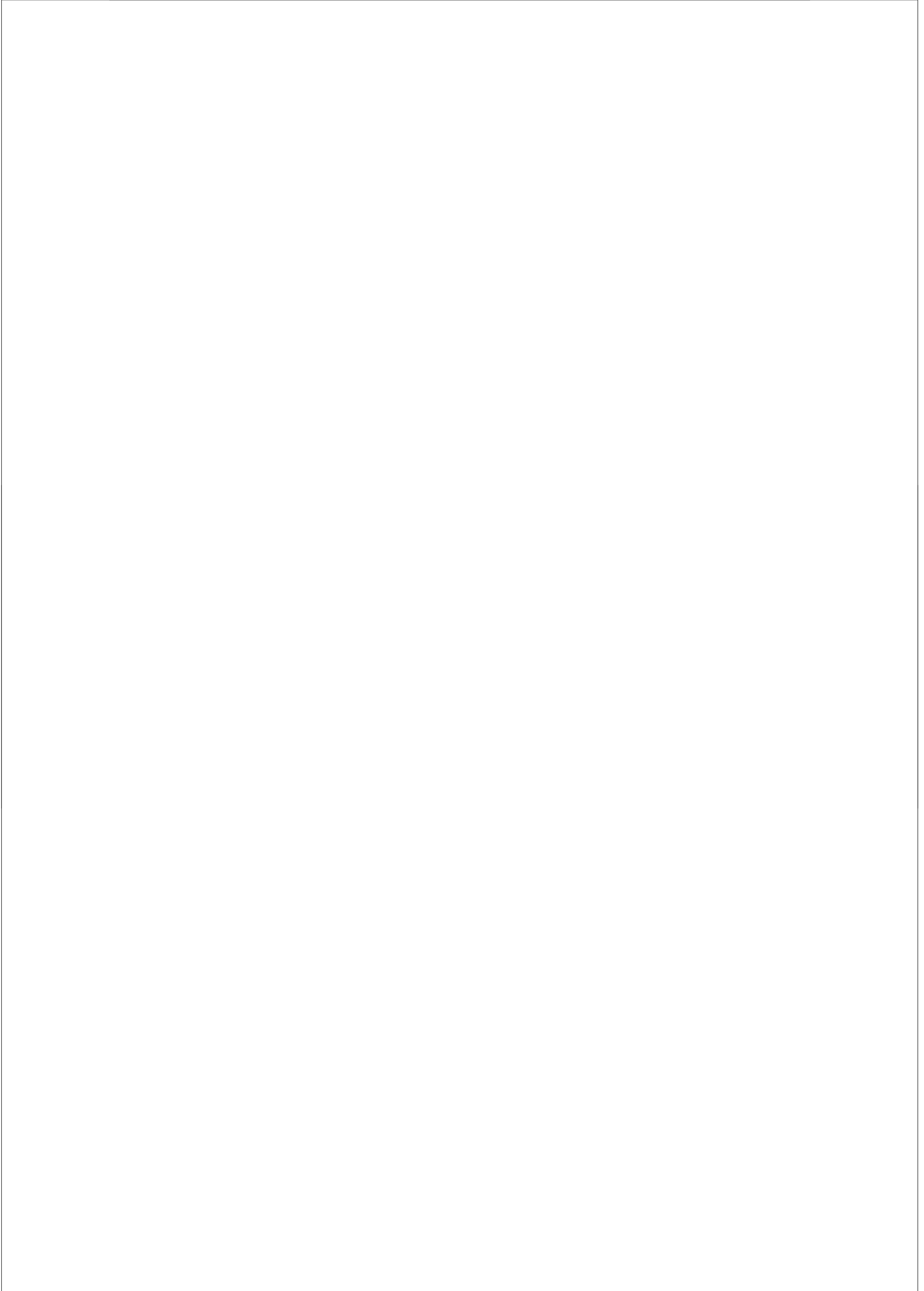
Catherine Werners-Butler



Curriculum vitae

Catharine Michelle Butler was born on September 13, 1969, in Delft, the Netherlands. She graduated from high school (Haags Montessori Lyceum) in 1988. She started her studies in Veterinary Medicine at the Faculty of Veterinary Medicine, University of Ghent, Belgium in 1991 and graduated as veterinarian in 1998. From 1998-2000 she did an internship in Large Animal Surgery and Anaesthesia at the Department of Large Animal Surgery and Anaesthesiology in Gent, Belgium. Another internship in General Equine Medicine was pursued at Diergeneeskundig Centrum Noord-Nederland, Emmeloord, the Netherlands between 2000-2001. In 2001 she started working as junior lecturer in Equine Internal Medicine, Department of Equine Sciences, University of Utrecht, where she subsequently did her residency from 2002-2006. This led to the title of Specialist KNMvD (Royal Dutch Veterinary Association), which was awarded to her in 2006. From 2006-2010 she worked as junior lecturer and researcher at the Department of Equine Sciences, Faculty of Veterinary Sciences, University of Utrecht. She worked a couple of months for the Animal Health Trust in Newmarket, United Kingdom before accepting a job as Associate Professor at the Large Animal Medicine and Surgery Programme, St. George's University, Grenada, West Indies, where she is responsible for teaching, research and clinical work. In 2012 she passed the ECEIM board exam and became a Diplomate of the European College of Equine Internal medicine (ECEIM).

Catharine Michelle Butler is geboren op 13 september 1969 in Delft, Nederland. In 1988 slaagde zij voor de middelbare school (Haags Montessori Lyceum). In 1991 begon zij haar studie Diergeneeskunde aan de Faculteit Diergeneeskunde in Gent, Belgie die zij afronde in 1998. Ze bleef nog 2 jaar werken aan de Faculteit Diergeneeskunde in Gent als intern bij de afdeling Chirurgie en Anaesthesiologie van de Grote Huisdieren. In 2000 besloot ze een internship Agemene Geneeskunde van het Paard te starten bij Diergeneeskundig Centrum Noord-Nederland te Emmeloord. In 2001 werd ze aangesteld als specialist in opleiding (SIO) bij de discipline Inwendige Ziekten van de Hoofdafdeling Gezondheidszorg paard aan de Faculteit Diergeneeskunde te Utrecht en dit resulteerde in de titel van Specialist Inwendige Ziekten van het Paard in 2006. Van 2006-2010 werkte ze als junior docent en onderzoeker bij dezelfde discipline en haar werk omvatte kliniekwerk, onderwijs en onderzoek. Na een paar maanden werkzaam te zijn geweest bij the Animal Health Trust in Newmarket (UK) in 2011 nam ze een aanbod aan om als Associate Professor te gaan werken bij de afdeling Large Animal Medicine and Surgery aan St. George's University te Grenada, West-Indies waar ze tot op heden werkzaam is. In 2012 werd het Europees Specialisten examen gehaald en is ze Diplomate of the European College of Equine Internal Medicine (ECEIM).



List of publications

List of publications

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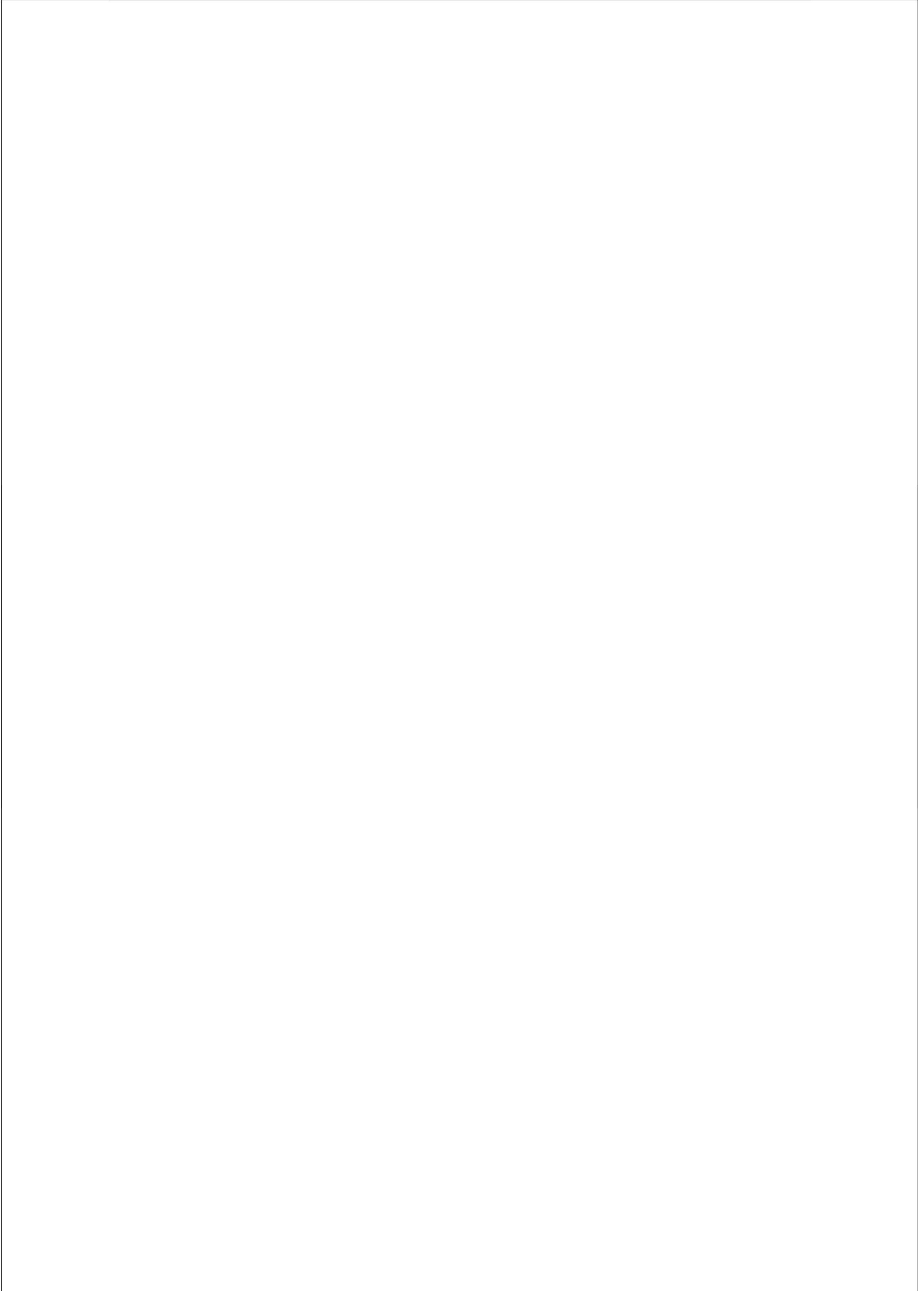
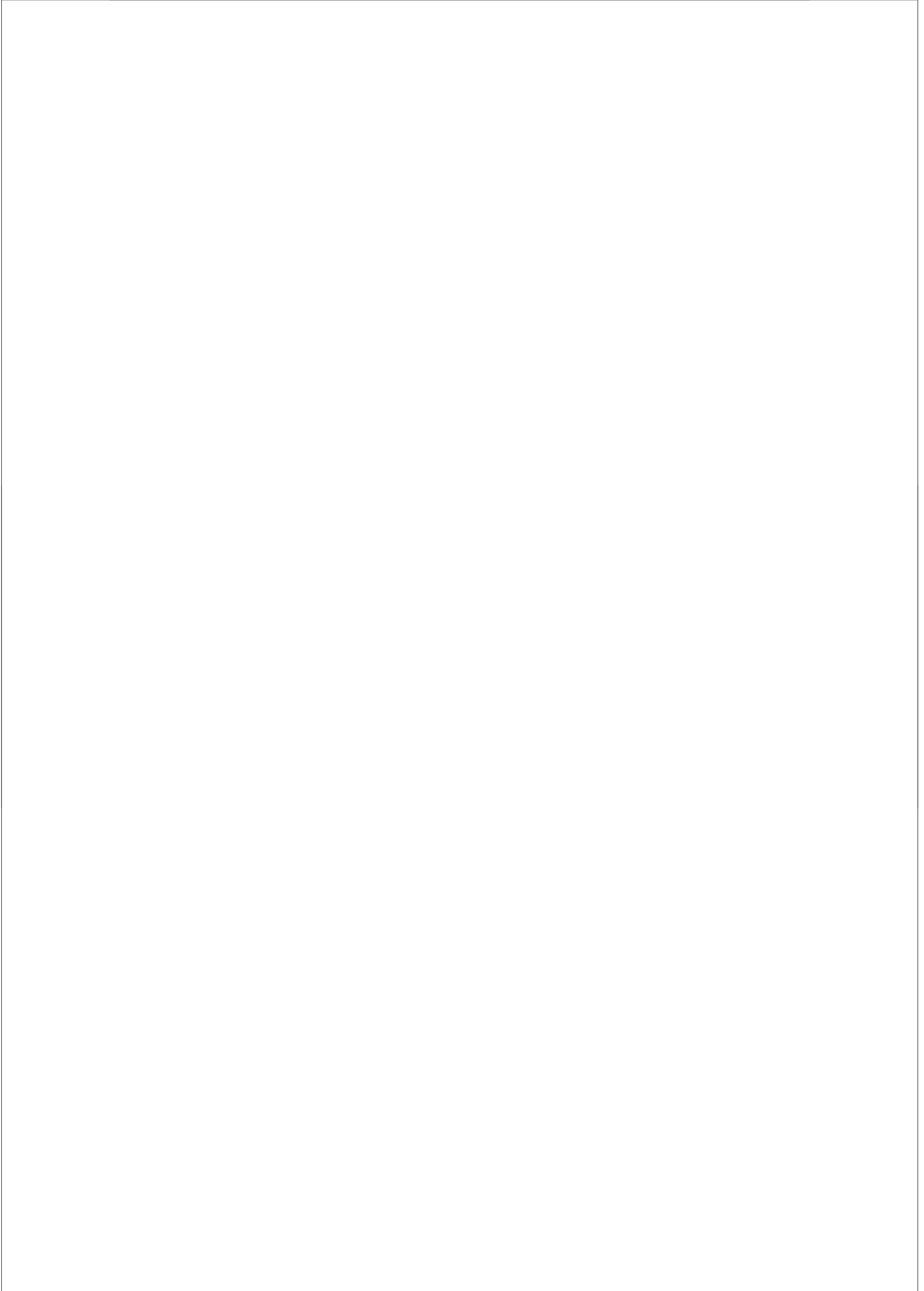




Colored illustrations

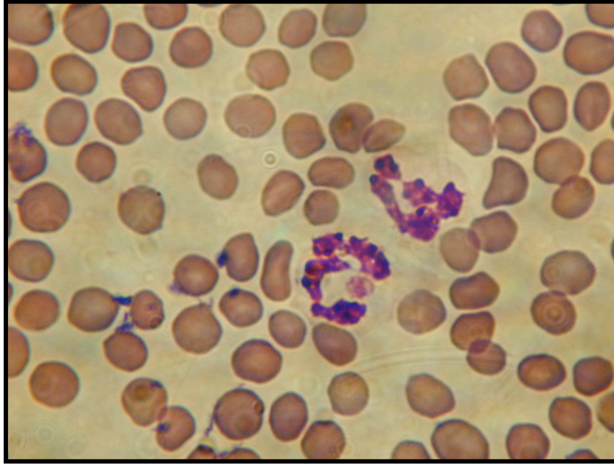


Fig. 1 Chapter IV

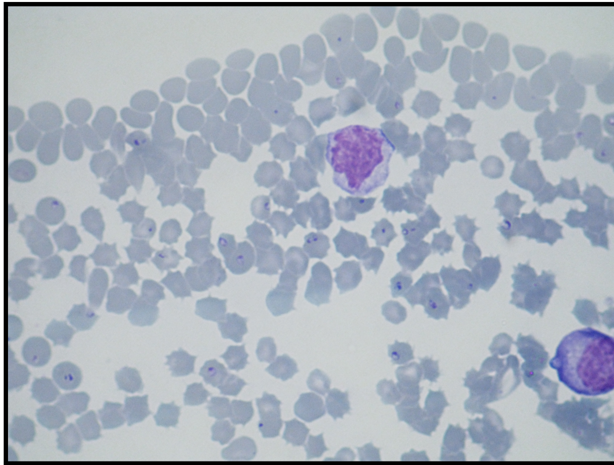


Fig. 2 Chapter V

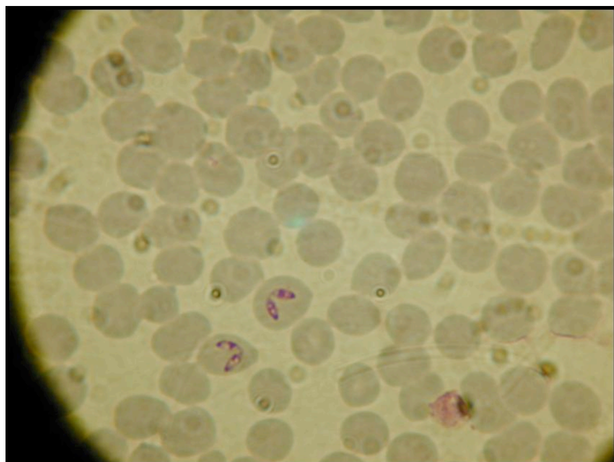
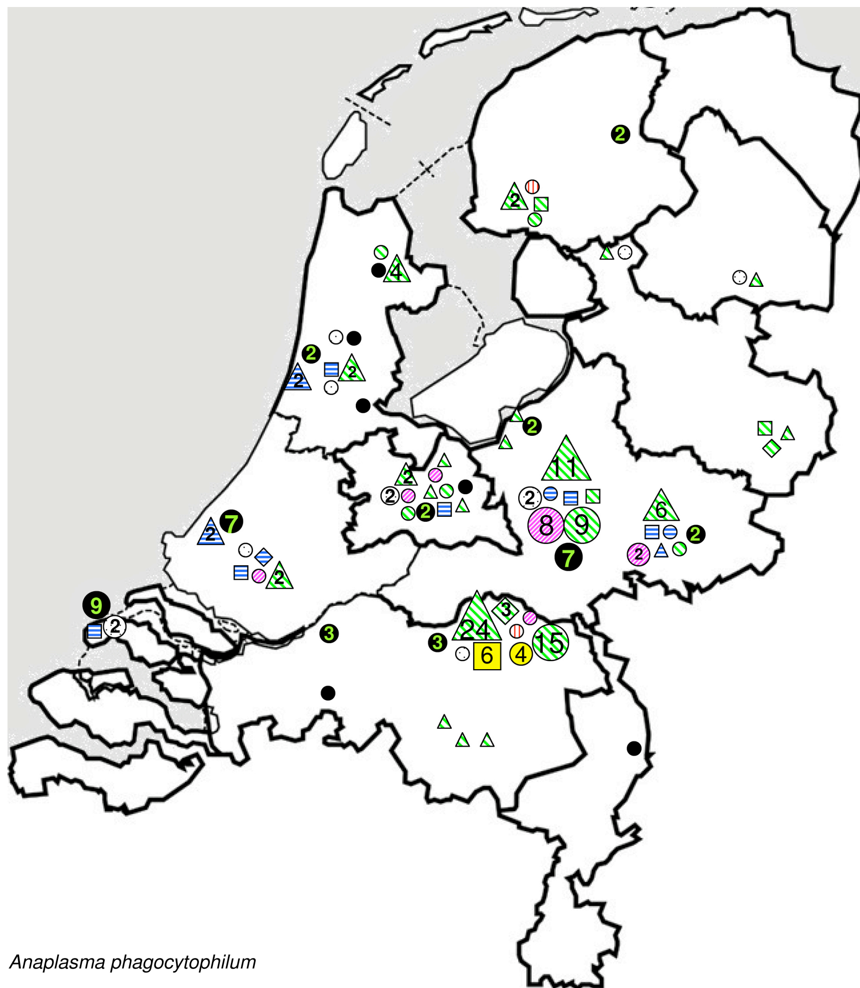


Fig. 3 Appendix I



Anaplasma phagocytophilum
Babesia caballi
Babesia divergens
Babesia microti
Babesia venatorum
Borrelia afzelii
Borrelia garinii
Borrelia sensu-stricto
Borrelia valaisiana
Ehrlichia schotti
Ricketsia helvetica
Theileria equi
Theileria equi-like
no specific DNA detected

Fig. 1 Chapter VI