Classification of ticks collected from horses in the Netherlands in 2008–2009 and identification of the (zoonotic) agents they contain

Catherine M. Butler[1,2], Marianne M. Sloet van Oldruitenborgh-Oosterbaan[1], Tom A. E Stout[1,3], Frans Jongejan[4,5], Arno H.Werners[6] and Dirk J. Houwers[7]

[1]   Department of Equine Sciences, Faculty of Veterinary Medicine, Utrecht University, Utrecht, the Netherlands
[2]   Large Animal Medicine and Surgery Department, School of Veterinary Medicine, St. George's University, True Blue, Grenada, West-Indies
[3]   Department of Production Animal Sciences, Faculty of Veterinary Science, University of Pretoria, Onderstepoort, South Africa
[4]   Utrecht Centre for Tick-borne Diseases, Faculty of Veterinary Medicine, Utrecht, University, Utrecht, the Netherlands
[5]   Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria, Onderstepoort, South Africa
[6]   Anatomy, Physiology and Pharmacology Department, School of Veterinary Medicine, St. George's University, True Blue, Grenada, West-Indies
[7]   Veterinary Microbiological Diagnostic Centre, Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, Utrecht, the Netherlands

Summary: This study shows which hard tick species (Ixodidae) were found on domestic horses in the Netherlands in 2008–2009, and what potential pathogens these ticks 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, *B. afzelii* in 22%, B. burgdorferi sensu stricto and *B. 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 *B. caballi* and *Theileria equi* in 1.5 and 3%, respectively. There were considerable regional differences suggesting focal distribution of these potential pathogens.

Keywords: tick, horse, PCR-RLB, zoonotic, emerging, Borrelia, Anaplasma, Babesia, Rickettsia, Ehrlichia

Introduction

Ticks play a significant role as vectors of several bacterial, protozoal and viral pathogens of worldwide human medical, veterinary and economic importance (*de la Fuente* et al. 2008, *Jongejan* et al. 2004). Among the various species of hard ticks endemic to Western Europe, *Ixodes ricinus* is the most frequently found (*Stanek* et al. 2004). In the Netherlands, *Ixodes ricinus* is the predominant species recovered from domestic animals (*Nijhof* et al. 2007) and is the most important vector of a number of emerging pathogens of veterinary and medical significance, including *Borrelia burgdorferi* sensu lato, Babesia spp., and Ehrlichia-Anaplasma spp. (*Estrada-Pena* et al.1999). Like other Ixodid ticks, *I. ricinus*' developmental cycle includes four stages (egg, larva, nymph, adult) and takes 2–3 years to complete (*Sonenshine* et al. 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* et al. 1991).

Although studies on the prevalence of tick-borne pathogens in tick vectors (*Ferquel* et al. 2006, *Reye* et al. 2010, *Wielinga* et al. 2009, *Wielinga* et al. 2006, *Hartelt* et al. 2008),

and vertebrate hosts (*Nijhof* et al. 2007, *Lommano* et al. 2012, *Passamonti* et al. 2010) 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. 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* et al. 2007, *Estrada-Pena* 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 *Borrelia burgdorferi* sensu lato

spp., *Theileria equi*, *Babesia caballi*, and *Anaplasma 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* 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 (*Butler* et al. 2016).

Classification and determination of sex and developmental stage

Ticks were classified using standard keys (*Estrada-Pena* 2004) and categorized 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, Rikkettsia, 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 (*Bekker* et al 2008, *Nijhof* et al. 2005, *Schouls* et al. 1999). Subsequently, the PCR products generated were applied to an RLB hybridization blot prepared with specific probes for the agents *B. burgdorferi* sensu lato (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 females and 5 males), 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.

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

The results of the PCR-RLB are summarized in Table 1. DNA from the pathogens of interest was detected in 95 (73%) of the 130 ticks examined. Borrelia spp. were the most commonly detected pathogens and were recovered from 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 *Theileria equi* (and the Theileria 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
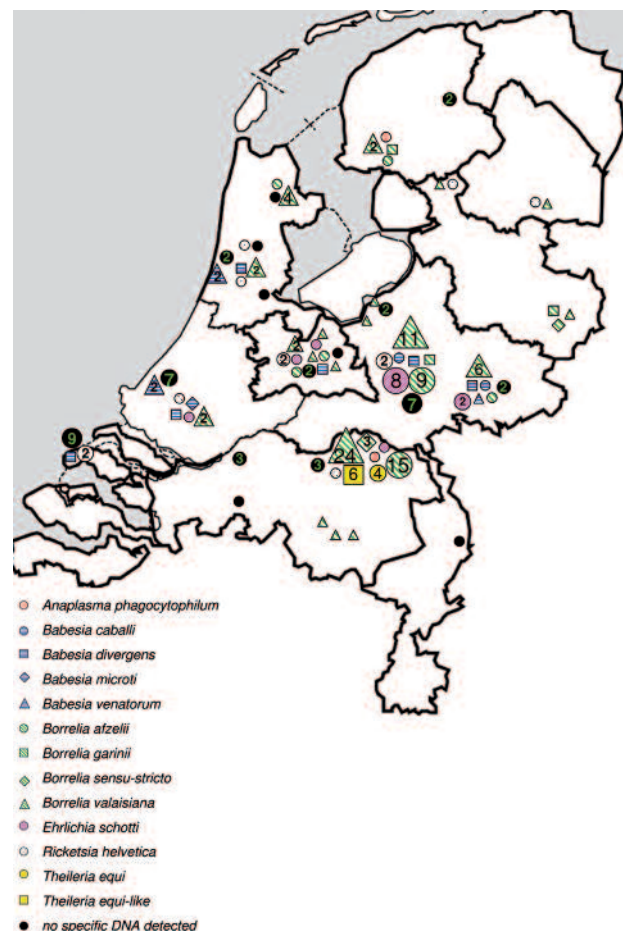

Fig. 1 Geographic distribution of the number of ticks with DNA from specific agents per region or location of collection (if no number is indicated it is 1).

case for previous studies of ticks collected from other (non-equine) host species in this part of Europe (*Nijhof* et al 2007, *Jaenson* et al. 2012, *Pavlidou* 2008). That 40% of collected ticks were nymphs was surprising in given that nymphs (and larvae) are generally considered to feed primarily on small and medium sized mammalian hosts (*Sonenschein* 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* et al. 2007). The two adult specimens of *H. marginatum* are also noteworthy; they were recovered from horses living more than 100 kilometers 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* 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* et al. 2004, *Formosinho* et al. 2006).

The geographic distribution of the submitted ticks is depicted in Fig 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 in 120 of the 130 ticks reported in the current article are discussed in the parallel article on horse and tick infection with Borrelia and *A. phagocytophilum* (*Butler* et al. 2016); the 10 extra ticks do not alter the general picture that *B. valaisiana* and *B. afzelii* were the most prevalent species and *B. burdorferi* sensu stricto much less common. The potential consequences in terms of clinical borreliosis are discussed in the other article, 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* et al. 2009) and dogs (*Nijhof* 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 (*Gray* et al. 2006, *Herwaldt* et al 2003). 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 horses, and which is a higher percentage than previously reported in the Netherlands or other Western European countries (*Nijhof* et al. 2007, *Wielinga* 2009, *Duh* et al. 2009, *Capelli* 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* et al. 2004), although chronic infections have been proposed to play a

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
Theilleria equi	2	2	0
Theilleria equi like	5	1	0
Total	70	80	2

A total 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 sensu stricto and *B. garinii* (n=1)
B. valaisiana, B. afzelii and *B. garinii* (n=1) with *B. valaisiana*
B. afzelii and *B. burgdorferi* sensu stricto (n=3)

role in sarcoidosis and chronic perimyocarditis (_Nilsson_ et al. 1999, _Nilsson_ et al. 2002). The prevalence of _R. helvetica_ DNA in the ticks in this study (9%) was lower than usually found in Europe (_Nijhof_ 2007, _Christova_ et al. 2003, _Hartelt_ et al. 2004), 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_ 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 the _Dermacentor 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. This is 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_ 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 (_Wielinga_ et al. 2006). In addition, the distribution of ticks containing this agent appeared to be rather focal (Fig. 1). It was recently reported that _E. schotti_ is identical to Neoehrlichia mikurensis (_Jahfari_ 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 (_Belongia_ 2002, _Thomas_ et al. 2001). Serological investigations in horses as well indicated a frequent co-infection with tick-borne pathogens (_Laus_ et al. 2013).

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.

Conflict of interest statement

None of the authors of this paper has a personal or financial relationship with other organizations or people that could inappropriately influence or bias the content of the paper.

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Erweiterte Zusammenfassung

Klassifizierung von Zecken und deren zoonotischen Keime bei Pferden in den Niederlanden aus den Jahren 2008-2009

Zecken spielen weltweit und speziesübergreifend eine wichtige Rolle als Vektoren für unterschiedliche Bakterien, Viren und Protozoen. In Westeuropa ist *Ixodes ricinus* die am häufigsten auftretende Zeckenart und diese ist verantwortlich für die Übertragung von *Borrelia burgdorferi* senso lato, *Babesia spp.* und *Ehrlichia-Anaplasma spp.*. Wie andere Zecken durchläuft I. ricinus in dem Entwicklungszyklus vier Stadien und dies innerhalb von 2−3 Jahren. Für diese Entwicklung ist in jedem Stadium eine Blutmahlzeit notwendig bei einem jeweils neuen Wirt. Adulte Zecken infizieren in der Regel große Säugetiere wie Rinder und Schafe, wogegen Larven und Nymphen bei kleinen oder mittelgroßen Wirbeltieren Blut saugen. Informationen zur Klassifizierung und Häufigkeit der unterschiedlichen Zecken-Spezies sowie zu der Prävalenz von Pathogenen in diesen Zecken sind rar. Aufgrund des Klimawandels breiten sich Zecken in Regionen aus, in welchen sie vorherig nicht das ganze Jahr überleben konnten und so auch dementsprechend eine Ausbreitung von vorherig nicht endemischen Erkrankungen auftreten kann. In dieser Studie sollte die Verteilung und Typisierung von Zecken aufgesammelt von Pferden in den Niederlanden erfolgen und ebenso erfasst werden, ob diese Zecken die Pathogene *Borrelia burgdorferi* sensu lato, Theileria equi, Babesia caballi,Anaplasma phagocytophilum, *Babesia divergens, Babesia bovis, Babesia microti, Babesia venatorum, Babesia canis, Rickettsia helvetica, Ehrlichia schotti* und *Anaplasma marginale* in sich tragen. Von Mai 2008 bis September 2009 wurden von Tierärzten eingesammelte Zecken eingesandt. Es handelte insgesamt um 130 Zecken von 56 Pferden. Es folgte die Bestimmung von Spezies, Geschlecht und Stadium. Die Zecken wurden in 70%igem Ethanol fixiert und das Screening für spezifischen Pathogenen erfolgte mittels PCR-RLB. Von den 130 Zecken wurden 68 als adulte *I. ricinus* (63 weibliche und 5 männliche) klassifiziert, 58 waren unreife *Ixodes spp.* (57 Nymphen und 1 Larve), 2 waren klassifiziert als adulte weibliche *D. reticulatus* und zwei als weibliche adulte *H. marginatum*. Bei 73 % der Zecken wurde DNA von den oben genannten Pathogenen identifiziert. Bei 72 Zecken lag DNA von Borrelia spp. vor und von diesen Zecken waren 28 noch mit weiteren Spezies infiziert. Die Inzidenz der pathogenen DNA unterschied

sich nicht zwischen adulten Zecken und Nymphen. Die geographische Verteilung der Pathogene unterschied sich und zwar vor allen bei *Theileria equi*, welche nur in einer südlichen Region des Landes nachgewiesen wurde.

Wie schon in vorherigen Studien nachgewiesen wurde der Hauptteil der Zecken als *Ixodes spp.* klassifiziert. Überraschenderweise waren 40% der eingesandten Zecken Nymphen, von welchen angenommen wurde, dass sie eher kleinere Säugetiere befallen. Beachtenswert ist die Klassifizierung von *D. reticulatus* und *Hyalomma marginatum*, was für eine aktuelle Neu-Ansiedelung dieser Zeckenarten in den Niederlanden spricht. Bei *Hyalomma* handelt es sich um eine Zecke, welche als Vektor für das Krim-Kongo-Fieber des Menschen und wahrscheinlich auch für das West Nile Fieber bei Mensch und Pferd von Bedeutung ist. Der Nachweis von DNA von unterschiedlichen Babesien-Spezies, welche für den Men-schen pathogen sind deutet daraufhin, dass das Risiko einer Babesiose beim Menschen ansteigt. Ein weiteres nachgewiesenes zoonotisches Pathogen war *R. Helvetica* (9%). Zum ersten Mal wurde DNA von *B. caballi, T. equi* und *T. equi*190lik in Ixodes-Zecken bei Pferden in den Niederlanden nachgewiesen. Da *Dermacentor reticulatus* – ein Vektor für die Piroplasmose – nachgewiesen wurde, sollte bei Pferden mit Fieber unbekannter Genese diese Infektion in Betracht gezogen werden. Die Ergebnisse dieser Studie zeigen, dass I. ricinus in den Niederlanden die am häufigsten auftretende Zecken-Spezies ist und dass diese Zecken eine Reihe zum Teil zoonotischer Pathogene in sich tragen. Des Weiteren wurde eine überraschend hohe Anzahl von Nymphen von den Pferden abgesammelt.