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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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Summary: 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* after the removal of one or more attached ticks (n = 120), which were subsequently examined for the presence of these agents. . All horses were examined at the time of tick removal and 6-12 weeks later; thirteen horses were examined again at 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 possibly 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.

Keywords: horse, Borrelia, Anaplasma, tick, PCR-RLB, ELISA, IFAT, sub-clinical infection, disease, Ixodes ricinus

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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 infections including arthritis (*Burgess* et al. 1986, *Hahn* et al. 1996), lameness (*Browning* et al. 1993), anterior uveitis (*Burgess* et al. 1986, *Priest* et al. 2012), neuroborreliosis (*Burgess* and *Mattison* 1987, *Imai* et al. 2011, *James* et al. 2010), low-grade fever and lethargy (*Magnarelli* et al. 1988, *Magnarelli* 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* et al. 2000b). Moreover, seroconversion to the agent is also seen in healthy subjects. In this respect, some investigators have suggested that an overlooked co-infection with *Anaplasma phagocytophylum* might explain the biological variation in the clinical manifestations described, and the difficulty of establishing a conclusive causal link between a Borrelia infection and disease in horses (*Chang* et al. 2000a, *Magnarelli* 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* et al. 2000a, *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 (*Carter* et al. 1994, *Magnarelli* and *Fikrig* 2005, *Manion* et al. 1998, *Marcus* et al. 1985). 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 (*Bacon* et al. 2003, *Johnson* et al. 2008, *Liang* et al. 2000); moreover, it is thought that C6 antibodies indicate active infection (*Philipp* et al. 2005). In Europe, however, several other Borrelia species besides *B. burgdorferi* sensu stricto are present, 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 (*Butler* et al. 2008, *Franzen* et al. 2005, *Franzen* 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 varying from 6 weeks to 23 months. Enrolment of individual horses into the study was permitted following removal of one or more attached tick(s) by an attending veterinarian. The ticks were subsequently tested for the presence of DNA of the two pathogens in order to get some indication of the risk of transmission associated with the biotopes of the horses. To investigate whether transmission had occurred, 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 (SNAP 4Dx, 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 a parallel article.

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 attached ticks, and the local veterinarian was called to remove these ticks after performing a general clinical examination and taking a blood sample from the jugular vein. 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 sent to the 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 11000 g 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 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 1000× magnification for the presence of inclusion bodies suggestive of tick-related pathogens.

Serology

A. 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 400× 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* 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.

B. 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* et al. 1999). The validity of this test has been evaluated in ten annual proficiency tests organized 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 (SNAP 4Dx; 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 (SNAP 3Dx) has been validated for the detection of *B. burgdorferi* antibodies in horses in the USA [21]. The SNAP 4Dx test can also detect antibodies to *A. phagocytophilum* in horses (*Maurizi* et al. 2009), although it has 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) hybridization (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 (*Bekker* et al. 2002, *Schouls* et al. 1999). Subsequently, RLB hybridization 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 *B. 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 at the 2nd sampling, only for animals that were initially seronegative. Haematology data for all horses are presented as mean +/- SD. Data were analyzed 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' (Language and Environment for Statistical Computing, R Team – R Foundation for Statistical Computing, Vienna, Austria 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 SNAP 4Dx tests; correlations between the various testing methods were assessed using kappa statistics.

Results

Sequential detection of antibodies to B. burgdorferi using ELISA and SNAP 4Dx

The results of the Elisa and the SNAP 4Dx 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 SNAP 4Dx (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 sero-negative 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.

Only one horse (no. 45) 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. 40) showed the opposite change (i.e. ELISA positive to negative), although it remained positive for C6 antibodies.

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

Table 1 Borrelia serology results in sequential serum samples recovered from 47 horses after a tick bite (nd = not determined; - = negative test result; + = positive test result; numbers indicate antibody titre), and Borrelia DNA extraction results in ticks after removal from the horse (- = negative test result; + = positive test result)

		Time point 1		Time point 2		Time point 3	
Horse	Tick	ELISA	SNAP 4Dx	ELISA	SNAP 4Dx	ELISA	SNAP 4Dx
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
9	-	0	-	0	-	nd	nd
10	-	1280	+	1280	+	1280	+
11	-	0	+	0	+	nd	nd
12	-	0	-	0	-	nd	nd
13	-	1280	+	1280	+	640	+
14	-	0	-	320	-	nd	nd
15	+	1280	+	1280	+	1280	+
16	-	0	-	0	-	nd	nd
17	+	0	-	0	-	nd	nd
18	+	0	-	0	-	nd	nd
19	+	320	-	0	-	nd	nd
20	+	320	+	320	+	nd	nd
21	+	0	-	0	-	nd	nd
22	+	0	-	0	-	nd	nd
23	+	1280	+	1280	+	1280	+
24	-	320	+	1280	+	1280	+
25	+	1280	+	1280	+	1280	+
26	+	0	-	0	-	nd	nd
27	+	320	+	320	+	nd	nd
28	+	0	-	0	-	nd	nd
29	+	0	+	320	+	nd	nd
30	+	0	-	0	-	nd	nd
31	-	0	-	0	+	nd	nd
32	+	0	-	0	-	nd	nd
33	+	0	-	320	+	nd	nd
34	+	0	-	0	-	nd	nd
35	+	320	+	320	+	nd	nd
36	-	0	-	320	+	nd	nd
37	+	320	-	1280	+	nd	nd
38	+	1280	-	320	+	320	+
39	+	320	-	0	-	nd	nd
40	+	1280	-	320	+	0	+
41	-	0	-	0	-	0	-
42	-	320	+	320	+	320	+
43	-	0	-	0	-	nd	nd
44	-	1280	+	1280	+	1280	+
45	-	0	-	320	-	1280	-
46	-	320	+	320	-	nd	nd
47	+	1280	+	1280	+	1280	+

Sequential detection of antibodies to A. phagocytophilum using IFAT and SNAP4Dx

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 SNAP 4Dx 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 SNAP 4Dx. Horse no. 33 was negative on both occasions by IFAT, whereas the SNAP 4Dx indicated a positive. Horse no. 40 showed a clear positive titre using the IFAT but was classified as negative via SNAP 4Dx. 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).

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 SNAP 4Dx 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. 45) showed symptoms potentially consistent with disease caused by Borrelia or Anaplasma, namely distal hind 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.

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*.

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*

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

a		SNAP 4Dx	
		Positive test result	Negative test result
iELISA	Positive test result	21	5
	Negative test result	5	16

Kappa value: 0.57

b		SNAP 4Dx	
		Positive test result	Negative test result
iELISA	Positive test result	19	7
	Negative test result	3	18

Kappa value 0.58

Table 3 Anaplasma serology results for 47 horses after a tick bite (- = negative test result; + = positive test result; numbers indicate antibody titre). None of the removed ticks was found positive for Anaplasma DNA

		Test time point 1		Test time point 2	
Horse	Age	IFAT 1	SNAP 4Dx	IFAT 2	SNAP 4Dx
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	+
9	7 years	0	-	160	+
10	6 weeks	0	-	0	-
11	16 years	>320	+	>320	+
12	6 years	0	-	0	-
13	2,5 years	0	-	0	-
14	4 years	>320	+	160	+
15	11 months	0	-	0	-
16	8 years	0	-	160	-
17	10 years	160	+	0	-
18	8 years	0	-	>320	+
19	8 years	0	-	>320	+
20	4 years	>320	+	160	+
21	19 years	0	-	0	-
22	7 years	0	-	0	-
23	18 years	0	-	0	-
24	12 years	0	-	0	-
25	15 years	0	-	0	-
26	10 years	0	-	0	-
27	8 years	0	-	160	-
28	7 years	0	-	0	-
29	19 years	0	-	0	-
30	6 years	160	+	>320	+
31	6 years	0	-	0	-
32	14 years	0	-	0	-
33	16 years	0	-	160	-
34	11 years	0	+	0	+
35	15 years	0	-	0	-
36	5 years	0	-	0	-
37	7 years	0	-	0	-
38	5 years	>320	+	>320	+
39	3 years	>320	+	>320	+
40	16 years	>320	+	320	+
41	18 years	>320	-	320	-
42	3 years	>320	+	320	+
43	15 years	0	-	0	-
44	12 years	0	-	0	-
45	19 years	160	+	160	+
46	15 years	0	-	0	-
47	15 years	0	-	0	-

(19/120: 15.8%), *B. garinii* (3/120: 2.5%) and *B. burgdorferi* senso stricto (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*.

Discussion

Overall, the results show that roughly half of the horses in this study had previously been exposed to *Borrelia* spp. 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 with these agents 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. 45, 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.

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* et al. 2000b).

Although a limited number of horses had antibodies to both pathogens, there was no proof of concurrent infection since none of the blood samples were positive for DNA of either pathogen. In short, the present results neither support nor disprove the possibility of a pathogenic interaction between concurrent *A. phagocytophilum* and Borrelia infections in horses. 'It is clear that detecting agents in a tick that has been removed from a horse does not necessarily mean that infection has taken place; it only means that the horse is exposed to a population of ticks that most likely contain these agents. It should be noted that tick populations strongly vary per geographic location, as do the agents they contain.

Most studies on Borrelia infections in horses have been performed in the U.S.A. where *B. burgdorferi* sensu stricto is the

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

a		SNAP 4Dx	
		Positive test result	Negative test result
IFAT	Positive test result	10	1
	Negative test result	1	35

Kappa value: 0.88

b		SNAP 4Dx	
		Positive test result	Negative test result
IFAT	Positive test result	12	3
	Negative test result	2	30

Kappa value: 0.75

Table 5 Haematological data categorized 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.

	Bor01 pos	Bor01 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
Thrombo	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; Thrombo = 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; Ana02 = Anaplasma titre test 2

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 *H. marginatum*.

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

only species present (*Bunikis* et al. 2004, *Piesman* and *Gern* 2004, *Steere* 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* et al. 2004). Moreover, the report that only *B. burgdorferi* sensu stricto is relatively resistant to the bactericidal effects of horse serum suggests that equine hosts may react differently to infections with different genospecies (*Kurtenbach* 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* sensu stricto was the least common with 1.6%; it is therefore assumed that the majority of detected anti-Borrelia antibodies were not induced by *B. burgdorferi* sensu stricto. 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 (*Krupka* et al. 2009, *Sillanpaa* et al. 2007). Nevertheless, given the reasonable correlation between the whole cell ELISA and the SNAP 4Dx, and the low prevalence of *B. burgdorferi* sensu stricto in the ticks, it can be 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 SNAP 4Dx 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, 11 and 31 tested positive for C6 alone. Moreover, three of the horses that became negative in the whole cell ELISA by the second sampling timepoint remained C6 positive. These results raise questions about the specificity of the SNAP 4Dx 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 sero-prevalence was high but there were no clinical signs, and the horses were infected mainly with *B. afzelii* (*Muller* 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 tissues in which it may persist for long periods while its DNA is hardly detected in the blood (*Chang* et al. 2000b). Overall, the results presented here support previous reports that most Borrelia-infections in horses, even those involving *B. burgdorferi* sensu stricto, remain sub-clinical (*Egenvall* et al. 2001, *Magnarelli* et al. 1988, *Muller* 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 SNAP 4Dx 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 infectious 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 summary, even if horses do become persistently infected, their role as agent reservoir should be comparatively minor.

Interestingly, the sero-prevalence of *A. phagocytophilum* (28%) in sampled horses was strongly contrasting with the incidence of its DNA (0.8%) in the removed ticks. This low incidence of *A. phagocytophilum* DNA is in line with earlier studies in other North-European countries (*Lommano* et al. 2012, *Schicht* 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 sero-prevalence 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, *Ixodes ricinus* was predominant, as expected, while finding one *Dermacentor reticulatus* was not surprising given that this tick species has recently become indigenous to the Netherlands (*Jongejan* et al. 2015). On the other hand, the two specimens of adult *Hyalomma marginatum* on two unrelated horses were interesting because this tick is not considered to be indigenous to North-Western Europe.

Conclusion

The high sero-prevalences 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 indicate that horses living in the Netherlands are at appreciable risk of tick-borne infections. On the other hand, only one horse showed clinical signs possibly consistent with tick-borne disease during the study period, even though a number clearly sero-converted. This suggests that the infections do not cause disease or that symptoms are sub-clinical. The low incidence of clinical disease may have been influenced by the predominance of *B. valaisiana* and *B. afzelii* over *B. burgdorferi* sensu stricto, and by the fact that the studied population consisted of recreational horses, rather than high level sport horses in which subtle clinical signs would most likely be

detected earlier. The discrepancy between a 25% sero-prevalence of *A. phagocytophilum* in horses and a very low occurence in ticks suggests that other vectors may exist for this parasite. Finally, this study confirms that sero-positivity 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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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

Borrelia burgdorferi und *Anaplasma phagocytophilum* in Zecken und deren equinen Wirte – Eine prospektive klinische und diagnostische Studie bei 47 Pferden nach Entfernung der blutsaugenden Zecke

Borrelien werden über die Zecke Ixodes ricinus übertragen. Ob ein direkter Zusammenhang zwischen der Borrelien-Infektion und einer klinischen Erkrankung besteht wird weiterhin diskutiert. Klinische Manifestationen welche beim Pferd den Borrelien zugeschrieben werden umfassen Arthritis, Lahmheit, vordere Uveitis, Neuroborreliose und Fieber sowie Lethargie. Allerdings ist der Nachweis der Borrelien ob per PCR oder durch einen serologischen Test nicht beweisend. Eine Serokonversion wird bei vielen gesunden Pferden nachgewiesen. Es besteht die Möglichkeit, dass die Co-Infektion mit *Anaplasma phagocytophilum* die Variationen der klinischen Manifestationen begründen könnte. Es wenig zu natürlichen *B. burgdorferi*-Infektionen beim Pferd bekannt. Im Gegensatz zu *B. burgdorferi* ist die Diagnose der *A. phagocytophilum* Infektion einfach beruhend auf klinischen Symptomen und dem Nachweis des Keimes im Blut. In der vorliegenden Studie sollte der klinische Verlauf bei Pferden, welche kürzlich von einer Zecke gebissen wurden über 6 Wochen bis zu 23 Monaten verfolgt und die Ergebnisse diagnostischer Untersuchungsverfahren verglichen werden. Die Pferde wurden in die Studie aufgenommen, nachdem eine Zecke von einem Tierarzt entfernt und eingesandt worden war.

Die Studienpopulation umfasste 47 Pferde. Nach einer klinischen Untersuchung wurde vor der Zeckenentfernung wurde eine Blutprobe entnommen. Die abgesammelte Zecke wurde in 70%igem Alkohol fixiert und versandt. Bei allen Pferden erfolgte eine zweite klinische Untersuchung einschließlich Blutprobenentnahme nach 6–12 Wochen. Bei 13 Pferden wurde eine dritte Untersuchung 9–23 Monate nach dem ersten Termin erneut einschließlich Blutprobenentnahme durchgeführt. Die Pferdebesitzer sollten während des gesamten Untersuchungszeitraums auf potentielle Symptome achten. Die Analyse der Blutproben umfasste Erstellung eines Differentialblutbildes, Blutausstrich einschließlich zytologischer Untersuchung, indirekter Antikörpernachweis für *A. phagocytophilum*, ELISA für *B. burgdorferi*, kommerziell erhältlicher Test für die Erfassung von Antikörper der IR6Region des Borrelien Membranprotein VlsE auch C6Antigen(SNAP4Dx) sowie eine PCR für Anaplasma- und Borrelia- DNA. Bei der Erstuntersuchung waren 44,7% der ELISA-Ergebnisse für *B. burgdorferi* seropositiv und zwar ≥1:320 und 19,1% der Ergebnisse lagen bei ≥1:1280. Zum zweiten Untersuchungszeitpunkt waren diese Ergebnis ähnlich, allerdings waren 5 zunächst positiv getestete Pferde nun negativ und 5 negativ getestete positiv. Beim SNAP-Test waren auch 44,7% beim ersten Untersuchungstag positiv und 53,2% bei der zweiten Blutprobe. Von den dreizehn Pferden, welche ein drittes Mal untersucht wurden, wiesen 11/13 einen Titer von ≥1:320 und 8/13 von ≥1:1280 auf. Der SNAP-Test wies 12/13 Pferde als positiv aus. Nur ein Pferd wies eine eindeutige Serokonversion von einem negativen Ergebnis der ersten Probe zu einem Wert von über 1:1280 auf, allerdings verblieb der SNAP-Test negativ. Bei 23,4% der Pferde lag zum Zeitpunkt der ersten Probennahme der Titer für A. phagocytophilum bei ≥1:160 und bei 17% bei ≥1:320. Proben 6–12 Wochen später genommen waren entsprechend zu 34% bzw. 19,1% positiv. Der SNAP4Dx-Test war bei der ersten Probennahme bei 23,4% und bei der zweiten Probe bei 29,8% der Pferde positiv. Bei 19,1% der Tiere wurden bei der ersten Probe Antikörper gegen *A. phagocytophilum* und *B. burgdorferi* nachgewiesen, dieser Prozentsatz sank auf 12,7% 6–12 Wochen später. Der SNAP4Dx war zum ersten Zeitpunkt bei 6,4% und bei der zweiten Probe zu 4,2% positiv für Antikörper beider Pathogene. Es zeigte sich keine Korrelation zwischen positivem serologischen Blutergebnis und den Risikofaktoren Alter, Geschlecht oder Zeckenbelastung.

Die Ergebnisse der Studie zeigen, dass annährend die Hälfte der Pferde vorherig Borrelien ausgesetzt waren und ein Viertel Anaplasmen. Die Diskrepanz zwischen der hohen Sero-Prävalenz gegenüber *A. phagocytophilum* und dem sehr seltenem Nachweis in der Zecke, deutet daraufhin, dass eventuell andere Vektoren für dieses Pathogen existieren.

Schlüsselwörter: Pferd, Borrelia, Anaplasma, Zecke, PCR-RLB, ELISA, IFAT, subklinische Infektion, *Ixodes ricinus*