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Detection of *Anaplasma phagocytophilum* in horses from Germany by molecular and serological testing (2008–2021)

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ABSTRACT

Background: Equine granulocytic anaplasmosis (EGA) is a tick-borne disease caused by *Anaplasma* (*A.*) *phagocytophilum*. In Germany, this pathogen is transmitted primarily by *Ixodes ricinus*. There is limited knowledge about its prevalence in horses in Germany. The aim of this retrospective study was to analyze the results of serological and molecular testing for *A. phagocytophilum* in horses which were done in a commercial laboratory in Germany over fourteen years. Additionally, risk factors were evaluated, and hematological abnormalities were addressed in horses with positive PCR results.

Methods: This retrospective study examined results of direct (Polymerase chain reaction [PCR]) and indirect (immunofluorescence antibody test [IFAT]) detection methods for *A. phagocytophilum* in horses on samples provided by German veterinarians and processed by the commercial laboratory LABOKLIN from 2008 to 2021. In horses with positive test results, a Complete Blood Count (CBC) and Serum Amyloid A (SAA) were also analyzed where possible.

Results: In total, 1217/4834 horses tested positive (PCR: 190/1246 horses, 15.2%; IFAT: 1036/3849 horses, 26.9%). Seasonality and location, as classified by federal state, had a statistically significant impact on PCR results ($P < 0.001$ for both). In horses with positive PCR results, hematological abnormalities were detected in 112/118 horses (95%), with thrombocytopenia (86%) and anemia (52%) representing the most common findings. The remaining 6/118 horses (5%) showed no hematological abnormalities on CBC. SAA was measured in 35 horses with positive PCR results, which exclusively showed marked elevation.

Conclusions: The seasonality of *A. phagocytophilum* infections confirmed by PCR testing was consistent with known peaks in vector activity in Germany. The high rate of horses with positive PCR results when compared to dogs and cats may be due to a lack of ectoparasite prophylaxis. Infections with *A. phagocytophilum* should be considered as a differential diagnosis in horses with cytopenia on CBC and SAA elevation, especially in the summer and after any possible tick exposure.

1. Introduction

EGA is a tick-borne disease caused by the obligate intracellular, Gram-negative bacterium *Anaplasma* (*A.*) *phagocytophilum*. Prior to 2001, this pathogen was classified as *Ehrlichia* (*E.*) *equi*, a member of the *E. phagocytophila* group which also includes the Human Granulocytic Anaplasmosis agent (Dumler et al., 2001). The bacterium is transmitted by ticks of the *Ixodes* (*I.*) *persulcatus* complex, in Germany mainly *I. ricinus* (Katavolos et al., 1998). While much less frequent, diaplacentar infections in horses after experimental infection (Gribble, 1969) and infections via blood transfusions in humans (Kemperman et al., 2008), dogs (Kemperman et al., 2008) and horses (Nyindo et al., 1978; Pusterla

Abbreviations: CBC, Complete blood count; CI, Confidence interval; EGA, Equine Granulocytic Anaplasmosis; IFAT, Immunofluorescence Antibody Test; IgG, Immunoglobulin G; OR, Odds ratio; PCR, Polymerase Chain Reaction; SAA, Serum amyloid A.

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and Madigan, 2013) have been reported.

Acute infections are diagnosed by PCR and/or microscopic detection of morulae in granulocytes in peripheral blood smears or buffy coats. The detection of morulae is less sensitive in horses as they can only be detected in peripheral granulocytes in the acute stage of the disease, at least two to four days after infection (Gribble, 1969; Dziegiel et al., 2013a). A positive PCR result has a high sensitivity and specificity for diagnosing acute infections (Courtney et al., 2004; Bakken and Dumler, 2006). IFAT is the preferred method for serological diagnosis in horses (Nyindo et al., 1978). A single positive antibody titre only indicates previous contact with the pathogen. As such, it is of limited diagnostic value but is of interest for epidemiology. In acute infections, antibody titres will remain negative until at least two to five days after morulae appear in peripheral blood (Diniz and Breitschwerdt, 2012). Antibodies may persist for months or years in dogs and humans (Klein et al., 1997; Egenvall et al., 2000; Kohn et al., 2011) and for up to two years in horses (Madigan and Gribble, 1987; Franzen et al., 2005). A four-fold rise in antibody titres over a testing period of four weeks indicates an acute infection with *A. phagocytophilum* (Sainz et al., 2015).

Infection with *A. phagocytophilum* in horses may result in a clinical or subclinical picture. The incubation period for natural infections is approximately one to three weeks (Madigan and Gribble, 1987). The most common clinical signs are fever, lethargy, anorexia, ataxia, and icterus, while the most common laboratory abnormalities are thrombocytopenia, anemia, and leukopenia (Reubel et al., 1998; Bermann et al., 2002; Franzen et al., 2005).

Anaplasma phagocytophilum appears to be especially prevalent in northern European countries such as Sweden (17–69 % of horses tested positive by PCR) (Engvall et al., 1996; Egenvall et al., 2001; Janzen et al., 2019) and Denmark (22 % positive tested horses using rapid ELISA testing) (Hansen et al., 2010). As for Central Europe, a lower prevalence was seen in the Czech Republic (5 % by PCR, 5–73 % by IFAT) (Hulinska et al., 2004; Praskova et al., 2011), the Netherlands (10% by PCR) (Butler et al., 2008), and Switzerland (4 % by IFAT) (Bretscher, 1991). In Germany, one previous study did PCR testing on six horses with symptoms consistent with EGA, one of which tested positive (17 %) (Dziegiel et al., 2013b). A second study described 14 horses with typical clinical signs of EGA, all of which tested PCR positive (Silaghi et al., 2011). There appear to be no larger scale studies at present.

As for other species, *Anaplasma phagocytophilum* was detected by PCR in 0.3–3 % (Hamel et al., 2012; Morgenthal et al., 2012; Bergmann et al., 2015; Schäfer et al., 2022) and by IFAT in 9–23 % (Hamel et al., 2012; Morgenthal et al., 2012) of German cats tested. In dogs in Germany, 4–6 % tested positive by PCR (Jensen et al., 2007; Kohn et al., 2011; Chirek et al., 2017) and 19–50 % tested positive by IFAT (Barutzki et al., 2006; Jensen et al., 2007; Schaarschmidt-Kiener and Müller, 2007; Kohn et al., 2011; Preyss-Jageler et al., 2016).

Overall, data on the prevalence of *A. phagocytophilum* in horses in Europe is limited and seems to vary depending on geographical location. Therefore, the aim of our study was to evaluate the rate of *A. phagocytophilum* infections in horses in Germany as diagnosed by direct (PCR) and indirect (IFAT) detection methods in a commercial laboratory. Additionally, possible seasonal, regional, and annual patterns were investigated as well as a potential correlation with age and sex of the horses. CBC and SAA were evaluated in horses with positive PCR results. We hope to increase clinical awareness of infections with *A. phagocytophilum* in horses.

2. Material and methods

Direct and indirect diagnostic methods for detection of *A. phagocytophilum* in horses were included in this retrospective study. Samples were sent to the commercial laboratory LABOKLIN (Bad Kissingen, Germany) by veterinarians in Germany between January 2008 and December 2021. The 95 % CI for the proportion of horses tested positive by PCR and IFAT were calculated by Wilson procedure including correction for continuity.

Direct detection methods include qualitative TaqMan real-time PCR (Applied Biosystems/Life Technologies, target gene: 60-kDa heat shock protein [HSP60]) on EDTA blood (3846 out of 3849 horses, 99.9%) or cerebrospinal fluid (3 out of 3849 horses, 0.1%). Two hundred µl of EDTA blood or cerebrospinal fluid were used for an automated total nucleic acid extraction using a commercially available kit ("MagNA Pure 96 DNA and Viral NA Small Volume Kit", Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. The resulting nucleic acid was eluted in a volume of 100 µl. Molecular testing for *Anaplasma phagocytophilum* was performed via TaqMan real-time PCR, targeting the 60-kDa heat shock protein (Gene Expression Assay, Applied Biosystems/Thermo Fisher Scientific, Waltham, USA) on a LightCycler® 96 instrument (Roche Diagnostics GmbH, Mannheim, Germany). Amplification consisted of an initial denaturation step at 95°C for 30 s, followed by 40 rounds of denaturation at 95°C for five seconds and annealing/extension at 60°C for 30 s. The PCR was applied as qualitative assay (negative/positive). Cq-values below 35 were considered positive. Each PCR run included a negative and a positive control as well as an extraction control in each sample, to check for nucleic acid extraction and PCR inhibition ("DNA Process Control Detection Kit", Roche Diagnostics GmbH, Mannheim, Germany).

IgG antibodies were detected in serum samples by IFAT according to the manufacturer's guidelines (MegaFLUO ANAPLASMA; MegaCor Diagnostik; > 1:40 positive). Sex, age and breed of the horses as well as the month and year of analysis were noted. Seasonality was defined as follows: spring (March – May), summer (June – August), autumn (September – November), winter (December-February). If several samples were submitted, only the results of either the first or the first positive sample of each horse were included in the statistical analysis. In horses with positive PCR results, any available results from hematological testing (ADVIA 2120i, Siemens Healthineers; Sysmex XT 2000iv, Sysmex Deutschland) or SAA (COBAS 8000, Roche Diagnostics) were also collected. Any thrombocytopenia below 60×10^9/l was confirmed by microscopic evaluation of peripheral blood smears. For regional analysis within Germany, Berlin was grouped with Brandenburg (Berlin-Brandenburg), Bremen with Lower Saxony, and Hamburg with Schleswig-Holstein.

Descriptive statistical analysis was carried out using SPSS for Windows (version 28.0; IBM). $P < 0.05$ was regarded as statistically significant. Binomial logistic regression was performed to determine the effect of sex, age groups, years of testing, regional distribution, and seasonality. For binominal logistic regression analysis, North Rhine-Westphalia, Lower Saxony/Bremen, Schleswig-Holstein/Hamburg, Mecklenburg Western Pomerania, Berlin-Brandenburg, and Saxony Anhalt were defined as northern federal states, whereas the reminder was classified as southern parts of Germany. Intact and castrated male horses were classified together as 'male' and compared to female horses in a binominal logistic regression analysis to examine a possible impact of sex. The median age of the horses included in the study was used to divide the study population according to the age of the horses tested (≤ 12 years and > 12 years, Table 2).

3. Results

In total, this study examined 4834 horses. This included 90 different breeds (most often Hanoverian horses (n = 327), Islandic horses (n = 305), Haflinger horses (n = 185)) and 35 mixed-breed horses. The breed was known for 881 of 4834 horses (18.2 %). One-hundred-and-ninety out of 1246 horses (15.2 %; 95 % CI [13.3 %, 17.4 %]) tested positive by PCR, however none of the three cerebrospinal fluid samples were positive. IFAT was performed on 3849 horses, of which 1036 (26.9 %; 95 % CI [25.5 %, 28.4 %]) tested positive (supplementary Table 1).

The highest rate of positive test results was seen in summer (119 out of 472; 25%), followed by spring (35 out of 237; 15 %) and autumn (36 out of 335; 11 %) (Fig. 1, Table 1). No PCR tests done in winter were

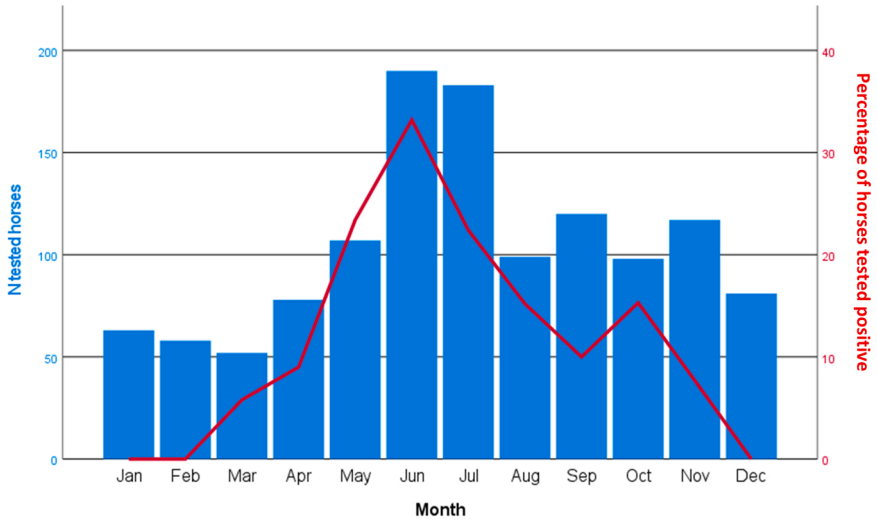

Fig. 1. Number (N) of horses tested for *Anaplasma phagocytophilum* by Polymerase Chain Reaction (PCR) and percentage of horses tested positive in the laboratory LABOKLIN (Bad Kissingen, Germany) from 2008 to 2021.

Table 1
Monthly distribution of test results for *Anaplasma phagocytophilum* in horses by direct (PCR) and indirect (IFAT) detection methods in the laboratory LABOKLIN (Bad Kissingen, Germany) from 2008 to 2021.

Month	PCR	IFAT
January	0/63 (0)	70/226 (31.0)
February	0/58 (0)	44/209 (21.1)
March	3/52 (5.8)	55/241 (22.8)
April	7/78 (9.0)	59/225 (26.2)
May	25/107 (23.4)	73/292 (25.0)
June	63/190 (33.2)	126/452 (27.9)
July	41/183 (22.4)	101/418 (24.2)
August	15/99 (15.2)	105/378 (27.8)
September	12/120 (10.0)	122/404 (30.2)
October	15/98 (15.3)	95/339 (28.0)
November	9/117 (7.7)	112/386 (29.0)
December	0/81 (0)	74/279 (26.5)
Total	**190/1246 (15.2)**	**1036/3849 (26.9)**
Chi-square test	***P* < 0.001**	**P = 0.263**

PCR: Polymerase chain reaction; IFAT: Immunofluorescence antibody test
PCR: $\chi^2 = 109.923$; $df = 11$; $P < 0.001$
IFAT: $\chi^2 = 13.474$; $df = 11$; $P = 0.263$

positive (0 out of 202, 0 %).

The age was known for 787 of 1246 horses tested by PCR (63.2 %; median 12 years; minimum 1 year, maximum 36 years; standard deviation 7.1 years) and 2295 of 3849 horses tested by IFAT (59.6 %; median 12 years; minimum 1 year; maximum 33 years, standard deviation 6.8 years). Older age > 12 years had a statistically significant impact on serology ($P < 0.001$) but not PCR results ($P = 0.346$, Table 2). The sex was known for 1014 of the 1246 horses tested by PCR (81.4 %; male 597/1014 [58.9 %]; female 417/1014 [41.1 %]) and in 3043 of the 3849 horses tested by IFAT (79.1 %; male 1805/3043 [59.3 %]; female 1238/3043 [40.7%]). Neither PCR ($P = 0.513$) nor serology results ($P = 0.791$) appeared to be influenced by sex in a statistically significant way (Table 2).

This study included horses from all federal states in Germany (Fig. 2, Fig. 3). The highest rates of horses with positive PCR results were seen in North Rhine-Westphalia (24 %), Saxony (21 %) and Mecklenburg Western Pomerania (20 %), while no horses from Saxony-Anhalt and Thuringia tested positive (Fig. 3). The highest percentage of horses with positive serology for *A. phagocytophilum* was seen in Saxony-Anhalt (37 %), Thuringia (32 %) and the Saarland (30 %). The lowest rates of detected antibodies were seen in Saxony (21 %), Berlin-Brandenburg (22

Table 2
Binominal logistic regression analysis in 729 horses tested for *Anaplasma phagocytophilum* by PCR and 2072 horses tested by IFAT, all with known sex, age, years, and months of testing from 2008 to 2021.

	B	*SE*	Wald	*P*	Odds Ratio	95%-CI for Odds Ratio	
						Lower bound	Upper Bound
PCR-testing							
Sex (male)	0.142	0.217	0.427	0.513	1.152	0.753	1.763
Age (> 12 years)	0.198	0.210	0.889	0.346	1.219	0.807	1.842
Years	**0.067**	**0.033**	**4.107**	**0.043**	**1.069**	**1.002**	**1.141**
Season (Summer)	**1.187**	**0.212**	**31.264**	**< 0.001**	**3.278**	**2.162**	**4.970**
Region (North)	**0.989**	**0.232**	**18.124**	**< 0.001**	**2.688**	**1.705**	**4.238**
Constant	-138.478	66.862	4.290	0.038	–	–	–
Antibody-testing (IFAT)							
Sex (male)	-0.027	0.101	0.070	0.791	0.974	0.799	1.186
Age (> 12 years)	**0.353**	**0.099**	**12.706**	**< 0.001**	**1.423**	**1.172**	**1.728**
Years	-0.010	0.012	0.616	0.433	0.990	0.967	1.015
Season (summer)	-0.035	0.106	0.105	0.746	0.966	0.784	1.190
Region (North)	-0.147	0.102	2.068	0.150	0.864	0.707	1.055
Constant	18.494	24.873	0.553	0.457	–	–	–

B: unstandardized regression weight; *SE:* standard deviation to the mean; PCR: Polymerase chain reaction; IFAT: Immunofluorescence antibody test
Variables entered: male, age > 12 years, year, summer, north
Degrees of freedom were 1 for all Wald statistics

Fig. 2. Numbers of horses tested positive for *Anaplasma phagocytophilum* by Immunofluorescence Antibody Test (IFAT) in the laboratory LABOKLIN (Bad Kissingen, Germany) from 2008 to 2021 (n positive/N total (%)).

%) and Bavaria (22 %) (Fig. 2).

The binomial logistic regression model included 729 out of the 1246 horses (58.5%) tested by PCR (117 out of 729 tested positive; 16.0 %) and was statistically significant ($\chi^2 = 54.457$, $df = 5$, $P < 0.001$). Correlations between predictor variables were low ($r < .70$), indicating that multicollinearity was not a confounding factor in the analysis. Of the five variables entered into the regression model, seasonality, ie. comparing summer to all other seasons ($P < 0.001$), regional distribution, ie. comparing northern to southern federal states ($P < 0.001$), and the year of testing ($P = 0.043$) contributed significantly in predicting positive test results for *A. phagocytophilum* by PCR, while the other variables showed no significant effect (Table 2). Horses were approximately three times more likely to test positive by PCR in summer (OR = 3.278) and in northern federal states (OR = 2.688). The likelihood of a positive PCR result for *A. phagocytophilum* increased by 6.9 % each year (OR = 1.069) (Table 2).

Of the 4834 horses tested for antibodies to *A. phagocytophilum*, 2072 (42.9 %) were included in the binominal logistic regression model (568 out of 2072 tested positive; 27.4 %). The binomial logistic regression model was statistically significant ($\chi^2 = 15.807$, $df = 5$, $P = 0.007$). Correlations between predictor variables were low ($r < .70$), indicating that multicollinearity was not a confounding factor in the analysis. Of the five variables entering the model, only age > 12 years had a statistically significant impact on predicting serological results ($P < 0.001$), while the other variables (seasonality, sex, year, and region) had no significant effect (Table 2). Horses older than 12 years had a 1.4-times higher likelihood of having detectable antibodies (OR = 1.423).

To evaluate seasonality, Odds Ratios were calculated for spring (PCR: OR = 0.955, 95 % CI = lower bound 0.641/upper bound 1.421; IFAT: OR = 0.865, 95 % CI = 0.720/1.039), summer (PCR: OR = 3.338, 95 % CI = 2.423/4.599; IFAT: 0.977, 95 % CI 0.839/1.138), autumn (PCR: OR = 0.592, 95% CI = 0.402/0.871; IFAT: OR = 1.171, 95 % CI =

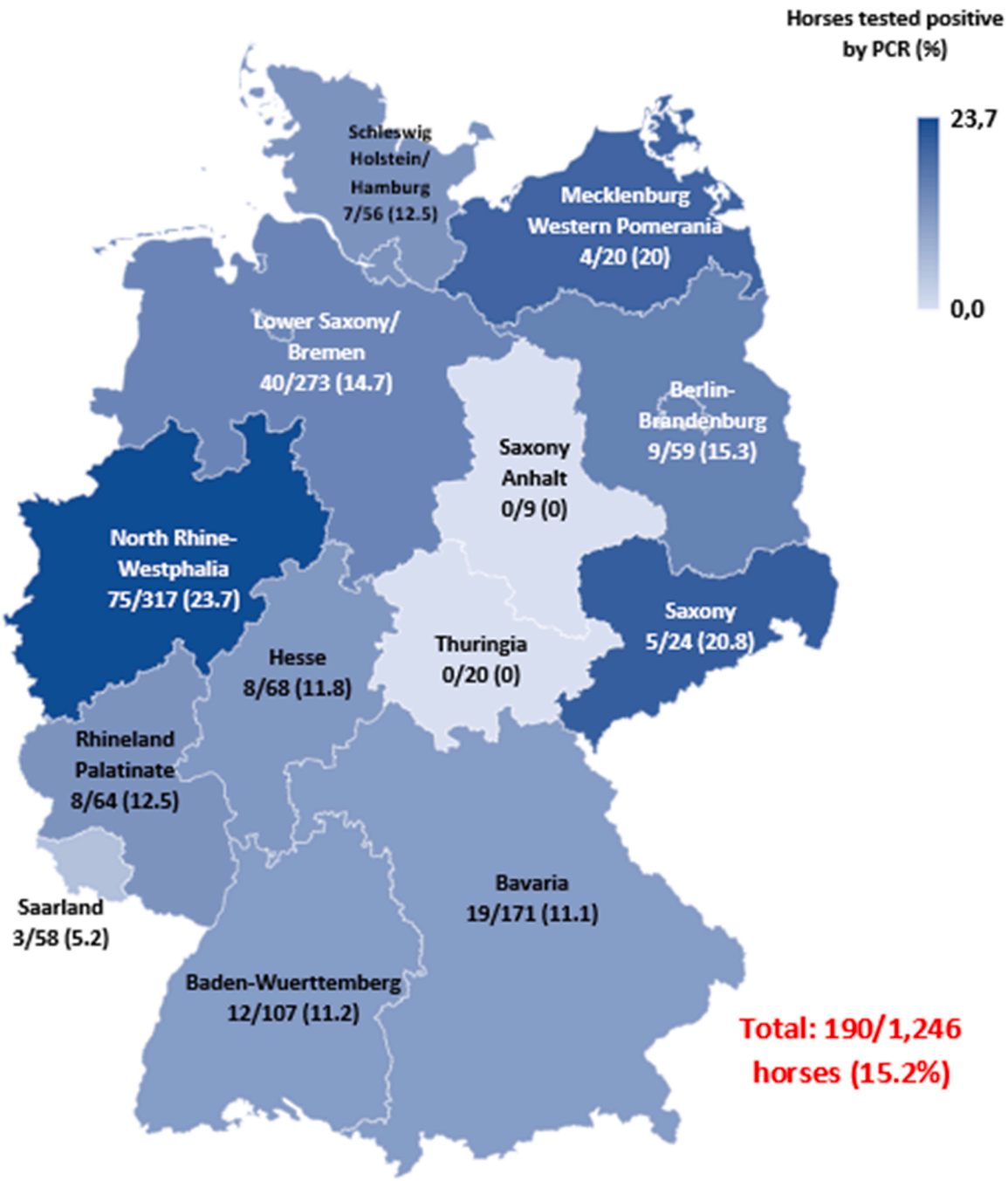

Fig. 3. Numbers of horses tested positive for *Anaplasma phagocytophilum* by Polymerase Chain Reaction (PCR) in the laboratory LABOKLIN (Bad Kissingen, Germany) from 2008 to 2021 (n positive/N total (%)).

1.003/1.366), and winter (IFAT: OR = 0.964, 96 % CI = 0.802/1.159).

Both PCR and antibody testing were done in 261 out of 4834 horses (5.4 %). Of this group, 133 horses had negative serology (51%), while antibodies were detected in 128 horses. Twenty-one horses had positive PCR results in the absence of any detectable antibodies. Nine horses had both positive PCR ad serology results/positive results in both diagnostic tests. Fisher's exact test revealed a statistically significant difference in results of PCR and serological testing ($P = 0.032$). There was a weak negative correlation between results of PCR and serological testing ($r = -0.137$, $P = 0.027$, $N = 261$).

A CBC was available in 118 out of 190 horses (62%) with positive PCR results for *A. phagocytophilum* (Table 3). The most common hematological abnormalities were as follows: thrombocytopenia was found in 102 out of 118 horses (86%), anemia in 61 (52%), and leukopenia in 22 horses (19%) (Table 3, Table 4). Morulae of *A. phagocytophilum* were detected in 57 out of 91 horses tested (62.6%). In six out of 118 horses (5%), no hematological abnormalities were detected (Table 3).

SAA was analyzed in 35 horses with positive PCR for *A. phagocytophilum*. Marked elevation was seen in 33 horses (reference range < 7 μg/ml; mean 638.6 μg/ml; median 713.2 μg/ml; minimum 139 μg/ml; maximum 1133 μg/ml; standard derivation 216.3 μg/ml). In the remaining two horses, SAA exceeded the upper reference range of the test (> 4000 μg/ml).

Table 3
Hematological abnormalities in Complete Blood Count in 112 horses with positive detection of *Anaplasma phagocytophilum* by PCR in the laboratory LABOKLIN (Bad Kissingen, Germany).

Parameter	Reference interval	Elevated	Decreased	Mean	Median	Min – Max	Std deviation
RBCs	6.0 – 12.0 × 10^{12}/l	3	39	6.7	6.4	3.4 – 15.3	1.73
HGB	110–170 g/l	3	67	110	108	61 – 286	26.7
HCT	0.3–0.5 l/l	3	61	0.30	0.29	0.17 – 0.7	0.07
WBCs	5.0 – 10.0 × 10^9/l	8	22	6.681	6.3	2.3 – 18.5	2.53
Seg	3.0 – 7.0 × 10^9/l	11	20	4.44	4.15	0.8 – 13.3	1.92
Band	0 – 0.6 × 10^9/l	0	0	0.004	0	0 – 0.3	0.03
Lym	1.5 – 4.0 × 10^9/l	4	57	1.65	1.5	0.4 – 5.7	0.95
Mono	0.04 – 0.4 × 10^9/l	51	2	0.5	0.4	0.0 – 2.6	0.37
Eos	0.04 – 0.3 × 10^9/l	2	61	0.09	0	0.0 – 1.2	0.14
Baso	0 – 0.15 × 10^9/l	2	0	0.02	0	0.0 – 0.3	0.05
THR	90 – 300 × 10^9/l	0	102	46.7	35.0	2 – 246	43.68

RBCs = red blood cells; HGB = hemoglobin; HCT = hematocrit; WBCs = white blood cells; Seg = segmented neutrophilic granulocytes; Lym = lymphocytes; Mono = monocytes; Eos = eosinophilic granulocytes; Baso = basophilic granulocytes; bands = banded neutrophilic granulocytes
Min = minimun, Max = maximum, Std deviation = standard deviation

Table 4
Hematological abnormalities in 118 horses with Complete Blood Count tested positive for *Anaplasma phagocytophilum* by PCR in the laboratory LABOKLIN (Bad Kissingen, Germany).

Hematological abnormality	Total	Mild	Moderate	Marked	Severe
Thrombocytopenia[a]	102/118 (86)	20/102 (20)	27/102 (27)	43/102 (42)	12/102 (12)
Anemia	61/118 (52)	47/61 (77)	11/61 (18)	3/61 (5)	–
Leukopenia	22/118 (19)	20/22 (91)	2/22 (9)	–	–
Leukocytosis	8/118 (7)	6/8 (75)	2/8 (25)	–	–
None	6/118 (5)	–	–	–	–

Thrombocytopenia: mild: > 50 – 90 × 10^9/l, moderate: > 30 – 50 × 10^9/l, marked: 10 – 30 × 10^9/l, severe: < 10 × 10^9/l
Anemia: mild: 0.25–0.29 l/l, moderate: 0.2 – 0.24 l/l, marked: 0.13 – 0.19 l/l, severe: < 0.13 l/l
Leukopenia: mild: > 3–4 × 10^{12}/l, moderate: > 2–3 × 10^{12}/l, marked: 1 – 2 × 10^{12}/l, severe: < 1 × 10^{12}/l
Leukocytosis: mild > 10 – 15 × 10^{12}/l, moderate: > 15 – 20 × 10^{12}/l, marked: > 20 – 25 × 10^{12}/l, severe: > 25 × 10^{12}/l
[a] Platelet count < 60 × 10^9/l was confirmed by manual count in peripheral blood smears.

4. Discussion

In this retrospective study, we analyzed the results of diagnostic tests on samples from 4834 horses which were sent in by veterinarians from Germany from 2008 to 2021. *A. phagocytophilum* was detected by PCR in 15.2% of these samples, and antibodies to the pathogen were detected in 26.9 % by IFAT. There is currently very limited data on seroprevalence and positive outcomes of PCR testing in Germany. In one 2013 study, one out of 5 clinically ill horses (17 %) was reported to have positive PCR results (Dziegiel et al., 2013b). A 2011 study reported 14 horses with typical clinical signs for EGA tested positive by PCR (Silaghi et al., 2011). Compared to the prevalence reported in dogs (PCR: 4–6 % (Jensen et al., 2007; Kohn et al., 2011; Chirek et al., 2017); IFAT/ELISA 19–50 % (Barutzki et al., 2006; Jensen et al., 2007; Schaarschmidt-Kiener and Müller, 2007; Kohn et al., 2011; Preyss-Jageler et al., 2016)) and cats (PCR 0.3–3 % (Hamel et al., 2012; Morgenthal et al., 2012; Bergmann et al., 2015; Schäfer et al., 2022); IFAT 9–23 % (Barutzki et al., 2006; Jensen et al., 2007; Schaarschmidt-Kiener and Müller, 2007; Kohn et al., 2011; Preyss-Jageler et al., 2016)) in Germany, horses have noticeably higher rates of positive PCR tests. While the horses in this study certainly are preselected by veterinarians, the same would be the case for these other species in most of the quoted studies. Higher risk of vector contact in horses due to a lack of ectoparasite prophylaxis and more extensive husbandry with mainly grazing except in winter months may contribute to the high numbers of affected horses. On the other hand, levels of seropositivity are comparable between all species, indicating a similar exposure to *A. phagocytophilum*.

Scandinavian countries reported an especially high prevalence of *A. phagocytophilum*. In Sweden, 17–69 % of horses had positive PCR results (Engvall et al., 1996; Egenvall et al., 2001; Janzen et al., 2019), and in Denmark, 22 % of horses tested positive in rapid ELISA testing (Hansen et al., 2010). In Central Europe, 5 % (PCR) and 5–73 % (IFAT) of horses tested positive in the Czech Republic (Hulinska et al., 2004; Praskova et al., 2011), 10 % (PCR) in the Netherlands (Butler et al., 2008) and 4 % (IFAT) in Switzerland (Bretscher, 1991). Our study showed higher rates of positive PCR results in northern federal states in Germany compared to more southern ones (OR = 2.688, Table 2). In northern federal states, 15.2 % of horses tested positive by PCR, which is similar to northern European countries but a higher prevalence than might be expected in Central Europe. This would be consistent with a possible trend of higher prevalence of *A. phagocytophilum* in northern Europe. However, most previous studies are based on samples taken before 2007 and more recent data is sparse.

Our study indicates a rising importance of *A. phagocytophilum* infections and/or rising awareness by veterinarians in Germany from 2008 to 2021, which is consistent with similar findings in dogs (Schäfer et al. 2022, in preparation) and cats (Schäfer et al., 2022). Possible causes include a change in climate or land use, or landscape heterogenicity (Janzen et al., 2019). However, a 10-year monitoring of *A. phagocytophilum* in *Ixodes ricinus* ticks in the German city of Hanover revealed stagnating infection rates of ticks (Blazejak et al., 2017). *Ixodes ricinus* is a crucial vector for *A. phagocytophilum* infections. In Germany, *I. ricinus* was found in all federal states but appeared to be concentrated in the northeastern federal states and Baden-Wuerttemberg (Rubel et al., 2021). The impact of vector activity on the prevalence of infection may be a topic for future studies, as might long-term monitoring of *A. phagocytophilum* in ticks in whole Germany.

In general, there was an agreement of rates of horses tested positive by PCR and IFAT in most of the individual federal states. The highest antibody detection rates were seen in Saxony-Anhalt (37%) and Thuringia (32%, Fig. 2), but none of these horses also tested positive by PCR (Fig. 3). However, the rates of PCR testing in these states were comparatively very low ($N = 9$ and $N = 20$, respectively).

In our study, we showed a statistically significant impact of

seasonality on positive PCR results in horses ($P < 0.001$), with higher rates in early summer (Fig. 1). This is consistent with known peaks in the vector activity of *I. ricinus* ticks in Germany occurring in late spring/early summer (Gethmann et al., 2020). There were no horses with positive PCR tests in winter, which may be because of a lower risk of vector contact due to both animals being kept in the stable most of the time and also reduced vector activity itself. Seasonality did not impact the results of serological testing in horses ($P = 0.746$). This is likely due to the prolonged persistence of antibody titres in horses after pathogen contact for at least two years, with the highest titres between day 19 and day 81 after infection (Van Andel et al., 1998).

A diagnosis of EGA should include seasonality of disease, tick exposure, clinical signs and typical laboratory abnormalities (Feige and Müller, 2007). Thrombocytopenia, anemia and leukopenia are the most common hematological abnormalities in EGA (Rikihisa, 1991; Franzen et al., 2005). Abnormalities are mostly mild to moderate (Joachim et al., 2022). This is consistent with our findings (Table 3, Table 4). Blood smears were evaluated in case of thrombocytopenia ($< 60 \times 10^9$/l). Morulae of *A. phagocytophilum* were detected in 57 out of 91 horses (62.6%). Morulae appear in granulocytes in peripheral blood during the acute stage of disease, starting two to four days post infection (Gribble, 1969; Dziegiel et al., 2013a). PCR is therefore considered the superior method for diagnosing EGA (Huhn et al., 2014). Antibodies against *A. phagocytophilum* are detectable from day 14 post infection and may persist for up to two years (Madigan and Gribble, 1987; Franzen et al., 2005). This may explain the lack of impact seasonality had on serological results in our study. A highly statistically significant impact for seasonality was however recognized in PCR testing (Table 1). Horses were three times more likely to test positive in summer than in their counterparts (OR = 3.278, Table 2). Horses > 12 years of age were more likely to have detectable antibodies (OR = 1.423, Table 2), which may be due to an expanded time frame with possible vector and pathogen contact compared to younger horses.

In the acute stage of the disease, PCR is recommended as the most sensitive diagnostic tool in routine diagnostics (Engvall et al., 1996). There are no reports of chronic or persistent infections in horses following natural infection with *A. phagocytophilum* in Europe (Pusterla and Madigan, 2013). Consequently, serological testing is mainly used as an epidemiological tool (Joachim et al., 2022). Elevation of SAA as an acute phase protein has been described in horses with *A. phagocytophilum* infection. SAA is a biomarker for acute inflammation and thus nonspecific. The elevations observed in our study may have other causes but are most likely linked to *A. phagocytophilum* infections as positive PCR results were obtained simultaneously.

The limitations of this study include its retrospective design. Information about living conditions, travel history or the indication for PCR and/or antibody testing (e. g. screening, clinically sick horses suspicious for EGA) was not available. These factors could influence the detection rates of horses tested positive for *A. phagocytophilum,* but their impact is probably limited due to the large number of horses included in the study. Horses were most likely tested for *A. phagocytophilum* by PCR due to clinical suspicion of EGA. Therefore, the study population may not be representative of the overall population of horses in Germany but rather was preselected by the submitting veterinarians. This would lead to higher rates of horses testing positive in our study and would also apply to similar studies in dogs and cats. Still, it would not affect seasonality or regional distribution. There was no information on any background history of travel within Europe or Germany. Additionally, horses were not tested for coinfections with other pathogens which could influence hematology findings.

5. Conclusions

The findings of 15 % and 27 % of horses testing positive by PCR and serology respectively, in a large cohort of horses in a nationwide study in Germany over a period of fourteen years, are substantial. Due to this high prevalence, EGA should be considered as a major differential diagnosis especially in early summer and (though less likely) in early spring and autumn. Hematological abnormalities (especially thrombocytopenia) support a suspicion for infection with *A. phagocytophilum*. Seasonality had a statistically significant impact on results of PCR testing with peaks correlating with the vector activity of *I. ricinus* ticks in Germany. The percentage of horses testing positive for *A. phagocytophilum* by PCR and/or IFAT differs in individual areas in Germany, with peaks seen in northern and western federal states.

Ethics approval and consent to participate

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CRediT authorship contribution statement

Ingo Schäfer: Conceptualization, Project administration, Methodology, Investigation, Writing – original draft. **Cornelia Silaghi:** Supervision, Writing – review & editing, Formal analysis. **Susanne Fischer:** Supervision, Writing – review & editing, Formal analysis. **Cedric Marsboom:** Methodology, Writing – review & editing. **Guy Hendrickx:** Methodology, Writing – review & editing. **Heidrun Gehlen:** Writing – review & editing. **Elisabeth Müller:** Project administration, Supervision, Validation, Writing – review & editing.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Ingo Schaefer reports a relationship with Laboklin Laboratory for Clinical Diagnostics GmbH & Co that includes: employment. Elisabeth Mueller reports a relationship with Laboklin Laboratory for Clinical Diagnostics GmbH & Co that includes: employment.

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Animal welfare statement

Not applicable.

Consent for publication

Not applicable.

Declaration of interest

Elisabeth Müller is the CEO of the commercial laboratory LABOKLIN (Bad Kissingen, Germany) and Ingo Schäfer an employee of the laboratory. This has not influenced the results of our study in any way.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the

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References

Bakken, J.S., Dumler, J.S., 2006. Clinical diagnosis and treatment of human granulocytotropic anaplasmosis. Ann. N.Y. Acad. Sci. 1078, 236–247.

Barutzki, D., De Nicola, A., Zeziola, M., Reule, M., 2006. [Seroprevalence of *Anaplasma phagocytophilum* infection in dogs in Germany]. Berl Munch Tierarztl Wochenschr 119, 342–347.

Bergmann, M., Englert, T., Stuetzer, B., Hawley, J.R., Lappin, M.R., Hartmann, K., 2015. Prevalence of selected rickettsial infections in cats in Southern Germany. Comp. Immunol. Microbiol. Infect. Dis. 42, 33–36.

Bermann, F., Davoust, B., Fournier, P.E., Brisou-Lapointe, A.V., Brouqui, P., 2002. *Ehrlichia equi* (*Anaplasma phagocytophila*) infection in an adult horse in France. Vet. Rec. 150, 787–788.

Blazejak, K., Janecek, E., Strube, C., 2017. A 10-year surveillance of Rickettsiales (*Rickettsia* spp. and *Anaplasma phagocytophilum*) in the city of Hanover, Germany, reveals *Rickettsia* spp. as emerging pathogens in ticks. Parasite Vector 10.

Bretscher, R., 1991. Serologische Untersuchungen zur Häufigkeit und Verbreitung der equinen Ehrlichiose in der Schweiz. Schweiz Arch. Tierheilkd. 133, 185.

Butler, C.M., Nijhof, A.M., Jongejan, F., van der Kolk, J.H., 2008. *Anaplasma phagocytophilum* infection in horses in the Netherlands. Vet. Rec. 162, 216–217.

Chirek, A., Silaghi, C., Pfister, K., Kohn, B., 2017. The occurence of *Anaplasma phagocytophilum* in canine blood donors in Berlin/Brandenburg (2006–2012): retrospective analysis of clinical data and relevance for transfusion medicine. Berl. Munch. Tierarzt Woche 131, 124–130.

Courtney, J.W., Kostelnik, L.M., Zeidner, N.S., Massung, R.F., 2004. Multiplex real-time PCR for detection of *Anaplasma phagocytophilum* and *Borrelia burgdorferi*. J. Clin. Microbiol. 42, 3164–3168.

Diniz, P.P., Breitschwerdt, E.B., 2012. *Anaplasma phagocytophilum* infection (canine granulocytotropic anaplasmosis). In: Greene, C.E. (Ed.), Infectious Diseases of the Dog and Cat. Elsevier, St. Louis, Missouri, pp. 244–254.

Dumler, J.S., Barbet, A.F., Bekker, C.P., Dasch, G.A., Palmer, G.H., Ray, S.C., Rikihisa, Y., Rurangirwa, F.R., 2001. Reorganization of genera in the families *Rickettsiaceae* and *Anaplasmataceae* in the order *Rickettsiales*: unification of some species of *Ehrlichia* with *Anaplasma*, *Cowdria* with *Ehrlichia* and *Ehrlichia* with *Neorickettsia*, descriptions of six new species combinations and designation of *Ehrlichia equi* and 'HGE agent' as subjective synonyms of *Ehrlichia phagocytophila*. Int. J. Syst. Evol. Microbiol. 51, 2145–2165.

Dziegiel, B., Adaszek, L., Kalinowski, M., Winiarczyk, S., 2013a. Equine granulocytic anaplasmosis. Res. Vet. Sci. 95, 316–320.

Dziegiel, B., Adaszek, L., Winiarczyk, M., Garcia-Bocanegra, I., Carbonero, A., Debiak, P., Winiarczyk, S., 2013b. Comparative analysis of 16S RNA nucleotide sequences of *Anaplasma phagocytophilum* detected in the blood of horses from various parts of Europe. J. Med. Microbiol. 62, 1891–1896.

Egenvall, A., Bonnett, B.N., Gunnarsson, A., Hedhammar, A., Shoukri, M., Bornstein, S., Artursson, K., 2000. Sero-prevalence of granulocytic *Ehrlichia* spp. and *Borrelia burgdorferi* sensu lato in Swedish dogs 1991-94. Scand. J. Infect. Dis. 32, 19–25.

Egenvall, A., Franzen, P., Gunnarsson, A., Engvall, E.O., Vagsholm, I., Wikstrom, U.B., Artursson, K., 2001. Cross-sectional study of the seroprevalence to *Borrelia burgdorferi* sensu lato and granulocytic *Ehrlichia* spp. and demographic, clinical and tick-exposure factors in Swedish horses. Prev. Vet. Med. 49, 191–208.

Engvall, E.O., Pettersson, B., Persson, M., Artursson, K., Johansson, K.E., 1996. A 16S rRNA-based PCR assay for detection and identification of granulocytic *Ehrlichia* species in dogs, horses, and cattle. J. Clin. Microbiol. 34, 2170–2174.

Feige, K., Müller, J.M.V., 2007. Equine granulozytäre Anaplasmose. Pferdespiegel 3, 114–116.

Franzen, P., Aspan, A., Egenvall, A., Gunnarsson, A., Aberg, L., Pringle, J., 2005. Acute clinical, hematologic, serologic, and polymerase chain reaction findings in horses experimentally infected with a European strain of *Anaplasma phagocytophilum*. J. Vet. Intern. Med. 19, 232–239.

Gethmann, J., Hoffmann, B., Kasbohm, E., Suss, J., Habedank, B., Conraths, F.J., Beer, M., Klaus, C., 2020. Research paper on abiotic factors and their influence on *Ixodes ricinus* activity-observations over a two-year period at several tick collection sites in Germany. Parasitol. Res. 119, 1455–1466.

Gribble, D.H., 1969. Equine ehrlichiosis. J. Am. Vet. Med. Assoc. 155, 462–469.

Hamel, D., Bondarenko, A., Silaghi, C., Nolte, I., Pfister, K., 2012. Seroprevalence and bacteremia [corrected] of Anaplasma phagocytophilum in cats from Bavaria and Lower Saxony (Germany). Berl. Munch. Tierarztl. Wochenschr. 125, 163–167 (In German).

Hansen, M.G., Christoffersen, M., Thuesen, L.R., Petersen, M.R., Bojesen, A.M., 2010. Seroprevalence of *Borrelia burgdorferi* sensu lato and *Anaplasma phagocytophilum* in Danish horses. Acta Vet. Scand. 52, 3.

Huhn, C., Winter, C., Wolfsperger, T., Wuppenhorst, N., Strasek Smrdel, K., Skuballa, J., Pfaffle, M., Petney, T., Silaghi, C., Dyachenko, V., Pantchev, N., Straubinger, R.K., Schaarschmidt-Kiener, D., Ganter, M., Aardema, M.L., von Loewenich, F.D., 2014. Analysis of the population structure of *Anaplasma phagocytophilum* using multilocus sequence typing. PLoS One 9, e93725.

Hulinska, D., Langrova, K., Pejcoch, M., Pavlasek, I., 2004. Detection of *Anaplasma phagocytophilum* in animals by real-time polymerase chain reaction. APMIS 112, 239–247.

Janzen, T., Petersson, M., Hammer, M., Aspan, A., Dinnetz, P., 2019. Equine Granulocytic Anaplasmosis in Southern Sweden: associations with coniferous forest, water bodies and landscape heterogeneity. Agric. Ecosyst. Environ. 285, 106626.

Jensen, J., Simon, D., Murua Escobar, H., Soller, J.T., Bullerdiek, J., Beelitz, P., Pfister, K., Nolte, I., 2007. *Anaplasma phagocytophilum* in dogs in Germany. Zoonoses Public Health 54, 94–101.

Joachim, A., Cavalleri, J.M.V., Berger, S., 2022. Equine anaplasmosis and equine piroplasmosis in Germany, Austria and Switzerland – previously anecdotal, now relevant? Schweiz Arch. Tierheilkd. 164, 35–50.

Katavolos, P., Armstrong, P.M., Dawson, J.E., Telford, S.R., 1998. Duration of tick attachment required for transmission of granulocytic ehrlichiosis. J. Infect. Dis. 177, 1422–1425.

Kemperman, M., Neitzel, D., Jensen, K., Gorlin, J., Perry, E., Myers, T., Miley, T., McQuiston, J., Eremeeva, M.E., Nicholson, W., Singleton, J., Adjemian, J., 2008. *Anaplasma phagocytophilum* transmitted through blood transfusion-Minnesota, 2007 (Reprinted from MMWR, vol 57, pg 1145-1148, 2008). J. Am. Med. Assoc. (300), 2718–2720.

Klein, M.B., Nelson, C.M., Goodman, J.L., 1997. Antibiotic susceptibility of the newly cultivated agent of human granulocytic ehrlichiosis: promising activity of quinolones and rifamycins. Antimicrob. Agents Chemother. 41, 76–79.

Kohn, B., Silaghi, C., Galke, D., Arndt, G., Pfister, K., 2011. Infections with *Anaplasma phagocytophilum* in dogs in Germany. Res. Vet. Sci. 91, 71–76.

Madigan, J.E., Gribble, D., 1987. Equine ehrlichiosis in northern California: 49 cases (1968-1981). J. Am. Vet. Med. Assoc. 190, 445–448.

Morgenthal, D., Hamel, D., Arndt, G., Silaghi, C., Pfister, K., Kempf, V.A., Kohn, B., 2012. Prevalence of haemotropic Mycoplasma spp., Bartonella spp. and Anaplasma phagocytophilum in cats in Berlin/Brandenburg (Northeast Germany). Berl. Munch. Tierarztl. Wochenschr. 125, 418–427 (In German).

Nyindo, M.B., Ristic, M., Lewis Jr., G.E., Huxsoll, D.L., Stephenson, E.H., 1978. Immune response of ponies to experimental infection with *Ehrlichia equi*. Am. J. Vet. Res. 39, 15–18.

Praskova, I., Bezdekova, B., Zeman, P., Jahn, P., 2011. Seroprevalence of *Anaplasma phagocytophilum* in horses in the Czech Republic. Ticks Tick. Borne Dis. 2, 111–115.

Preyss-Jageler, C., Muller, E., Straubinger, R.K., Hartmann, K., 2016. Prevalence of antibodies against *Borrelia burgdorferi*, *Anaplasma phagocytophilum*, and *Leptospira interrogans* serovars in Bernese Mountain Dogs. Tierarzt Prax. Ausg. K. Kleintier Heimtiere 44, 77–85.

Pusterla, N., Madigan, J.E., 2013. Equine granulocytic anaplasmosis. J. Equine Vet. Sci. 33, 493–496.

Reubel, G.H., Kimsey, R.B., Barlough, J.E., Madigan, J.E., 1998. Experimental transmission of *Ehrlichia equi* to horses through naturally infected ticks (*Ixodes pacificus*) from Northern California. J. Clin. Microbiol. 36, 2131–2134.

Rikihisa, Y., 1991. The tribe *Ehrlichieae* and ehrlichial diseases. Clin. Microbiol. Rev. 4, 286–308.

Rubel, F., Brugger, K., Chitimia-Dobler, L., Dautel, H., Meyer-Kayser, E., Kahl, O., 2021. Atlas of ticks (Acari: Argasidae, Ixodidae) in Germany. Exp. Appl. Acarol. 84, 183–214.

Sainz, A., Roura, X., Miro, G., Estrada-Pena, A., Kohn, B., Harrus, S., Solano-Gallego, L., 2015. Guideline for veterinary practitioners on canine ehrlichiosis and anaplasmosis in Europe. Parasit. Vectors 8, 75.

Schaarschmidt-Kiener, D., Müller, W., 2007. Labordiagnostische und klinische Aspekte der kaninen Anaplasmose und Ehrlichiose. Tierärztl. Prax. 35 (K), 129–136.

Schäfer, I., Kohn, B., Müller, E., 2022. *Anaplasma phagocytophilum* in domestic cats from Germany, Austria and Switzerland and clinical/laboratory findings in 18 PCR-positive cats (2008-2020). J. Feline Med. Surg. 24, 290–297.

Silaghi, C., Liebisch, G., Pfister, K., 2011. Genetic variants of *Anaplasma phagocytophilum* from 14 equine granulocytic anaplasmosis cases. Parasit. Vectors 4, 161.

Van Andel, A.E., Magnarelli, L.A., Heimer, R., Wilson, M.L., 1998. Development and duration of antibody response against *Ehrlichia equi* in horses. J. Am. Vet. Med. Assoc. 212, 1910–1914.