Evaluation of potential predictor variables for PCR assay diagnosis of *Anaplasma phagocytophilum* infection in equids in Northern California

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OBJECTIVE
To identify clinical or clinicopathologic variables that can be used to predict a positive PCR assay result for *Anaplasma phagocytophilum* infection in equids.

ANIMALS
162 equids.

PROCEDURES
Medical records were reviewed to identify equids that underwent testing for evidence of *A phagocytophilum* infection by PCR assay between June 1, 2007, and December 31, 2015. For each equid that tested positive (case equid), 2 time-matched equids that tested negative for the organism (control equids) were identified. Data collected included age, sex, breed, geographic location (residence at the time of testing), physical examination findings, and CBC and plasma biochemical analysis results. Potential predictor variables were analyzed by stepwise logistic regression followed by classification and regression tree analysis. Generalized additive models were used to evaluate identified predictors of a positive test result for *A phagocytophilum*.

RESULTS
Total lymphocyte count, plasma total bilirubin concentration, plasma sodium concentration, and geographic latitude were linear predictors of a positive PCR assay result for *A phagocytophilum*. Plasma creatine kinase activity was a nonlinear predictor of a positive result.

CONCLUSIONS AND CLINICAL RELEVANCE
Assessment of predictors identified in this study may help veterinarians identify equids that could benefit from early treatment for anaplasmosis while definitive test results are pending. This information may also help to prevent unnecessary administration of oxytetracycline to equids that are unlikely to test positive for the disease. (*Am J Vet Res* 2018;79:637–642).

Some of the first reports of equids infected with *Anaplasma phagocytophilum* were from Northern California; however, clinical cases have more recently been reported in several different countries.[1–7] In the initial retrospective studies[1,3] describing the clinical disease, equids were described as febrile, with mean body temperatures ranging from 40.1°C to 41.8°C. Most affected equids had clinical signs of ataxia and edema, and evidence of petechial hemorrhages was common. Laboratory changes identified included leukopenia, anemia, and thrombocytopenia, although specific platelet counts and ranges and the biochemical findings associated with the disease were not reported.[1,3]

In the aforementioned studies,[1,3] diagnosis was typically made on the basis of clinical signs and the presence of inclusions (described as *Ehrlichia* sp inclusions) in granulocytes on Giemsa-stained slides of blood smears.[1,3] Other research has described the use of a PCR assay–based test to detect *A phagocytophilum* in equids and other species.[8–11] Increasingly, reported clinical cases of *A phagocytophilum* infection have been diagnosed by PCR assay, as have other common infectious diseases in equids.[12] Analysis by PCR assay may potentially allow diagnosis at earlier or later stages of disease when clinical signs are less severe and the inclusions are not identified in blood smears.[13] In 1 study,[13] the PCR signal was detected 2 to 3 days before appearance of clinical signs, whereas diagnostic inclusion bodies were first noted 2 to 3 days after the onset of fever.

There are some reports[14,15] of equids with atypical and often severe clinical signs associated with *A phagocytophilum* infection. A presumptive diagnosis of *A phagocytophilum* infection is often made at the initial veterinary visit on the basis of clinical signs, history, and laboratory findings, and treatment is started. However, there has not been a rigorous statistical evaluation of the historical, clinical, or clinicopathologic variables associated with clinical cases of *A Phagocytophilum* infection in equids. Many diseases other than *A phagocytophilum* infection can cause fever, leukopenia, and thrombocytopenia in equids.[16–18] Identification of variables specifically as-

sociated with *A phagocytophilum* infection is needed to aid equine practitioners in practically assessing the risk of disease in a given patient before definitive test results are complete.

The purpose of the retrospective study reported here was to evaluate variables as potential predictors for definitive diagnosis of *A phagocytophilum* infection in equids by PCR assay. We hypothesized that the geographic location where the animal resided at the time of testing as well as specific clinical and clinicopathologic variables would be significantly associated with *A phagocytophilum* infection.

Materials and Methods

Criteria for selection of cases

A retrospective, computer-generated search of medical records at the Loomis Basin Equine Medical Center, Penryn, Calif, between June 1, 2007, and December 31, 2015, was conducted to identify equids that were tested by PCR assay for *A phagocytophilum* infection. All equids that tested positive for *A phagocytophilum* infection and had CBC and plasma biochemical analysis results available were included in the case-control design study. For each test-positive animal (case equids), 2 time-matched equids that tested negative for *A phagocytophilum* (control equids; 1 tested before and 1 after the case animal) were selected for inclusion. When multiple equids were tested sequentially and had positive results, the controls were selected sequentially from the groups of equids with negative test results before and after that set. In cases when an equid tested negative for *A phagocytophilum* but did not have other laboratory test results available, the next sequentially identified *A phagocytophilum*–negative equid was selected.

Procedures

Demographic data obtained from the medical records for each equid included age, sex, species, breed, month of diagnosis, and the town nearest to the residence of the equid at the time of testing as well as its elevation above sea level, longitude, and latitude (determined by use of a mapping coordinates website,[19] cross-checked with additional sources). Physical examination findings on admission including body (rectal) temperature, heart rate, respiratory rate, decreased gastrointestinal sounds (yes or no), presence of edema (yes or no), signs of colic (yes or no), and presence of neurologic deficits (yes or no) were recorded, and laboratory data including CBC and plasma biochemical analysis results were collected. Information regarding outcomes was obtained by a combination of medical records review and owner follow-up when available. Diagnosis was recorded for equids of the control group.

From 2007 through 2014, diagnostic real-time PCR assays were performed at a university diagnostic laboratory.[a] From 2014 through 2015, DNA extraction was performed with a semiautomated nucleic acids purification system[b] and isolation kits[c] used according to the manufacturer's instructions, and the products were analyzed by real-time PCR assay.[d] The *A phagocytophilum* assay was purchased from the diagnostic laboratory[a] and targeted the major surface protein 2 gene at approximately 100 bp. The same *A phagocytophilum* PCR assay primers, probes, and protocols were used for all samples.

Statistical analysis

All relevant data were entered into a spreadsheet, and analysis was completed with a statistical software package.[e] Data are reported as median and range. Potential predictor variables were first analyzed by stepwise logistic regression to identify variables that were linear predictors of the log odds of the outcome of interest (positive or negative result for the *A phagocytophilum* PCR assay). Second, classification and regression tree analyses[20] were used to determine which predictors and thresholds best distinguished between equids that had positive and negative test results for the disease. Finally, generalized additive models were used to evaluate predictors identified with the first 2 models, allowing for a possible nonlinear relationship between a predictor and the log odds of a positive or negative test result. Values of $P < 0.05$ were considered significant; in some models, variables that did not meet significance but were close to this value were retained if they were considered to add to the overall model.

Results

The records search resulted in inclusion of 54 case and 108 control equids in the study. The median age of case equids (35 mares, 18 geldings, and 1 horse of unrecorded sex) was 12 years (range, 3 to 24 years; no age was reported for 4 animals). This group included 52 horses (18 Arabians, 11 American Quarter Horses, 3 Mustangs, 3 Rocky Mountain Horses, 2 American Paints, 2 Appaloosas, and 13 horses of other breeds) and 2 donkeys. The control equids comprised 69 geldings, 36 mares, and 3 horses of unrecorded sex with a median age of 12 years (range, 1 to 30 years); there were 31 Arabians, 16 American Quarter Horses, 12 American Paints, 7 Morgans, 6 Miniature Horses, 6 Warmblood-type horses, and 4 Missouri Fox Trotters.

The geographic locations and clinical examination findings for both groups were summarized **(Table 1)**. Results of available CBC and plasma biochemical analyses were tabulated **(Table 2)**. Neutropenia (12/50 [24%] and 29/98 [30%] in the case and control groups respectively), lymphopenia (44/50 [88%] and 74/98 [76%]), and thrombocytopenia (40/50 [80%] and 43/98 [44%]) were common in both groups. Four equids in the case group were noted to have neurologic signs and 3 were described as having petechiae. A total of 8 equids in the control group were noted to have neurologic signs and 1 was described as having petechiae.

In the final generalized additive model, none of the physical examination findings were identified as

Table 1—Geographic location information and relevant clinical examination findings for 162 equids that underwent testing for *Anaplasma phagocytophilum* infection by PCR assay between June 1, 2007, and December 31, 2015, in a retrospective case-control study to identify predictors of a positive test result.

	Cases (n = 54)		Controls (n = 108)	
Variable	**Result**	**No. of equids**	**Result**	**No. of equids**
Geographic location				
Elevation (meters above sea level)	490 (123 to 984)	52	374 (16 to 984)	105
Longitude (°W)	−120.967 (−121.193 to −120.818)	52	−121.077 (−122.015 to −120.685)	105
Latitude (°N)	38.899 (38.669 to 39.262)	52	38.854 (37.051 to 39.219)	105
Physical examination findings				
Rectal temperature (°C)	39.0 (35.6 to 40.8)	42	38.9 (37.0 to 40.9)	90
Heart rate (beats/min)	51 (32 to 84)	40	48 (32 to 80)	86
Respiratory rate (breaths/min)	20 (12 to 54)	33	17 (8 to 72)	82
Decreased gastrointestinal sounds	8 (15%)	54	34 (31%)	108
Edema	7 (13%)	54	15 (14%)	108
Colic signs	6 (11%)	54	22 (20%)	108
Neurologic deficits	4 (7%)	54	8 (7%)	108

Animals with positive and negative PCR assay results were identified as case and control equids, respectively. Results are represented as median (range) or number (%) of equids with signs present. Not all equids had all data available.

Table 2—Selected cell counts and results of plasma biochemical analysis for the same 162 equids as in Table 1.

	Cases (n = 54)		Controls (n = 108)		
Variable	**Result**	**No. of equids**	**Result**	**No. of equids**	**Reference range**
CBC					
WBCs (cells/µL)	4,710 (2,510–12,400)	51	5,360 (1,330–20,380)	99	5,400–14,300
Neutrophils (cells/µL)	3,740 (490–8,770)	50	3,775 (90–13,810)	98	2,300–9,500
Lymphocytes (cells/µL)	670 (70–5,600)	50	1,000 (210–5,530)	98	1,500–7,700
Platelets (cells/µL)	61,000 (6,000–188,000)	50	106,500 (19,000–348,000)	98	100,000–400,000
Hematocrit (%)	31 (13–48)	50	31 (20–62)	97	32–53
Plasma biochemical analysis					
Sodium (mmol/L)	129 (117–136)	47	132 (123–138)	99	126–146
Potassium (mmol/L)	3.5 (2.6–4.5)	49	3.7 (1.1–5.5)	98	2.5–5.2
Total CO_2 (mmol/L)	27 (16–33)	48	28 (18–32)	99	20–33
CK (U/L)	129 (43–1,245)	49	198 (78–1,006)	99	120–470
Glucose (mg/dL)	128 (67–170)	49	118 (53–278)	98	65–110
Calcium (mg/dL)	11.4 (9.6–13.6)	49	11.8 (8.4–13.7)	99	11.5–14.2
BUN (mg/dL)	13 (9–100)	49	15 (9–30)	99	7–25
Creatinine (mg/dL)	1.3 (0.8–3.8)	48	1.2 (0.6–9.0)	100	0.6–2.2
AST (U/L)	240 (125–780)	49	243 (132–632)	99	175–340
Total bilirubin (mg/dL)	3.5 (0.1–7.2)	49	2.4 (0.2–6.2)	99	0.5–2.3
GGT (U/L)	15 (8–45)	49	16 (7–61)	99	5–24
Albumin (g/dL)	3.1 (2.0–3.9)	49	3.2 (2.1–4.3)	99	2.2–3.7
Globulin (g/dL)	3.9 (2.7–5.7)	49	3.7 (1.6–5.5)	100	2.7–5.0
Total protein (g/dL)	7.0 (5.7–8.3)	49	7.0 (4.0–9.2)	99	5.7–8.0
Fibrinogen (mg/dL)	400 (200–1,298)	28	389 (100–649)	53	200–400
Sodium (mmol/L)	129 (117–136)	47	132 (123–138)	99	126–146

Results are represented as median (range).
AST = Aspartate transaminase. CK = Creatine kinase. GGT = γ-Glutamyltransferase.

significant predictors of a positive PCR assay result for *A phagocytophilum*. However, the geographic location in which the horse resided at the time of testing was significantly associated with this result, with latitude having a positive linear relationship (P = 0.002) with increasing log odds of this outcome. Longitude had a nonlinear relationship associated with increasing log odds as direction moved eastward until the point of 121° west and was retained as contributing to the overall model, although the cutoff for significance was not reached (P = 0.063). Analysis of CBC data did not identify platelet count or neutrophil count as having a significant association with a positive test result. However, the total lymphocyte count was a significant (P = 0.01) linear predictor, with lower lymphocyte count more likely to be associated with a positive PCR assay result. Multiple predictor variables were identified on analysis of the plasma biochemical data. A higher total bilirubin concentration (P = 0.021) and lower sodium concentration (P = 0.002) were both linear predictors of a positive PCR assay result. Low CK activity was a nonlinear predictor (P = 0.008) of a positive result, up to a cutpoint of 750 U/L.

Fifty-three of 54 (98%) case equids survived to the end of treatment, and 102 of 108 (94%) control equids survived to the end of treatment. Oxytetracycline was administered as the sole treatment for case equids at a dosage of 6.6 mg/kg, IV, every 24 hours for 5 days (3/54 equids) or 3 days (1/54 equids). Doxycycline (10 mg/kg, PO, q 12 h for 10 to 14 days) was used as the sole treatment for 8 of 54 case equids, and minocycline (4 mg/kg, PO, q 12 h for 14 days) was given as the sole treatment to 1 of 54 equids. Of the remaining 41 case equids, 37 received oxytetracycline (6.6 mg/kg, IV, q 24 h) for 1 to 5 days followed by doxycycline (10 mg/kg, PO, q 12 h) or minocycline (4 mg/kg, PO, q 12 h) for 7 to 14 days. In addition to the described antimicrobials, other treatments provided for case equids included flunixin meglumine (PO or IV; n = 41), phenylbutazone (PO; 2), IV fluid administration (7), and oral fluid administration (9).

Three of 54 case equids responded to initial treatment for the infection and then relapsed with clinical signs approximately 14 days after the medication was discontinued. Of these 3 animals, 2 had received oxytetracycline (6.6 mg/kg, IV, q 24 h; duration of 4 days for one and 2 days for the other). The third horse had received a single IV oxytetracycline treatment followed by 7 days of minocycline (4 mg/kg, PO, q 12 h) administration. Following the relapse, 2 of these patients were treated with oral minocycline administration at the described dosage for 14 days, and 1 received IV oxytetracycline treatment at the described dosage for 5 days. The clinical signs in all 3 animals resolved following the second course of treatment.

Of the 108 equids in the control group, 21 (19%) had a definitive diagnosis. These included *Salmonella* infection (5 [24%]), septic peritonitis (3 [14%]), neoplasia (3 [14%]), equine coronavirus infection (2 [10%]), *Corynebacterium pseudotuberculosis* infection (2 [10%]), large colon impaction (2 [10%]), strangulating intestinal lesion (1 [5%]), bacterial pneumonia (1 [5%]), diskospondylitis (1 [5%]), and clostridial myositis (1 [5%]). The remaining 87 equids in the control group did not have a definitive diagnosis. Only 7 of those without a definitive diagnosis were treated with antimicrobials. The remaining equids in the control group received only supportive care, which typically included flunixin meglumine. Other treatments for control group equids included IV fluids (28/108) and fluids administered via stomach tube (13/108).

Discussion

Previous reports[1,21] have focused on the changes in circulating concentrations of platelets, leukocytes, and RBCs in equids with *A phagocytophilum* infection relative to established reference ranges. Although these changes are consistent across most reports of anaplasmosis in equids, these same abnormalities are identified in many ill equids.[16–18] Practitioners typically evaluate animals with signs of illness and prioritize potential causes of disease on the basis of clinical examination results and available laboratory data. Despite the abnormalities in platelet, leukocyte, and RBC concentrations identified in equids with *A phagocytophilum* infection, results of the present study indicated that the total lymphocyte concentration may be a better predictor of this disease when considering the variables evaluated.

An increase in plasma total bilirubin concentration was identified as a predictor of *A phagocytophilum* infection in this study. Although primary liver disease can be associated with a high circulating total bilirubin concentration, very few equids in this study had liver enzyme activities above the reference ranges, and the abnormalities were mild. Anorexia is also associated with high bilirubin concentrations in equids, and it may be that the anorexia in equids with *A phagocytophilum* infection is more profound than in equids with some other clinical conditions.[22] However, many of the equids in the control group of our study had serious clinical diseases and had been ill for prolonged periods when evaluated. Previous clinical reports[7,15] have also identified hyperbilirubinemia, and specifically high indirect bilirubin concentrations, in equids with anaplasmosis. Sepsis has been shown to interfere with uptake of bilirubin into the liver in laboratory animals,[23] and this may have also played a role in the finding for equids with *A phagocytophilum* infection in the present study. One weakness of the study was that unconjugated and conjugated bilirubin were not measured separately. A future study measuring these separate components of total bilirubin concentration could help to further define the cause of hyperbilirubinemia in most equids with *A phagocytophilum* infection.

The finding that a decrease in plasma sodium concentration was predictive for a positive *A phagocytophilum* PCR assay result was consistent with a relative free water excess. Hyponatremia has been identified in other cases of *A phagocytophilum* infection in equids.[7] Two common reasons for this change would be alterations in renal function or increased free water intake.[24] Measures of renal function appeared similar between case and control equids in the present study, suggesting that increased free water intake may be more likely than impaired free water excretion. Many equids in this study were described as having a decreased appetite, but owners may monitor water intake less carefully than consumption of feed. It is possible that case equids consumed greater amounts of water than control equids. Gross lipemia was not observed in the collected plasma samples, and hyperproteinemia was rarely present, suggesting that pseudohyponatremia was unlikely.[24] Future studies should focus on water intake and excretion by equids with *A phagocytophilum* infection as well as on measurement of the controllers of sodium and water balance (vasopressin, renin, aldosterone, and angiotensin II).

The finding that a decrease in plasma creatine kinase activity was a significant predictor of *A phagocytophilum* infection was unexpected.

Colic signs appeared to be more common in control equids than in case equids, and this may have resulted in greater muscle activity and increased enzyme activity. However, it is interesting to note that many of the case equids had creatine kinase activities below the laboratory reference range. Low circulating activities of this enzyme have been described in a variety of clinical diseases and may be associated with lack of physical activity or with disease processes.[25] Low creatine kinase values have been associated with a poor prognosis in human patients with critical illness.[26] Given the successful recovery of nearly all case equids in the present study, it would have been difficult to explore associations between the severity of the decrease in creatine kinase activities and prognosis in this study.

The geographic location where equids resided at the time of examination (specifically latitude, with longitude also contributing to the overall model) was significantly associated with a positive PCR assay result for *A phagocytophilum*, and this was an expected finding. The veterinary practice where the study took place is located at the base of the Sierra Foothills but equids are evaluated and treated from within a radius of approximately 200 miles. The proposed tick vector for *A phagocytophilum* likely plays a role in the regions where the disease is observed.[27] Considering that the organism is transmitted by different ticks in different parts of the world, it is likely that the findings related to geographic data described in the present study would only be relevant to this area.[28–30] Differences in strains from various parts of North America and their relationship to specific tick vectors have been explored.[31]

Previous data suggest that geographic variations in host susceptibility may be more important than the competence of the tick vector in determining transmission of some *A phagocytophilum* strains.[31] Veterinarians should be familiar with the factors affecting tick distribution in their specific regions. For example, many practice regions may only detect cases of *A phagocytophilum* infection in specific zones of their practice, and this information can be used to map out areas with a higher index of suspicion for the disease. Likewise, certain breeds of horses may be used for activities that are more likely to take place in types of terrain where vectors may be present. For example, horses training for endurance or trail riding are often Arabians, and this may lead to such breeds having a greater likelihood of the disease.

The clinical features identified in case equids of the present study were different than those found in previous studies where inclusion bodies in granulocytes were used for the diagnosis.[1,3] Specifically, ataxia, edema, and petechial hemorrhages were uncommon in the present study and could rarely be used to identify clinical cases. The differences in clinical signs of affected equids in our study, compared with those in previous reports, could have been attributable to a number of factors. For equids in the present study, the diagnosis was made by PCR assay, rather than by examination of blood smears, and therefore our study may have included animals with different stages or severities of disease. It would be expected, however, that the study would still have included some equids with more severe signs of the disease, including ataxia and edema, despite the use of a more sensitive diagnostic test. Differences in strain virulence might also have been a factor that influenced this finding. Further research should evaluate whether veterinarians in specific regions are more likely to identify particular clinical findings in equids with *A phagocytophilum* infection. The findings of the present study would have been stronger with more equids, and particularly with equids from different parts of the world. Also, inclusion of a larger group of donkeys would have allowed analysis as a subset to determine if there are specific differences in predictors of the disease between this species and horses.

This study did not compare the results of PCR assay testing for *A phagocytophilum* infection and examination of blood smears because the authors' practice relies exclusively on PCR assay results in diagnosis of the disease. The preference for PCR assay over traditional blood smears is multifactorial but is primarily owing to its ability to detect the organism earlier in the course of disease.[9,29,32] The high sensitivity and specificity of the PCR assay method also makes it an ideal test.[29] The advantages of blood smear examination include lower cost and immediate availability of the results when the test is performed in-house.

This study provided important information regarding factors that can help veterinarians to identify equids that are likely to test positive for *A phagocytophilum* infection. Oxytetracycline is often administered before testing has been completed, but its unnecessary use should be avoided, and equids lacking significant risk factors for the disease should not be given oxytetracycline until test results are available. Our results suggested that total lymphocyte count and specific plasma biochemical test results (total bilirubin concentration, total sodium concentration, and creatine kinase activity) were useful predictors of a positive *A phagocytophilum* PCR assay result in equids. The geographic location of the animal may also be helpful but is expected to be unique to a given practice area.

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Footnotes

a. Real-time PCR Research and Diagnostics Core Facility, University of California-Davis, Davis, Calif.
b. KingFisher Duo, Thermo Fisher Scientific, Waltham, Mass.
c. KingFisher Pure DNA Blood Kit, Thermo Fisher Scientific, Waltham, Mass.
d. PikoReal Real-time PCR system, Thermo Fisher Scientific, Waltham, Mass.
e. SAS, version 9.3, SAS Institute Inc, Cary, NC.

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