SURVEY OF *ANAPLASMA PHAGOCYTOPHILUM* ANTIBODIES IN CAPTIVE PRZEWALSKI'S HORSES (*EQUUS FERUS PRZEWALSKII*)

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Abstract: *Anaplasma phagocytophilum* (formerly *Ehrlichia equi*) is a tick-borne pathogen of domestic horses and the causative agent of Equine Granulocytic Anaplasmosis. After the occurrence of clinical anaplasmosis in a Przewalski's horse (*Equus ferus przewalskii*) housed at the Smithsonian Conservation Biology Institute in 2008, opportunistic serosurveillance of the herd was initiated. From 2008 to 2014, 57 serum samples were collected from 27 individuals (10 males; 17 females). Using indirect immunofluorescent antibody assays assay for anti-*Anaplasma phagocytophilum* antibodies, it was determined that the prevalence was 53%. No significant sex differences were identified. A statistical association between increasing age and seropositive status suggests cumulative risk of exposure to *Anaplasma phagocytophilium*. After exclusion of four clinical cases of anaplasmosis, it was found that 22 – 57% of those sampled each year were seropositive and clinically normal, suggesting that the majority of Przewalski's horses develop subclinical or self-limiting anaplasmosis after exposure to *A. phagocytophilium*.

Key words: *Anaplasma phagocytophilum*, *Equus ferus przewalskii*, IFA, indirect immunofluorescent antibody assay, Przewalski's horse.

BRIEF COMMUNICATION

The Przewalski's horse (P-horse; *Equus ferus przewalskii*) is a subspecies of the wild horse (*Equus ferus*) that is native to central Asia and became extinct in the wild in the late 1960s primarily due to habitat loss, hunting, and competition with domestic livestock.[4] With the assistance of successful ex-situ conservation programs, the species has been successfully reintroduced to Mongolia and China, and is now classified as endangered by the International Union for Conservation of Nature.[4]

The Smithsonian Conservation Biology Institute (SCBI) in Virginia, USA has contributed to P-horse ex-situ conservation for nearly four decades. Clinical disease associated with *Anaplasma phagocytophilum* infection in a captive P-horse at SCBI first occurred in 2008, and four other occurrences of clinical anaplasmosis were subsequently identified.[11] *Anaplasma phagocytophilum* is a tick-borne, non-contagious pathogen of carnivores, ruminants, humans, and domestic horses.[12] It is the causative agent of Equine Granulocytic Anaplasmosis (EGA), and is seasonally transmitted by the *Ixodes* spp. tick vector.[2,5,6,9] The SCBI facility has 3100 acres of deciduous forest, cultivated agricultural fields, and pastures, of which approximately 1000 acres are used to house collection animals, including herds of P-horses. Ticks of varying species are present throughout the SCBI property, including *I. scapularis*, and *A. phagocytophilum* is known to be endemic in this region.[1] From 2008 to 2014, this study was initiated to investigate the seroprevelance of *A. phagocytophilum* in this captive population to understand the disease exposure. This report evaluates those data retrospectively to describe patterns of exposure and titer development of *A. phagocytophilium* in P-horses.

From 2008 to 2014, 57 blood samples were collected from 27 P-horses housed at SCBI. Samples were collected opportunistically at different times of the year when animals were restrained for examinations or procedures. Chemical restraint with a variety of anesthetic drugs was employed throughout the study period, and from 2013 onward physical restraint with a hydraulic mechanical restraint device (Fauna Hydraulic TAMER, Fauna Research Inc., Red Hook, New York 12571, USA) was also employed for awake or sedated mare examinations. After collection, blood was allowed to clot and centrifuged for separation of serum within 0.5 to 3 hr of collection. Blood was kept cool on ice or in refrigeration until centrifugation and testing. Testing for antibodies to *A. phagocytophilum* was via indirect immunofluorescent antibody assay (IFA) performed by the University Tennessee College of Veterinary Medicine Diagnostic Laboratory Services (UTDLS; Knoxville, Tennessee 37996, USA). The UTDLS laboratory considers titers ≥ 1:80 to be moderate to high in level of antibody and likely more indicative of current or recent exposure. For the purposes of this manuscript, titers ≥ 1:80 will be referred to as positive.

Of the 27 P-horses sampled, 10 were male and 17 were female (Table 1). If an individual was sampled more than once in a year, then only the last sample of the year was tabulated into the data. Eleven P-horses were sampled once, eight were sampled twice, four were sampled three times, two were sampled four times, and two were sampled five times. When first sampled, five P-horses were subadult (<3 yr), 13 were considered adult (≥ 3 yr and < 20 yr), and nine were considered geriatric (≥ 20 yr); although one P-horse from each age group entered the next age group on subsequent sampling. The median age at first sampling was 17.4 yr (range 1.8-32.1 yrs) with the median age of 15.7 yr (range 1.8-28.6 yrs) for female P-horses and the median age of 18.3 yr (1.8-32.1 yrs) for males.

It was found that 30 – 67% of the SCBI herd sampled each year were seropositive for *A. phagocytophilum*, with an overall 53% (30 of 57; 95% confidence interval, 40 – 60%) seroprevalence from 2008 – 2014 (Table 2). The variation of seroprevalence between years may be influenced by surveillance bias as sample collection from P-horses was opportunistic. Titer persistence for individuals between years was variable, but the majority of individuals, once seropositive, persisted above the cut-off point. A minority of individuals had titers that waned with the individual becoming seronegative. Fisher's exact test was utilized to detect any differences in sex or age group of individual antibody-positive P-horses at the time that individual initially seroconverted or at the time of first sampling if that individual never seroconverted. Results were considered statistically significant at $P < 0.05$. A similar number of males (seven of 10 [70%]) and females (10 of 17 [58%]) were *A. phagocytophilum* antibody positive, and no significance was found between sexes ($P = 0.69$). By age group, zero of four (0%) subadult P-horses, eight of 13 (62%) adults, and nine of 10 (90%) geriatrics were antibody positive. Geriatric horses were significantly more likely to be seropositive than subadult horses ($P = 0.005$), but there was no difference in titer status between adults and either subadult ($P = 0.08$) or geriatric horses ($P = 0.179$). This association between seropositive status and increasing age suggests cumulative risk of exposure over time.

Excluding the four clinical occurrences of anaplasmosis where IFAs were measured, 22 – 57% of the SCBI herd sampled each year and 49% (26 of 53) overall were seropositive, but asymptomatic for *A. phagocytophilium* infection during 2008 – 2014.[11] These results reveal that the majority of P-horse exposed to *A. phagocytophilium* are subclinical and may develop self-limiting anaplasmosis. Reports in domestic horses suggest a similar pattern in that clinical cases of EGA occur sporadically, but serological surveys indicate that the chance of exposure to the

causative agent in horses may be common, particularly in enzootic areas.[3,8] Genetic variation may influence clinical outcome as *A. phagocytophilum* displays genetic heterogeneity with varied clinical disease in cattle, horses, humans, and sheep in North America and Europe.[10,12] Particular *A. phagocytophilum* variants have caused low morbidity in one species and high morbidity in another.[10] One study, in Germany, showed that high genetic variation can exist within a small geographic areas by sequencing five different pathogenic *A. phagocytophilum* variants in 14 domestic horse EGA cases.[10] In the eastern United States, white tailed deer (*Odocoileus virginiansus*) are established reservoir hosts for an apathogenic variant (Ap-V1) of *A. phagocytophilum*, and multiple other wild mammals are reservoir competent, including the white-footed mouse (*Peromyscus leucopus*), raccoon (*Procyon lotor*), and gray squirrel (*Sciurus carolinensis*).[7,12] All of these wild mammals are pervasive on the SCBI landscape, so inherent genetic variation of *A. phagocytophilum* could be cause for the variable clinical response in P-horses.

Due to the relatively high rates of positive serology, *A. phagocytophilum* should be considered enzootic to P-horses when housed in pastures in an enzootic region with known *A. phagocytophilum* and *Ixodes* spp. EGA is considered enzootic in domestic horses in the Czech Republic where one study found that 100% of P-horses (10/10) tested were seropositive to *A. phagocytophilum* by IFA with no reported disease.[8] It is unclear why there were no clinical cases of anaplasmosis in these P-horses. Similarly, the P-horses at SCBI are in a region that is considered enzootic for *A. phagocytophilum*. Their access to large pastures that are shared with native wildlife reservoirs for anaplasmosis and tick vectors puts them at increased risk of exposure to this pathogen. As *A. phagocytophilum* has a worldwide distribution with suitable tick vectors, it could be a future threat to in-situ conservation efforts for P-horses in Mongolia

and China.[2] While the prevalence of clinical anaplasmosis is low (four clinical cases out of 30 positive titers), morbidity can be severe and debilitating.[11] Understanding P-horse immunologic response to *A. phagocytophilum* should be pursued. The authors recommend PCR testing with genetic sequencing to confirm clinical anaplasmosis, serology to monitor population exposure, and further study of anaplasmosis as a component of ex-situ and in-situ P-horse conservation efforts.

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Table 1. Indirect fluorescent antibody (IFA) assay results for anti-*Anaplasma phagocytophilum* antibodies in captive Przewalski's horses (*Equus ferus przewalskii*) at the Smithsonian Conservation Biology Institute in Virginia, USA between 2008-2014.

ID #	Sex	Year of Birth	IFA Titers[a]						
			2008	2009	2010	2011	2012	2013	2014
1	M	1977	--	1280	640	--	640	--	--
2	F	1981	--	1280	--	--	80	--	--
3	F	1982	--	--	40	--	80	--	--
4	F	1983	--	--	--	320	--	--	--
5	F	1986	--	--	--	< 20	--	--	--
6	M	1988	80	--	--	--	--	--	--
7	M	1988	160	--	< 20	< 20	160[b]	--	--
8	M	1988	--	< 20[b]	--	--	< 20	--	320
9	F	1989	--	--	640	--	--	--	--
10	F	1990	640	--	640	--	--	--	--
11	M	1990	< 20	640	--	--	--	320	--
12	M	1990	< 20	640	320	--	--	320	< 20
13	F	1991	640	--	--	--	--	--	--
14	F	1991	--	--	640	--	--	--	--
15	F	1994	--	< 20	1280	--	320	1280[b]	640
16	M	1999	--	320	80	--	160	< 20	--
17	F	2001	--	--	< 20	--	--	< 20	--
18	F	2003	--	< 20	--	--	--	--	--
19	F	2006	--	--	80	--	--	< 20	--
20	M	2006	--	--	--	--	< 20	< 20	--
21	F	2006	--	--	--	--	--	--	40
22	F	2007	--	--	< 20	--	--	--	--
23	F	2008	--	--	< 20	--	--	< 20	--
24	M	2008	--	--	< 20	--	< 20	< 20	--
25	M	2009	--	--	--	--	--	< 20	--
26	F	2010	--	--	--	--	< 20	--	320
27	F	2012	--	--	--	--	--	--	< 20[b]

[a] University Tennessee College of Veterinary Medicine Diagnostic Laboratory Services, Knoxville, Tennessee 37996, USA.
[b] This individual had clinical anaplasmosis at time of this titer.

Table 2. Summarized results of indirect fluorescent antibody (IFA) assay for anti-*Anaplasma phagocytophilum* antibodies in 27 captive Przewalski's horses (*Equus ferus przewalskii*) at the Smithsonian Conservation Biology Institute in Virginia, USA between 2008-2014.[a]

Year	Total in whole herd	Total tested	IFA titers[c]									
			< 1:80 (negative)		≥ 1:80 (total positive)			1:80	1:160	1:320	1:640	1:1280
			n	%	n	%	95% CI[d]	n	n	n	n	n
2008	19	6	2	33	4	67	36 – 98	1	1	0	2	0
2009[b]	25	8	3	37	5	63	35 – 91	0	0	1	2	2
2010	25	14	6	43	8	57	34 – 80	2	0	1	4	1
2011	24	3	2	67	1	33	0 – 70	0	0	1	0	0
2012[b]	24	10	4	40	6	60	34 – 86	2	2	1	1	0
2013[b]	25	10	7	70	3	30	4 – 56	0	0	2	0	1
2014[b]	23	6	3	50	3	50	18 – 82	0	0	2	1	0
Totals		57	27	47	30	53	40 – 60	5	3	8	10	4

[a] Individuals are repeated between years, but not within a year.

[b] One included individual in this year had clinical anaplasmosis.

[c] University Tennessee College of Veterinary Medicine Diagnostic Laboratory Services, Knoxville, Tennessee 37996, USA.

[d] CI, confidence interval.