J Vet Intern Med 2005;19:232–239

Acute Clinical, Hematologic, Serologic, and Polymerase Chain Reaction Findings in Horses Experimentally Infected with a European Strain of *Anaplasma phagocytophilum*

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Six horses were experimentally infected by administration of horse blood containing a Swedish strain of *Anaplasma phagocytophilum.* The polymerase chain reaction (PCR) signal was consistently detected 2–3 days before appearance of clinical signs and persisted 4–9 days beyond abatement of clinical signs, whereas diagnostic inclusion bodies were 1st noted on average 2.6 ± 1.5 (SD) days after onset of fever. Clinical signs and hematologic changes were largely indistinguishable from those previously reported for diseases caused by *A phagocytophilum* (formerly *Ehrlichia equi*—''Californian agent'') and the human-derived human granulocytic ehrlichiosis agent. Horses 1st demonstrated antibody response 12–16 days after inoculation, 2 cases of which were still febrile, and serotiters rapidly peaked within 3–7 days of clinical illness. One horse died during the acute stage of disease, but initial clinical signs and hematologic changes were similar to those of other infected horses. This report shows that, despite minor genetic differences, a European equine-derived strain of *A phagocytophilum* may be similar in pathogenicity to the Californian agent. The PCR used holds promise to widen the diagnostic window and would also be diagnostic during the initial days of clinical disease when inclusions in neutrophils in blood smears are not yet apparent.

Key words: *Ehrlichia equi*; Equine granulocytic ehrlichiosis; Human granulocytic ehrlichiosis; Sudden death.

Equine granulocytic ehrlichiosis (EGE) is a tick-borne infectious disease caused by the obligate intracellular bacterium *Anaplasma phagocytophilum*, previously called *Ehrlichia equi.*[1] *A phagocytophilum* includes agents formerly named *Ehrlichia equi, Ehrlichia phagocytophila,* and the human granulocytic ehrlichiosis (HE) agent. The variant of *A phagocytophilum* that is pathogenic to cattle (formerly *E phagocytophila*) has been reported to induce seroconversion but no clinical disease when infused into horses.[2] Similarly, infusion of horse-pathogenic variants of the bacterium into cattle or lambs results in seroconversion in the absence of clinical disease.[3–5] Thus, cross-species differences in pathogenicity within this bacterial species appear to exist. The variants of *A phagocytophilum* that have been described to be pathogenic to horses will, for the rest of this paper, be described as ''equine-derived Californian agent'' (formerly *E equi*), ''equine-derived European agent'' (Swedish and Swiss strains), and the ''HGE agent'' (which includes European and American human-derived strains).

EGE was 1st described in the United States 1969,[6] and has since been reported in many countries, including Brazil, Germany, Switzerland, Sweden, and the United Kingdom.[7–11] Diagnosis of acute EGE is usually based on typical clinical signs, such as high fever, depression, inappetence, and distal limb edema and ataxia, coupled with the detection of cytoplasmic inclusion bodies in the neutrophilic and occasionally eosinophilic granulocytes.[6,12] However, these clinical signs are not pathognomonic for the disease, and horses with the disease may be positive for inclusions in the granulocytes for only a portion of the clinical illness. Although serology is the standard method available to confirm exposure to the organism, polymerase chain reaction (PCR) analysis for detection of the organism is being introduced as a more sensitive tool in routine diagnostic work.

The incidence of clinical disease in naturally infected horses is not known but it appears that subclinical infection with *A phagocytophilum* in endemic areas is common, because many horses develop titers to this agent without signs of disease.[12–14] Gribble[6] 1st described experimental infection with *A phagocytophilum* in horses in which clinical signs and pathologic changes were studied by using Californian isolates of the bacterium. Subsequently, other groups have examined aspects of this infection with Californian isolates.[5,15–17] Despite these latter studies, none involved more than a few animals, and all had varying aims and designs. Thus, comprehensive details on clinical and clinicopathologic findings including detection by PCR, particularly closer documentation of temporal events and relationships in a larger group of uniformly managed horses infected with the equine-derived Californian agent are lacking. Further, experimental infection of horses with European isolates of equine-derived *A phagocytophilum* has been only scantily studied.[3,18] In contrast, a number of studies have been performed where horses have been inoculated with the human-derived HGE agent, which results in clinical changes largely indistinguishable from EGE disease.[5,19–23]

EGE is commonly diagnosed in Sweden. This paper is part of a longer-term study addressing whether persistence of infection with *A phagocytophilum* can occur in horses. Horses were infected experimentally and followed over a 4-month period with subsequent postmortem examination. The aim of this part of the study was to infect horses with a genetically well-defined European (Swedish) equine-derived strain of *A phagocytophilum* and to study the acute clinical disease stage over a 3-week period by recording the alterations in clinical, hematologic, and serologic findings.

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Submitted March 29, 2004; Revised June 25, 2004; Accepted October 26, 2004.

0891-6640/05/1902-0013/$3.00/0

Further, the detectable granulocytic inclusions, PCR signal, and serologic response were related temporally to the occurrence of clinical signs of disease. The findings were compared with reports of disease in horses caused by the equine-derived Californian agent and by the HGE agent.

Materials and Methods

Animals

Five Standardbred horses aged 5–10 years, and one 19-year-old Warmblood riding horse were used in this study. The Standardbreds had just retired from racing careers. All horses were clinically healthy based on a detailed clinical examination and routine blood chemistry and CBC. Horses were negative for antibodies to *A phagocytophilum* (National Veterinary Institute, Uppsala, Sweden) and had negative or negligible titers to equine herpes virus 1 and 4 and to equine viral arteritis. Serologic status against equine infectious anemia (EIA) was not performed because all horses were raised in Sweden, which is free of the EIA virus. The horses were housed in individual stalls in a shared room, without contact with other horses, in the research animal area of the Department of Large Animal Clinical Sciences, Swedish University of Agricultural Sciences, Uppsala, Sweden. Hospital staff attended the horses, which were allowed daily access to outdoor, vegetation-free, sand paddocks. The study was carried out in winter-spring conditions and was approved by the Ethical Committee of The Swedish National Board for Laboratory Animals (reference number C153/99).

Experimental Infection

All infective blood used for the experiment originated from a clinical case of EGE in a Swedish horse. Blood for infection in this series of experiments was collected from 2 experimentally infected horses during the acute phase of infection; donor 1 was infected with blood from the clinical case, as described by Franzén and others,[18] and donor 2 was the 1st horse infected in this current group of experimental horses. Blood for experimental infection to serve as donor blood for the present study was harvested in sodium citrate-containing blood bags during the acute phase of illness, and held at −70°C. The infective blood was thawed in cold running water and then infused into the recipient horses through the jugular vein (day 0). Horses 1 and 2 were infected with the blood from donor 1 and the other 4 horses were infected with blood from donor 2. The infective dose was calculated to be 20×10^6 infected neutrophils (horses 1 and 2) or 6×10^6 infected neutrophils (horses 3–6).

Clinical Evaluation and Sampling of Horses

All horses were examined clinically on a daily basis. The evaluation included a complete clinical examination with special focus on gait, signs of neurologic disease, or lameness or ataxia while moving in the stall and paddock. Blood samples for microscopic examination of inclusions and PCR evaluation were obtained on a daily basis, whereas samples for CBC and serology were obtained during the period when horses showed signs of disease, for the most part daily or on alternate days. Blood was withdrawn from the jugular vein via catheter or needle puncture to tubes containing ethylenediaminetetraacetic acid (EDTA) for CBC and into plain glass tubes for serology.

CBC

Total hemoglobin, red blood cell count, and total and differential white blood cell and thrombocyte counts were analyzed electronically.[a] Microscopic evaluation of blood smears was used to detect inclusion bodies in the neutrophilic granulocytes indicative of *Anaplasma* sp within those cells. Blood smears were stained with Giemsa for light microscopy and with Acridin orange for fluorescent microscopy. At least 200 neutrophils in every blood sample were examined for presence of inclusions.

DNA Sequence and Data Analysis of the Inoculum

DNA sequencing reactions with fluorescently labeled dideoxynucleotide technology[b] were applied. The PCR and sequencing primers are listed in Table 1. Sequencing reaction products were separated and data were collected by using an ABI 3100 automated DNA sequencer.[c] The sequence was fully determined for both strands of each DNA template to ensure maximum accuracy of the data. Sequences were edited, assembled, and analyzed by using Vector NTI Suite 8.[d] DNA extracted from blood sampled at the acute phase stage of infection from donor 1 and from all 6 infected horses in the study were sequenced for the 16S rRNA (1,428 base pairs [bp]), *groESL* (1,339 bp), and *ankA* (1,735 bp) genes. Sequences for each gene were identical between the sampled horses. The 16S rRNA gene sequence (GenBank accession number AY527213) proved to be identical to the *A phagocytophilum* sequenced from Swedish horses as described by Johansson and others[28] and Engvall and others,[29] and to the HGE agent. The *ankA* and the *groESL* gene sequence (GenBank accession number AY529487 and AY529489, respectively) were compared to sequences deposited in GenBank.[e]

PCR for A phagocytophilum

DNA from EDTA blood samples was extracted by using a QIAamp Blood Extraction Kit.[f] For PCR, the primers used, as described by Goodman and others,[30] were GER3 (TAGATCCTTCTTAACGGAAGGGCG) and GER4 (AAGTGCCCGGCTTAACCCGCTGGC).[g] To prevent contamination, the preparation of reaction mixtures, DNA extraction, amplification, and detection of PCR products were all performed in different laboratory rooms. Additionally, aerosol-resistant filter pipette tips were used throughout the experiment. Amplification of *A phagocytophilum* 16S rRNA was carried out in a 50-μL reaction mixture of 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 4 mM $MgCl_2$, 0.2 mM each of deoxynucleoside triphosphates,[h] 0.25 mM of each primer, and 1 U of AmpliTaq Gold DNA polymerase[i] and 10 μL of extracted DNA as a template. The reaction mixture was subjected to amplification by using an automated DNA thermal cycler.[j] The amplification reaction was started by a heating step at 95°C for 8 minutes. A touchdown protocol followed, where each cycle involved heating to 95°C for 15 seconds, cooling to 68°C for 30 seconds, and heating again to 72°C for 1 minute; the annealing temperature was decreased to 58°C over 20 cycles. After the touch-down cycles, 40 additional cycles of 95°C for 15 seconds, 55°C for 30 seconds, and 72°C for 1 minute were performed. A nontemplate control, with the DNA template substituted with water in the reaction mixture, a positive *A phagocytophilum* blood control sample, and a negative *A phagocytophilum* blood control sample were always included in each PCR run. Amplicons were visualized on 1.5% agarose gels with 100-bp ladders as molecular weight markers.[k]

Serology

Sera were analyzed by an immunofluorescence assay (IFA) for the presence of antibodies to *A phagocytophilum*.[14,31] Briefly, 2-fold dilutions of fresh sera were added to slides precoated with *E equi* antigen.[l] Bound antibodies were visualized by fluorescein isothiocyanate (FITC)–conjugated rabbit anti-horse immunoglobulin[m] when slides were examined by fluorescence microscopy. Samples not proving positive at 1:10 or at either of the dilutions 1:40 or 1:80 were deemed negative. If a sample proved positive at any of the 3 initial dilutions, the serum was titrated further until end-point titer was reached. The sera initially were tested at the dilutions 1:10 and 1:40. If samples were positive at any of these dilutions, the sera were titrated with 2-fold dilutions until the end points were reached. In this test, an IFA titer ≥ 1:40 was considered positive. The test is accredited according

Table 1. Primer sequences for polymerase chain reaction (PCR) and sequencing of the 16S rRNA, *ankA*, and *groESL* heat shock operon partial genes of *Anaplasma phagocytophilum*.

Gene and Primer Name	Functions	Nucleotide Sequences of Primers (5′–3′)	Product Size (bp)	Reference
16S rRNA				
fD1	PCR and sequencing	AGAGTTTGATCCTGGCTCAG	1,428	24
rP2	PCR and sequencing	ACGGCTACCTTGTTACGACTT		24
880f	Sequencing	ATTAGATACCCTGGTAG		25
800r	Sequencing	CTACCAGGGTATCTAAT		25
1050f	Sequencing	TGTCGTCAGCTCGTG		25
1050r	Sequencing	CACGAGCTGACGACA		25
EB4F	Sequencing	GTGCCAGCAGCCGCGGTAATAC		26
EHR521R	Sequencing	CTTTAACTTACCGAACCGCCTACA		26
Epank				
AQ2F3 (1812f)	PCR and sequencing	GAAGAAATTACAACTCCTGAAG	1,735	26
Epank 3597r	PCR and sequencing	TAGGGCCTTCACTTTGAGGA		This study
LA6f (2252f)	Sequencing	GAGAGATGCTTATGGTAAGAC		27
AQ2R1 (2491r)	Sequencing	AAGCCGTCTTACCATAAGCAT		26
Epank 2798f	Sequencing	CATCCTTGGGTAGTGGCCTAG		This study
Epank 2798r	Sequencing	CTAGGCCACTACCCAAGGATG		This study
GroESL				
HS43	PCR and sequencing	ATAGCTAAGGAAGCATAGTC	1,339	26
groESL 1404r	PCR and sequencing	CGTCGTCATTCTTGCCTTTT		This study
groESL 558f	Sequencing	TGTCTGCRAATGGAGACAAG		This study
groESL 691r	Sequencing	GATATCCGCGATCAAACTGC		This study

bp, base pairs.

to the standard EN ISO/IEC 17025[n] with inclusion of positive and negative controls. The limit for seropositivity was originally set by calibrating the test according to results obtained from external laboratories.

Results

Clinical Findings

All horses developed signs of EGE, which in each horse consisted of at least 6 of the following signs: fever; depression (interpreted from changes of reduced alertness, somewhat lowered head position, and reduced interest in surrounding events); partial or total loss of appetite; increased heart rate; increased respiratory rate; ataxia, stumbling, or unwillingness to move; and edema in distal limbs. One of 2 horses receiving the higher does of infective blood died suddenly and unexpectedly 2 days into clinical illness. Before death clinical and clinicopathologic findings were indistinguishable from those of the other horses, and the general findings on postmortem examination resembled changes observed by Gribble.[6] Details of gross, microscopic, immunohistochemical, and PCR findings at postmortem examination are being reported elsewhere. Because of loss of this animal at day 2 of the acute illness, this horse is from here on excluded from this report. The other horse given the higher infective dose (horse 1) had a similar course and severity of acute clinical disease compared to the other 4 horses. The onset of clinical signs varied from 6 to 9 days after infection. The horse given the higher infectious dose had the shortest incubation period. The febrile period varied between 5 and 10 days (as represented by horse 3; Fig 1), and in some cases other signs persisted for several days after fever subsided. The 1st sign in all horses was fever, often accompanied with depression and increased respiratory rate. Thereafter, an increased heart rate and decreased or total lack of appetite were noted. Ataxia occurred on days 2–4 after initial onset of clinical disease in all horses and distal limb edema generally occurred 4 days into clinical disease, and was the sign most delayed in onset. Duration of signs is shown in Figure 2. In 2 of the horses, a transient grade II/V systolic heart murmur over the left heart base developed during the febrile stage and disappeared thereafter. In 2 horses, fever was biphasic, which in the 2nd fever spike in 1 horse (horse 6) was associated with a mild catheter-associated thrombophlebitis. By 22 days after infection, all abnormal signs associated with EGE had fully abated in all the surviving horses.

CBC

All horses developed a distinct but transient leucopenia, neutropenia, and a marked lymphopenia during the febrile stage of the infection. A marked thrombocytopenia also developed in all horses and persisted longer than the leucopenic phase (Fig 3). Mild anemia was also noted during the infection in all horses. The most consistent hematologic alterations were the distinct lymphopenia and thrombocytopenia. Inclusions in neutrophils developed in all horses 2.6 days (range 1–5 days, SD $\pm$ 1.5 days) after onset of clinical signs, and once present persisted for 7.6 days (range 6–10 days; with horse 3 shown in Fig 1). The percentage of neutrophils with inclusions varied over time and between horses (range 0.5–16%).

PCR

The results of the PCR test became positive 3 days after inoculation in 1 horse (the horse with higher infectious

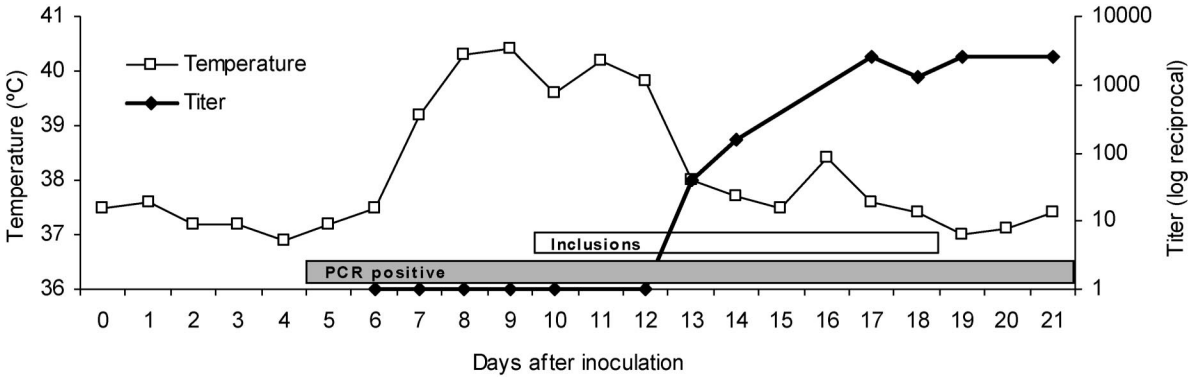

Fig 1. Rectal temperature, reciprocal immunofluorescence assay titers, polymerase chain reaction test, and ehrlichial inclusions over the initial 3 weeks after inoculation (day 0) in a representative horse (horse 3) experimentally infected with a European (Swedish)-derived *Anaplasma phagocytophilum* strain.

dose) and 5–6 days after inoculation in the other 4 horses, and remained positive through 18–21 days after inoculation in all horses. The results of the PCR test were positive in all horses 2–3 days before clinical signs appeared and remained positive for 4–9 days after rectal temperatures returned to within the reference range. In all horses, the results of the PCR became positive from 4 to 7 days before inclusions were found in the neutrophils.

Serology

All horses seroconverted by 12–16 days after inoculation, reaching maximal titers within 3–7 days from when seropositivity (cutoff value 1 : 40, National Veterinary Institute, Uppsala, Sweden) was identified (Fig 1). Two of the horses were still febrile or had other clinical signs at the time of seroconversion. Maximum titers were 1 : 1,280 to 1 : 5,120, and confirmed as true peak values because horses were monitored for an additional 14 weeks (unpublished data).

Discussion

We were able to induce clinical disease in all inoculated horses. All 5 horses that were followed through the entire acute phase of disease had a very similar clinical picture

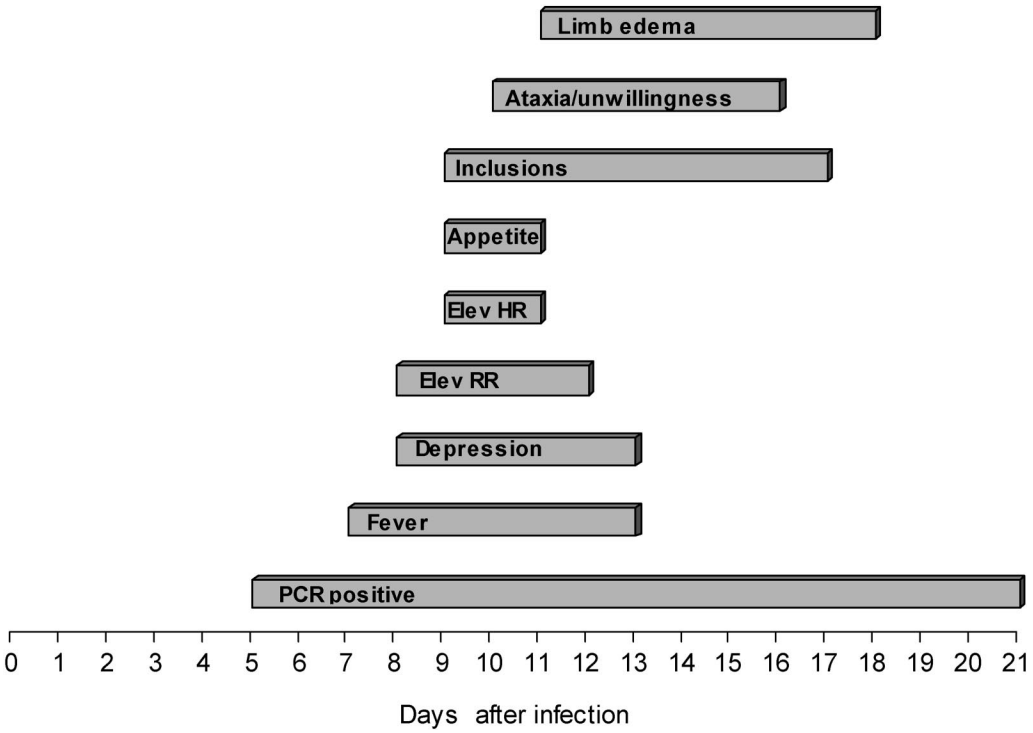

Fig 2. Days of onset and duration of clinical signs, compared to inclusion presence and polymerase chain reaction–positive signal in 5 horses experimentally infected with a European (Swedish)-derived *Anaplasma phagocytophilum* strain. Elev HR, heart rate increase; Elev RR, respiratory rate increase; Ataxia/unwillingness, ataxia and unwillingness to move or stumbling.

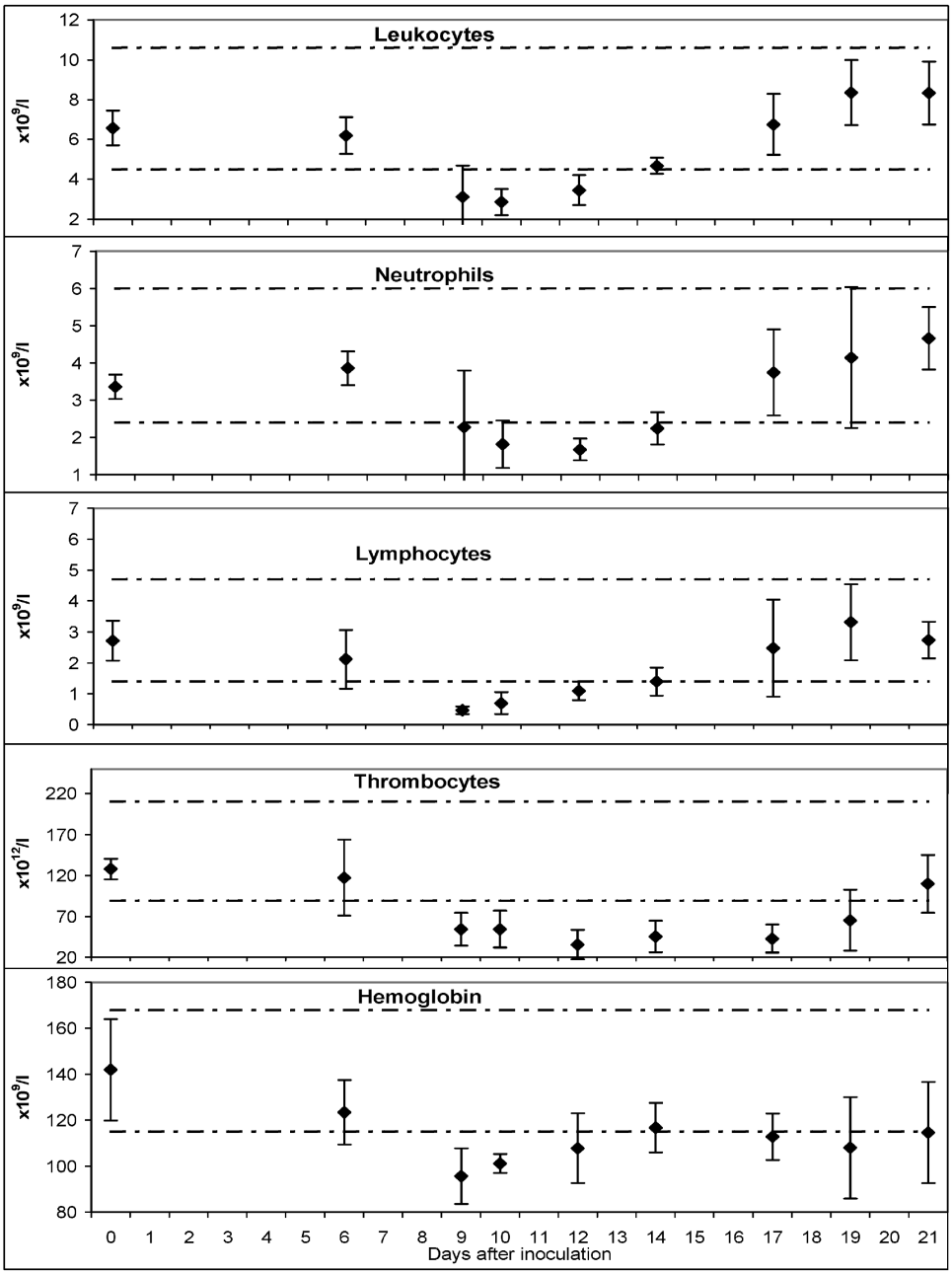

Fig 3. Mean value and 1 standard deviation for total white blood cell, neutrophil, lymphocyte, and thrombocyte count, and total hemoglobin in 5 horses experimentally infected with a European (Swedish)-derived *Anaplasma phagocytophilum* strain. Day 0 is the day of inoculation. The dashed horizontal lines on the graphs represent reference ranges for each parameter.

of fever, depression, ataxia, and distal limb edema, which were the same clinical signs described in the early work of Gribble.[6] However, in contrast to Gribble's[6] pioneering study, clinically detectable icterus was not observed in the horses in the present study, and in 2 of these horses, a transient grade II systolic heart murmur was detected during the febrile stage. The mild anemia noted was probably insufficient to cause the murmur, which, based on its transient appearance, location, and character, was most likely due to physiologic turbulence. The hematologic response was consistent with previous reports[6,12] of early thrombocytopenia, lymphopenia, neutropenia, and mild anemia. Although this study lacked a negative control horse given uninfected blood, the potential clinical effects of blood infusion alone occur at the time of infusion, with the red cells being destroyed within 1 week,[32] which was in advance of the onset of clinical disease in the group of horses in this study. Thus, the clinical illness we observed, which appeared fully after the major influence of any transfusion influence, reflected the acute infection process with EGE. Although unlikely, it cannot be discounted that blood transfusion alone can marginally influence the kinetics of the immune and hemato-

logic response in acute infection with EGE when compared to natural infection.

Blood tests to facilitate diagnosis of EGE during the clinical illness have traditionally relied on the presence of intracytoplasmic inclusions (morula) in the circulating neutrophilic granulocytes, with only the recent introduction of PCR analysis for presence of the organism's DNA. Diagnostic inclusions in neutrophils were detectable on average 2.6 days after appearance of 1st clinical signs of disease, which is a minor but clinically important difference to earlier suggestions that inclusions occur simultaneously with onset of fever.[6,12] Because blood smears were examined with a high degree of scrutiny with similar methodology to that in the work of Gribble,[33] our findings appear to reflect a valid, and clinically relevant difference from earlier reports. However, in our work, positive confirmation of inclusions was set as definitive identification of *A phagocytophilum* inclusions. Very small or single inclusions described by Gribble[33] are not recorded as positive in our laboratory because of the risk of toxic or other cytoplasmic changes being mistaken for early inclusions. More recently, different PCR systems have been developed to detect *A phagocytophilum* for research purposes and are even coming into use in routine diagnostic work in selected laboratories. Thus, although the presence of neutrophilic inclusions as a diagnostic tool is specific for this disease, it lacks diagnostic sensitivity, because inclusions have a limited window of appearance in the course of the disease. In this model of disease, with the European equine-derived strain, diagnosis based on detection of inclusions during the initial days of fever can be negative, whereas the PCR was positive in all horses even in advance of appearance of clinical signs. This indicates that in clinical cases, the diagnosis may be missed during the 1st days of fever if presence of clearly identifiable inclusions is used as the sole diagnostic tool.

All horses seroconverted by 12–16 days after infection, which was 6–8 days after onset of initial clinical signs of fever. At the point of detection of seroconversion, all horses were still positive for inclusion bodies and 2 of the 5 horses were still febrile. Further, 3 horses attained maximal titers while inclusion bodies were still present. All horses reacted serologically very similarly to this experimental infection. This may be due to the similar infective dose as well as similar care and housing of the horses when infected and thus may not necessarily be applicable to natural infection with ticks when horses are on pasture.

If the antibody titer rises as rapidly under natural infection as in our experimentally infected horses, the 1st of paired samples needs to be taken as early as possible into the illness if seroconversion is to be documented. This is because antibody titer can peak after as early as 10 days into clinical illness, and the time frame from seroconversion to attaining peak titer was 3–7 days (mean 4.4 days) in all cases. However, in natural tick infection, the infectious dose is presumably smaller or delivered over a longer time, which might induce a less rapid antibody response. Comparison between intravenous inoculation versus infection via infected ticks in experimental HGE-agent infection in horses showed the incubation period to be significantly longer, with appearance of clinical signs and seroconversion delayed in tick-infected horses.[22] However, the severity of clinical signs, the course of disease, and mean geometric titers in convalescent sera taken at 30 days after infection were similar regardless of route of inoculation. In a separate study in which 3 horses were exposed to *A phagocytophilum*–infected ticks, 2 of the horses developed clinical signs on days 18 and 25, respectively, after 1st exposure.[13] Seroconversion was detected in the 2 horses 4 and 7 days, respectively, after onset of clinical signs of disease, remarkably similar to the time frame in the present study. The 3rd horse remained clinically unaffected and failed to seroconvert despite being PCR positive to the organism. These 2 abovementioned studies support the possibility that IV inoculation also mimics tick-borne inoculation in the infective process. A separate study intended to follow antibody kinetics in acute infection showed that 3 of 10 horses studied had already reached maximal or next to maximal titer at the 1st occasion of blood sampling in acute illness and with simultaneous presence of inclusions. Another 2 horses also were seropositive, although not at peak level, at 1st sample.[34] The above observations, when combined with results of the present study, suggest that demonstration of a 4-fold rise in antibody titer to confirm diagnosis of acute EGE can require initial sampling early in the course of clinical disease. Equally troubling for use of serology as a diagnostic tool is the high prevalence of seropositivity in horses from areas where EGE is endemic, despite absence of history of clinical disease.[14,31]

With the ability of the HGE agent to infect horses, the horse has been used as an animal model for human infection, perhaps at the expense of attention to equine-derived isolates and EGE. To characterize the infectious agent used in this study the 16S rRNA gene and the *groESL* and the *ankA* genes were partly sequenced. The Swedish horse-derived European strain of *A phagocytophilum* had a 16S rRNA nucleotide sequence identical to that of the HGE agent, but showed minor differences to the originally reported Californian agent of *A phagocytophilum* (previously named *E equi*). The HGE-identical 16S rRNA gene sequence also has been reported from horses with EGE in the eastern United States.[35] By comparing the sequences of the *groESL* and the *ankA* genes to sequences deposited in GenBank, it is clear that our inoculate clusters together with *A phagocytophilum* isolated in Europe, and not in the United States. Despite minor genetic variation between Californian and European isolates of *A phagocytophilum* seen in this study and also reported by others,[26,27,36] it appears that the organisms in horses act clinically and biologically in a similar manner, because the clinical signs observed in this experimental infection mimic naturally occurring EGE in the United States[37] and in Europe,[8–11] and, furthermore, mimic the clinical disease described in horses after experimental infection with human-derived HGE agent.[3,19]

Footnotes

[a] Cell-Dyn 350, Abbot Laboratories, Abbot Park, IL

[b] Dye terminator cycle sequencing ready reaction kit, Applied Biosystems, Foster City, CA

[c] ABI 3100 automated DNA sequencer, Applied Biosystems, Foster City, CA
[d] Vector NTI Suite 8, InforMax, Frederick, MD
[e] GenBank accession numbers AF020521, AF100883, AF100884, AF100885, AF100886, AF100887, AF100888, AF482759, AF100889, AF100890, AF356512, AF100891, AF100892, AF100893, AF100894, AF047897, AF100882, AF153716, AF033101, AF482760, EPU96735, AF548385, AF548386, EPU96730, EPU96729, AF172159, AF172158, AY219849, ESU72628, ESU96728, AF172160, AF172161, AF172162, AF172163, and EEU96727
[f] QIAamp Blood Extraction Kit, Qiagen, GmbH, Hilden, Germany
[g] GER3 and GER4 primers, MedProbe, Oslo, Norway
[h] Deoxynucleoside triphosphates, Applied Biosystems, Stockholm, Sweden
[i] AmpliTaq Gold DNA polymerase, Applied Biosystems, Foster City, CA
[j] PTC-200, MJ Research, Waltham, MA
[k] 100 Base-pair ladder, Amersham Biosciences, Uppsala, Sweden
[l] *Ehrlichia equi* antigen, Protatek, St Paul, MN
[m] FITC-conjugated rabbit anti-horse immunoglobulin, Cappel ICN Pharmaceuticals, Inc, Aurora, OH
[n] Available at www.iso.org.

Acknowledgments

We thank Ulla-Britt Wikström and Sara Johansson for excellent laboratory support. This research was supported by the Swedish Horserace Totalizator Board (AB Trav och Galopp).

References

1. Dumler JS, Barbet AF, Bekker CP, et al. 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 2001;51:2145–2165.

2. Pusterla N, Lutz H, Braun U. Experimental infection of four horses with *Ehrlichia phagocytophila.* Vet Rec 1998;143:303–305.

3. Pusterla N, Pusterla JB, Braun U, Lutz H. Experimental cross-infections with *Ehrlichia phagocytophila* and human granulocytic *Ehrlichia*–like agent in cows and horses. Vet Rec 1999;145:311–314.

4. Stuen S, Artursson K, Olsson Engvall E. Experimental infection in lambs with an equine granulocytic ehrlichia species resembling the agent that causes human granulocytic ehrlichiosis (HGE). Acta Vet Scand 1998;39:491–497.

5. Pusterla N, Aandersson RJ, House JK, et al. Susceptibility of cattle to infection with *Ehrlichia equi* and the agent of human granulocytic ehrlichiosis. J Am Vet Med Assoc 2001; 218:1160–1162.

6. Gribble DH. Equine ehrlichiosis. J Am Vet Med Assoc 1969; 155:462–469.

7. Mesquita Neto F de, Ajauro T, Vieira RM, Gouveia AMG. *Ehrlichia equi* in horses from the municipality of Betim (Brazil). Arq Bras Med Vet Zootec 1984;36:203–207.

8. Bücher G, Grandras R, Apel G, Friedhoff KT. Der erste fall von ehrlichiosis beim pferd in Deutchland. Dtsch Tierärtztl Wochenschr 1984;91:408–409.

9. Hermann M, Bauman D, Lutz H, Wild P. Erster diagnostizierter fall von equiner Ehrlichiose in der Schweiz. Pferdeheilkunde 1985;1: 247–250.

10. Björsdorff A, Johnssson A, Sjöström A-C, Madigan J. *Ehrlichia equi*–infektion diagnostiserad hos häst. Svensk Veterinärtidning 1990;42:357–360.

11. Korbutiak E, Schneiders D. Equine granulocytic ehrlichiosis in the UK. Vet Rec 1994;135:387–388.

12. Madigan JE. Equine ehrlichiosis. Vet Clin North Am Equine Pract 1993;9:423–428.

13. Reubel GH, Kimsey RB, Barlough JE, Madigan JE. Experimental transmission of *Ehrlichia equi* to horses through naturally infected ticks (*Ixodes pacifinus*) from northern California. J Clin Microbiol 1998;36:2131–2134.

14. Egenvall A, Franzen P, Gunnarsson A, et al. 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 2001;49:191–208.

15. Nyindo MBA, Ristic M, Lewis GE, et al. Immune response of ponies to experimental infection with *Ehrlichia equi.* Am J Vet Res 1978;39:15–18.

16. Corstvet RE, Gaunt SD, Karns PA, et al. Detection of humoral antigen and antibody by enzyme-linked immunosorbent assay in horses with experimentally induced *Ehrlichia equi* infection. J Diagn Invest 1993;5:37–39.

17. Barlough JE, Madigan JE, DeRock E, Bigornia L. Nested polymerase chain reaction for detection of *Ehrlichia equi* genomic DANN in horses and ticks (*Ixodes pacificus*). Vet Parasitol 1996;63:319–329.

18. Franzén P, Artursson, K, Gunnarsson A, et al. Experimentally induced equine granulocytic ehrlichiosis with a Swedish *Ehrlichia* sp. isolate. VIII International Conference on Equine Infectious Diseases, Dubai, 1998.

19. Madigan JE, Richter PJ, Kimsey RB, et al. Transmission and passage in horses of the agent of human granulocytic ehrlichiosis. J Infect Dis 1995;172:1141–1144.

20. Barlough JE, Madigan JE, DeRock E, et al. Protection against *Ehrlichia equi* is conferred by prior infection with the human granulocytic ehrlichia (HGE agent). J Clin Microbiol 1995;33:3333–3334.

21. Chang Y-F, Novocel V, Dubovi E, et al. Experimental infection of the human granulocytic ehrlichiosis agent in horses. Vet Parasitol 1998;78:137–145.

22. Pusterla N, Leutenegger CM, Chae JS, et al. Quantitative evaluation of ehrlichial burden in horses after experimental transmission of human granulocytic *Ehrlichia* agent by intravenous inoculation with infected leukocytes and by infected ticks. J Clin Microbiol 1999;37: 4042–4044.

23. Pusterla N, Madigan JE, Asanovich KM, et al. Experimental inoculation with human granulocytic *Ehrlichia* agent derived from high- and low-passage cell culture in horses. J Clin Microbiol 2000; 38:1276–1278.

24. Weisburg WG, Barns SM, Pelletier DA, Lane DJ. 16S ribosomal DNA amplification for phylogenetic study. J Bacteriol 1991;173: 697–703.

25. Drancourt M, Bollet C, Raoult D. *Stenotrophomonas africana* sp. nov., an opportunistic human pathogen in Africa. Int J Syst Bacteriol 1997;47:160–163.

26. Björsdorff A, Bagert B, Massung RF, et al. Isolation and characterization of two European strains of *Ehrlichia phagocytophila* of equine origin. Clin Diagn Lab Immunol 2002;9:341–343.

27. Chae J, Foley JE, Dumler JS, Madigan JE. Comparison of the nucleotide sequences of 16S rRNA, 444 *Ep-ank*, and *groESL* heat shock operon genes in naturally occurring *Ehrlichia equi* and human granulocytic ehrlichiosis agent isolates from northern California. J Clin Microbiol 2000;38:1364–1369.

28. Johansson KE, Pettersson P, Uhlen M, et al. Identification of the causative agent of granulocytic ehrlichiosis in Swedish dogs and horses by direct solid phase sequencing of PCR products from the 16S rRNA gene. Res Vet Sci 1995;58:1109–112.

29. Olsson Engvall E, Pettersson B, Persson M, et al. A 16S rRNA–based PCR assay for detection and identification of granulocytic *Ehrlichia* species in dogs, horses and cattle. J Clin Microbiol 1996;34: 2170–2174.

30. Goodman JL, Nelson C, Vitale B, et al. Direct cultivation of

the causative agent of human granulocytic ehrlichiosis. N Engl J Med 1996;334:209–215. Erratum. N Engl J Med 1996;335:361.

31. Madigan JE, Hietala S, Chalmers S, DeRock E. Seroepidemiologic survey of antibodies to *Ehrlichia equi* in horses of northern California. J Am Vet Med Assoc 1990;196:1962–1964.

32. Kallfelz FA, Whitlock RH, Schultz RD. Survival of ^{59}Fe-labeled erythrocytes in cross-transfused equine blood. Am J Vet Res 1978;39: 617–620.

33. Gribble DH. Equine Ehrlichiosis. Davis, CA: University of California; 1970. Thesis.

34. Artursson K, Gunnarsson A, Wikström U-B, Olsson Engvall E. A serological and clinical follow-up in horses with confirmed equine granulocytic ehrlichiosis. Equine Vet J 1999;31:473–477.

35. Madigan JE, Barlough JE, Dumler JS, et al. Equine granulocytic ehrlichiosis in Connecticut caused by an agent resembling the human granulocytotropic *Ehrlichia.* J Clin Microbiol 1996; 34:434–435.

36. Massung RF, Owens JH, Ross D, et al. Sequence analysis of the *ank* gene of granulocytic ehrlichiae. J Clin Microbiol 2000; 38: 2917–2922.

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