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Transmission of *Anaplasma phagocytophila* (Human Granulocytic Ehrlichiosis Agent) in Horses Using Experimentally Infected Ticks (*Ixodes scapularis*)

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With 1 table

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Summary

Most human granulocytic ehrlichiosis (HGE) studies carried out in horses use needle inoculation of infected leucocytes or cell cultures. This route of inoculation does not accurately reflect natural infection of the tick-borne agent. To investigate whether tick transmission influences the course of granulocytic ehrlichiosis in the horse model, experimental transmission through infected laboratory-reared *Ixodes scapularis* ticks was attempted into two healthy horses. One additional horse served as negative control and was exposed to uninfected ticks. Eleven days after exposure to nymphal or adult ticks infected with *Anaplasma phagocytophila* (HGE agent) the two horses developed severe clinical and laboratory signs consistent with granulocytic ehrlichiosis. Bacteraemia was determined at various time points in the two horses by observation of morulae within neutrophils and by detection of *A. phagocytophila* genomic DNA by PCR of peripheral blood leucocytes. Further, both horses seroconverted. In contrast the control horse stayed uninfected. The results demonstrate that *A. phagocytophila* can be experimentally transmitted by infected nymphal and adult ticks and that the agent is able to produce a severe disease, similar to naturally occurring cases. Therefore, tick transmission is highly reproducible and can be successfully used in the equine animal model in order to study HGE.

Introduction

Human granulocytic ehrlichiosis (HGE) is a recently recognized tick-borne infectious disease in the United States and Europe caused by a rickettsial agent of the *Anaplasma* (*Ehrlichia*) genus (Bakken and Dumler, 2000). Very recently the agent of HGE, *E. phagocytophila* and *E. equi* have been grouped into a single species and named *A. phagocytophila* (Dumler et al., 2001). *Anaplasma phagocytophila* produces a similar febrile disease in humans and horses (Barlough et al., 1995; Madigan et al., 1995, 1996; Chang et al., 1998a). The horse has been suggested as an appropriate animal model to study HGE because the disease is highly reproducible and characterized by clinical and laboratory findings and pathology similar to that observed in humans (Lepidi et al., 2000). In this model, horses have mostly been infected by the intravenous route using fresh blood from human patients, blood stabilates from a horse infected with human blood, or *in vitro* cultured *A. phagocytophila* (Barlough et al., 1995; Madigan et al., 1995, 1996; Chang et al., 1998a; Pusterla et al., 2000). However, experimental transmission of the human strain of *A. phagocytophila* into horses using ticks, similar to the field situation, has not been described. The purpose of the present study was to attempt experimental transmission of *A. phagocytophila* into horses by using laboratory-reared *Ixodes scapularis* ticks that had previously been fed on infected mice.

Materials and Methods

Ehrlichial strain

The Webster strain of *A. phagocytophila* was originally isolated from a human patient in northwest by inoculation into HL-60 promyelocyte cell line (Goodman et al., 1996; Asanovich et al., 1997). To prepare the inoculum for mice, *A. phagocytophila* were propagated in the HL-60 cell line in RPMI 1640 supplemented with 2 mM L-glutamine and 1% foetal bovine serum at a final concentration of 2×10^5 cells/ml. The cultures were maintained in a 5% CO_2 incubation chamber at 37°C and examined every 3 days by Romanowsky staining (LeukoStat, Fisher Scientific, Pittsburgh, PA, USA) of cytocentrifuged cells for the presence of characteristic morulae. The agent was propagated by limited passage into uninfected HL-60 in order to use the cells for inoculation.

Tick maintenance and mice inoculation

We established a colony of *I. scapularis* ticks from larvae provided by Dr Joseph Piesman (Division of Vector-Borne Infectious Diseases, Centers for Disease Control and Prevention, Public Health Service, US Department Health and Human Services, Ft. Collins, CO, USA). The ticks were maintained at 22°C and 98% humidity in vials housed in 30 cm^3 Plexiglas boxes under standard conditions of long day photophase. Beakers containing saturated solutions of $CuSO_4$ maintained the humidity within these boxes. Subadult ticks were allowed to feed on either HGE agent-infected or HGE agent-free white-footed mice (*Peromyscus leucopus*; Genetic Stock Center, Department of Biological Sciences and Institute of Biological Research and Technology, University of South Carolina, Columbia, SC, USA).

The pathogen-free mice were infected by intraperitoneal inoculation of 200 μl of phosphate-buffered saline containing 1×10^6 infected HL-60 cells (not more than 1.2×10^6 total cells) with the Webster strain of *A. phagocytophila*. Infection was determined 7 days post-inoculation by testing mouse blood using PCR. Thereafter, only PCR positive mice were infested with ticks. Control ticks were allowed to feed on mice inoculated intraperitoneally with 1×10^6 uninfected HL-60 cells. Each mouse was then infested with either 50–100 larvae or 10–15 nymphs. Mice were then transferred singly to standard wire-mesh bottomed cages suspended over pans of water and observed until recovery. Replete larvae and nymphs were collected from the pans of water below each animal and allowed to molt into nymphs and adults, respectively, before being used as experimental challenge for horses. The procedures for inoculation and care of the mice and the horses were approved by the Animal Care and Use Administrative Committee at the University of California, Davis, and the animal holding facilities are accredited by the American Association for the Accreditation of Laboratory Animal Care.

The infection status of the ticks after molting was assessed by PCR testing nymphs and adult ticks originating from the same cohort as the ticks used to challenge the horses. We tested 10 nymphs and 10 adults that fed either upon infected or upon uninfected mice as larvae and as nymphs, respectively. Seven nymphal (70%) and eight adult (80%) ticks previously fed upon experimentally infected mice tested PCR positive, whereas, none of the 10 nymphal and 10 adult ticks fed upon uninfected mice was PCR reactive.

Horse transmission

Three clinically normal and ectoparasite-free horses named Horse A (14-year-old Quarter Horse gelding), Horse B (16-year-old Thoroughbred mare) and Horse C (20-year-old Quarter Horse mare) were used. The horses were housed in isolation stalls that were vector proof at the Center of Equine Health, University of California, Davis, and were seronegative and PCR-negative for *A. phagocytophila* prior to tick challenge.

To infect a horse via the bites of infectious ticks we shaved a 10 cm diameter area on the withers over which we glued a 20 cm diameter tennis hat by the brim to the surrounding hair. The tennis hat was fitted with a closure in one seam across the crown. Ticks placed under this hat through the closure, kept sealed and taped shut, thus had unrestricted access to 100 cm^2 of shaved skin. Ticks thus contained were checked twice daily and removed when found detached and replete. On day 1 hats were glued in the preceding manner to the horses. On day 2 they were infested with ticks as follows: Horse A received 30 infected nymphs, Horse B received 30 infected adults (15 each males and females), and Horse C received 30 non-infected nymphal ticks. On day 3 all ticks except adult males had attached, day 5 rapid feeding phase began, day 7 nymphs had started to drop off and the remaining nymphs and adult ticks had stopped feeding by day 8. Horse A developed 26 bite sites, Horse B 15 and Horse C 28.

The horses underwent a thorough clinical examination before experimental infection and thereafter for 30 days. General attitude and behaviour, appetite, rectal temperature, heart and respiratory rates, icterus, petechiae, intestinal motility, faecal consistency, limb swelling and reluctance to move were assessed twice daily.

Blood was collected from all horses into 10 ml evacuated glass tubes with and without anti-coagulant (Vacutainer, Becton Dickinson, Franklin Lakes, NJ, USA) beginning on the day of experimental infection (day 0) and daily thereafter for 30 days. The leucocyte, erythrocyte, and platelet counts were determined and blood smears were examined for characteristic inclusion bodies (morulae). *Anaplasma phagocytophila* antibodies were detected by an indirect immunofluorescent-antibody assay using *A. phagocytophila* of equine origin essentially as described elsewhere and the cutoff titre for a positive serological response was set at ≥ 10 (Madigan et al., 1990).

PCR amplification of A. phagocytophila from blood and tick

DNA obtained from peripheral blood leucocytes and ticks was extracted with the QIAmp tissue kit, according to the manufacturer's instructions (Qiagen, Santa Clara, CA, USA). Prior to extraction each individual tick was placed in 100 μl of buffered phosphate solution in an Eppendorf tube and mechanically homogenized by using sterile scissors. The templates were examined for the presence of *A. phagocytophila* genomic DNA by nested PCR as described elsewhere (Barlough et al., 1996). Current cycling parameters were as follows: pre-heat treatment at 94°C for 5 min and then 35 cycles of 94°C for 1 min, 60°C for 2 min, and 72°C for 1.5 min, followed by a final extension at 72°C for 7 min. The expected fragment size for the nested-round PCR was 928 base pairs. PCR products were visualized in ethidium bromide-stained 1.5% agarose gels.

Results

Horse A remained clinically asymptomatic until a fever of 38.8°C, depression and anorexia developed on day 11 post-exposure (p.e.). The fever lasted for 4 days and reached the maximal temperature of 40°C by day 12 p.e. Shortly thereafter icterus (lasting 4 days), petechiation on oral and nasal mucosa and third eyelids (lasting 6 days), distal oedema on all four limbs (lasting 5 days) and reluctance to move (lasting 3 days) were noted (Table 1). The clinical signs lasted up to day 18 p.e. The most dramatic haematological change was a severe decrease in the number of platelets to 10 000/μl (normal range 100 000–300 000/μl) beginning on day 13 p.e. and lasting until day 21 p.e. The leucocyte, neutrophil, lymphocyte and erythrocyte counts decreased considerably at the time at which the fever occurred and then rebounded on day 19 p.e. *Anaplasma phagocytophila* inclusion bodies were first observed in approximately 2% of the peripheral blood neutrophils at the end of the febrile period (day 14 p.e.). The infection rate progressively increased to 26% by day 20 p.e., before rapidly decreasing to 1% by day 21 p.e. (duration of ehrlichaemia 8 days). The nested PCR became positive on day 8 p.e. and remained positive through day 23 p.e. (duration of PCR detection 16 days). Horse A seroconverted by day 14 p.e. with a titre of 10. The titre increased and reached 320 by the end of the study.

Horse B remained clinically asymptomatic until fever (39.4°C), depression and anorexia of 3-days duration developed on day 11 p.e. The maximal temperature was recorded on

Table 1. Clinical, haematological, serological and molecular findings in horses experimentally exposed to *Ixodes scapularis* ticks harbouring the Webster strain of *A. phagocytophila*

Horse	Incubation period (days)	Clinical findings	Lowest haematological findings[a]	Duration of infection (days)	
				LM[b]	PCR[c]
Horse A	11	Fever, apathy, anorexia, icterus, petechiation, limb oedema, reluctance to move	Leucopenia (3700/μl) Lymphopenia (222/μl) Neutropenia (2000/μl) Erythrocytopenia (4.7×10^6/μl) Thrombocytopenia (10 000/μl)	14–21	8–23
Horse B	11	Fever, apathy, anorexia, petechiation, limb oedema	Leucopenia (5100/μl) Lymphopenia (608/μl) Neutropenia (2100/μl) Erythrocytopenia (6.4×10^6/μl) Thrombocytopenia (9000/μl)	12–15	8–24
Horse C	0[d]	Within normal limits	Within normal limits	0	0

[a]Reference values: leucocytes 5400–14 300/μl, neutrophils 2260–8580/μl, lymphocytes 1500–7700/μl, erythrocytes $6.8–12.9 \times 10^6$/μl, platelets 100 000–350 000/μl.
[b]Detection of *A. phagocytophila* inclusion bodies via light microscopy.
[c]Detection of *A. phagocytophila* genomic DNA via nested PCR.
[d]Infection and clinical signs not detected at any interval.

the first and last day of pyrexia. Petechiation (lasting 3 days) and oedema on all four limbs (lasting 2 days) were noted shortly thereafter (Table 1). The clinical signs lasted up to day 15 p.e. Platelet counts declined from day 13 p.e. and reached the lowest value of 9000/μl by day 15 p.e. The leucocyte, neutrophil, lymphocyte and erythrocyte counts decreased considerably at the time at which the fever occurred and returned to normal by day 16 p.e. Neutrophil inclusion bodies were first observed by day 12 p.e. (3%) and could be detected until day 15 p.e. (duration of bacteraemia 4 days). The highest infection rate of 14% was determined on day 14 p.e. Peripheral blood leucocytes were positive by nested PCR from day 8 through day 24 p.e. (duration of PCR detection 17 days). Horse 2 seroconverted by day 14 p.e. and reached a titre of 320 by the end of the study period.

Horse 3 differed from the other two horses in that no clinical, haematological or serological abnormalities were detected within 30 days post-infection, nor were inclusion bodies observed in leucocytes and PCR was negative.

Discussion

In its natural occurrence, *A. phagocytophila* is transmitted to humans or animals by *Ixodes* spp. ticks. Transmission of the bovine and equine strain of *A. phagocytophila* by naturally infected *I. ricinus* and *I. pacificus*, respectively, have established these ticks as vectors of the agent in the United States and in Europe (MacLeod, 1936; Richter et al., 1996; Reubel et al., 1998). The detection of *A. phagocytophila* in *Ixodes* spp. was first reported by Pancholi et al. (1995). Since then, many studies have shown the presence of *A. phagocytophila* in *Ixodes* spp. from the United States and Europe mostly by PCR detection (Chang et al., 1998b; Cinco et al., 1998; Parola et al., 1998; Schauber et al., 1998; Kramer et al., 1999; Petrovec et al., 1999; Pusterla et al., 1999). Mice have been proposed as a reservoir host for *A. phagocytophila* and have been shown in laboratory to acquire and transmit *A. phagocytophila* by *Ixodes* spp. ticks (Telford et al., 1996; Hodzic et al., 1998a,b; Katavolos et al., 1998). Furthermore, mice have been used successfully to study the pathogenesis and immunity of *A. phagocytophila* (Martin et al., 2000). However, experimentally infected mice lack clinical and some haematological signs, making the comparison of clinical signs and lesions difficult. Horses, like humans, seem to be accidental hosts in the life cycle of *A. phagocytophila*. Because of the susceptibility of horses to *A. phagocytophila* and to the clinical, laboratory and pathological findings similar to that observed in humans, equids have been suggested as an appropriate animal model (Lepidi et al., 2000). However, in this animal model horses have never been infected by ticks, the natural route of transmission. The purpose of our study was to determine the susceptibility of horses to *A. phagocytophila*-infected *I. scapularis* ticks reared in laboratory in order to optimize the experimental infection protocol and to enhance reproducibility of this animal model.

The tick transmission in our study produced a severe disease independently of the tick stage. Natural cases of equine granulocytic ehrlichiosis (EGE) induced by human and equine isolates of *A. phagocytophila* are characterized by high fever, limb oedema, icterus, petechiation, reluctance to move, ataxia, leucopenia, erythrocytopenia, thrombocytopenia, and inclusion bodies within neutrophils (Madigan and Gribble, 1987; Madigan et al., 1996). Thus, in our experimental study peak and duration of fever, depression, anorexia, icterus, petechiation and distal limb oedema were comparable with natural infections. Less common signs such as reluctance to move and ataxia were only observed in one horse (Horse A), probably reflecting individual variability. The incubation time of 11 days observed in Horse A and Horse B was comparable with the 8-days incubation time reported in a horse experimentally infected with *E. equi* through laboratory-reared *I. pacificus* ticks (Richter et al., 1996). The severe haematological findings, such as leucopenia, erythrocytopenia, thrombocytopenia and bacteraemia observed in both horses were consistent with naturally occurring cases of EGE. Detection of *A. phagocytophila* genomic DNA by nested PCR was possible 4–6 days before the appearance of morulae until 2–9 days after the last detection of inclusion bodies. This is in accordance with the PCR detection time (11–17 days) reported in horses experimentally infected by *E. equi* harbouring ticks (Richter et al., 1996; Reubel et al., 1998). Furthermore, the seroconversion time and the titre at the end of the study were similar

between Horse A and Horse B consistent with experimental tick infections (Richter et al., 1996; Reubel et al., 1998). The data from Horse A and Horse B were in most instances comparable with that reported in horses intravenously inoculated with the same strain of *A. phagocytophila* (Madigan et al., 1995; Pusterla et al., 2000). While the disease severity was similar between both routes, the tick route was associated with a longer incubation and seroconversion time, which probably reflects the slower kinetics of the agent between tick vector and equine host.

In conclusion, we report the first transmission of the Webster strain of *A. phagocytophila* into horses by using laboratory-reared *I. scapularis* ticks. Despite the small number of horses infected, *A. phagocytophila* can be reproducibly transmitted by infected nymphal and adult ticks and is able to produce a severe disease, similar to naturally occurring cases. As the tick and the intravenous route of infection generate similar clinical and laboratory findings, both routes can be successfully used in the equine animal model in order to study HGE.

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