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ENDOGENOUS TRANSPLACENTAL TRANSMISSION OF *NEOSPORA HUGHESI* IN NATURALLY INFECTED HORSES

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ABSTRACT: Over a 2-yr study period, we investigated possible endogenous transplacental transmission of *Neospora hughesi* in 74 mare and foal pairs following the diagnosis of neuronal neosporosis in a weanling foal. Presuckle and postsuckle serum of each foal, serum and colostrum of each periparturient mare, and serum of each mare and foal pair, collected at 3-mo intervals thereafter, were tested for *N. hughesi* using an indirect fluorescent antibody test (IFAT). Furthermore, whole blood and colostrum samples and placentae were tested for the presence of *N. hughesi* by real-time PCR. The mares' seroprevalence at foaling based on IFAT (titer ≥ 160) was 52 and 6% in 2006 and 2007, respectively. Colostral antibodies against *N. hughesi* were detected in 96 and 11% of the mares in the 2-yr study. With the exception of 3 foals, all remaining foals were born seronegative to *N. hughesi.* Passive transfer of colostral antibodies to *N. hughesi* was documented in 15 foals. Three foals born from 2 different mares had presuckle antibodies at a titer ranging from 2,560 to 20,480. All 3 foals were born healthy. Two foals were born to the same dam that also gave birth to the weanling diagnosed with neuronal neosporosis in 2005. The third foal was born to a second mare with no previous foaling history at the farm. Seroconversion was documented in 10 foals and 9 mares over the 2-yr study. All blood and colostrum samples tested PCR negative for *N. hughesi.* Only 1 placenta collected in 2007 from the mare with the 2 congenitally infected foals tested PCR positive for *N. hughesi.* In conclusion, *N. hughesi* persisted in this population via endogenous transplacental infection.

The epidemiology of *Neospora* spp. in horses is poorly understood; reports of neosporosis are sparse and include 2 aborted fetuses (Dubey and Porterfield, 1990; Pronost et al., 1999) and a congenitally infected foal and 9 adult horses with visceral or neural neosporosis (Gray et al., 1996; Lindsay et al., 1996; Marsh et al., 1996; Daft et al., 1997; Hamir et al., 1998; Cheadle et al., 1999; Finno et al., 2007; Wobeser et al., 2009). Subclinical infection is relatively common among horses in several parts of the world, with seroprevalence studies reporting 10–35% of healthy adult horses testing seropositive to *Neospora* spp. (Dubey, 2003). Although the ecology of *Neospora* spp. associated with horses is still unresolved, many comparisons have been drawn with *Neospora caninum,* the causative agent of ascending neuromuscular disease in dogs and abortion in cattle worldwide. Definitive hosts of *N. caninum* include the domestic dog and the coyote (Gondim, 2006). These 2 carnivores shed oocysts of *N. caninum* in feces, and, after sporulation, these oocysts are infectious to intermediate hosts such as cattle. Potential sources of horizontal infection amongst cattle also include colostrum or milk from infected animals, infected placentae, and fetal fluids (Ho et al., 1998; Davison et al., 2001; Moskwa et al., 2007). It appears, however, that transplacental transmission is of major importance in the spread of *N. caninum* in cattle and accounts for as much as 95% of seropositive animals in endemically affected herds (Barr et al., 1993; Anderson et al., 1995, 1997; Dubey et al., 2007). The risk of transplacental transmission of *Neospora hughesi* in a cohort of mares and foals on 4 California farms with a history of EPM has been reported (Duarte et al., 2004). The latter study found no serologic or histologic evidence of in utero infection with *N. hughesi;* however, the prevalence of this protozoal parasite was low in the study population. In an attempt to determine if transplacental transmission of *N. hughesi* occurs between latently infected broodmares and their fetuses, we studied this transmission route in a herd of horses with a previously diagnosed case of neuronal neosporosis and also high seroprevalence of antibodies against *N. hughesi.*

MATERIALS AND METHODS

Farm and study horses

The study was triggered by a neurologically affected 4-mo-old Percheron filly referred to the William R. Pritchard Veterinary Medical Teaching Hospital, School of Veterinary Medicine, University of California at Davis, in August of 2005 (Finno et al., 2007). The filly was diagnosed with neuronal neosporosis based on the presence of symmetrical spinal cord signs involving the thoracic and pelvic limbs and a positive serum (1,280) and CSF (20) titer by indirect fluorescence antibody test (IFAT) against *N. hughesi.* Serum from the filly's dam also tested positive for antibodies against *N. hughesi* (640) by IFAT. Preliminary data from the farm was collected in the fall of 2005. Sixty-three randomly selected resident horses out of the population of 180 were tested by IFAT; 14% of horses were seropositive (titer ≥ 160) against *N. hughesi.*

The study farm is located in northern California and houses approximately 180 Percheron horses on 48.6 ha. The study was performed over 2 foaling seasons (2006 and 2007), and all resident pregnant mares were enrolled in the study. The age of the broodmares ranged from 4 to 13 yr (median = 4.5 yr). Only 2 resident mares were born and raised on the premises, and the remaining 56 mares had been purchased from Canada and the eastern and western United States 1 to 3 yr prior to the start of the study. During the 2006 foaling season, 27 mare and foal pairs were studied. During the 2007 foaling season, the number of mare and foal pairs increased to 47. Sixteen broodmares were enrolled in both years.

Sample collection and testing

Whole blood and colostrum samples were collected from each mare at the beginning of the study, which coincided with the foaling day. Additional blood samples were collected at 3-mo intervals thereafter, for a total period of 12–24 mo, depending on whether the mare was enrolled in 1 or 2 foaling seasons. Whole blood and colostrum were used for the PCR detection of *N. hughesi* (Pusterla et al., 2006), while serum and colostrum were used for the antibody detection of *N. hughesi,* as previously described

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(Packham et al., 2002). Each placenta was inspected for gross abnormalities, and 2.5 × 2.5 cm pieces were collected from areas of the placenta coinciding with the pregnant and the non-pregnant horn, the body, and the cervical star area for PCR analysis. Presence of *Neospora* spp. for PCR positive placentae was further confirmed via sequence analysis of the PCR product and immunohistochemical analysis, as previously reported (Marsh et al., 1996). Whole blood samples were collected from newborn foals at the time of delivery (pre-colostrum sample), 24–48 hr post-foaling (post-colostrum), and approximately every 3 mo thereafter for both *N. hughesi* PCR and antibody detection. Additional whole blood samples, collected at 48 hr post-foaling, were used to determine total plasma IgG concentrations using a commercial kit (SNAP Foal IgG Test, IDEXX Laboratories, Westbrook, Maine) in order to rule out failure of passive transfer.

Serum from mares and foals and colostrum samples were tested for antibodies against *N. hughesi* using the indirect fluorescent antibody test (IFAT), as described previously (Packham et al., 2002). Two-fold dilutions of serum and colostrum were prepared, starting at a dilution of 1:40. The reciprocal end-point titer was the last dilution with evidence of distinct, whole-parasite fluorescence. A reciprocal titer of ≥160 for *N. hughesi* was used to designate a positive IFAT result (Packham et al., 2002).

RESULTS

Seventy-four foals were born to 58 mares over the 2-yr study period. All foals were born healthy and remained healthy throughout the study. Presuckle serum samples were collected from all 74 foals, 71 of which had no detectable antibodies to *Neospora hughesi* (<40) in serum. Three foals, born to 2 different mares, had pre-colostral antibody titers ranging from 2,560 to 20,480 (Table I), consistent with endogenous transplacental *N. hughesi* infection. Two of these foals were born to the same dam that gave birth to the weanling filly diagnosed with EPM in 2005. The third foal with suspected endogenous transplacental *N. hughesi* infection was born to a second mare with no previous foaling history at the farm. Adequate transfer of colostral antibodies (IgG concentrations ≥ 800 mg/dl) was documented in all foals at 48 hr of age. After ingestion of colostrum, 15 foals had positive titers (≥ 160) to *N. hughesi,* ranging from 160 to 20,480, while the remaining foals remained seronegative to *N. hughesi.* Among the 12 foals with colostrally acquired antibodies to *N. hughesi* and no evidence of transplacental infection, the titers to *N. hughesi* ranged from 160 to 1,280 (median = 60).

Antibodies to *N. hughesi* (titer ≥ 160) were detected in 14/27 (52%) and 3/47 (6%) mares at foaling in 2006 and 2007, respectively. The serum titers to *N. hughesi* ranged in these mares from 160 to 10,240 (median = 320). Serum titers of antibody against *N. hughesi* in the 2 mares that gave birth to the transplacentally infected foals were among the highest recorded (640 to 10,240).

Colostral antibodies against *N. hughesi* (titer ≥ 160) were detected in 26/27 (96%) and 5/47 (11%) of the mares in 2006 and 2007, respectively, at titers ranging from 160 to 40,960 (median = 320). Titers against *N. hughesi* in colostrum were either higher (24 colostral samples) or equal (7 colostral samples) to the titers in peripheral blood of the same mares. Further, 14 mares with detectable antibodies to *N. hughesi* in colostrum had serum titers below the cutoff titer of 160.

All 12 foals with documented passive transfer of anti–*Neospora hughesi* colostral antibodies became seronegative (<40) on their first 3-mo re-sampling. This was in marked contrast to the 3 foals with suspected transplacental infection. These foals remained seropositive to *N. hughesi* during the entire 12-mo follow-up period with titers ranging from 160 to 5,120 (Table II). With the exception of the 2 seropositive mares that gave birth to the foals with presuckle antibodies to *N. hughesi,* the remaining seropositive mares became seronegative at the 6-mo follow-up. The 2 above mentioned mares remained seropositive during the entire study period with titers ranging from 160 to 1,280 (Table II). These mares were followed for 2 additional pregnancies in order to investigate further transplacental transmission of infection. The older mare gave birth to 1 additional healthy foal that showed evidence of transplacental infection in 2008. This mare was lost to follow-up after being sold later that year. The second mare gave birth to an additional 2 healthy foals, both of which had detectable anti–*N. hughesi* serum antibody collected before ingestion of colostrum. Collectively, these 3 additional foals had pre-suckle antibody titers ranging from 640 to 1,280 (Table II).

Seroconversion, defined as a rise in antibody concentration from undetectable to a titer ≥160, was documented in 9 unrelated mares and 10 foals with titers ranging from 160 to 320 (median = 160). Several clusters of seroconversion were observed in June of 2006 (4 horses), in March of 2007 (3), and in February of 2008 (10; Table III). None of the foals that seroconverted had prior evidence of passive transfer of specific colostral antibody or

TABLE I. Serum and colostral antibody titers to *Neospora hughesi* in mare and foal pairs on a California farm.*

Pair number (year)	Foal serum presuckle IFAT	Foal serum postsuckle IFAT	Mare serum IFAT	Mare colostrum IFAT
1 (2006)	<40	80	160	160
2	<40	80	80	160
3	<40	80	<40	320
4	<40	40	160	160
5	<40	80	40	160
6	<40	320	1,280	2,560
7	<40	40	80	160
8	<40	160	80	<40
9	<40	640	160	320
10	<40	160	640	1280
11	<40	160	160	160
12	<40	1,280	320	2,560
13	<40	80	80	160
14†	20,480	20,480	10,240	40,960
15	<40	40	40	160
16	<40	<40	160	640
17	<40	80	80	640
18	<40	160	80	320
19	<40	<40	160	160
20	<40	80	80	320
21	<40	160	160	640
22	<40	640	80	320
23	<40	80	40	320
24	<40	160	320	1,280
25	<40	160	640	640
26	<40	640	640	640
27	<40	40	80	320
28 (2007)	2,560	5,120	640	2,560
29	<40	40	160	160
30†	2,560	5,120	640	5,120
31	<40	<40	80	160
32	<40	<40	40	160

* IFAT, indirect fluorescent antibody test.
† Same mare that also gave birth to the weanling foal diagnosed with EPM in 2005.

TABLE II. Temporal serum titers in 2 mares and their 3 foals with suspected transplacental *Neospora hughesi* infection on a California farm.*

Mare/foal (pair, year)	Post-foaling IFAT	3 mo IFAT	6 mo IFAT	9 mo IFAT	12 mo IFAT
Mare (14, 2006)†	10,240/40,960‡	640	640	640	640
Foal (14, 2006)	20,480/20,480§	640	320	320	320
Mare (28, 2007)‖	640/2,560	320	640	640	640
Foal (28, 2007)	2,560/5,120	2,560	5,120	2,560	2,560
Mare (30, 2007)†	640/5,120	1,280	320	640	640
Foal (30, 2007)	2,560/5,120	5,120	640	640	640
Mare (33, 2008)†	640/2,560	—#	—	—	—
Foal (33, 2008)	640/1,280	—	—	—	—
Mare (34, 2008)‖	1,280/640	—	—	—	—
Foal (34, 2008)	1,280/2,560	—	—	—	—
Mare (35, 2009)‖	5,120/640	—	—	—	—
Foal (35, 2009)	640/1,280	—	—	—	—

* IFAT, indirect fluorescent antibody test.
† Thirteen-year-old mare, which also gave birth to the weanling foal diagnosed with EPM in 2005.
‡ Titer serum/titer colostrums.
§ Titer presuckle/titer postsuckle.
‖ Five-year-old mare.
Follow-up titers not available.

transplacental exposure to *N. hughesi.* The foals were between 9 and 12 mo of age at onset of antibody detection, while the age of the broodmares ranged from 4 to 10 yr.

All blood and colostrum samples tested PCR negative for *N. hughesi.* Only 1 placenta collected in 2007 from the mare with the 2 congenitally infected foals tested PCR positive for *N. hughesi.* The ITS-1 sequence generated from the PCR-positive placenta showed 100% homology to a *N. hughesi* ITS-1 sequence isolated from a horse in California (GenBank AF038859). Immunohistochemistry of this same placenta using *N. caninum* polyclonal antisera revealed rare positive reacting round to oval structures compatible with protozoal tachyzoites lying within the stroma of placental villi in the absence of any appreciable inflammatory cell response.

DISCUSSION

The present study, which represents the first attempt to investigate transplacental infection in horses with *N. hughesi,* was initiated following the diagnosis of neural neosporosis in a 4-mo-old filly. We suspected that this filly had been infected transplacentally from its seropositive dam. Further, the study population was selected based on a high seroprevalence to *N. hughesi* determined the year before initiating this study. Two broodmares from a pool of 58 study animals were found to be latently infected. During the study period, these 2 mares gave birth to 3 healthy foals that showed evidence of endogenous transplacental infection based on high presuckle serum antibody to *N. hughesi.* An additional 3 foals were born from these 2 mares during the 2008 and 2009 foaling season, and these foals also had evidence of endogenous transplacental infection. The study population was unique in regard to age and origin. The recently acquired broodmares were young, with a median age of 4.5 yr, and had been purchased from several U.S. states and Canada. One of the latently infected mares was 13-yr-old and originated from Iowa, while the other mare was a 5-yr-old that had been purchased from Canada. Furthermore, for most of the mares, the 2 study years represented their first (6 mares) or second (35 mares) foaling.

The life cycle and biology of *N. hughesi* and its interaction with horses have remained poorly investigated because of the sporadic nature of infection. Parallels, however, can be drawn between *N. hughesi* and the closely related *N. caninum,* a causative agent of ascending neuromuscular disease in dogs and abortion in cattle. Cattle have been shown to be susceptible to infection through ingestion of *N. caninum* oocysts contaminated feed and water; moreover, it is widely accepted that herd infections with *N. caninum* are primarily maintained by repeated transplacental transmission (Dubey et al., 2007). The mechanism of reactivation of latent *Neospora* spp. infection is unknown but seems to be linked to the immune status of the dam (Anderson et al., 1997; Williams et al., 2009). The dam's immune system must be sufficiently modulated by the pregnancy to allow the parasite to reactivate and differentiate back to the tachyzoite stage, which

TABLE III. Seroconversion to *Neospora hughesi* in 9 broodmares and 10 foals following natural exposure on a California farm.

Mare/foal	Age	Date	Serum titer IFAT
Mare	10 yr	April 2006	160
Mare	5 yr	June 2006	160
Mare	5 yr	June 2006	160
Mare	4 yr	June 2006	160
Mare	9 yr	June 2006	160
Mare	6 yr	May 2007	160
Mare	4 yr	May 2007	160
Mare	4 yr	May 2007	160
Foal	1 yr	February 2008	160
Foal	1 yr	February 2008	320
Foal	1 yr	February 2008	160
Foal	1 yr	February 2008	320
Foal	10 mo	February 2008	160
Foal	10 mo	February 2008	160
Foal	10 mo	February 2008	320
Foal	9 mo	February 2008	160
Foal	9 mo	February 2008	160
Foal	9 mo	February 2008	160
Mare	9 yr	April 2008	160

can then migrate to the fetus, thus allowing the parasite to be transmitted through several generations of the same family (Innes, 2007). Similar transmission routes are suspected for *N. hughesi,* although no domestic or sylvatic definitive hosts have yet been identified. Our study results showed that exposure to *N. hughesi* occurred via repeated endogenous transplacental transmission from 2 persistently infected broodmares. These 2 mares were considered to be latently infected based on the presence of high serum antibody titers to *N. hughesi,* determined on several occasions throughout the study period. Together, these 2 mares gave birth to 6 healthy foals that showed evidence of transplacental infection over a 4-yr period (2006–2009). Four of these foals were fillies and will have the potential to infect their progeny if they become breeding animals in the future. In cattle, the probability of transplacental infection, determined by measurement of precolostral antibody status of calves born from seropositive dams, has been shown to be 0.95 (Davison et al., 1999). Further, cows remain infected for life and transmit *N. caninum* infection to their offspring in several consecutive pregnancies or intermittently (Boulton et al., 1995; Fioretti et al., 2000). Based on the study results, the authors conclude that *N. hughesi* infection can be transmitted over successive generations of horses by the endogenous transplacental route from a latently infected dam to her offspring.

It is of interest to note that the short-term outcome of transplacental infection had no clinical effect on the foals, with the exception of the foal diagnosed with neuronal neosporosis in 2005. It remains unknown if the latter foal developed the disease secondary to reactivation of a latent *N. hughesi* infection or secondary to horizontal transmission. In cattle, the outcome of *N. caninum* infection following recrudescence of latent infection depends upon the age of the fetus, the magnitude of the parasitemia, and the particular strain involved (Buxton et al., 2002). In the study foals, timing of transplacental infection most likely occurred in the third trimester of gestation based on serological results and lack of abortion. This assumption is supported by the birth of clinically normal foals and the presence of presuckle antibodies to *N. hughesi.* Immunologic competence of the equine fetus with functional B lymphocytes is present by day 200 of gestation (Perryman et al., 1980). Furthermore, sequential serum titers to *N. hughesi* did not reveal any rise in antibody levels in the 2 latently infected mares as an indirect temporal indicator of higher antigen stimulus, as is generally observed in cattle (Paré et al., 1996, 1997).

At the time of foaling, 17 mares had positive antibody titers to *N. hughesi.* Evidence of seropositive status provides no information about the route of infection, viability of the parasite or how recently the infection occurred. However, the highest titers were found in the 2 mares that gave birth to the transplacentally infected foals. In comparison with the other seropositive mares, the latently infected broodmares maintained high antibody titers to *N. hughesi* throughout the study period and during subsequent pregnancies. High serologic titers in cattle have been significantly associated with precolostral seropositivity in their calves (Paré et al., 1996; Fioretti et al., 2000). This was in marked contrast to this study, where the additional seropositive mares maintained detectable antibodies for up to 6 months post-foaling.

The majority of the foals with documented passive transfer of colostral antibodies to *N. hughesi* were born to mares with high titers ($\geq$160) against *N. hughesi* in serum, or colostrum, or both. Colostrally acquired anti–*N. hughesi* antibodies were short lived in foals that did not have evidence of endogenous transplacental infection, which is in agreement with the results of previous studies in cattle (Dubey and Schares, 2006). Seroconversion, not associated with ingestion of colostrum, was documented in 19 mares and foals during the 2 foaling seasons. Of interest was the clustering of seropositive animals by age and month, with a distinct temporal difference between broodmares (June and May) and foals (February). Exposure in these horses is likely to have occurred via the ingestion of sporulated *N. hughesi* oocysts from the environment.

Sporadic abortion or abortion storms are the most common manifestations of bovine neosporosis. A less common manifestation is infection of the central nervous system, seen mainly in calves less than 4 mo of age (Anderson et al., 1995; Dubey and Shares, 2006). Abortion due to *Neospora* spp. has been occasionally reported in horses, and several serosurveys have implicated *Neospora* spp. as a possible abortogenic pathogen (Dubey and Porterfield, 1990; Pronost et al., 1999; McDole and Gay, 2002; Pitel et al., 2003; Villalobos et al., 2006). The failure to detect *N. hughesi* by PCR in the majority of the placental samples from the latently infected broodmares in the present study is not unusual for congenital neosporosis, because the number of organisms is often low (Barr et al., 1993). The true clinical impact of horses infected with *N. hughesi* remains to be determined beyond the sporadic development of central nervous infection. However, it is likely that latently infected animals may be at a higher risk of developing clinical disease following immunosuppression (Finno et al., 2007). Long-term follow-up studies are needed to determine the impact of transplacental infection on the overall well-being of horses.

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