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Detection of *Leptospira* spp. in the Aqueous Humor of Horses with Naturally Acquired Recurrent Uveitis

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Leptospiral organisms have long been presumed to be associated with the presence of equine recurrent uveitis. This project was undertaken to determine the presence of *Leptospira* spp. in the aqueous humor of horses with uveitis to determine if there was an association with inflammation. Thirty horses were determined to have recurrent uveitis based on clinical evaluation or history. Sixteen horses were judged clinically and historically to be free of uveitis and were used as controls. Aqueous humor samples were cultured and evaluated by PCR for the presence of *Leptospira* DNA. Serum was collected and evaluated for the presence of antibodies against five serovars in a leptospirosis panel. Twenty-one of 30 horses with recurrent uveitis and one of 16 uveitis-free horses were positive by PCR for the presence of *Leptospira* DNA. Six of these 21 horses with uveitis were culture positive for leptospires from the aqueous humor. Serologic results did not correlate well with the presence of *Leptospira* DNA or organisms in the aqueous humor. *Leptospira* spp. are present in a high percentage of horses with naturally occurring recurrent uveitis.

Recurrent uveitis is a leading cause of blindness in horses worldwide. The signs (4, 17, 20) and lesions (3, 4, 10) illustrate the severity of the clinical course. The inflammation is nongranulomatous in nature and recurs at unpredictable intervals. The cornea, lens, vitreous body, and retina may also be involved. The inciting cause is usually not identified. Proposed causes include parasites, neoplasia, trauma, and infectious agents (4).

Leptospirosis has long been cited as a cause of equine recurrent uveitis, particularly *Leptospira interrogans* serovar pomona (1, 2, 8, 11, 20). Uveitis typically occurs months to years after naturally acquired (18, 19) or experimentally induced (15, 22) infection with serovar pomona. The purpose of this study was to determine the presence of leptospires in the aqueous humor of horses with uveitis to determine if there was an association with inflammation.

MATERIALS AND METHODS

Thirty horses that ranged in age from 3 to 25 years (mean age, 13.7 years) were diagnosed with recurrent uveitis, either because they had lesions which were considered diagnostic of this syndrome or had a history of recurring episodes of uveitis. Sixteen horses that ranged in age from 2 to 15 years (mean age, 6.9 years) were selected as controls. These horses did not have any history of, or any ocular signs compatible with, recurrent uveitis. The gender ratios for the control group and horses with uveitis were similar (63% male, 37% female). Horses with uveitis included the following breeds: Thoroughbred, 3; Standardbred, 1; Appaloosa, 5; Paso Fino, 1; Quarter Horse, 12; Paint, 1; grade, 1; Arabian, 4; American Miniature, 1; Thoroughbred cross, 1. The horses were from various regions of northern California.

The eyes of all horses were evaluated with a transilluminator, a slit lamp biomicroscope, and a direct ophthalmoscope when possible. Some horses with uveitis were to be euthanized due to the severity of their ophthalmic condition. All control horses had severe nonocular problems and were being euthanized. For sample collection, some horses with uveitis were anesthetized with intravenous injections of xylazine (1.1 mg/kg of body weight) and ketamine (2.2 mg/kg). The remainder as well as all the control horses were euthanized with an overdose of sodium pentobarbital. Aqueous centesis was done with a 27-gauge butterfly catheter and a 3-ml syringe. About 1.5 ml was obtained from each eye. For 27 of the 30 horses with uveitis and 12 of the 16 control horses, about 0.3 ml of this aqueous sample was placed into a transport medium and then transferred to the modified Tween 80-albumin medium described previously (9) and cultured at 27°C. The remainder was frozen immediately for PCR evaluation. Blood was collected from 28 of the 30 horses with uveitis and all the control horses for serologic testing for *Leptospira* spp. The eyes were enucleated from the horses that had been euthanized, placed in Bouin's solution, and routinely prepared for microscopic examination.

Culture. Aqueous humor (0.3 to 0.6 ml) was inoculated into the transport medium, which consisted of 87 mg of KH_2PO_4 and 644 mg of Na_2PO_4/liter and 1% bovine serum albumin. A 1:10 dilution of inoculated transport medium was then added to the culture medium, PLM-5 (Centeon, Kankakee, Ill.) along with 0.167% BBL agar and 200 μg of 5-fluorouracil/ml. Samples were incubated at 27°C for at least 2 months. Once a week, 5 μl of culture was viewed under an ×40 dark-field microscope for the presence of leptospires. A compact zone of growth approximately 1 cm from the meniscus was visible after 1 week in all positive cultures. The presence of *Leptospira* spp. in this zone was confirmed by microscopy.

Serology. The microscopic agglutination test was used to determine the serological titers of the serum samples collected from the horses. The test was performed by the California Veterinary Diagnostic Services Laboratory Systems at the University of California-Davis campus.

Primer design for enzymatic amplification. Because *Leptospira* spp. have been associated with uveitis (1, 2, 15), a comparison of known 16S gene sequences of pathogenic leptospires was made using the GCG software. Four oligonucleotide primers were designed to amplify a conserved region found in *L. interrogans* (accession no. X17547), *Leptospira borgpetersenii* serovars hardjobovis and sponselee (accession no. U12670), *L. borgpetersenii* 1627 serovar burgas (accession no. U12669), *Leptospira pomona* serovar kennewicki (accession no. 71241), and *L. interrogans* serovars canicola, moulton (accession no. 17547), fainei (accession no. U60594), and inadai (accession no. Z21634). The outer primer set corresponds to bases 428 to 450 (5′ AGGGAAAAATAAGCAGCGATGTG 3′) and is complementary to bases 981 to 999 (5′ ATTCCACTCCATGTCAAGCC 3′), giving a 571-bp product. The internal or nested primer set corresponds to bases 552 to 570 (5′ GAAAACTGCGGGCTCAAAC 3′) and is complementary to bases 925 to 940 (5′ GCTCCACCGCTTGTGC 3′), resulting in a 370-bp product. The base numbering is arbitrarily assigned based on the sequence of *L. interrogans.*

Detection of PCR products. The aqueous humor was boiled for 10 min and centrifuged for 5 min, and the supernatant was stored at −20°C. PCR amplification for the presence of leptospiral DNA was performed in a total volume of 100 μl. Thirty microliters of supernatant was mixed with a solution consisting of sterile water, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 130 μM (each) dATP, dTTP, dCTP, and dGTP, 5 U of *Taq* DNA polymerase, and 50 pmol of primers. Each sample was overlaid with 30 ml of sterile mineral oil to

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eliminate evaporation. Initial denaturation was at 94°C for 4 min and was followed by 30 cycles of annealing at 94 and 55°C and extension at 72°C for 1 min each on an MJ thermocycler. One-tenth of the volume was used as the template for nested amplification under the same conditions using the primers described above. Ten microliters (1/10 volume) of the PCR mixture from both rounds of amplification was mixed with loading dye and analyzed by electrophoresis in a 1% agarose gel (data not shown).

Statistical analysis. The horses were classified into groups with and without uveitis. These groups were compared with respect to gender, breed, and status of the PCR, culture, and serologic titer to serovars pomona, grippotyphosa, hardjo, icterohemorrhagiae, and canicola using the Fisher exact tests. The actual titer values were compared using Wilcoxon rank sum tests. Stepwise logistic-regression models were used to select a subset of those factors that were most predictive of uveitis status. This analysis was run twice, once using all factors as predictors and once excluding predictors that were insignificant in the initial analysis and had substantial numbers of missing values. The latter analysis was run in order to minimize the number of subjects that were detected on account of missing values.

RESULTS

PCR. Twenty-one (70%) of the horses with uveitis ($n = 30$) had leptospiral DNA in the aqueous humor detectable with PCR. Fifty-five samples were collected from these horses, of which 30 (55%) were positive. Ten horses were positive bilaterally, and seven were positive unilaterally (23.3%). For four horses, either only one eye was present or permission was given to sample only one eye, and three of these eyes (75%) were positive. The association between a positive PCR result and uveitis was significant ($P < 0.0011$). In contrast, only one eye of the 29 eyes sampled (3.4%) from the 16 control horses (6.25%) was positive with PCR.

Culture. Six of 27 horses with uveitis (22.2%) and none of 12 control horses tested were culture positive for *Leptospira* from the aqueous humor. The horses with positive cultures were also positive by PCR (eight of nine eyes). The association between a positive culture, however, and uveitis was not significant ($P = 0.151$) due to the low sensitivity of this test. Preliminary characterization of the isolates was performed by the National Leptospirosis Reference Center in Ames, Iowa. Four of the cultures were identified as serovar pomona. The other two isolates, though identical, did not match any of the serovars either serologically or genetically and thus are still unidentified.

Serology. Twenty-four of 28 horses with uveitis (85.7%) and 10 of 16 control horses (62.5%) had serologic titers for *Leptospira* of ≥1:100. Sixteen of the horses with uveitis (57.1%) and two of the control horses (12.5%) had titers of ≥400. By Fisher's exact method and logistic regression, the positive titer for serovar pomona (≥1:100) was significant ($P = 0.01$ and 0.0001, respectively). The positive titers for pomona, grippotyphosa, and hardjo were significant by the Wilcoxon tests ($P = 0.0019$, 0.039, and 0.04, respectively).

There was no significant association between the breed, gender, or age and the presence of uveitis.

DISCUSSION

Leptospiral DNA was detected in the aqueous humor of 21 of 30 horses with uveitis by PCR. Only that of 1 of 16 control horses was positive. Comparisons between antibody titers in aqueous humor and serum have led to the conclusion that an elevated aqueous humor titer suggests local antibody production, supporting a diagnosis of leptospire-associated uveitis (5; K. Schwink, Abstr. 21st Ann. Meet. Am. College Vet. Ophthalmol., p. 164–165, 1990). Although aqueous humor antibody titers were not determined as a comparison to serological values in this study, the presence of the organisms in the aqueous humor supports a diagnosis of leptospira-associated uveitis.

The eye has been classically considered an immune-privileged site (12). Investigations for the presence of onchocerciasis, leptospirosis, toxoplasmosis, and brucellosis were frequently unrewarding (12). It had commonly been accepted that, although these diseases play a role in the initiation of ocular inflammation, subsequent development of hypersensitivity and autoimmune responses were responsible for the recurrence of the uveitis in the clinical cases and that evidence of the initial insult may be difficult to find (12).

By PCR assay, leptospiral DNA was found in the aqueous humor of 21 of 30 horses. This technology has been utilized for the rapid detection of leptospiral infections in cattle urine (7) and in blood, cerebrospinal fluid, urine, and aqueous humor from human beings (14, 16; F. Merien, P. Perolat, E. Mancel, D. Persan, and G. Baranton, Letter, J. Infect. Dis. **168:**1335–1336, 1993). The diagnosis of leptospirosis had previously relied on culturing of the organism(s), serologic methods (microscopic agglutination technique), or dark-field microscopy, which is positive only at certain stages of the infection. Culturing of leptospires is often not successful, and it is slow and susceptible to contamination (16).

Serologic studies have been performed to evaluate the prevalence of leptospiral antibody titers in horses with and without uveitis (6, 13). Reliance on the use of serology as a means of establishing a diagnosis is uncertain, and opinions differ as to what constitutes a significant leptospiral titer. A positive serological reaction does not necessarily indicate current or recent infection because titers may persist for more than 7 years after infection (21).

The positive serologic results of our study were not predictive of the aqueous PCR status. Two horses were positive by the aqueous humor PCR and culture but were serologically negative. Notwithstanding, the cultured isolates were not serologically reactive with any of the serovars used in the microscopic agglutination test and thus may reflect an as yet unidentified leptospiral serovar. This suggests that there is a need to expand the number of serovars which are being evaluated serologically or that some horses are not mounting a systemic immune response to these organisms.

Although leptospiral organisms are difficult to grow in culture media, 22% of the aqueous humor samples from horses with uveitis were culture positive in this study. Brem et al. (1, 2) have also reported the culturing of leptospires from horses with uveitis. It is important to note that all of the positive cultures were also positive by PCR. In this study it is apparent that PCR is a more reliable tool for detecting the presence of leptospires in equine recurrent uveitis.

We found no significant association between breed and uveitis, contrary to another study in which the Appaloosa was found to be at greater risk (6). This may have been due to the relatively small number of horses in our study.

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