

DETECTION OF LEPTOSPIRA INTERROGANS IN FIXED EQUINE EYES
AFFECTED WITH END-STAGE EQUINE RECURRENT UVEITIS

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The undersigned, appointed by the dean of the Graduate School, have examined the thesis entitled:

DETECTION OF *LEPTOSPIRA INTERROGANS* IN FIXED EQUINE EYES
AFFECTED WITH END-STAGE EQUINE RECURRENT UVEITIS

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CHAPTER 1

INTRODUCTION - EQUINE RECURRENT UVEITIS

Equine recurrent uveitis (ERU) is the most frequent cause of blindness in horses worldwide, having approximately an 8% prevalence in horses in the USA, with some studies reporting up to a 15% prevalence.^{4,5,24} The disease is initiated by a single uveitis episode, which is followed by waxing and waning episodes of uveitis. It is the chronic and recurrent nature of the disease that differentiates ERU from the primary ocular and systemic forms of equine uveitis. Horses affected with ERU commonly develop cataracts, synechia, glaucoma and phthisis bulbi secondary to the uveitis and will often become irreversibly blind as a result.¹¹ Economic losses associated with ERU are significant, and many blind horses are euthanized for ethical reasons.

Many factors have been associated with the onset of clinical uveitis in horses. These include parasites, neoplasia, trauma and infectious agents. *Leptospira interrogans* has been implicated as an etiologic agent in some cases of ERU.^{1,18,27} Horses experimentally or naturally infected with *L. interrogans* were shown to develop clinical uveitis 1 to 2 years after infection.^{1,27} Numerous studies have directly connected *L. interrogans* and ERU by identifying the bacteria in fresh aqueous and vitreous humor samples of affected horses.^{9,12,28} A recent study by Hartskeerl *et. al.* characterized leptospires via bacterial culture from 32.2% of intraocular samples collected from 501 Western European horses with ERU.¹² Another study performed in Western Europe in 2004 revealed 52.8% of equine vitreous humor samples with ERU were positive via culture for *Leptospira* and 71% were positive by PCR.²⁸ Although extensive studies have been performed in

Western Europe, only one study confirming the presence of *Leptospira* organisms in ERU affected equine ocular tissue has been performed in the United States. In this Californian study, *Leptospira* DNA was detected by end-point PCR in fresh samples of aqueous humor from 70% of horses with clinical ERU and in 6% of normal control horses.⁹ Horses with ERU also have increased antibodies to *Leptospira* in vitreous humor as compared to serum antibodies; this suggests that there is a persistent ocular infection with *Leptospira*.²⁹ A strong positive relationship has also been documented between leptospiral seroreactivity, uveitis and blindness in horses.⁸ Cross-reactivity between antigens of equine cornea, lens and leptospire has been documented using immunoblotting techniques.^{18,20} Research suggests that local immune mechanisms contribute to the pathogenesis of equine recurrent uveitis.¹⁴ The findings of predominantly CD4+ lymphocytes, increased IL-2 and IFN γ and decreased IL-4 are suggestive of a cell mediated (Th1) immune response.^{10,23} Expression of major histocompatibility complex II expression in the trabecular meshwork, non-pigmented ciliary body epithelium and retina of affected horses supports the notion that immune regulation plays a key role in ERU.^{13,23} The autoimmune nature of ERU has been demonstrated experimentally by the induction of recurrent uveitis in horses following injection of interphotoreceptor retinoid-binding protein and S-angiten.^{6,7}

CHAPTER 2

EXPERIMENTAL PURPOSE AND HYPOTHESIS

The purpose of this study was to determine the frequency of detection of *L. interrogans* DNA and antigen in fixed equine ocular tissues affected with end-stage ERU using real-time PCR and immunohistochemistry. Our hypothesis was that *Leptospira* DNA and antigen would be detectable in fixed archival equine ocular tissue affected with ERU.

Scientific impact of this study is multi-factorial. This study introduces two techniques which could be tools for diagnostic pathologists or laboratory diagnosticians who might want to determine leptospiral involvement in cases of ERU when only fixed tissue is available. It also will provide insight into the role of *Leptospira* in chronic, end stage ERU. Currently, no epidemiologic studies exist linking *Leptospira* and ERU in the Midwestern or Eastern United States. Since most ERU horses (90%) in this study are from the Midwest and Eastern areas of America, a new demographic of ERU horses will be represented. New insight in the etiology and pathogenesis of ERU as a single disease entity and as an animal model for human recurrent uveitis might also be provided.

CHAPTER 3

DETECTION OF *LEPTOSPIRA INTERROGANS*

1. MATERIAL AND METHODS

Samples:

Samples were fixed in 10% neutral buffered formaldehyde, dehydrated in ascending concentrations of ethanol and xylene, and embedded routinely in paraffin. Sections of 5 micron thickness were prepared from tissue blocks. Thirty equine globes were obtained. Controls included (1) ten normal equine eyes and (2) ten equine eyes with a non-recurrent form of uveitis. The experimental group consisted of ten eyes with ERU. A positive control sample of *L. interrogans* infected porcine kidney was also included. Ocular histopathologic evaluation to confirm presence or absence of ERU in experimental and control groups was performed by a board-certified veterinary pathologist (R.R.D.).

DNA Extraction:

DNA extraction was performed on paraffin-embedded, formalin-fixed equine ocular sections. The sample of *Leptospira* infected porcine kidney was also extracted and served as a positive control for DNA extraction. Two individual 5 micron sections from each tissue specimen were deparaffinized in xylene, rehydrated in descending concentrations of ethanol and transferred to 1.7 ml tubes. For each of the DNA extraction steps strict protocols were followed to avoid cross-contamination of samples.

The pelleted tissue was rinsed with 100% ethanol, centrifuged at 12,000 x g and air dried. Tissue was digested overnight with proteinase K^a at 56 °C. Extraction of DNA was performed using the MagneSil Genomic, Fixed Tissue System^a according to the manufacturer's instructions. Samples of DNA with a final volume of 25 µL were stored at -20 °C until analysed.

DNA Quantification:

To ensure adequate total DNA concentration in the samples for further experiments, DNA quantification was performed on all equine ocular samples. The primers^b F (forward) 5'-GGA GCA GAC ATC AAA TAA GTA GG-3' and R (reverse) 5'-CCA TAA CCA TCA CCA TGA TAG G-3' were used to target the ubiquitous equine membrane associated transporter gene.¹⁶ A product size of 100 bp was targeted and SYBR green was used to monitor and quantify double stranded DNA levels. Real-time quantitative PCR was performed on a Stratagene Mx4000^c using SYBR green reagents.^d The PCR reaction contained 5 µl of template in a final volume of 20 µl. Each primer was used at a final concentration of 200 nM. The amplification protocol consisted of 15 min at 95°C followed by 40 cycles of amplification (denaturation at 94°C for 15 sec, annealing at 60°C for 1 min and extension at 76°C for 30 sec) after which the reaction was stopped (95°C for 2 min) and melted (55°C-96°C) with plate readings every 1°C.

Real-Time PCR:

All samples were subjected to real-time PCR for *Leptospira*. The primers^b and probes^b for this PCR were designed from alignments of available *Leptospira* 16S rDNA partial

sequences obtained from the GenBank nucleotide sequence database as described previously.²⁵ The PCR primers F 5'-CCC GCG TCC GAT TAG-3' and R 5'-TCC ATT GTG GCC GRA GAC AC-3' were located between the positions 191 and 278 of the *Leptospira* 16S ribosomal subunit gene (GenBank accession DQ991474) with an expected product size of 87 base pairs (bp). The probe, 5'- (FAM)CTC ACC AAG GCG ACG ATC GGT AGC-3'(3IABlkFQ), had the fluorescent reporter dye, 6-carboxy-fluorescein (FAM) located at the 5' end of the probe and the quencher Iowa Black FQ located at the 3' end and corresponds to positions 225 - 248 of GenBank accession DQ991474. The PCR was performed on a Stratagene Mx4000^c and was carried out using 5 µL of sample DNA, extraction control (porcine kidney) or known positive and negative DNA controls added to the 45 µL mastermix^e providing final concentrations of 200 nM of each primer and 200nM of the FAM- IABlkFQ labeled probe as previously described.²⁵

Immunohistochemistry:

Paraffin-embedded, formalin-fixed equine ocular sections and the section of *Leptospira* infected porcine kidney were positioned on slides, hydrated and placed in Tris buffer for at least 5 minutes prior to staining. Slides were treated with 3% H₂O₂ for 15 minutes, washed, treated with Proteinase K for 5 minutes, washed, subjected to Protein Block^f for 5 minutes and drained. Deparaffinized sections were incubated in rabbit anti-*Leptospira* cocktail antibody^g (a mixture of antibodies against *L. interrogans* serovars *pomona*, *hardjo*, *icterohaemorrhagiae*, *canicola*, *grippotyphosa* and *bratislava*) at a 1:1600 concentration for 30 minutes. Negative controls were treated with rabbit IgG antibody^h at

a 1:1000 dilution for 30 minutes. Subsequently, sections were incubated with a biotinylated secondary antibodyⁱ for 20 min and a streptavidin-biotin complex reagentⁱ for 20 min, both at room temperature followed by a tris buffer rinse. Development proceeded for 5 minutes with NOVA Red^j. NOVA Red chromagen is readily discernable from black-brown melanin pigment present in equine globes. Slides were counterstained in Mayer's hematoxylin and Azure blue, then dehydrated and cover-slipped. Additional controls were also included via omission of the primary antibody or both primary and secondary antibodies. The staining intensity and distribution of *Leptospira* antigen was evaluated by a masked board certified veterinary pathologist (J.R.T.). Immunostaining from an average of three high-power (x200) microscopic fields was assessed using the following semi-quantitative grading scale: grade 0, none or occasional positive cells only; grade 1, sparse positive cells; grade 2, moderate positive cells; and grade 3, intense positive staining cells.

Sources and Manufacturers:

- a. Promega Corporation, Madison, WI.
- b. Integrated DNA Technologies Inc., Coralville, IA
- c. Stratagene Inc., La Jolla, CA
- d. Qiagen QuantiTect SYBR Green PCR Kit, Valencia, CA
- e. TaqMan Universal PCR Mastermix Mix, Applied Biosystems, Foster City, CA
- f. DakoCytomation, Carpinteria, CA
- g. National Veterinary Services Laboratories (NVSL), Ames, IA
- h. Sigma, St. Louis, MO

- i. LSAB2 System-HRP, DakoCytomation, Carpinteria, CA
- j. Vector Laboratories, Burlingame, CA

2. RESULTS

The presence of amplifiable DNA was confirmed on all equine ocular tissue samples following extraction by performing PCR for an equine genomic target. Amplifiable DNA was identified in 29 of 30 samples after initial extraction with one normal control sample being negative. This sample was extracted a second time and DNA PCR was repeated, confirming amplifiable DNA was present in the second extraction and in the final extraction of 30 of 30 samples. Real-time PCR evaluation for *Leptospira* DNA was negative for all equine ocular samples and for the negative DNA control. The extraction control (porcine kidney) and positive DNA control were positive, showing amplification of the 87 bp target *Leptospira* product. Immunohistochemistry indicated that 2 of 10 eyes from the ERU affected experimental group and the section of *Leptospira* infected porcine kidney exhibited positive immunoreactivity. Positive results were repeatable on multiple sections. Of the positive horses in the ERU group, grade 2 focal immunoreactivity was noted in the corneal epithelium of one horse and in the uveal tract (inflammatory cells within the ciliary body) of another horse. A representative photomicrograph is shown in Fig. 1A and Fig. 1B. The control sample of porcine kidney exhibited multi-focal areas of mixed grade 2 and grade 3 immunoreactivity. Positive samples were confirmed by repeating IHC on a minimum of 3 sections from the same sample. The 8 additional samples from the experimental (ERU) group were negative as were all samples from control groups and the negative controls (Fig. 1C). Statistical

evaluation using Fisher's Exact Test revealed no significant difference ($p=0.1$) in the detection of *Leptospira* antigen between the experimental (ERU) group and the control groups.

3. DISCUSSION

Our hypothesis that *Leptospira* DNA would be detectable in fixed archival equine ocular tissue affected with ERU was rejected. Our hypothesis that *Leptospira* antigen would be detectable in fixed archival equine ocular tissue affected with ERU was accepted.

However, *Leptospira* antigen had low prevalence in ocular tissues from ERU affected horses. Detection of *Leptospira* components (DNA, antigen) was lower in this study compared to previously reported evaluations of fresh ocular tissue from horses with ERU.

There are several explanations for decreased leptospiral DNA and antigen detection in the fixed equine ocular samples evaluated in this study. Although amplifiable DNA was confirmed in all equine samples by SYBR green PCR and presence of *Leptospira* DNA was detected in the extraction control (fixed porcine kidney), target DNA fragmentation may have occurred as a process of fixation and resulted in negative results by real-time PCR for *Leptospira*. Fixation may have damaged antigenic portions of *Leptospira* bacteria present in sections of equine ocular tissue. Although the control section of porcine kidney was processed in an identical manner and revealed strong positive staining for *Leptospira* sp. antigen, uncontrolled sample variation of formalin fixation time and time spent in paraffin blocks also may have affected our results. In previous studies, *Leptospira* DNA and antibodies toward *Leptospira* have been isolated from

aqueous and vitreous humor samples. Although a small amount of aqueous and vitreous humor is preserved by fixation, *Leptospira* organisms could have been lost during the fixation process if they were present in small numbers and isolated solely in the aqueous/vitreous compartments.

All eyes included in this study were in the chronic or end-stage categories of ERU, whereas the majority of ocular tissue used in previous studies^{2,8,11,16,27} detecting *Leptospira* sp. was from horses affected with active, earlier stages of ERU. The immune-mediated etiology of ERU is well documented and widely accepted. Recent publications have demonstrated immunogenic potential of small components of *Leptospira* sp., suggesting the bacteria may play a role in the initiation of some cases ERU.^{13,14,19,25} In the ERU affected equine tissue samples used in this study, *Leptospira* may have been present initially, and subsequently cleared by the immune system with no organisms present in ocular tissue with end-stage disease. Alternatively, *Leptospira* may have never been present in ERU affected eyes evaluated in this study.

The detection of leptospiral antigen in two ERU affected eyes is a trend unique to our study but was not statistically significant when compared to control groups. This finding may be due to true bacterial presence or could be related to antigenic similarity between equine ocular tissues and *Leptospira* bacteria which has been previously documented with immunoblotting techniques.^{13,18,19} In these studies, a protein epitope involved in an antigenic relationship with equine cornea and lens was located internal to the surface of *Leptospira* bacteria.¹⁹ One positive case in this study revealed *Leptospira*

immuoreactivity of the cornea, which was consistent anatomically with the previous literature while the other positive case showed increased staining of inflammatory cells within the ciliary body of the uveal tract.

A discrepancy noted in this study was that *Leptospira* antigen was detected in two samples but *Leptospira* DNA was not detected by PCR (a more sensitive testing method). A possible explanation for this finding would be that different sections of the same eye were used for immunohistochemistry and PCR with bacteria only being present in certain sections. Alternatively, antigenic similarity between equine ocular tissues and *Leptospira* bacteria¹⁸ could have caused false positive reactivity with *Leptospira* antibodies.

Epidemiologic studies to link *Leptospira* and ERU in the Midwestern or Eastern United States do not exist. In epidemiologic studies of human leptospirosis, there is significant evidence showing that certain geographic areas have a higher incidence of ocular disease.^{20,21} In order to determine if geographic variation of *Leptospira* involvement in ERU is present, additional studies are needed to compare *Leptospira* incidence in fresh and fixed ocular tissues from horses in different geographic localities.

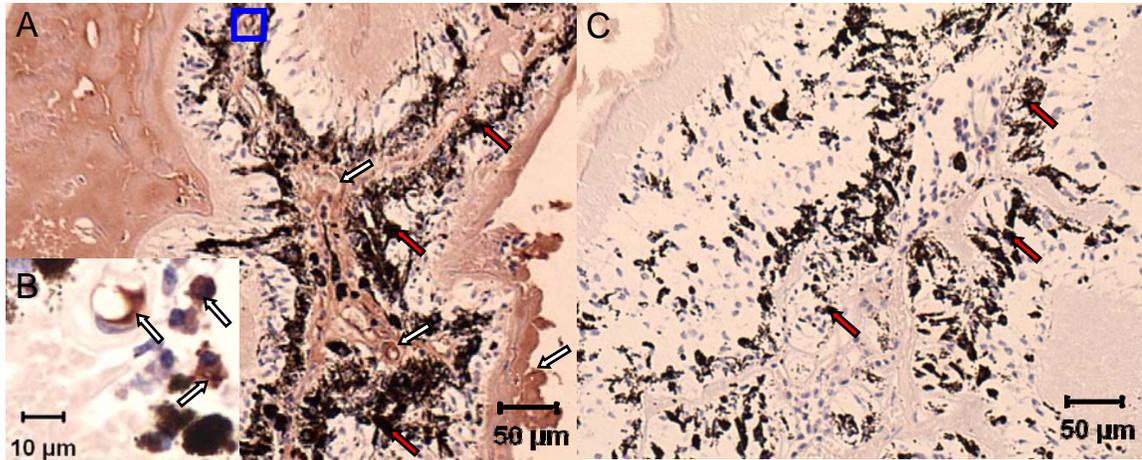


Figure 1. A: Representative photomicrograph demonstrating positive immunoreactivity (open arrows) with *Leptospira* antibody for antigens in the ciliary body of an ERU affected horse. B: (Inset) Close up of the same ciliary body (blue box from A) containing *Leptospira*-positive inflammatory cells (open arrows). C: Photomicrograph demonstrating the negative control (rabbit IgG applied instead of primary antibody cocktail) for the same ERU affected horse. Note black melanin pigment in both positively stained images and negative control (red arrows).

REFERENCES

1. Bernard WV: 1993, Leptospirosis. *Vet Clin North Am Equine Pract* 9:435-444.
2. Brem S, Gerhards H, Wollanke B, et al.: 1999, [35 leptospira isolated from the vitreous body of 32 horses with recurrent uveitis (ERU)]. *Berl Munch Tierarztl Wochenschr* 112:390-393.
3. Deeg CA, Ehrenhofer M, Thurau SR, et al.: 2002, Immunopathology of recurrent uveitis in spontaneously diseased horses. *Exp Eye Res* 75:127-133.
4. Deeg CA, Kaspers B, Gerhards H, et al.: 2001, Immune responses to retinal autoantigens and peptides in equine recurrent uveitis. *Invest Ophthalmol Vis Sci* 42:393-398.
5. Deeg CA, Pompetzki D, Raith AJ, et al.: 2006, Identification and functional validation of novel autoantigens in equine uveitis. *Mol Cell Proteomics* 5:1462-1470.
6. Deeg CA, Thurau SR, Gerhards H, et al.: 2002, Uveitis in horses induced by interphotoreceptor retinoid-binding protein is similar to the spontaneous disease. *Eur J Immunol* 32:2598-2606.
7. Dwyer AE, Crockett RS, Kalsow CM: 1995, Association of leptospiral seroreactivity and breed with uveitis and blindness in horses: 372 cases (1986-1993). *J Am Vet Med Assoc* 207:1327-1331.
8. Faber NA, Crawford M, LeFebvre RB, et al.: 2000, Detection of *Leptospira* spp. in the aqueous humor of horses with naturally acquired recurrent uveitis. *J Clin Microbiol* 38:2731-2733.
9. Gilger BC, Malok E, Cutter KV, et al.: 1999, Characterization of T-lymphocytes in the anterior uvea of eyes with chronic equine recurrent uveitis. *Vet Immunol Immunopathol* 71:17-28.
10. Gilger BC, Michau TM: 2004, Equine recurrent uveitis: new methods of management. *Vet Clin North Am Equine Pract* 20:417-427, vii.
11. Hartskeerl RA, Goris MG, Brem S, et al.: 2004, Classification of leptospira from the eyes of horses suffering from recurrent uveitis. *J Vet Med B Infect Dis Vet Public Health* 51:110-115.
12. Kalsow CM, Dwyer AE: 1998, Retinal immunopathology in horses with uveitis. *Ocul Immunol Inflamm* 6:239-251.
13. Lucchesi PM, Parma AE: 1999, A DNA fragment of *Leptospira interrogans* encodes a protein which shares epitopes with equine cornea. *Vet Immunol Immunopathol* 71:173-179.
14. Lucchesi PM, Parma AE, Arroyo GH: 2002, Serovar distribution of a DNA sequence involved in the antigenic relationship between *Leptospira* and equine cornea. *BMC Microbiol* 2:3.
15. Mariat D, Taourit S, Guerin G: 2003, A mutation in the MATP gene causes the cream coat colour in the horse. *Genet Sel Evol* 35:119-133.
16. Niedermaier G, Wollanke B, Hoffmann R, et al.: 2006, [Detection of leptospira in the vitreous body of horses without ocular diseases and of horses with equine recurrent uveitis (ERU) using transmission-electron microscopy]. *Dtsch Tierarztl Wochenschr* 113:418-422.

17. Parma AE, Cerone SI, Sansinanea SA: 1992, Biochemical analysis by SDS-PAGE and western blotting of the antigenic relationship between *Leptospira* and equine ocular tissues. *Vet Immunol Immunopathol* 33:179-185.
18. Parma AE, Santisteban CG, Villalba JS, Bowden RA: 1985, Experimental demonstration of an antigenic relationship between *Leptospira* and equine cornea. *Vet Immunol Immunopathol* 10:215-224.
19. Parma AE, Sanz ME, Lucchesi PM, et al.: 1997, Detection of an antigenic protein of *Leptospira interrogans* which shares epitopes with the equine cornea and lens. *Vet J* 153:75-79.
20. Rathinam SR: 2002, Ocular leptospirosis. *Curr Opin Ophthalmol* 13:381-386.
21. Rathinam SR, Rathnam S, Selvaraj S, et al.: 1997, Uveitis associated with an epidemic outbreak of leptospirosis. *Am J Ophthalmol* 124:71-79.
22. Romeike A, Brugmann M, Drommer W: 1998, Immunohistochemical studies in equine recurrent uveitis (ERU). *Vet Pathol* 35:515-526.
23. Schwink KL: 1992, Equine uveitis. *Vet Clin North Am Equine Pract* 8:557-574.
24. Smythe LD, Smith IL, Smith GA, et al.: 2002, A quantitative PCR (TaqMan) assay for pathogenic *Leptospira* spp. *BMC Infect Dis* 2:13.
25. Verma A, Artiushin S, Matsunaga J, et al.: 2005, LruA and LruB, novel lipoproteins of pathogenic *Leptospira interrogans* associated with equine recurrent uveitis. *Infect Immun* 73:7259-7266.
26. Williams RD, Morter RL, Freeman MJ, Lavignette AM: 1971, Experimental chronic uveitis. Ophthalmic signs following equine leptospirosis. *Invest Ophthalmol* 10:948-954.
27. Wollanke B, H. G, S. B, et al.: 2004, Etiology of equine recurrent uveitis (ERU): Autoimmune disease or intraocular leptospiral infection? *Pferdheilkunde* 20:327+.
28. Wollanke B, Rohrbach BW, Gerhards H: 2001, Serum and vitreous humor antibody titers in and isolation of *Leptospira interrogans* from horses with recurrent uveitis. *J Am Vet Med Assoc* 219:795-800.