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## Role of Intraocular Leptospira Infections in the Pathogenesis of Equine Recurrent Uveitis in the Southern United States

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ROLE OF INTRAOCULAR *LEPTOSPIRA* INFECTIONS  
IN THE PATHOGENESIS OF EQUINE RECURRENT UVEITIS IN THE SOUTHERN  
UNITED STATES

A Thesis

Submitted to the Graduate Faculty of the  
Louisiana State University and  
Agricultural and Mechanical College  
in partial fulfillment of the  
requirements for the degree of  
Master of Science

In  
The School of Veterinary Medicine  
Through  
The Department of Veterinary Clinical Sciences

By

Florence Polle  
DrMedVet, Ecole Nationale Vétérinaire de Toulouse, 2007  
August 2012

To my parents, who always let me make my own choices.

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## ABSTRACT

To investigate the role of intraocular leptospiral infections in horses with Equine Recurrent Uveitis (ERU), ocular fluid samples were collected from donated and client-owned horses with a history and ocular findings consistent with chronic ERU. Additionally, eyes were harvested from horses with normal ophthalmic examinations as a control group. Blood samples were obtained for *Leptospira* serology using microscopic agglutination test (MAT). Aqueous and vitreous humor samples were aseptically obtained and submitted for aerobic culture and *Leptospira* culture, PCR and MAT.

Twenty-one control horses (40 eyes) and 31 ERU horses (46 eyes) were available for study. Serology results were available for 48/52 horses: 16/21 control and 23/27 affected horses were positive for at least one serovar; Bratislava was the most common serovar for both groups. *Bacillus* sp. and *Micrococcus* sp. were cultured from one control eye; *Streptococcus* sp. (n=1) and *Leptospira* (n=6) from eyes with ERU. *Leptospira* isolated belonged to serogroup pomona (n=4) and grippotyphosa (n=2). PCR results were positive in 14/31 (45%) horses diagnosed with ERU; no control horses were positive by PCR (p=0.0001). MAT was positive for 17/24 of ERU horses (71%) and 1/21 (4.7%) of normal horses (p<0.0001). Horses with ERU had a high prevalence of *Leptospira* infection based on PCR and MAT results from intraocular fluids compared to controls. *Leptospira* infection should be considered as a cause of ERU in the southern United States. The diagnosis of these intraocular infections was not aided by serology and required specific, invasive sampling of ocular fluid.

## INTRODUCTION

Equine Recurrent Uveitis (ERU), also called “Moon blindness”, “Periodic ophthalmia” or “Iridocyclitis” is characterized by recurrent episodes of ocular inflammation and is the main cause of blindness in horses. The inflammation primarily affects the vascular tunic of the eye or uvea.

The syndrome was initially thought to be caused by changes in the moon and has been described since the early days of veterinary medicine. In the 4<sup>th</sup> century BC, Vegetius in *Artis Veterinariae sive Mulomedicinae* wrote about an ERU-like syndrome. Vegetius thought the cyclic nature of the inflammation was associated with changes in the phase of the moon and called it “morbus lunaticus” or “moon blindness” (Paglia et al. 2004). Italians described a similar syndrome during the renaissance; then throughout Europe during the following centuries. Dr. James Wardrop described most of the clinical and pathologic features of ERU in 1819 in *An Essay on the Diseases of the Eye of the Horses*. In the United States, Dr. J. Carver in 1818 blamed poor ventilation and hygiene as the cause of periodic ophthalmia and criticized the practice of blowing powdered glass into the eye as a cure.

Much speculation occurred about the causes of ERU. Prevailing theories included infectious causes, hereditary predisposition, climate, toxins, parasites and thyroid deficiency. In the 1940s, ERU was first connected to *Leptospirosis* in Germany. Since then, *Leptospirosis* has been linked to ERU in many countries but the pathogenesis remains unclear.

ERU has a worldwide distribution and is responsible for substantial economic losses to the equine industry. The equine industry in the US is worth an estimated 112 billion dollars yearly. Economic losses result from veterinary care, disrupted training, decreased performance, loss of value and loss of use because of blindness. The estimated cost of ERU because of its high prevalence could be up to 200 million dollars yearly. Many clinical and pathological features of ERU are similar to recurrent uveitis in humans. For this reason, and because of the high economic impact of the disease, ERU has been studied extensively.

In this manuscript, we will review the literature on ERU, with an emphasis on *Leptospirosis* as a possible etiology. We will then present the results of our study investigating the role of bacterial infection in the development of ERU in Louisiana and neighboring states.

## CHAPTER ONE. REVIEW OF THE LITERATURE

### 1.1. The normal equine eye

The equine eye is the largest eye of all terrestrial mammals. Approximate globe dimensions are 42 to 44 mm from the anterior to posterior axis, 45 to 50 mm vertically and 50 to 54 mm horizontally. The globe is composed of three basic layers: the fibrous tunic (the cornea and sclera) that give the eye a constant shape and form; the uvea (the choroid, iris and ciliary body) that modify both external and internal light, provide nourishment and remove waste; and the inner nervous coat (the retina and optic nerve). The three tunics contain the inner transparent media of the eye: aqueous humor, lens and vitreous humor which function to transmit and refract light and keep the globe distended. The iris divides the globe into an anterior and a posterior chamber, which communicate through the pupil. The anterior chamber volume is approximately  $3.04 \pm 1.27$  mL. Mean vitreous volume is  $26.15 \pm 4.87$  mL (Gilger 2005).

#### 1.1.1. The uvea

ERU primarily involves the uveal tract: the iris and ciliary body comprise the anterior uvea and the choroid comprises the posterior uvea. The uveal tract provides most of the blood supply to the internal contents of the eye and is directly linked to the systemic circulation; therefore, systemic diseases can also affect the uvea.

The choroid and ciliary body are both attached to the internal surface of the sclera while the iris originates from the anterior portion of the ciliary body and extends centrally to form a diaphragm in front of the lens. The central opening of the iris, the pupil, is horizontally oval and bordered by variable sized black masses called granula iridica or corpora nigra. These corpora nigra may act as a light barrier or shade and also augment pupillary constriction. The iris of most horses is golden to dark brown but can also be blue, white or heterochromic, especially in color dilute breeds. The iris is broken down into a central pupillary zone and a peripheral ciliary zone, separated by a collarette. The iris and ciliary body contain heavily pigmented connective, vascular and muscle tissue.

The ciliary body produces aqueous humor through active secretion and ultrafiltration of plasma. As viewed from the vitreous cavity, the ciliary body is divided into the anteriorly positioned pars plicata and the posteriorly positioned pars plana. As the name suggests, the pars plicata is characterized by a folded or pleated appearance, with approximately 100 ciliary processes extending into the posterior chamber. From the ciliary processes, zonular fibers extend and connect to the equatorial region of the lens. The pars plana is a relatively smooth, flat portion that extends from the pars plicata to the most peripheral extension of the retina. The entire inner surface of the ciliary body (the surface in contact with the vitreous body) is lined with a double row of epithelial cells. The innermost epithelial cell layer (from the perspective of the vitreous cavity) is nonpigmented and is referred to as the nonpigmented epithelium (NPE) of the ciliary body. The second epithelial cell layer is heavily melanotic and is referred to as the pigmented epithelium of the ciliary body (Fig. 1). Tight junctions between NPE cells are thought to represent the epithelial portion of the blood-aqueous barrier. Deep to the two-layer ciliary body

epithelium, each ciliary process has a central portion of connective tissue and a vascular plexus, which is fenestrated, allowing leakage of plasma into the ciliary body stroma. The epithelial portion of the blood-aqueous barrier filters this plasma, removing virtually all protein and cells. Thus the aqueous humor represents an ultrafiltrate of plasma.

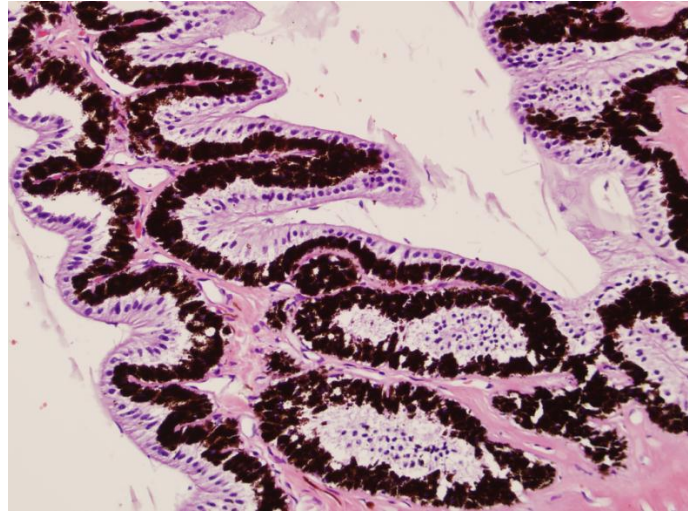


Figure 1: Normal histologic section of the ciliary processes demonstrating pigmented and non-pigmented epithelium (HE stain, 20x magnification).

The choroid is the posterior extension of the uvea, joining the ciliary body at the ora serrata. The choroid's main function is to provide blood supply to the retina. The tapetum fibrosum is a specialized reflective layer of the choroid located in the superior fundus. The tapetum is visible because the retinal pigmented epithelium overlying the tapetum does not contain melanin. Choroidal vasculature consists of larger vessel exteriorly and medium size vessel interiorly. The choriocapillaris is the inner most layer of the choroid and is composed of a thin capillary network (Carastro 2004).

### **1.1.2. Normal immunology**

The eye is an immune privileged site in the same way as the nervous system. The mammalian eye has adapted the intraocular immune effector mechanism to limit the intensity and extent of the local response to antigen challenge. This situation of immunological tolerance is fundamental to the integrity of the healthy eye (Gelatt 2007). Intraocular immune privilege is determined by a number of mechanisms, including the blood-ocular barrier, the absence of lymphatic drainage in the eye, immunoinhibitory cytokines in the ocular fluids and the phenomenon of anterior chamber associated immune deviation. These mechanisms function to protect sensitive

intraocular tissues from the effects of uncontrolled T cell driven inflammation, and their impairment exposes the uvea to the possibility of immune mediated insult (Barnett et al. 2004). The mechanisms by which immune tolerance is broken and inflammatory disease arises are unresolved.

## **1.2. Epidemiology of Equine Recurrent Uveitis**

### **1.2.1. Prevalence**

ERU is a spontaneous disease with a worldwide distribution. The prevalence is usually high although it varies with geographical location. This variation might be explained by the diversity of etiologic agents and breeds and their heterogenic geographical distribution.

In Europe, the prevalence seems to vary widely from up to 30% in central Europe (Cross 1966) to 1-2.5% in the UK (Barnett et al. 2004). An older study from the UK showed a prevalence of 0.09% (Gelatt 2007). More recent studies estimated the prevalence around 1% (Mellor et al. 2001; Rocha et al. 2004). In central Europe, the prevalence of ERU in the early 20<sup>th</sup> century was reported to reach 70% in areas with clay soil and frequent flooding, while in drier areas the prevalence was around 5%. More recent reports document a prevalence of 8-10% in Germany (Deeg et al. 2002; Spiess 2010).

In the United States, prevalence was observed to be >1% in NY (Dwyer et al. 1995). In a review of the Veterinary Medical Data Base records from January 1986 through December 1990, examination data on 79,037 horses were obtained. Diagnosis of uveitis was made in 860 (1.1%) (McLaughlin et al. 1992). Authors estimate the prevalence of ERU in the United States to be between 8 and 15%, with 1-2% of horses having disease severe enough to threaten vision (Gilger et al. 2004).

In India, uveitis was reported in 4.8% of 500 horses from the Indian army (Thangadurai et al. 2010). The blindness rate from ERU was estimated to be as high as 12% in regions of Africa (Choyce 1964).

### **1.2.2. Breed and genetic susceptibility**

In the USA, one study (Dwyer et al. 1995) showed that the odds of finding uveitis were 8.3 times greater in Appaloosas than in all other breeds combined. Appaloosa horses were also more likely to have severe lesions and to be blind. In the study reviewing examination data on 79,037 horses from the Veterinary Medical Data Base records, Appaloosa horses were significantly more likely than the total population to have uveitis while Standardbreds and Thoroughbreds were significantly less likely to have uveitis (McLaughlin et al. 1992). In a retrospective study performed in New York state on cases from 1975 to 1987 (Angelos et al. 1988), Appaloosas had a significantly higher risk of developing uveitis (OR = 6.4) relative to Thoroughbreds, while Standardbreds had a significantly lower risk of developing uveitis relative to Thoroughbreds. Within Appaloosas, horses with a lighter hair coat are more likely to be affected than horses with

a darker coat (Gilger 2005). Some authors have found that horses with a dark or dappled hair coat were more often affected (Gilger 2005).

In Europe, breed analysis showed that certain breeds of Trotter and Warmblood horses were more likely to have uveitis (Alexander et al. 1990). Warmblood and draft horses were also reported to be more likely to have posterior uveitis (Gilger 2005). Although Appaloosas are not a common breed, they also seem to have a predisposition to ERU in Europe (Spiess 2010). In one study, Thoroughbred type horses were less likely to be affected than Warmbloods or ponies (Gilger 2005).

ERU susceptibility has long been suspected to have a heritable component. This may be linked to equine leukocyte antigen (ELA) haplotypes influencing the occurrence and expression of autoimmune intraocular inflammatory disease. A strong association with the MHC1 haplotype ELA-A9 with ERU in German Warmblood horses has been identified (Gilger 2005). A similar association was found in particular Appaloosas breeding lines (Dwyer et al. 1995). Other non-ELA genes may be involved in creating the permissive genetic background necessary for developing the disease. This predilection may be similar to the well-known association of MHC type with uveitis in human beings.

### **1.2.3. Age and sex**

Studies have failed to show a clear association between age and ERU. In one study, younger animals between one and four years of age were predominantly affected (Alexander et al. 1990), whereas another study showed that horses older than 15 years of age were more likely to be affected (Gilger 2005). Most studies fail to show a relationship, but it is generally admitted that most horses develop the disease between 4 and 8 years of age (Gilger 2005), at the prime of their performance. In two studies, males were shown to be more likely to have ERU (Alexander et al. 1990; Szemes et al. 2000), but most studies to date have not found the incidence to be higher in males than females (Alexander et al. 1990; Wollanke et al. 2001).

### **1.2.4. Etiology**

Single uveitic episodes can be secondary to trauma, or secondary to a systemic disease process. Anterior uveitis is a common finding in foals with septicemia or rhodococcal pneumonia (Reuss et al. 2009; Leiva et al. 2010). Uveitis may also accompany chronic or severe corneal disease (axonal reflex). Specific viral causes of uveitis include equine influenza virus, equine herpes virus 1, equine viral arteritis, and equine infectious anemia virus. Bacterial causes include *Leptospira* spp., *Brucella* spp., *Borrelia burgdorferi*, *Streptococcus* spp., *Rhodococcus equi* and *E. coli* (Gilger 2005). *Borrelia burgdorferi* has been implicated as causing panuveitis in a pony (Burgess et al. 1986). *Brucella abortus* has been suggested as a cause of ERU but surveys of affected and control horses could not find a correlation (Davis et al. 1950). *Streptococcus equi* and influenza virus have been clinically implicated in uveitis, but are not thought to play a significant role in ERU (Roberts 1971; Martin 2010). Some studies have documented an

association between *Leptospira* and ERU in some geographic locations; this will be discussed later in this work.

*Toxoplasma gondii* is a protozoan parasite that can infect horses although clinical disease is rare. Few case reports have demonstrated elevated titers to *Toxoplasma* in horses with chorioretinitis (Eugster et al. 1976) and in one horse with optic nerve atrophy (Sellon et al. 2007). However, one study in India showed no correlation between positive titers and ocular lesions (Chhabra et al. 1980) and another study in horses with ERU showed no correlation with positive titers (Alexander et al. 1990).

*Onchocerca cervicalis* microfilaria has long been thought to be a major cause of ERU. *O. cervicalis* is spread by *Culicoides* spp., and causes dermatitis in horses. It is thought that the microfilariae migrate along vessels through subcutaneous tissue to the eyelids, then into the conjunctiva, cornea and uvea (Sellon et al. 2007). The importance of *O. cervicalis* may vary with geographic region: the incidence of the larvae in England is too low to account for ERU, while in the US, it was at one time found in 50 to 90% of the horses depending on the region (Stannard et al. 1975; Lloyd et al. 1978; Lyons et al. 1981). The routine use of broad-spectrum anthelmintics has markedly decreased *O. cervicalis* as a major cause of uveitis.

### **1.3. Diagnosis and clinical signs**

#### **1.3.1. Clinical presentation**

Diagnosis of ERU is often based on the patient's history and compatible clinical signs. A complete ophthalmologic examination should be performed under sedation to rule out other diseases that can present with similar clinical signs and confirm the diagnosis of uveitis. An auriculopalpebral block that paralyzes the upper eyelid will allow more complete examination of a painful eye.

Horses can have isolated episodes of uveitis that should be differentiated from the chronic, recurrent form. Treatment should be continued until all signs have resolved for at least one month as premature cessation of treatment can result in recurrence of clinical signs. Other diseases that can mimic ERU include all causes of painful eyes: ocular trauma, corneal ulceration, keratitis, glaucoma and intraocular tumors.

Classic recurrent uveitis presents as distinct acute episodes of inflammation. Examination reveals classic acute clinical signs but may also show ocular changes that occur with chronicity. Flare-ups of uveitis can be seasonal and occur only once a year. The severity of the next attack and the duration of the quiescent period between attacks are unpredictable and severity varies between individuals. ERU is bilateral in more than 80% of Appaloosa horses and in 38% of horses with no predisposing factors (Gilger 2005).

Subclinical or insidious uveitis often presents as an incidental finding or as blindness in the late stages. No outward signs of discomfort are generally noted by the owner even in advanced cases.

Common changes include vitreal liquefaction, focal or diffuse cataract formation, changes in iris pigmentation and synechiae formation. This type of uveitis is most commonly seen in Appaloosa, Warmblood and draft breed horses (Gilger et al. 2004; Gilger 2005).

The most common clinical sign in classic acute ERU is pain, presenting as photophobia, lacrimation and blepharospasm. The eyelid might be swollen and tightly closed, making examination difficult. Some degree of enophthalmos due to globe retraction can be present.

A low intraocular pressure (IOP) is a constant and important sign and may be obvious even by digital tonometry. Although the IOP is typically reduced during inflammation due to lowered aqueous production and increased outflow, inflammatory debris and formation of synechiae and inflammatory membranes can create mechanical resistance to normal outflow, resulting in uveitic (secondary) glaucoma. The increase in IOP alters the optic nerve function and results in degeneration of retinal ganglion cells eventually leading to blindness (Annear et al. 2012).

Other common signs of acute uveitis include (Fig. 4 and 5):

- Conjunctival and episcleral hyperaemia, with or without chemosis
- Corneal edema and neovascularization
- Aqueous flare, fibrin, hypopyon and hyphema.
- Miosis
- Rubeosis iridis; iridial swelling; iridal color change
- Cloudy, yellow-green vitreous

The sequelae to uveitis indicate previous attacks and include the following (Fig. 6, 7 and 8):

- Anterior and posterior synechiae in 1/3 of cases and 40% of Appaloosas
- Pigment on the anterior lens capsule
- Anterior capsular and cortical cataracts. In one study, the risk of cataract development in a horse with uveitis was 42 times higher than in horses without ERU (McLaughlin et al. 1992).
- Lens luxation can occur following zonular fiber degradation
- Darkened iris
- Atrophy of corpora nigra
- Vitreal debris, fibrous strand and liquefaction
- Retinal degeneration, often peripapillary and retinal detachment resulting in blindness
- Phthisis bulbi or glaucoma

The association between peripapillary choroidal degeneration and ERU is unclear (Matthews et al. 1990). In ponies experimentally infected with *Leptospira* spp. peripapillary chorioretinitis developed along with anterior uveitis (Williams et al. 1971). One recent study showed that



depigmented punctate chorioretinal foci were not indicative of or associated with ERU (Mathes et al. 2012).

### 1.3.2. Additional tests

Complete physical examination and minimum database blood work should be performed in horses with acute uveitis to rule out any underlying disease that could cause the uveitis or be worsened by treatment. Isolated ocular disease does not result in CBC or acute inflammatory protein changes in horses (Labelle et al. 2011), and if such changes are observed, a non-ocular inflammatory focus should be suspected.

Serologic diagnosis for possible etiologic agents such as *Leptospira*, *Toxoplasma*, *Brucella* or Lyme disease can be performed. Although early studies showed that a positive serology to *Leptospira* was indicative of *Leptospira*-induced uveitis, this finding has been challenged recently (Faber et al. 2000). More invasive diagnoses, such as aqueocentesis or vitreocentesis (Fig. 2 and 3), are thought to be more specific and sensitive in detecting the initiating agent. Cytology, culture, antibody detection or molecular diagnosis can be performed on ocular fluid, but the number of tests performed is often limited by the volume obtained. Conjunctival biopsy can be performed to detect *Onchocerca* microfilaria. Recently, one study demonstrated that pigment epithelium-derived factor (PEDF) was significantly down-regulated in sera of horses with ERU and proposed that it could be used as a uveitis biomarker (Zipplies et al. 2009).

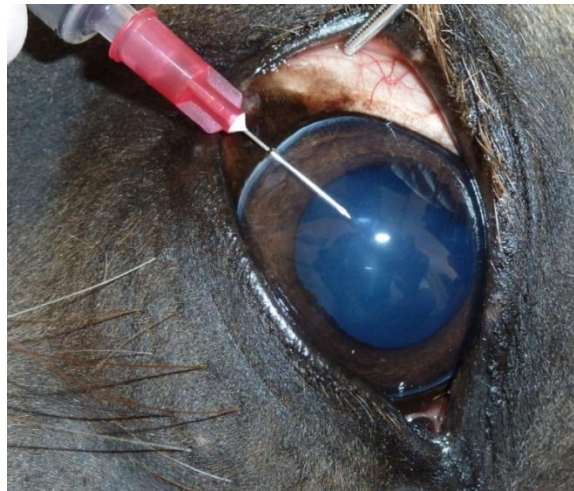


Figure 2: Aqueous paracentesis. The bulbar conjunctiva is grasped with thumb forceps near the site of entry, and a 25- to 30-gauge needle is directed through the limbal cornea or subconjunctival limbus parallel to the iris, avoiding the lens.

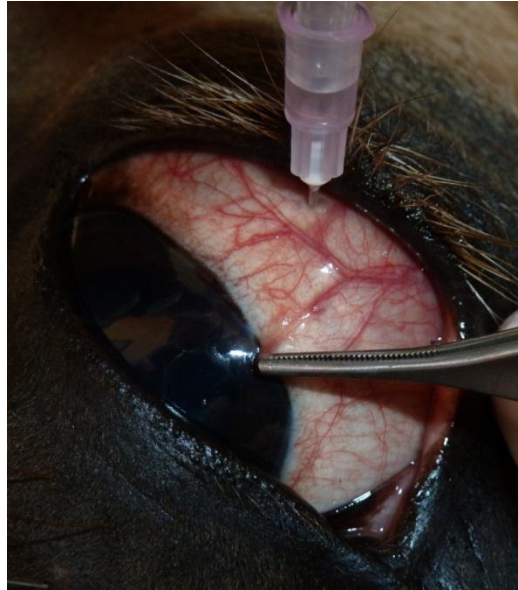


Figure 3: Vitreous paracentesis. A 20 gauge needle is inserted through the conjunctiva and sclera through the pars plana, 10mm posterior to the dorsal limbus. The needle should be directed toward the optic nerve to avoid the lens.

### **1.3.3. Pathology of ERU**

In early ERU, congestion of uveal vessels and inflammatory cellular infiltrates are observed. Neutrophils are the first cells infiltrating the uvea and can result in hypopyon when accumulated in the anterior chamber. They are soon replaced by lymphocytes, plasma cells and macrophages. With time and further recurrence, organization of the lymphocyte infiltrate is evident. Nodules in the ciliary body and iris are composed of B lymphocytes in the center and T lymphocytes in the periphery (Deeg et al. 2002). The diffuse infiltrating population is also composed of T cells and a high percentage is CD4 (Gilger et al. 1999).

The findings of infiltration of lymphocytes and plasma cells into the nonpigmented ciliary epithelium, a thick acellular hyaline membrane adherent to the inner aspect of the nonpigmented epithelium (Fig. 10) and eosinophilic linear inclusions in the nonpigmented ciliary epithelium (Fig. 11) are considered diagnostic of ERU (Cooley et al. 1990). The retina and choroid are involved in most horses with ERU (Deeg et al. 2002), with scattered foci of T-lymphocyte infiltration and retinal degeneration.

### **1.4. Treatment, prevention and prognosis of ERU**

The goal of treatment is to control pain, reduce inflammation to limit ocular damage and preserve vision. Because in most cases an initiating agent cannot be identified, treatment is most often symptomatic. Severe cases might necessitate frequent treatment, up to every hour. Because of the

pain associated with uveitis and the frequency and length of treatment, a subpalpebral lavage system is often placed to facilitate administration of drugs. Medications should be slowly reduced in frequency once clinical signs abate. Therapy can last for weeks or months and should not be stopped abruptly to prevent recurrence.

#### **1.4.1. Medical treatment (Table 1)**

##### **1.4.1.1. Anti-inflammatory drugs**

Anti-inflammatory drugs are the mainstay of ERU. Steroids are more effective in controlling the inflammation than non-steroidal anti-inflammatory agents, but because of the significant side effects associated with systemic steroids in horses, a combination of topical steroids and systemic nonsteroidal anti-inflammatory drugs (NSAIDs) are commonly used.

Local steroid administration includes the use of topical solutions or ointments and subconjunctival injections of long-acting agents. Corticosteroids are strongly contraindicated when ulceration of the cornea is present and horses should be examined thoroughly for the presence of corneal defects prior to initiation. Subconjunctival injection of corticosteroids provides long-term control of the inflammation and decreases the intensity of treatment. However a major drawback to subconjunctival injection is that the drug cannot be removed once injected and could cause serious complications if corneal ulceration arises during the course of treatment. Topical NSAIDs can be used in conjunction with steroids topically or instead of them when corneal ulceration is present.

Topical steroids do not penetrate the posterior segment of the eye. Systemic administration of anti-inflammatory drugs is necessary when treating posterior uveitis. Although steroids can be used, NSAIDs are safer and often preferred. Flunixin meglumin has better ocular penetration than other NSAIDs; however, a recent study has shown that the COX-2 selective NSAID, firocoxib (Equioxx®), also had excellent intraocular penetration and might be safer for long-term use (Hilton et al. 2011).

Anti-inflammatory drugs can also be used during quiet periods in an attempt to prevent recurrence. Aspirin seem to be well-tolerated long-term and is often the NSAID of choice. Similarly, topical steroids can be continued as a once daily treatment but horses should be monitored closely for corneal ulcer development. The efficacy of these preventive measures has not been proven.

##### **1.4.1.2. Cycloplegics and mydriatics**

The pupil in uveitis usually exhibits severe miosis and posterior synechiae are a frequent sequelae. To limit pain associated with ciliary muscle spasm and prevent permanent vision compromise caused by synechiae formation, mydriatics such as atropine (1-4%) should be administered topically and to effect. Atropine also narrows the capillary inter-endothelial cell junctions to reduce capillary plasma leakage. Although the effect of atropine usually lasts several days (up to 14 days) in healthy horses, it can be reduced to just a few hours during severe

inflammation. The pupil should be kept dilated until complete resolution of the clinical signs. Of the domestic species, the horse appears most sensitive to topical mydriatics, especially 1% atropine. Daily monitoring of fecal output, gut sound and mydriasis of the opposite eye is recommended to monitor for systemic absorption. Scopolamine 0.25% is a strong mydriatic that can be used sporadically to break down posterior synechiae.

#### 1.4.1.3. Antibiotics

Antibiotics, both topical and systemic, are often of limited value in treatment of ERU. However, treatment can be attempted if systemic signs are present or if a specific etiologic agent is isolated. Topical antibiotics are only recommended in cases where a corneal ulcer is present. Systematic use of topical antibiotics in uveitis cases could result in disequilibrium of the normal corneal flora and predispose to keratomycosis.

If leptospirosis is suspected, systemic therapy can be initiated with an appropriate antibiotic. Little is known about antimicrobial susceptibility of *Leptospira* spp. and there are no standardized methods for testing. Studies from other animal species indicate that the bacteria is susceptible in vitro to doxycycline, penicillin, ampicillin, oxytetracycline, streptomycin, cefotaxime, erythromycin and fluoroquinolones (Weese 2009). In one study, the administration of oral doxycycline resulted in steady state serum concentration; however it did not result in appreciable concentration of the drug in the aqueous or vitreous of normal eyes (Gilmour et al. 2005). Enrofloxacin at 7.5 mg/kg intravenously resulted in aqueous concentration above the MIC of *Leptospira* spp. (Kim et al. 2006; Divers et al. 2008) and might be the best option for treatment of horses with suspected acute leptospirosis with ocular involvement. However, it is important to note that antibiotic treatment has never been shown to prevent recurrence.

In one study, intravitreal injection of 4 mg of gentamicin in horses with ERU that had a positive serology for *Leptospira* spp. resulted in a dramatic reduction in recurrences (17/18 eyes had no further episodes) (Pinard et al. 2005). However, 4/9 eyes that were visual pre-injection lost vision, possibly due to retinal toxicity. Complications from the procedure can include subconjunctival or vitreal hemorrhage, endophthalmitis, lens damage, retinal detachment and retinal toxicity resulting in vision loss.

#### 1.4.1.4. Other

Injection of the anterior chamber with tissue plasminogen activator (TPA) can be performed to accelerate fibrinolysis in cases with severe fibrin accumulation in the anterior chamber. A 27g needle is inserted at the limbus and 25 to 150 µg of TPA is injected. TPA should be avoided if recent hemorrhage is present.

Severe corneal edema can be controlled by the use of a topical hyperosmotic solution, such as 5% NaCl. Increased intraocular pressures in cases with secondary glaucoma can be treated with topical carbonic anhydrase inhibitors and beta blockers.

Anthelmintic treatment can be performed on suspected cases of *Onchocerca cervicalis*. However, the rapid kill of microfilaria can precipitate a uveitic episode and should therefore be done with caution. It has been advocated to use ivermectin in association with corticosteroids after the acute inflammation subsides.

#### 1.4.1.5. Limitations

Many horses with ERU will develop corneal ulceration as a result of iatrogenic and self-inflicted trauma or secondary to corneal degeneration. These ulcers can have devastating consequences if they are not recognized and treated promptly because the corneal immunity is compromised by steroid administration. Owner should be educated on how to recognize ulcers and to not self-medicate without veterinary advice. Difficult horses should have a subpalpebral lavage system placed to minimize the risk of trauma and ensure adequate drug delivery. Finally, the affected eye(s) should be protected using a mask.

Symptomatic medical treatment can be expensive and extremely time consuming. Many horses require lifelong treatment in order to control recurrence. Most horses will develop disease bilaterally and progress to blindness even if excellent care is provided.

### 1.4.2. Surgical treatment

Surgical treatment of uveitis has progressed dramatically over the past decade.

#### 1.4.2.1. Vitrectomy

Vitrectomy has been performed on human patients with uveitis for more than 20 years both for diagnostic and treatment purposes (Barnett et al. 2004). The technique was first applied to horses with ERU in Germany in 1989. The vitreous humor contains inflammatory mediators, cells and possibly antigens that are thought to perpetuate the inflammation. The procedure has been described in detail elsewhere (Fruhauf et al. 1998; Keller et al. 2005; Spiess 2010). Briefly, a scleral incision is performed at the level of the pars plicata and a vitrectomy device is inserted into the posterior chamber. The vitreous is then incised and aspirated and the posterior segment is irrigated with a dilute gentamicin solution. The vitrectomy cutter can be visualized and guided through the dilated pupil.

Several studies performed in Germany on more than 1200 eyes showed that 94-98% of horses do not show further recurrence of uveitic episodes after surgery (Gilger 2005; Von Borstel et al. 2005; Tóth et al. 2006). The visual prognosis is good providing that the retina and lens are intact, and that there is no severe inflammatory damage. In one study, 14% of eyes became non-visual after surgery due to retinal detachment or progression of cataract (Von Borstel et al. 2005). If significant changes are present before surgery, the visual prognosis is guarded but surgery can still be performed in an attempt to conserve the eye. The goal is to stop recurrence of uveitis and discontinue medical treatment. In one study, the eye could be saved in 95% of horses where phthisis bulbi was present (Tóth et al. 2006). Even in glaucomatous eyes, the IOP could be decreased post-surgery.

Complications associated with vitrectomy include intraocular hemorrhage, but the risk can be decreased to less than 1% if CO2 laser is used to perform the sclerotomy as opposed to a blade. Other complications include retinal detachment and cataract formation in 10 to 14% of the cases (Von Borstel et al. 2005; Tóth et al. 2006). However, the rate of complications seems to decrease drastically with experience: in one study from Munich, retinal detachment occurs in less than 1% of the cases and cataract formation in 3% (Gilger 2005). Severe complications necessitating enucleation (endophthalmitis, panuveitis) occurred in less than 2% of cases (Tóth et al. 2006).

One study was performed in the US showed less promising results: only 37% had decreased clinical signs after surgery and only 24% conserved vision (Brooks et al. 2001). Cataract formation occurred in 46% of the cases. The author concluded that vitrectomy is not recommended for the treatment of horses with ERU in the US.

The differences in success rate probably reflect surgical technique and experience, as well as case selection criteria. In one study from Germany, the majority of horses testing positive for antibodies against *Leptospira interrogans* in the vitreous (40/47; 82.5%) showed no further episodes of ERU, while 6/7 (85.7%) of horses testing negative continued to experience episodes of ERU (Tömördy 2010). The author concluded that vitrectomy should be used in horses with *Leptospira*-induced uveitis and that vitreal and aqueous humor samples should be tested by microscopic agglutination test (MAT) before performing the procedure. This difference of outcome depending on the etiology could also explain why the procedure has such a low success rate outside of Europe.

#### 1.4.2.2. Cyclosporine implants

Cyclosporine A (CsA) is a neutral cyclic undecapeptide that is presently used in the prevention of rejection of organ transplants. It regulates cytokine gene transcription, specifically interleukin-2, subsequently inhibiting T lymphocyte proliferation. It also inhibits nitric oxide synthesis induced by IL-1, lipopolysaccharides and TNF- $\alpha$ . CsA is a lipophilic compound that does not penetrate the cornea or sclera; therefore it cannot be used topically for intraocular diseases. Systemic administration of CsA is also not practical because of very high costs and potential for severe systemic side effects. CsA has also been showed to be bactericidal against *Leptospira* and other microorganisms in vitro (Gilger et al. 2006).

Ocular implants are becoming more popular: they allow continuous delivery of drugs to the target tissue while bypassing the blood aqueous barrier and the cornea. They also considerably decrease the amount of time spent medicating for the owner and increase comfort for the animal.

One study looked at the efficacy of episcleral implants in an attempt to decrease the invasiveness of the procedure. The implant was found to have no efficacy in controlling the disease probably because CsA does not cross the sclera (Gilger et al. 2006).

Intravitreal implant

A polyvinyl alcohol/silicone-coated intravitreal CsA sustained delivery device was found to be well tolerated in normal horses (Gilger et al. 2000). The device is 2 x 3 mm and release 4µg/day of CsA in the vitreous for an estimated 5 years. A full thickness incision is made through the sclera and the pars plana to insert the implant. The stem of the device is then sutured into the scleral incision. In equine eyes with experimentally-induced uveitis, the intravitreal CsA implant decreased the duration and severity of inflammation, cellular infiltration, tissue destruction and level of transcription of pro-inflammatory cytokines (Gilger et al. 2000; Gilger et al. 2001). One study performed on horses with naturally occurring uveitis showed that the implant prevented recurrence in 81% of horses. Only 3/16 horses had recurrences but those were shorter, less severe and less frequent (0.36 episodes/year vs. 7.5 episodes per year prior to surgery) (Gilger et al. 2001). Another study showed no more recurrences in 65% of cases with 78.2% being visual 12 months after surgery (Wilkie et al. 2001). Complications included intraocular hemorrhage in 44% of the cases, cataract formation (6 to 9%), secondary glaucoma (6%) and retinal detachment (6 to 13%) (Gilger et al. 2001; Wilkie et al. 2001).

#### Suprachoroidal implant

Recently, an implant allowing delivery of cyclosporine directly to the ciliary body was developed. The implant is a 6mm diameter disk placed into the suprachoroidal space under a scleral flap (Gilger et al. 2006). Adequate CsA concentrations were obtained 30 to 45 days after implantation of the device. The rate of recurrence was significantly reduced (from 0.54 to 0.09 episodes per month) as was the rate of blindness post implantation. Fifteen percent of eyes lost vision in a mean of 14 months post implantation. Significantly fewer complications such as retinal detachment were observed compared with intravitreal implants. Clinically, the implants release CsA for about 24 months.

Ideal candidates for CsA implantation are horses with uveitis that are well controlled by traditional medical treatment but suffer from frequent recurrences. Surgery should not be performed on horses with active inflammation. The implants are not currently commercially available and only a few institutions perform the procedure.

#### 1.4.2.3. Other

Phacoemulsification for the treatment of cataract secondary to ERU can be performed in an attempt to restore vision. Results are often poor due to the presence of new blood vessels on the iris and lens capsule. These vessels can bleed during surgery resulting in severe hyphema. In addition, surgical trauma usually precipitates further uveitic episodes. Cataract surgery can however be combined with another procedure such as cyclosporine implantation or pars plana vitrectomy.

When vision is lost and the eye is still painful, enucleation might be necessary for the animal's comfort. Enucleation can be performed using a trans-palpebral or trans-conjunctival approach; a prosthesis can be placed within the orbit to improve the cosmetic outcome. Alternatively,

intrasceral prosthesis placement can be performed in cases in which the fibrous tunic of the eye is healthy enough to support the prosthesis.

### **1.4.3. Vaccination**

Vaccination against leptospirosis is a controversial treatment that has been advocated in horses with ERU. In developed countries, vaccination is common for cattle, pigs and domestic dogs. Most bovine and porcine vaccines contain serovar hardjo and pomona. No leptospiral vaccine has been commercially developed for horses. The efficacy of a leptospiral vaccine in horses is difficult to verify as the acute leptospirosis usually is clinically inapparent, the protection of a vaccine is serogroup specific, and latency period until uveitis develops may be years. Thus, long-term studies following vaccinated and unvaccinated horses would be necessary to demonstrate a protective effect of the vaccine.

In one study in Germany, vaccination with a stable-specific killed vaccine (serovar Grippotyphosa) was performed in two stables with a high incidence of ERU (Wollanke et al. 2004). Seventy-six horses received at least the first vaccination and one booster injection. In all vaccinated ponies and horses, a significant humoral response was observed and no adverse reactions were seen. In both barns, no additional horses developed uveitis 5 years and 6 months after vaccination.

A study performed in the United States used an inactivated multivalent porcine *Leptospira* vaccine on 41 horses with ERU (Rohrbach et al. 2005). After the second vaccination, the interval between acute episodes increased significantly but the overall progression of the disease was unchanged. Data suggested that the vaccine had better efficacy if it was given during the active phase of the disease. The hypothesis is that active inflammation allows better intraocular penetration of antibodies. The study did not conclude a beneficial effect of vaccination.

Other measures such as a change of environment could decrease the horse exposure to the initiating agent and possibly decrease the rate of recurrence. Good preventative care such as regular foot care, deworming and vaccination could also minimize episodes (Gilger 2002). Anecdotic evidence also indicates that the use of multivalent vaccines, especially against Equine Influenza Virus, Equine Herpes Virus and *Streptococcus equi* might precipitate a uveitic episode (Cutler 2006). Some authors recommend vaccinating horses with ERU over several weeks (Gilger 2002).

### **1.4.4. Prognosis**

Prognosis will vary depending on the extent of ocular damage present when the horse is first diagnosed by a veterinarian. Visual prognosis for ERU should be at best guarded. Blindness is a common endpoint of ERU, attributable to additive consequences of multiple episodes of disease. Horses that become blind are difficult to manage and useless for any purposes except breeding, and are therefore often euthanized.



Vision was lost in one or both eyes in 44% of uveitis horses in one study (Dwyer et al. 1995). In another study, 20% of horses with ERU were blind bilaterally and 36% unilaterally (Gilger 2005). In cases of unilateral uveitis, if no inflammatory episode is noticed in the contralateral eye within 2 years, it is unlikely that uveitis will develop in that eye.

Serology for *Leptospira* serovar pomona and breed can be used for prognostic evaluation of the likelihood of blindness occurring in one or both eyes. The likelihood of having blindness in at least one eye within 11 years of the first attack was 100% for seropositive Appaloosas, 72% for seronegative Appaloosas, 51% for seropositive non-Appaloosas and 34% for seronegative non-Appaloosas. In seropositive horses, the odds of blindness were 4.4 times greater than in seronegative horses with ERU. In Appaloosa horses, the odds of blindness were 3.8 times than in non-Appaloosas with uveitis (Dwyer et al. 1995).

### **1.5. Immunology of ERU**

A dysregulated immune response was long suspected as a cause of ERU. Research has focused on the identification of infectious agents that may induce uveitis, such as bacteria, and particularly *Leptospira interrogans*, viruses and parasites. However many aspects of ERU point toward an immune mediated disease, such as the recurrence of inflammation, the positive response to corticosteroids and cyclosporine, and the lack of success of antibiotics (Deeg 2008).

The majority of infiltrating cells in the uvea of horses with ERU were identified as T cells, with a predominance of CD4+ T cells with a Th1 phenotype (Romeike et al. 1998; Gilger et al. 1999; Deeg et al. 2001). Analysis of mRNA collected from the eyes of ERU horses demonstrated the presence of mRNA for the Th1 cytokines Interleukin-2 and Interferon- $\gamma$  (Gilger et al. 1999). The observation of a deviant MHC class II antigen expression on resident ocular cells suggests that aberrant immune regulation plays a role in ERU (Romeike et al. 1998). The cells form characteristic lymph-follicle like structures in the uvea. Further studies showed that these T cells proliferate after stimulation with some intraocular autoantigen such as S-Ag or Interphotoreceptor Retinoid-Binding Protein (IRBP). Both proteins are expressed in the retina and pineal glands, and pinealitis has been reported in experimental and naturally occurring uveitis (Caspi et al. 1988; Kalsow et al. 1993; Kalsow et al. 1999). Septal areas of pineal glands from horses with uveitis had clusters of MHC class II antigen-expressing cells and T lymphocytes (Kalsow et al. 1993; Kalsow et al. 1999). Chemokines have been isolated from the ciliary epithelium that may play a role in the recruitment and activation of leukocytes in diseased eyes (Gilger et al. 2002). The importance of the inflammatory process in the pathogenesis of ERU is highlighted by the success of immunosuppressive therapy: treatment of affected horses with cyclosporine implants resulted in both a reduction of cytokines levels and improvement of the clinical disease (Gilger et al. 2000).

### 1.5.1. Targeted autoantigens

Current concepts to explain the origin and perpetuation of autoimmune diseases include molecular mimicry, bystander activation and epitope spreading (Deeg 2008). These mechanisms could appear independently or together and might even interact.

Several autoantigens probably participate in the pathogenesis of ERU. IRBP and S-Ag have been identified early on in the investigation of the disease (Deeg et al. 2001). Malate dehydrogenase (MDH) and Cellular Retinaldehyde-Binding Protein (cRALBP) were recently identified as autoantigens with 2DE Western blots using the retinal proteome as autoantigenic source (Deeg 2009). Both proteins induced uveitis with high incidence in Lewis rats (71% and 89%, respectively). Retinal architecture was widely destroyed (Deeg et al. 2006). MDH-induced uveitis also had a marked inflammatory component driven by invading CD3+ T cells (Deeg et al. 2008). In the horse, MDH injection did not induce uveitis despite considerable autoantibody formation and autoaggressive MDH-specific T cells. The same observation was made regarding S-Ag auto antigen (Deeg et al. 2004), which failed to induce uveitis in most horses inoculated despite being considered a major uveitic autoantigen in rats (Caspi et al. 1988). Horses also developed a high titer of anti-S-Ag antibodies and autoreactive T cells, but these T cells did not overcome the blood-retinal barrier. In contrast, CRALBP injection caused uveitis in 100% of horses and relapses could be induced in a predictable manner (Deeg et al. 2006), as it was possible with IRBP. These findings underscore the limitations of rodent models in investigations of ERU, and that data obtained in one animal model cannot be transferred to other species without further consideration.

Major retinal autoantigens, such as S-Ag, IRBP and cRALBP remained stably expressed during all stages of ERU, although their physiological expression sites within the retina were destroyed by inflammation. This finding explains why uveitic attacks persist even in severely damaged eyes.

### 1.5.2. Molecular mimicry

The hypothesis of an initiating infectious agent as trigger for an autoimmune disease is known as molecular mimicry: the immune response is directed against an infectious agent first, and then after clearing the infection, is falsely directed to a similar epitope of the target tissue (Thurau et al. 1997). Immune response to *Leptospira* proteins can be measured in eyes with ERU, although it is unclear if the response is generated in the eye or reflects the leakage of the blood-retinal barrier (Halliwell et al. 1985; Deeg et al. 2001; Deeg et al. 2007). A study from 1985, demonstrated partial antigenic identity between equine cornea and *Leptospira* (Parma et al. 1985). The same authors demonstrated in 1987 (Parma et al. 1987) that horses inoculated with *Leptospira* had antibodies in their serum, tears and aqueous humor, that cross-reacted with cornea. A DNA fragment from *L. interrogans* pomona codes for a 90kDa protein which cross-reacts strongly with a 66kDa equine corneal protein (Lucchesi et al. 1999). This same fragment was then found in other strains of *Leptospira*, belonging to serovars canicola,

icterohaemorrhagiae, pomona, pyrogenes, wolfii, bataviae, sentot, hebdomadis and hardjo (Lucchesi et al. 2002).

Two immunogenic lipoproteins of *Leptospira*, LruA and LruB, are expressed in the eye of uveitic horses and cross react with equine ocular tissue. LruA antiserum reacted with lens and ciliary body extracts, whereas LruB reacted very strongly with retinal extract (Verma et al. 2005). Lens proteins reacting with LruA antiserum were identified to be  $\alpha$ -crystallin B and vimentin, and retinal proteins reacting with LruB antiserum to be  $\beta$ -crystallin B2 (Verma et al. 2010). Recently, a novel leptospiral protein, LruC, was identified. LruC-specific antibody levels were increased in eye fluids and sera of uveitic horses (Verma et al. 2012). The role of molecular mimicry in the development of ERU is under further investigation.

### **1.5.3. Differentially regulated candidates in uveitis target tissues**

The molecular processes leading to retinal degeneration and blindness still remain unknown. One group tried to identify several differentially regulated proteins that are part of the pathway involved in the immune response by exploration of the intraocular proteomes of horses with and without ERU (Deeg et al. 2007; Hauck et al. 2007). One candidate that is up-regulated in the retina in ERU is complement component C3. Intraocular complement activation had a significant impact on disease activity in experimental autouveitis in rats (Jha et al. 2006; Jha et al. 2006). The pigment epithelium-derived factor (PEDF), produced by the RPE cells and retinal Mueller glial cells (RMG), is significantly down-regulated in uveitis to around 20% of the normal expression level (Deeg et al. 2007). PEDF operates as a regulator of inflammatory factors and suppresses endothelial permeability by protecting tight junction proteins. Down-regulation of PEDF is associated with appearance interferon- $\gamma$  in ERU (Hauck et al. 2007). It has been shown that RMG are activated in the disease process (Deeg et al. 2007; Hauck et al. 2007), as evidence by up-regulation of vimentin and glial fibrillary acidic protein and down-regulation of glutamine synthetase. Activated RMG down-regulate PEDF and express interferon  $\gamma$ , a TH-1 cytokine.

## **1.6. Leptospirosis and ERU**

### **1.6.1. Leptospirosis**

Leptospirosis is presumed to be the most widespread zoonosis in the world (Levett 2001). Clinical signs often include fever, renal and hepatic insufficiency, pulmonary manifestation and reproductive failure. Humans are incidental hosts and clinical presentation can vary between subclinical or mild infection (90% of cases) to severe illness characterized by jaundice, acute renal failure and bleeding (Weil's disease).

#### **1.6.1.1. Bacteriology**

Leptospirosis is a zoonotic bacterial disease with worldwide distribution caused by spirochetes of the genus *Leptospira*. The genus infects virtually all species of mammals, as well as reptiles and amphibians. The first formal report of the disease in humans was made by Adolf Weil in the 19<sup>th</sup> century. Subsequently, leptospirosis was identified in dogs, livestock and later in horses in 1947.

The genus *Leptospira* belongs to the family *Leptospiraceae*, order *Spirochaetales* that includes the genus *Borrelia* and *Treponema*, which are also pathogens. Leptospires are thin, tightly coiled and highly mobile spirochetes with hooked ends. They are normally about 0.1 to 0.2 µm in diameter and 6-20µm long. Two periplasmic flagella with polar insertions are located in the periplasmic space and confer both translational and non-translational forms of movement. Leptospires have a distinctive double-membrane architecture, sharing the characteristics of both Gram-positive and Gram-negative bacteria. The cytoplasmic membrane is closely associated with a peptidoglycan cell wall, which is overlaid by an outer membrane. Within the outer membrane, the LPS constitute the main antigen for *Leptospira*. It is structurally similar to Gram-negative bacteria LPS, but has a lower endotoxic potential, being up to 12 times less lethal for mice when compared with *E. coli* LPS (Adler et al. 2010). This is possibly related to the unusual features of its lipid A component. Despite the Gram-negative characteristics of leptospires, they do not stain well with conventional bacteriologic dyes. Therefore, other techniques, such as darkfield microscopy, silver staining or immunologic staining have been developed for the identification of leptospires.

Leptospires are obligates aerobes with an optimum growth temperature of 28-30°C. They grow in simple media which is usually enriched with vitamins B2 and B12, long-chain fatty acids as an energy source, and ammonium salts. The most widely used medium is based on the oleic acid, bovine serum albumin and polysorbate Ellinghausen, McCullough, Johnson, and Harris medium (EMJH). Growth of leptospires is often slow and can take up to 6 months. In semisolid growth media, growth reaches a maximum density in a zone beneath the surface where the oxygen tension is optimum (Dinger's ring).

Prior to 1989, the genus *Leptospira* was divided into two species based on phenotypic characteristics: *L. interrogans* comprising all pathogenic strains, and *L. biflexa* containing the saprophytic strains isolated from the environment. Both are divided into numerous serovars (over 60 for *L. biflexa* and over 200 for *L. interrogans*) defined by agglutination after cross-absorption with homologous antigen (Levett 2001). Serovars that are antigenically related have been grouped into serogroups. The phenotypic classification has been replaced by a genotypic one constituted of genomospecies that is more taxonomically correct. The genus *Leptospira* includes 13 pathogenic species (*L. alexanderi*, *L. alstonii*, *L. borgpetersenii*, *L. inadai*, *L. interrogans*, *L. fainei*, *L. kirschneri*, *L. licerasiae*, *L. noguchi*, *L. santarosai*, *L. terpstrae*, *L. weilii*, *L. wolffii*) and saprophytic species (*L. biflexa*, *L. meyeri*, *L. yanagawae*, *L. kmetyi*, *L. vanthielii*, *L. wolbachii*). The genomospecies of *Leptospira* do not correspond to the previous two species, and pathogenic and saprophytic serovars occur within the same genomospecies. Thus, neither serogroup nor serovar reliably predict the species of *Leptospira*. The serological classification is still widely used because of the lack of simple DNA-based identification methods (Levett 2001).

#### 1.6.1.2. Diagnostic testing

The definitive diagnosis of leptospirosis is problematic in that the fastidious nature of the organism leads to difficulties in the culture of this bacterium. Some serovars may take up to 6

months to culture before positive results are obtained. In addition, spirochetes die rapidly in tissue samples or body fluids unless maintained at 4°C. For successful isolation, fresh fluid or tissue homogenate should be inoculated in a special growth media (most often Ellinghausen, McCullough, Johnson, and Harris medium). For this reason, culture is not useful as a routine test for diagnosis of individual patients, but remains important for epidemiological purposes. Isolates can then be characterized using cross agglutination absorption protocol or more modern techniques such as 16S rRNA sequencing or multiple locus sequence typing.

Several PCR protocols for detection of leptospiral DNA have been developed. Most protocols are genus specific and detect both pathogenic and non-pathogenic species of *Leptospira*. Improved sensitivity has been achieved by quantitative PCR either using TaqMan probes or SYBR green fluorescence (Smythe et al. 2002; Adler et al. 2010).

Leptospire do not stain well with traditional haematoxylin and eosin techniques, but can be identified in fluids using dark-field microscopy or fluorescent antibody techniques. Giemsa and silver stain can be used on tissue section, although this is not always reliable.

As a result of the difficulties and limitation of direct identification of leptospire, diagnosis is often based on serology. The microscopic agglutination test (MAT) remains the gold standard for diagnosis. Both the sensitivity and specificity of MAT are very high; however, it is labor intensive with wide inter-laboratory variability. Cross-reaction commonly occurs and latent infections might not be identified. Paired titers are more useful, with a fourfold increase indicating recent infection. ELISA tests have been developed using a wide variety of antigen preparations. These ELISA assays obviate the need for the maintenance of live cultures necessary to perform MAT. However, the sensitivity and specificity of the ELISA test are lower than those of MAT.

#### 1.6.1.3. Leptospirosis in horses

Animals (including humans) can be divided into maintenance hosts and accidental hosts. Pathogenic leptospire persistently colonize the kidneys from reservoir animals, which eliminate the bacteria in the urine and contaminate the water and environment. The most important maintenance hosts are small mammals, which may transfer infection to domestic farm animals, dogs and humans. Different rodent species may be the reservoir of different serovars, but rats are generally maintenance hosts for the serovars of the serogroup icterohaemorrhagiae and ballum, and mice are host for the serogroup ballum. Dairy cattle may harbor serovars hardjo, pomona or grippotyphosa, pigs may harbor pomona, tarssovi, or bratislava, sheep may harbor hardjo and pomona and dogs canicola. Bratislava has been suspected to be host-adapted in horses. Host adapted serovars usually do not cause significant disease.

Leptospirosis is transmitted by the contact of abraded skin or mucous membranes with water or soil contaminated with urine from reservoir animals. Leptospirosis typically follows a biphasic course with a septicemic phase lasting 4-7 days, followed by an immune phase, characterized by

antibody production and excretion of leptospire in the urine. Most complications of leptospirosis are associated with localization of the bacteria within the tissues during the immune phase.

Leptospirosis is most commonly subclinical in horses, although it has been associated with several syndromes. Serovar bratislava in one study was not associated with hematologic or biochemical alteration but reproductive disorders (Pinna et al. 2010). Abortion in the last trimester is one of the most common manifestations of leptospirosis in horses. Abortions are most often sporadic but extreme weather conditions, especially flooding, can be responsible for clusters of cases (Kinde et al. 1996). The most common isolate involved is serovar pomona type Kennewicki but other serovars have been isolated from equine fetuses. The skunk is believed to be the maintenance host for this serovar, but raccoons, white tail deer and opossum can also be infected. One genetic variant seems to predominate in case of equine abortion whereas other genetic variants can also be found in wildlife (Timoney et al. 2011). The prevalence of *Leptospira*-induced abortion is between 2.5% to 4.4% (Donahue et al. 1991; Donahue et al. 1995). Several other rare syndromes have been reported such as acute renal failure (Hogan et al. 1996; Frellstedt et al. 2008) and respiratory failure in foals (Broux et al. 2012). One group found a possible relation between exercise induced pulmonary hemorrhage and seropositivity to serovar Copenhageni (Hamond et al. 2011).

Although clinical disease is rare, exposure to leptospire is common in horses. Horses are fed roughage, which is almost inevitably contaminated by rodent urine. Because horses are not vaccinated, the presence of these antibodies can only be explained by the occurrence of subclinical infection. The incidence is significantly higher in warmer climates due to longer survival of leptospire in the environment. The disease is seasonal with peak incidence in summer or fall in temperate region and during rainy seasons in warm-climate regions.

The prevalence of leptospiral exposure has been studied extensively (Table 1). Seroprevalence increases with age (Lees et al. 1994; Pilgrim et al. 1999) and exposure to the outdoors (Barwick et al. 1998; Barwick et al. 1998; Blatti et al. 2011) especially during the spring and fall season (Baverud et al. 2009; Jung et al. 2010; Blatti et al. 2011). In general, the chances of being seropositive rises by approximately 10% with each year of life (Blatti et al. 2011). Thoroughbreds and Standardbreds were also less exposed than other breeds, possibly because of their decreased exposure to outdoors compared to more rustic breeds (Pilgrim et al. 1999). The high seroprevalence in healthy horses indicates that they are often exposed to or infected with *Leptospira* spp. without developing signs of disease. Therefore, other laboratory and clinical data should always be taken into consideration when interpreting serological test results for *Leptospira* spp.

### **1.6.2. Leptospiral uveitis in human medicine**

Adolf Weil first reported ophthalmic complications of systemic leptospirosis in 1886. A significant concentration of serovar specific LPS was observed in the aqueous humor of human patients with leptospiral uveitis, suggesting an endotoxin mediated process (Priya et al. 2008).

Leptospiral uveitis commonly appears within 10 to 40 days after the febrile illness, but may be delayed up to several years. The prognosis for vision in ocular leptospirosis is often good, with complete recovery. Although the microagglutination test is considered to be the reference test for serologic diagnosis of leptospirosis, a negative result does not rule out the disease because the patient might be infected with a serotype absent from the battery of testing antigens. In a few cases, serology of the aqueous humor can remain negative. Recurrence is not a common feature but might be under diagnosed because some uveitic episodes are so mild that they can go unnoticed (Moro 1960). However, in one study from the south Pacific, the rate of recurrence was 46% (Mancel et al. 1999). The reported incidence varies from 2 to 40% (Levett 2001). A study prospectively following patients with leptospirosis in India showed that 18% developed uveitis, but only 20% of them had visual signs and none had recurrence (Pappachan et al. 2007). Recently, a large cluster of cases of uveitis was reported in India following an outbreak of leptospirosis after heavy flooding: in 73 patients with leptospiral uveitis, the pattern of ocular involvement was unilateral in 35 and bilateral in 38. In 95% of the cases, panuveitis was observed (Rathinam et al. 1997).

Table 1: Seroprevalence of *Leptospira* in horses

Country or State	Seroprevalence	Main serovar	Reference
United Kingdom	25.2%	Bratislava	Vet. Rec., 2007
Netherlands	72%	Copenhagi	Houwens, 2011
Portugal	37%	Australis	Rocha, 2004
Brazil	67% - 74%	Icterohaemorrhagiae	Hashimoto, 2007 Jorge, 2011
Korea	25%	Sejroe	Jung, 2010
Mongolia	2-31%	Bratislava, Hardjo	Odont, 2005
Australia	33%	Pomona	Slatter, 1982
Switzerland	58.5%	Pyrogenes	Blatti, 2011
Italy	11.4%	Bratislava	Cerri, 2003
Northern Ireland	89.1%	Bratislava	Ellis, 1983
California	27.3%	Pomona	Verma, 1977
Ohio	33.6%	Bratislava	Pilgrim, 1999
NY	56%	Bratislava	Barwick, 1998
Kentucky	10-68%	Bratislava	Williams, 1994

### 1.6.3. Evidence for leptospirosis as a cause of ERU

An association between leptospirosis and ERU was first demonstrated in Germany by Rimpau in 1947 (Rimpau 1947). It was further substantiated by the observation that in 2 farms where outbreaks of clinical leptospirosis were observed, most horses developed ERU within 24 months (Roberts 1958). Subsequently, Williams et al. inoculated 2 groups of ponies with *L. interrogans* serovar pomona and observed ocular changes over several years. Sixty one percent of eyes developed recurrent uveitis resulting in varying degrees of ocular damage (Williams et al. 1971).

#### 1.6.3.1. Europe

One study from the UK showed that the prevalence of *Leptospira* titers among cases with uveitis (11.1%) was not significantly different from the control cases (9%) (Matthews et al. 1987).

Brem et al. was the first group to report the isolation of live leptospire from vitreous samples of 4 horses affected by ERU (Brem et al. 1998) in Germany. Vitreous material from 42 and serum samples from 40 horses were tested for antibodies to *Leptospira* by MAT and positive titers were found in 81% of vitreous samples, 83% of sera, and 91% of horses. The same authors were able to isolate *Leptospira* in 35 out of 130 vitreous samples (26.9 %) in a later study (Brem et al. 1999). These isolates belong to the grippotyphosa serogroup (n = 31) and to the australis serogroup (n = 4). Vitreous samples and one serum sample from each horse were also tested for leptospiral antibodies using MAT. Seventy percent of vitreous samples and 82% of serum samples were positive.

Another study in 1998 showed that *Leptospira* titers were higher in ocular fluid than in serum in 57% of 150 horses undergoing vitrectomy (Wollanke et al. 1998). In this study, 50/227 horses with ERU had serum titers  $\geq 1:800$ , vs. 24/97 normal horses (25%). In undiluted vitreous samples from 20 horses with clinically normal eyes, no antibody titers to *Leptospira* could be detected. Among 150 horses with ERU, 60% had positive vitreous antibody titers.

The largest study of its kind was performed by Wollanke et al. in 2004, where vitreous samples from 426 eyes suffering from ERU were evaluated (Wollanke et al. 2004). In serum samples, there were no significant differences in the occurrence and level of antibodies against leptospire between sound horses and horses with ERU. In 3/54 (6 %) of vitreous samples from normal eyes and in 382/426 (90 %) of vitreous samples from eyes suffering from ERU, MAT testing could detect antibodies. Calculation of the Goldman-Witmer Coefficient proved intraocular antibody production in 34/36 (94 %) eyes ( $C > 8$ ). Positive culture results were observed in 189/358 (53 %) vitreous samples from eyes suffering from ERU and in none of 41 vitreous samples from sound eyes. Positive culture results were seen in vitreous samples from eyes with a history of ERU of only a few weeks as well as in samples from eyes with a history of ERU for several years. In 18/189 (9.5 %) of horses with positive culture, serology was negative. PCR showed leptospiral DNA in 39/55 (71 %) of vitreous samples from eyes with ERU. Re-examined vitreous samples showed decreasing antibody titers with increasing intervals after vitrectomy and one year after surgery, MAT failed to detect antibodies. The authors stated that in horses suffering from ERU an intraocular leptospiral infection is present, which causes infection-associated autoimmune phenomena until the intraocular leptospiriosis is eliminated by vitrectomy.

In 2004, one group isolated leptospire from 32.2% of the intraocular samples collected from 501 horses (Hartskeerl et al. 2004) originating from various European countries. Seventy-eight percent of the isolates belonged to serogroup grippotyphosa, 14.2% to serogroup australis, 3.6% to serogroup sejroe, 2.5% to serogroup pomona and 1.5% to serogroup javanica. A more recent



study showed that in 66% of 90 horses with ERU, leptospire could be detected by PCR in vitreous humor (Von Borstel et al. 2010).

In one study, detection of leptospire by electron microscopy in the vitreous humor was attempted but was only successful in less than 10% of cases (Niedermaier et al. 2006), suggesting that histological methods are not adequate for the diagnosis of ocular infections.

#### 1.6.3.2. North America

In a study from NY state (Dwyer et al. 1995), 56% of horses with uveitis were seropositive to serovar pomona versus 9% of horses without uveitis. Seropositive horses were 13 times more likely to have uveitis. Horses with uveitis that were seropositive were 4.4 times more likely to be blind than horses that were seronegative. In this study, 30% of seropositive horses without complaint of uveitis also had evidence of subclinical uveitis on ophthalmic examination.

In another study from California (Faber et al. 2000), 70% of horses with uveitis and 3% of normal horses had leptospiral DNA in the aqueous humor, detectable by PCR. Culture was positive in 6 affected horses (22.2%): 4 of the isolates were identified as serovar pomona, and 2 were unidentified. There was no difference between the rate of seropositivity of ERU horses (85.7%) and control horses (62%). Fifty-seven percent of ERU cases had titers above 400 vs. 12% of the controls. Positive titers for serovar pomona were also significantly associated with uveitis. In a small study from Quebec, 3/12 eyes with ERU were PCR positive for *Leptospira* (Pinard et al. 2005).

In a histological study, no leptospiral DNA could be detected by PCR in fixed eyes affected with end-stage ERU (Pearce et al. 2007). Two out of ten ERU eyes exhibited positive immunoreactivity to leptospiral antigens, but the difference with control was not significant. The authors hypothesized that the DNA could have been fragmented or lost during the fixation process or that the organism was not present anymore because of the late stage of the disease.

However, a more recent study from Gilger et al. failed to identify bacterial DNA in the aqueous humor of horses with ERU from the southeastern United States (Gilger et al. 2008). Only 2 horses with ERU had evidence of intraocular antibody production. No significant difference was found in titers of *Leptospira* antibodies in serum or aqueous humor between ERU and normal horses. The authors concluded that the continued presence of *Leptospira* did not play a direct role in the pathogenesis of ERU in this area of the United States.

#### 1.6.3.3. Other

One study out of Iran compared PCR and antibody detection using ELISA on serum of 31 horses with ERU and 30 healthy horses. In horses with ERU, 22.5% of serum samples were positive by PCR and 16.3% by ELISA (Kojouri et al. 2009). None of the control horses were positive, supporting a role of leptospirosis in ERU in this area of the world as well.

In Brazil, 199 horses were studied (Braga et al. 2011). A total of 107 (53.8%) horses were seropositive for *Leptospira*, 54 had high ( $\geq 800$ ) titers, of which 44 were against serovar Icterohaemorrhagiae. Forty-two out of these 44, plus 40 seronegative horses (titers  $\leq 100$ ) were given detailed ophthalmic examinations. Over 90% of seropositive horses had ophthalmic alteration consistent with uveitis, whereas 80% of seronegative horses had no abnormal findings. Thus ocular alterations were significantly more frequent in seropositive horses.

In summary, leptospirosis has been linked to ERU in numerous geographical areas but not in the southeastern US. Live bacteria could be cultured from eyes at all stages of uveitis, suggesting that the bacteria adapt well to this nutrient poor environment and are able to escape the immune response. The exact role of leptospirosis in ERU has not been elucidated. Hypotheses include a direct effect of the bacteria, endotoxin mediated or molecular mimicry inducing an auto-immune response to ocular antigens.

## CHAPTER TWO. ROLE OF INTRAOCULAR LEPTOSPIRA INFECTIONS IN THE PATHOGENESIS OF EQUINE RECURRENT UVEITIS IN THE SOUTHERN UNITED STATES

### 2.1. Materials and methods

**Animals:** Twenty-one horses with no history of or clinical findings compatible with ERU or ocular inflammation, which were donated to Louisiana State University, were used as control horses (group 1; n=21). Adult horses with clinical signs and history compatible with ERU, donated to LSU because of blindness, chronic pain or loss of use, were studied prospectively (group 2; n=15). The medical records of all horses in which uveitis had been diagnosed and that underwent aqueocentesis or vitreocentesis for diagnosis purposes at the Louisiana State University Veterinary Teaching Hospital between September 2006 and March 2012 were reviewed (group 3; n=17). Horses were considered to have ERU if the history was consistent with recurrent episodes of intraocular inflammation and three or more of the following clinical signs were present on ophthalmic examination: low intraocular pressure, corneal edema, scarring or neovascularization, aqueous flare, hypopyon, miosis, corpora nigra atrophy, iris hyperpigmentation and fibrosis, synechiae, cataract, vitreal cellular infiltrate and retinal degeneration. The use of animals was approved by the Louisiana State University Institutional Animal Care and Use Committee.

**Ophthalmic examination:** Ophthalmic examinations were performed on all horses by a board-certified ophthalmologist. Menace, dazzle, palpebral, oculocephalic and pupillary light reflexes were determined. Horses were sedated with xylazine (0.3-0.5mg/kg). The palpebral branch of the auriculopalpebral nerve was blocked bilaterally by injecting 1.5 mL of 2% lidocaine when necessary for complete examination. Schirmer tear testing and fluorescein staining were performed. Intraocular pressure was determined using a Tono-Pen (Oculab®) after topical anesthesia of the cornea using 0.5mL of proparacaine hydrochlorate. The eyelid margin, cornea, anterior chamber, iris and lens were evaluated by direct examination and biomicroscopy using a slit-lamp (Kowa SL-14®). Tropicamide (0.5 mL per eye) was applied topically to dilate the pupil and allow examination of the vitreous and ocular fundus by indirect ophthalmoscopy. Eyes were classified as being in an early stage if no severe chronic changes were present and the vision was intact. Chronic stage was defined as eyes with advanced disease but no significant vision loss (early cataractous changes, synechiae, corneal scarring). Eyes where vision was lost irreversibly as a result of chronic uveitic changes (such as mature or hypermature cataract, retinal detachment, glaucoma or phthisis bulbi) were classified as end-stage. Eyes were also classified as in an acute or quiet phase based on the presence of aqueous flare and cells, vitreal cellular accumulation or fibrin formation.

**Sample collection and processing:** Blood was collected by jugular venipuncture. Serum was separated within 2 hours after collection and refrigerated.

Horses from group 1 and 2 were euthanized prior to ocular sample collection by IV injection of a barbituate euthanasia solution (10mL/50kg) after sedation with xylazine (0.5-0.7mg/kg). Following euthanasia, approximately 2 mL of aqueous humor was collected after aseptic surgical preparation of the eye by inserting a 25-gauge needle through the limbus into the anterior chamber, then slowly aspirating (Fig. 2). Vitreous humor was then collected by inserting an 18-gauge needle 10 mm caudal to the dorsal limbus and aspirating 2 mL of vitreous humor from the central vitreous body (Fig.3). The eyes were then enucleated using a transconjunctival technique and placed in 10% formalin.

For sampling of ocular fluids, horses in group 3 were either sedated with a combination of an  $\alpha$ 2-agonist and butorphanol (for sampling of non-visual eyes) or anesthetized with an  $\alpha$ 2-agonist followed by administration of ketamine and diazepam (for sampling of visual eyes). Aqueous or vitreous humor was sampled aseptically using varying techniques depending on the procedure performed: diagnostic aqueocentesis, pars plana vitrectomy, or posterior chamber injection of antibiotics.

*Leptospira* antibody titers: One milliliter of aqueous humor (or vitreous when aqueous volume was not sufficient) was frozen at  $-80^{\circ}\text{C}$  immediately after collection until analyzed. One ml of serum was also analyzed immediately after collection. The microscopic agglutination test (MAT) was used to determine antibody titers in the serum and aqueous/vitreous humor samples collected. Samples were evaluated for antibodies against serovars pomona, grippotyphosa, icterohaemorrhagiae, canicola, and hardjo. Titers were reported as the reciprocal of the highest dilution in which 50% of the leptospire were agglutinated. Titers  $\geq 100$  were considered diagnostic. Testing was performed by the Louisiana Animal Disease Diagnostic Laboratory at the Louisiana State University Campus. Horses with antibody titers in ocular fluid that were higher than antibody titers in serum were defined as having intraocular antibody production. Two different criteria were evaluated: ratio of 1 if ocular titer/serum titer  $> 1$  and ratio of 4 if ocular titer/serum titer  $> 4$ .

*Leptospira* qPCR: A minimum of 0.5 ml of aqueous humor, vitreous humor or both when available was collected in EDTA and frozen at  $-80^{\circ}\text{C}$  until processing. The samples were shipped overnight on dry ice to the Real-time PCR Research and Diagnostics Core Facility at UC Davis, California. Real-time PCR (qPCR) was used to detect *Leptospira* DNA in samples obtained. This test is a genus-specific test, and evaluates for numerous genomospecies including: interrogans, kirschneri, sanatosai, noguchii, weilii, and borgpetersenii and their associated serovars. DNA was extracted from samples using standard phenol-chloroform extraction and quantified by spectrophotometry to ensure uniform DNA concentration between samples. Sample DNA was used as a template and amplified by real-time PCR. A pair of single-stranded DNA primers (forward and reverse) was used to amplify a target sequence of *Leptospira* DNA (proprietary primers, UC Davis). A TaqMan probe (fluorescent probe) is added to visualize PCR products during amplification. Utilizing a Quiagen<sup>®</sup> thermal cycler, the reaction parameters are as follows:  $95^{\circ}\text{C}$  for 5 minutes (for the first cycle, 2 minutes for additional cycles),  $60^{\circ}\text{C}$  for one minute, then

72°C for 2 minutes for a total of 40 cycles. An ABI Prism 7700 spectrophotometer was used to visualize the PCR product. Curves of fluorescence per sample were generated and compared to a standard curve of control DNA.

*Leptospira* culture: One milliliter of fresh aqueous and/or vitreous was placed in 9ml of transport media immediately after collection. The transport medium was composed of 87mg KH<sub>2</sub>PO<sub>4</sub>, 664mg of Na<sub>2</sub>HPO<sub>4</sub> and 1% bovine serum albumin. One hundred microliters from the inoculated transport medium was deposited on the surface of EMJH (Johnson and Harris modification of Ellinghausen-McCullough) culture medium containing 0.1mg/mL of 5-Fluorouracil to suppress contaminants. Prepared media was autoclaved to reduce the chance of obtaining saprophytic *Leptospira* growth. Samples were sent overnight on ice to the Infectious Bacterial Diseases Research Unit, Ames, IA. Upon receipt, sample were transferred to semi-solid EMJH medium and incubated at 29°C. Plates were examined for growth after 7, 14, 21 and 28 days of incubation and thereafter monthly for 6 months using darkfield microscopy. Isolates were typed to the serogroup level by microagglutination. Cultures were reported as negative if no growth was observed after 6 months.

Aerobic culture: Aqueous and vitreous were collected on a sterile swab and submitted to the Louisiana Animal Disease Diagnostic Laboratory at the Louisiana State University Campus for aerobic culture. Samples were cultured on Blood agar plate and McConkey agar plate at 35-37°C. Media were examined for growth every 24 hours. If no growth was observed after 72 hours, the results are reported as “No Bacteria Isolated”.

Histology: Samples were fixed in 10% neutral buffered formaldehyde, dehydrated in ascending concentration of ethanol and xylene and routinely embedded in paraffin. Section of 5-micron thickness were prepared from tissue blocks and evaluated by a single observer (R.C.) following routine hematoxylin and eosin (HE) stain.

Statistical analysis: The horses were classified into control and uveitis groups. Gender, breeds and status of the serology, qPCR, culture, ocular titers and ocular antibody production of both groups were compared using the Fisher exact test. The actual serum titer values for each serovars and the age were compared using Wilcoxon rank sum tests. The association between combinations of variables including culture results, status of serology, presence of intraocular antibodies, ocular antibody production, qPCR, blindness, and presence of unilateral or bilateral disease was tested by use of the Fisher exact test. SAS was used for statistical analysis. Values of  $p \leq 0.05$  were considered significant.

## **2.2.Results**

### **2.2.1. Horses**

In the control group, 21 horses (40 eyes) were studied. Age ranged from 1.5 to 21 years (mean 9.3 years, median 9 years). The group was composed of 9 geldings, 9 mares and 3 stallions. Breeds represented included American Quarter Horse (n=9), Thoroughbred (n=6), Paint Horse

(n=3), Arabian (n=2) and Tennessee Walking Horse (n=1). Horses originated from the states of Louisiana (n=20) and Mississippi (n=1). The most common cause for donation included lameness, chronic laminitis and soft tissue tumors.

Group 2 (ERU donation) was composed of 14 horses (25 affected eyes), age 6 to 26 years (mean 16 years, median 17 years). Breeds represented included American Quarter Horses (n=10), Appaloosas (n=2), Thoroughbred (n=2) and Arabian (n=1). Ten mares, 2 geldings and 2 stallions were present. Horses originated from the states of Louisiana (n=7), Mississippi (n=3), Texas (n=3) and Missouri (n=1).

Groups 3 (ERU client-owned) was composed of 17 horses (29 affected eyes, 21 sampled eyes), age 2 to 20 years (mean and median 10 years). Breeds represented included American Quarter Horses (n=11), and one of each of the following: Thoroughbred, Appaloosas, Dutch Warmblood, Shire, Paint Horse and Morgan. The group consisted of eight geldings, eight mares and 1 stallion. Horses originated from Louisiana (n=12), Arkansas (n=2), Kansas (n=1), Mississippi (n=1) and Texas (n=1).

Group 2 and 3 were grouped as a uveitis group. Mean age was 12.7 years, median 13 years. There was no significant difference in the breed, age, or sex distribution between uveitis and control horses.

In the horses admitted to the LSU VTHC between September 2006 and March 2012, 38% were American Quarter Horses, 42% were Thoroughbreds and 2.6% were Appaloosas. In the uveitis group, 70% were American Quarter Horses, 9.6% were Thoroughbreds and 9.6% were Appaloosa horses.

### **2.2.2. Ophthalmic examination**

The ocular findings for horses with uveitis are detailed in Table 2. In all cases, at least 3 signs of uveitis were present, which combined with history, allowed us to make a diagnosis of ERU in those horses.

A total of 31 horses with 54 affected eyes were examined. Disease was bilateral in 23 horses and unilateral in 8 horses. Twenty of the horses examined were non-visual in one or both eyes at the time of first examination (seven bilaterally, thirteen unilaterally) (Fig. 4 to 8).

Fourteen eyes out of 54 were in a quiet phase (26%) and 40 eyes (74%) had evidence of active inflammation at the time ocular samples were taken. Nineteen eyes out of 54 (35.2%) were in early stage of the disease, ten eyes (18.5%) were in chronic stage and 25 eyes (46.3%) were end-stage.

A total of 46/54 uveitic eyes were sampled. Twelve of these eyes were in a quiet phase (26%) and 34/46 had evidence of inflammation (74%). Seventeen eyes had early stage disease (37%), 9 were in a chronic phase (19.5%) and 20 had end stage disease (43.5%).

In the control group, non-significant ocular findings included: iris to iris persistent pupillary membranes, heterochromia iridis, punctate incipient cortical cataract, cystic corpora nigra and mild contact keratitis in 2 cases with a periocular neoplasm.



Figure 4: Miosis, iris color change and corneal neovascularization during an acute episode of uveitis (Horse 322 OD).

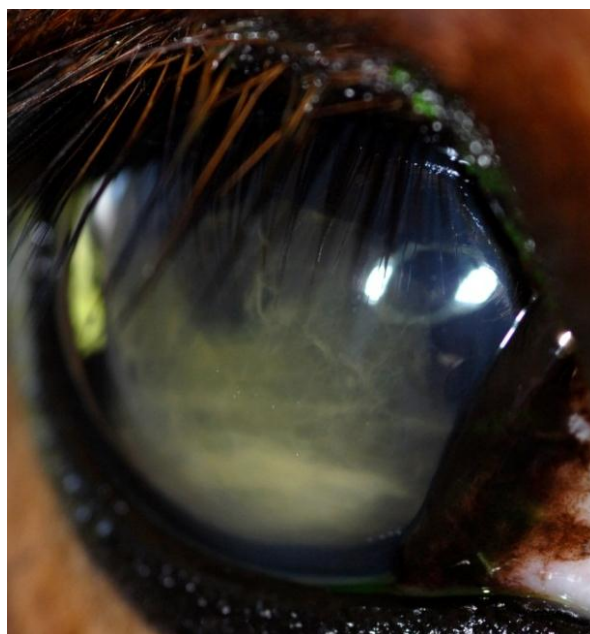


Figure 5: A horse with acute uveitis demonstrating fibrin in the anterior chamber (Horse 351 OD).

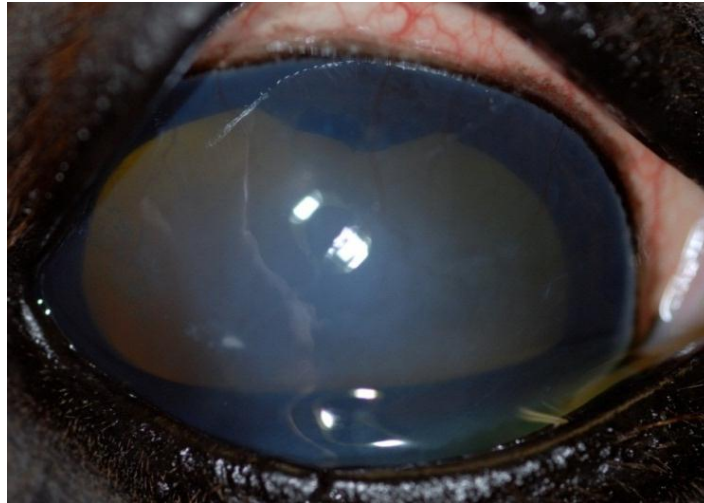


Figure 6: A horse with chronic ERU, showing glaucoma, posterior lens luxation, mydriasis, atrophy of the corpora nigra, corneal edema and neovascularization (Horse 357 OD).



Figure 7: Dyscoric pupil, corpora nigra atrophy, posterior synechiae, corneal scar and hypermature cataract in an eye with chronic ERU (Horse 333 OS).





Figure 8: Chronic end-stage ERU in an eye with phthisis bulbi, dense hypermature cataract, corneal edema, scarring and vascularization (Horse 357 OS).

Table 2: Ophthalmic examination findings in horses with ERU.

<b>Ophthalmic Findings</b>	<b>Number of eyes affected in Group 2</b>	<b>Number of eyes affected in Group 3</b>
<b>Glaucoma</b>	1	0
<b>Phthisis bulbi</b>	10	6
<b>Low IOP</b>	16	20
<b>Corneal edema or scarring</b>	12	5
<b>Aqueous flare or cells</b>	16	18
<b>Iris color change</b>	11	2
<b>Corpora Nigra atrophy</b>	12	2
<b>Lens luxation</b>	5	0
<b>Cataracts</b>	19	7
<b>Vitreous degeneration/Vitritis</b>	2	16
<b>Hyperemia of the optic nerve head</b>	1	1
<b>Haab's striae</b>	2	1
<b>Keratic precipitates</b>	7	8
<b>Corneal neovascularization</b>	8	8
<b>Posterior synechiae</b>	14	12
<b>Anterior synechiae</b>	5	0
<b>Hypopyon, fibrin</b>	3	5
<b>Episcleral injection</b>	5	11
<b>PIFM</b>	6	4
<b>Miosis</b>	5	8
<b>Fundus depigmentation/ scars</b>	1	2
<b>ONH atrophy</b>	0	1
<b>Peripapillary retinal folds</b>	0	5
<b>Retinal detachment</b>	0	5

### 2.2.3. Histological findings

Histology was performed on 25 affected eyes from group 2, 5 affected eyes from group 3 and all 40 control eyes. Examination was also performed in 2 unaffected eyes from horses from group 2 with unilateral disease with no abnormal histological findings.

Control eyes: Corneal vascularization of the superficial corneal stroma was present in 3 eyes (including 2 cases with mild contact keratitis from a periocular neoplasm). Seven eyes had evidence of mild lymphoplasmacytic conjunctivitis close to the limbus, bilaterally except for 1 eye. Foci of posterior scleral mineralization were noted in four eyes. In four eyes, a single focus of lymphoplasmacytic inflammation was noted in the ciliary body (n=2) or choroid (n=2). No other inflammatory changes were noted in those eyes. Focal cortical cataract was observed in 2 eyes.

Affected eyes: One eye had buphthalmia and 9 had phthisis bulbi. Twenty-three eyes had corneal vascularization. Seven eyes showed focal mild LP conjunctivitis. Scleral degenerative changes were noted in 15 eyes (hyalinization and/or mineralization). In one case, focal mineralization of the extraocular muscles was noted. Pre-iridal fibrovascular membranes were evident in 22 eyes, in 6 cases bilaterally. Pre and post iridal membranes were seen in 10 eyes. Some end-stage eyes were difficult to assess due to collapse of the anterior chamber. Synechiae were noted in 12 eyes (anterior in 10, posterior in 2). Seven eyes had evidence of retrocorneal membrane formation (Fig. 9). No lens was available in sections from 2 eyes. Focal cortical cataract was noted in 3 eyes, and advanced hypermature cataract with foci of mineralization in 23 eyes from 14 horses. Luxation and/or rupture of the lens capsule were noted on histopathology in 5 eyes.

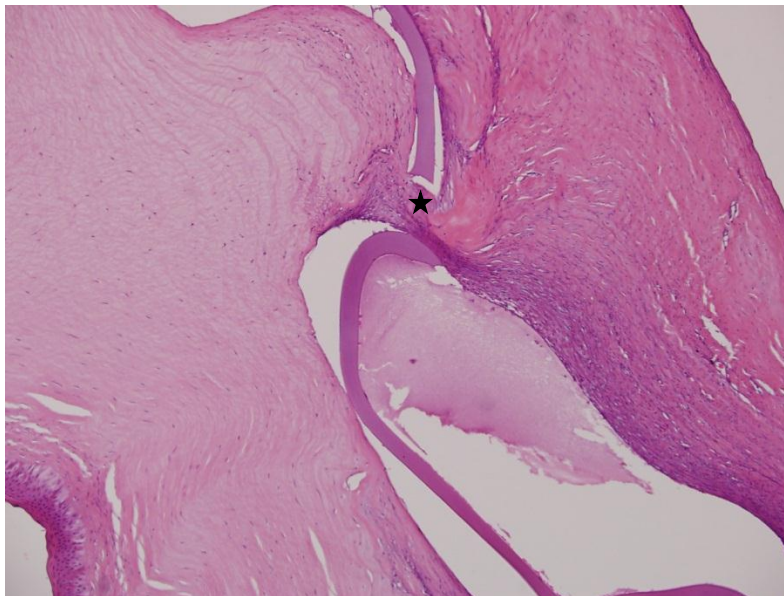


Figure 9: Break in Descemet's membrane (star) with associated retrocorneal membrane in an eye with end stage ERU (HE stain, 10x magnification).

The distribution of inflammation was described as:

- Anterior uveitis only (n=2) or anterior uveitis with associated vitritis (n=2), if the ciliary body and iris only were affected.
- Panuveitis (n=1) if the choroid was also involved
- Endophthalmitis (n=3) when panuveitis spread to the aqueous/vitreous humor and retina
- Panophthalmitis (10) when the inflammation also involved the fibrous tunic (cornea and sclera)
- End-stage (n=10) when the advanced state of phthisis bulbi made identification of all intraocular structures difficult.

Three histological criteria have been described for the diagnosis of ERU: linear eosinophilic inclusions in non-pigmented epithelium of the ciliary body (Fig. 10), accumulation of lymphocytes, plasma cells within the non-pigmented ciliary body epithelium (Fig. 11) and presence of hyaline, acellular material adherent to the inner aspect of the non-pigmented ciliary body epithelium (Fig. 12). Twenty-eight eyes with uveitis met all of the criteria. In 2 eyes with phthisis bulbi, the intraocular structures were too atrophied and disorganized in order to meet all of the necessary criteria.

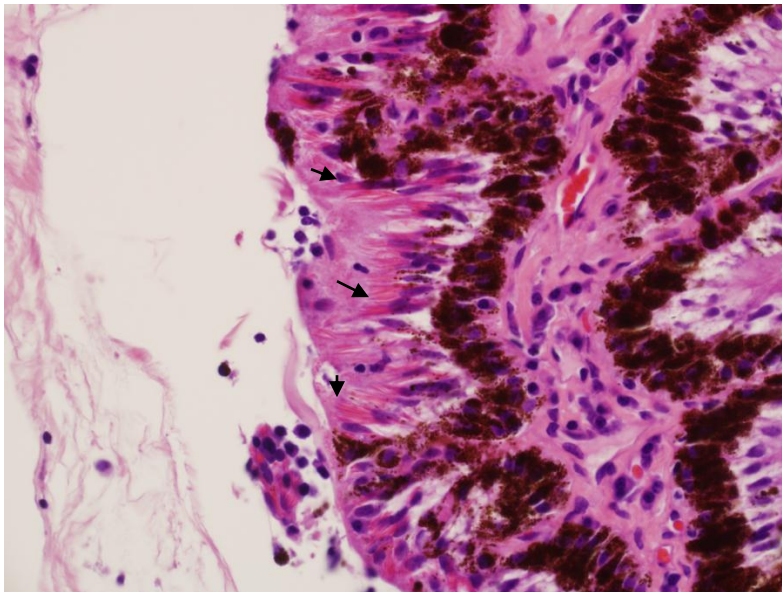


Figure 10: Eosinophilic linear inclusions (arrows) in the nonpigmented epithelium of the ciliary body in an eye with chronic ERU (HE stain, 50x magnification).

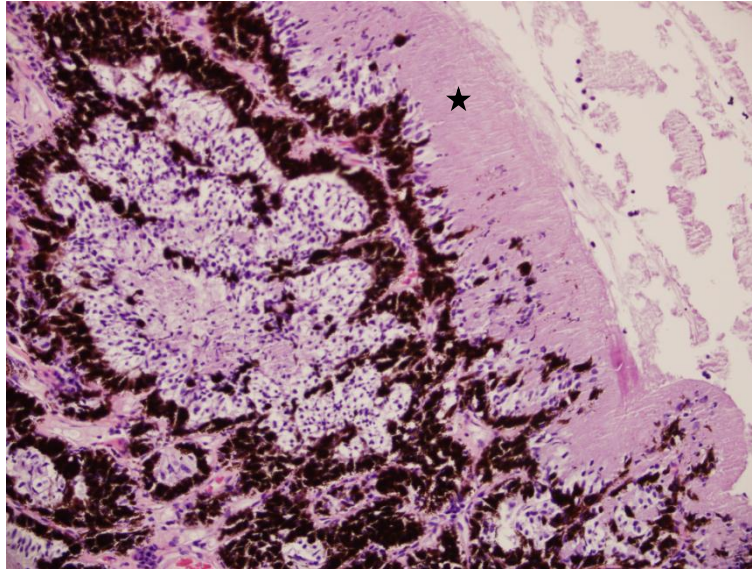


Figure 11: Hyaline material (star) along the nonpigmented epithelium of the ciliary body in an eye with end stage ERU (HE stain, 20x magnification).

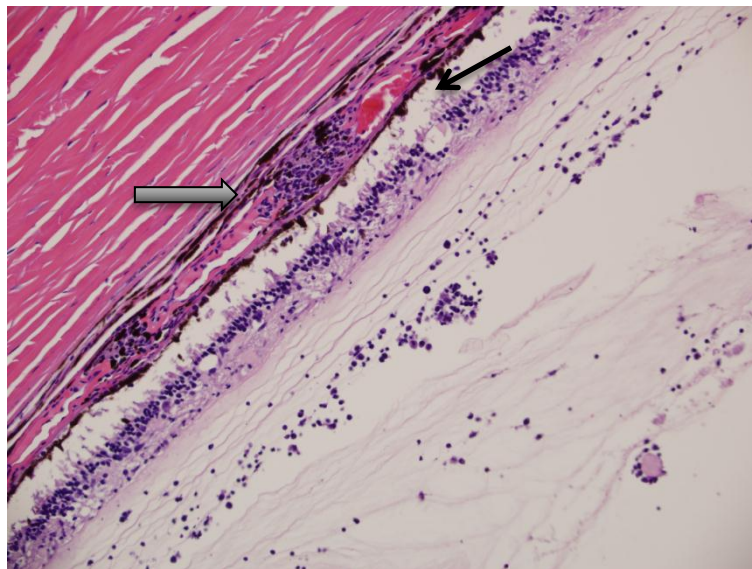


Figure 12: Lymphocytic follicle formation in the choroid (large arrow) and retinal atrophy (small arrow) with inflammatory infiltrate in an eye with end stage ERU (HE stain, 20x magnification).

Additionally, linear eosinophilic inclusions were identified in non-traditional locations: retina (7 eyes), optic nerve head (5 eyes) and both (8 eyes). In 4 eyes, recognizable retinal tissue was not identified and in 4 eyes no sections of ONH were available for evaluation. In general, with phthisical eyes and subsequent atrophy of the non-pigmented epithelium, it was harder to find inclusions and more hyaline material and less lymphocytic/plasmacytic inflammation was noted.

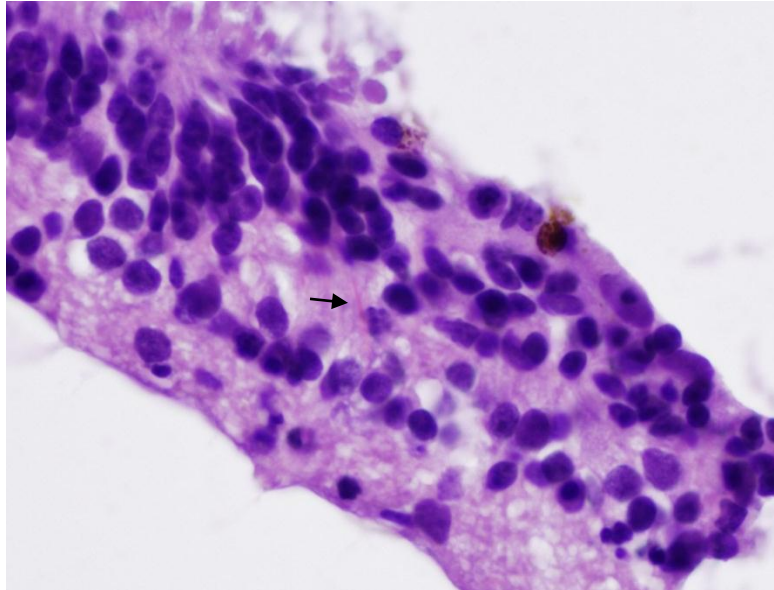


Figure 13: Eosinophilic linear inclusion in the retina (arrow) in an eye with chronic ERU (HE stain, 50x magnification).

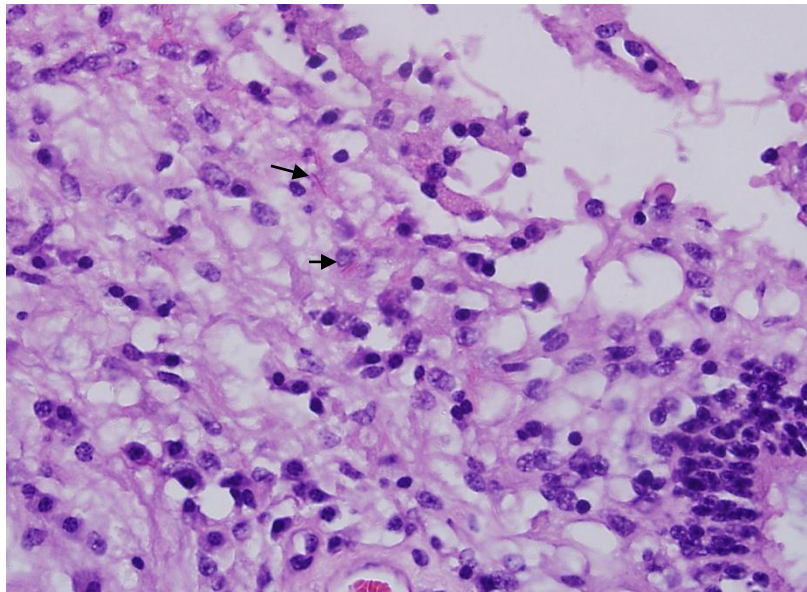


Figure 14: Linear eosinophilic inclusions (arrows) in the optic nerve head in an eye with end stage ERU (HE stain, 60x magnification).

#### 2.2.4. Serology

Serology was performed in all control horses and 27/31 horses with ERU. The number of uveitis horses (23 out of 27; 85%) and control horses (16 out of 21; 76%) that had serologic titers for

*Leptospira*  $\geq$  1:100 for at least one serovar was not significantly different ( $p=0.21$ ). The number of uveitis horses (19 out of 27; 73%) and control horses (7 out of 21; 33%) that had serologic titers for *Leptospira*  $\geq$  1:100 for more than one serovar was significantly different ( $p=0.009$ , OR: 4.7 (95% CI: 1.3-16.2)) (Fig. 15). Thirteen horses with uveitis and 8 control horses had a titer  $\geq$ 400. This difference was not significant ( $p=0.18$ ). Horses with uveitis had significantly higher titers for serovar grippotyphosa ( $p=0.05$ ) and pomona ( $p=0.006$ ) and were more likely to be seropositive to serovar pomona ( $p=0.008$ ) and grippotyphosa ( $p=0.05$ ) (Table 3). All titers were  $\leq$ 800.

Table 3: Number of horses (%) with positive serum titers to different serovars and mean titers, determined by MAT

Serovar	Number of Horses		Mean titer	
	Control	Uveitis	Control	Uveitis
Bratislava	13/21 (61.9%)	19/27 (70.3%)	190	203
Canicola	5/21 (23.8%)	5/27 (18.5%)	61	29
Grippotyphosa	1/21 (4.7%)*	7/27 (25.9%)*	9*	74*
Hardjo	6/21 (28.5%)	6/27 (22.2%)	52	55
Icterohaemorrhagiae	3/21 (14.2%)	7/27 (25.9%)	23	70
Pomona	2/21(9.5%)*	12/27 (44.4%)*	19*	152*

Values followed by \* indicate a significant difference between control and uveitis horses.

### 2.2.5. Culture

Aerobic culture was positive in 1 control horse (*Bacillus* spp. and *Micrococcus* spp.) and 1 uveitis horse (*Streptococcus* spp.). In both cases, contaminants were suspected, as all are part of the normal conjunctival flora. Bacteria were not identified by gram stain performed on histological sections.

Six of 28 horses with uveitis (21.4%) and none of the control horses ( $n=21$ ) had a positive leptospiral culture of ocular fluid. This difference was significant ( $p=0.03$ ). Four isolates were identified as belonging to the serogroup Pomona, and two to serogroup Grippotyphosa. All horses with positive culture had bilateral ERU and the four horses that had both eyes sampled were positive in one eye only. Three eyes were quiet and 3 had active inflammation at the time of sampling. One eye was end stage, 3 were in a chronic stage and two had early changes of ERU.

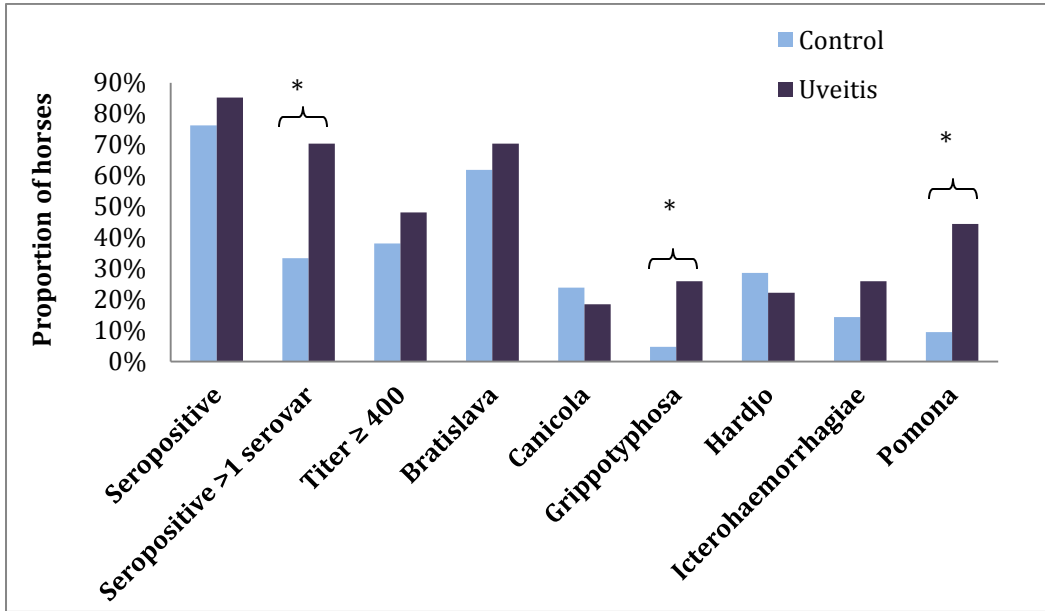


Figure 15: Serology results in control and uveitis horses as determined by MAT. Stars (\*) indicate values that are significantly different between uveitis and control horses.

### 2.2.6. Real-Time PCR

Real-Time PCR was performed on all eyes sampled (46 eyes in 31 horses with uveitis and 40 eyes from 21 control horses). Fourteen of 31 horses with uveitis (45.2%) and none of the control horses (n=21) had positive *Leptospira* qPCR on ocular fluid. This difference was statistically significant (p=0.0001). Forty-six eyes from horses with ERU were sampled and 21 were positive (45.6%). Seven horses were positive bilaterally, one unilaterally, and 6 horses only had one eye sampled.

All but one horse with a positive qPCR had bilateral disease. Four out of the 21 eyes with a positive qPCR result were in a quiet phase (19%) and 17 eyes had evidence of active inflammation (81%). Ten of 21 eyes with a positive qPCR result were in an early stage, 6 were chronic and 5 had end-stage disease.

### 2.2.7. Ocular MAT and antibody production

Ocular titers to *Leptospira* were determined in 24 horses with uveitis and all control horses. In two of the horses with uveitis, serum titers were not measured; therefore local antibody production could not be calculated. Seventeen of 24 horses with uveitis (70.8%) and 1 of 21 control horses (4.7%) had positive *Leptospira* titers in ocular fluid. This difference was significant (p<0.0001, OR: 48.6 (95% CI: 5.4-435.2)). Twenty-seven of 36 (75%) uveitic eyes and 1/40 control eyes (2.5%) had positive titers by MAT (p<0.0001).

Thirteen of 22 horses with ERU (59%) and 1/21 (4.7%) control horse had evidence of local intraocular antibody production (*Leptospira* titer in ocular fluid/serum titer>1). This difference was significant (p<0.0001, OR: 28.8 (95% CI: 3.2-255.7)). Twenty-two out of 33 (66.7%) eyes with uveitis and 1/40 (2.5%) control eye had evidence of intraocular antibody production (p<0.0001, OR: 71.5 (95% CI: 8.7-587)).

Using a four folds ratio (*Leptospira* titer in ocular fluid/serum titer>4) as definitive evidence of local antibody synthesis, 9/22 horses and 12/33 eyes with ERU had definitive local antibody synthesis. None of the control horses had a ratio >4. This difference was significant (p<0.0001)

The most commonly detected antibody in ocular fluid was against serovar bratislava (21/36 eyes, mean titer 377, range 0-6400), followed by hardjo (18/36, mean titer: 183, range: 0-1600) and pomona (13/35 eyes, mean titer 457, range 0-6400). The highest titer recorded was to serovar bratislava and pomona (6400), and the highest mean titer to serovar pomona. The one control horse with positive ocular MAT had antibodies against serovar hardjo (titer: 100).

Commonly, antibodies to more than one serovar appeared to be locally produced (8 horses and 13 eyes). The most common serovar that demonstrated local antibody production was hardjo (12 eyes) followed by pomona (11 eyes). Local antibody synthesis to all 6 serovars was detected.

Table 4: Results of ocular tests in uveitis and control, by eyes and by horses.

	Horses		Eyes	
	Uveitis	Control	Uveitis	Control
<i>Leptospira qPCR</i>	14/31 (45.2%)*	0/21 (0%)*	21/46 (45.6%)*	0/40 (0%)*
<i>Leptospira</i> Culture	6/28 (21.4%)*	0/21 (0%)*	6/41 (14.6%)*	0/40 (0%)*
<i>Leptospira</i> Antibodies (MAT)	17/24 (70.8%)*	1/21 (4.7%)*	27/36 (75%)*	1/40 (2.5%)*
Ocular Antibody production	13/22 (59%)*	1/21 (4.7%)*	22/33 (66.7%)*	1/40 (2.5%)*

Stars (\*) indicate values within a row that are significantly different between control and uveitis horses. Ocular antibody production is calculated using a ratio of 1.



### 2.2.8. Agreement between tests (Fig. 15)

There was no significant association between results of the qPCR and being seropositive ( $p=0.31$ ), being seropositive to multiple serovars ( $p=0.14$ ), or being seropositive to any individual serovar. None of the serology results were significantly associated with culture results either.

The presence of intraocular antibodies was not statistically associated with culture ( $p=0.44$ ) or qPCR ( $p=0.23$ ) results in an individual eye (Table 5). There was no significant association between ocular antibody production (ratio of 1) and results of the PCR ( $p=0.26$ ) or culture ( $p=0.33$ ) in an individual eye. Using a ratio of 4, no association could be found between ocular antibody production and the result of qPCR ( $p=0.28$ ) or culture ( $p=0.19$ ). Results of culture and PCR were significantly associated ( $p=0.04$ ) in an individual eye. Results for horses with a positive culture are reported in Table 6.

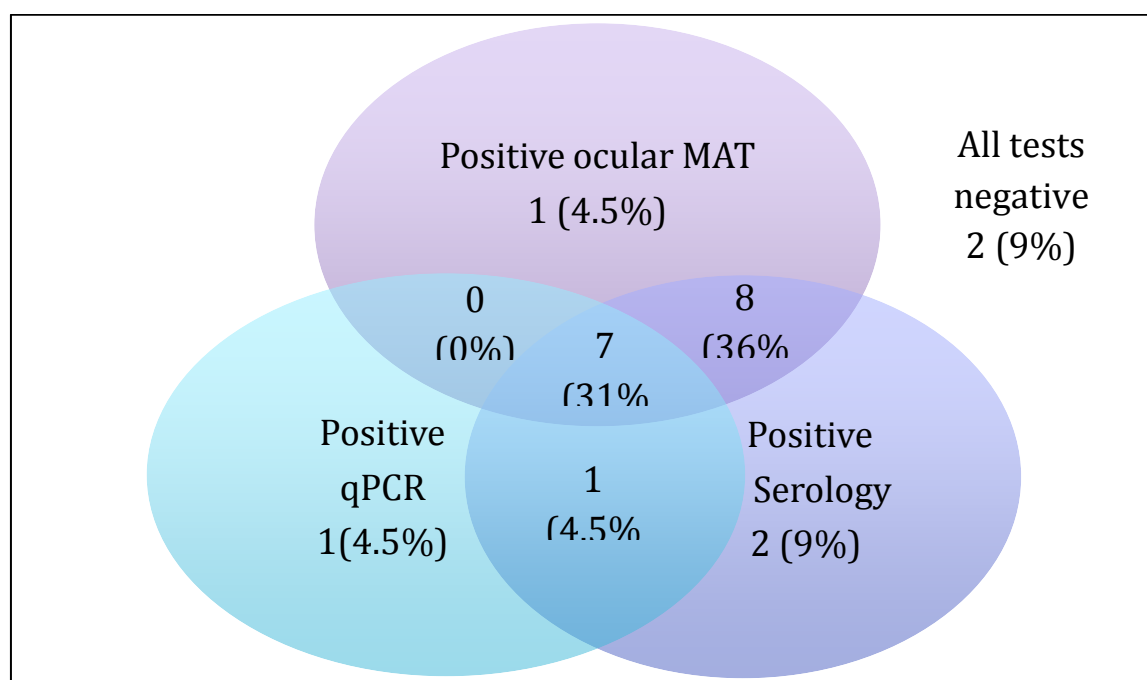


Figure 16: Venn diagram showing data from patients with uveitis whose ocular samples were tested by qPCR and MAT ( $n=22$ ).

### 2.2.9. Prognosis

Eyes with positive culture were not more likely to be blind than eyes with a negative culture ( $p=0.22$ ), nor were eyes with a positive qPCR ( $p=0.11$ ). There was however, a significant association between blindness and positive ocular MAT ( $p=0.03$ ).

Eyes with evidence of active inflammation were not more likely to have positive qPCR results ( $p=0.16$ ), culture ( $p=0.33$ ) or local antibody production ( $p=0.3$ ) than eyes in a quiet phase.

Table 5: Agreement between qPCR and Antibody production in ocular fluid of eyes with ERU. A ratio of 1 between serum and ocular titer was used for the determination of antibody production

	qPCR +	qPCR -
Ocular Antibody +	10/14 (71.4%)	12/19 (63.2%)
Ocular Antibody -	4/14 (28.5%)	7/19 (36.8%)

Table 6: Association between results of culture, qPCR, serology and ocular MAT.

Culture isolate	qPCR	Serology	Ocular MAT	Antibody production
<b>Grippotyphosa</b>	Negative	Grippotyphosa, Hardjo	Grippotyphosa	Negative
<b>Grippotyphosa</b>	Positive	Negative	NP	NP
<b>Pomona</b>	Positive	Bratislava, Canicola, Icterohaemorrhagiae Pomona	NP	NP
<b>Pomona</b>	Positive	Bratislava, Pomona	Bratislava, Grippotyphosa	Grippotyphosa
<b>Pomona</b>	Positive	Bratislava, Canicola, Grippotyphosa Icterohaemorrhagiae Pomona	Negative	Negative
<b>Pomona</b>	Positive	Bratislava, Pomona	Bratislava, Grippotyphosa, Pomona	Grippotyphosa, Pomona

NP: not performed

Horses with bilateral disease were not statistically more likely to have *Leptospira* antibodies in ocular fluids or antibodies produced in ocular fluid (regardless of the ratio used). There was a significant association between bilateral disease and PCR results ( $p=0.03$ ). All the horses with positive culture had bilateral disease; however the difference did not reach statistical significance ( $p=0.1$ ).

Table 7: Results of ocular tests in horses with unilateral and bilateral disease.

	Bilateral disease	Unilateral disease	P value
<i>Leptospira</i> qPCR	13/23 (56.5%)	1/8 (12.5%)	0.03
<i>Leptospira</i> Culture	6/20 (21.4%)	0/8 (0%)	0.1
<i>Leptospira</i> Antibodies	13/18 (72.2%)	4/6 (66.7%)	0.37
Ocular antibodies production	10/17 (58.8%)	3/5 (60%)	0.39

### 2.3. Discussion

The results of our study support a role of *Leptospira* in the pathogenesis of ERU in the Southern United States. Leptospire were isolated by culture in 21% of horses, bacterial DNA was detected by qPCR in 45% of horses, and anti-*Leptospira* antibodies were present in the ocular fluid of 70% of horses.

As reported previously (Ellis et al. 1983; Williams et al. 1994; Houwers et al. 2011), serologic evidence of exposure to *Leptospira* was very common in the southern United States, both in horses with uveitis (85%) and in control horses (76%). A high exposure rate was expected because the warm and humid climate of our area is ideal for the growth of leptospire and horses are frequently exposed to stagnant water. Antibodies against all six serovars tested were detected both in control and uveitis horses. The most common antibodies in serum were directed against serovar bratislava followed by serovar pomona. Serovar bratislava has been hypothesized to be host adapted in horses, because of its high prevalence in different geographical areas and low pathogenicity (Ellis et al. 1983; Pinna et al. 2010). Horses that had uveitis were more likely to be seropositive to more than one serovar. Seropositivity to multiple serovars could represent true exposure to different serovars or cross-reactivity between serovars. Serovars with low titers usually indicates cross reactivity, whereas a high single MAT titer of  $\geq 800$  is accepted as indicative of current infection in horses. One study evaluating the association between serovars which had a titer  $\geq 1600$  concluded that multiple exposure is common and probable in horses from NY state (Barwick et al. 1998). Only one horse with uveitis in our study had a titer of 800 or above, which could be indicative of recent exposure or infection. From our results, it is possible exposure to multiple serovars increase the risk of developing uveitis after leptospiral infection. Horses with uveitis had significantly higher titers to serovar pomona and grippotyphosa. Seropositive horses to serovar pomona were 13.2 times more likely to have uveitis than seronegative horses in another study from North America (Dwyer et al. 1995). These results might indicate that serovar pomona and grippotyphosa are involved in the pathogenesis of ERU. However, the antibodies detected in serum are not always directed against the serovar isolated on

culture: in people, the ability of convalescent phase MAT titers to predict the infecting serogroup may be as low as 40% (Levett 2001).

The result of serology failed to predict the result of ocular tests in our study and two horses with ocular antibody production and one horse with a positive PCR had a negative serology. Similarly, most studies have failed to confirm a significant association between ERU and leptospiral seroreactivity (Halliwell et al. 1985; Matthews et al. 1987; Gerhards et al. 1999; Faber et al. 2000). Faber et al. found no correlation between serologic results and the presence of leptospiral DNA in aqueous humor, and in another study, 4 of 41 horses with ERU and positive vitreous *Leptospira* culture were seronegative (Gerhards et al. 1999). We confirmed serologic testing has both low sensitivity and low specificity in the diagnosis of *Leptospira*-associated uveitis, and is of limited value.

A large proportion of horses with uveitis (70%) had anti-*Leptospira* antibody titer >100 in ocular fluid. This was similar to the proportion reported in studies from Germany: 67% (Gerhards et al. 1999), although the titers reported in this study were much higher (409,600) than in our study (maximum titer of 6400). In normal eyes, the blood-ocular barrier prevents antibodies from reaching the intraocular medium, therefore preventing an inflammatory response that could be damaging to the eye. There is controversy as to whether a positive ocular MAT is indicative of local antibody production due to infection or just leakage of antibodies from the serum through a damaged blood-ocular barrier (Baarsma et al. 1991). It is known that horses that have repeated bouts of ocular inflammation can have aqueous humor titers lower or equal to serum titers (Gilger 2005). A higher titer in the aqueous humor than in the serum is thought to evidence intraocular antibody production and therefore true infection. This was found in 59% of horses with ERU in our study. Other studies have advocated that definitive intraocular production of antibodies should be based on a 3 or 4 folds difference between serum and vitreous humor antibody titers (Wollanke et al. 2001; Gilger et al. 2008). This was found in 40% of horses with ERU in our study. Similarly, Wollanke et al. in Germany showed that 63% of horses with ERU had ocular antibody synthesis (Wollanke et al. 2001). These results were very different from the results of Gilger et al., who found only one horse with ERU had definitive intraocular antibody production. Intraocular antibody synthesis can be detected more accurately by determination of the Goldman-Witmer coefficient (GWC):

$$\frac{\text{Aqueous } Leptospira \text{ IgG} \times \text{Total serum IgG}}{\text{Total ocular IgG} \times \text{Serum } Leptospira \text{ IgG}}$$

or calculation of the antibody index, that compare the ratio of serum/aqueous concentration of *Leptospira* Ig to that of albumin which is never synthesized in the eye (Davidson et al. 1987; Furr et al. 2011):

$$\frac{Q_{Leptospira \text{ Ab}}}{Q_{Albumin}}$$

Both these techniques require determination of IgG concentrations, which cannot be measured by MAT and were not measured in our study. Several studies have looked at the local production of antibodies in infectious uveitis. Although theoretically, a GWC of 1 or above indicates local antibody production, many authors use a coefficient of 2 or 3 as definitive evidence to account for variability of IgG measurement (Baarsma et al. 1991; Abe et al. 1996; Fekkar et al. 2008). Few studies have calculated a true GWC in horses with ERU: in one study, 94% of horses with ERU had a GWC>8 (Wollanke et al. 2004). It is likely that using this method in our study, even more horses would have shown evidence of local antibody production. Measuring albumin concentration in ocular fluid can also help estimating the extent of the blood ocular barrier breakdown, since albumin is not synthesized in the eye. Another mean to differentiate the role of infection and inflammation in ocular antibody elevation would have been to include a third group of horses with inflammatory ocular diseases that were not ERU.

Leptospiral DNA was detected in the ocular fluid of 14 out of 31 horses with ERU by qPCR. This was a lower detection rate than was reported by other groups in the US (70%) (Faber et al. 2000) and Germany (71%) (Wollanke et al. 2004). However, a recent study from the southeastern US could not detect bacterial DNA in aqueous humor of horses with ERU using a universal bacterial PCR (Gilger et al. 2008). The different results could be explained by the lack of sensitivity of the PCR protocol used in their study for leptospiral DNA. Another study of horses with ERU in Midwestern US failed to detect leptospiral DNA in fixed ocular tissue using PCR. Some of the first PCR protocol developed for leptospirosis were found to lack sensitivity: a magnetic immunocapture PCR assay for the detection of leptospires in bovine urine did not detect organisms in 24% of culture positive samples (Taylor et al. 1997). However, when used on ocular fluid, molecular diagnosis techniques seem to be more reliable than culture (Faber et al. 2000; Wollanke et al. 2004). Real-Time PCR is both more sensitive and specific than traditional PCR (Wong et al. 2005). Recently, a Taqman assay for the detection of pathogenic *Leptospira* was found to have an analytical sensitivity of 10 copies/reaction, and a clinical specificity and sensitivity of 99.5% and 96.4%, respectively, when compared to culture (Slack et al. 2007). To determine if the difference between studies in North America is the result of geographical and population difference or the result of non-standardized diagnostic tests, a multi-center study using the same protocol should be performed.

Detection of leptospires by culture constitutes the definitive diagnosis; however it is a difficult and long process due to the fastidious nature of the organism. The isolation rate of *Leptospira* in our study (21.4%) was similar to the results in California reported by Faber et al. (22%) (Faber et al. 2000). In their study, 4/6 isolates belonged to the serogroup pomona and 2 isolates could not be typed. In Europe, higher isolation rates (26-53%) were reported, the most common serovar isolated being grippotyphosa (Brem et al. 1999; Hartskeerl et al. 2004; Wollanke et al. 2004). Our study confirmed that pomona is the most common serovar isolated from eyes with ERU in the United States, but also demonstrated for the first time that serovar grippotyphosa is involved in

the pathogenesis of ERU outside of Europe. This finding was also corroborated by the fact that horses with ERU had higher serum titers than control horses to both serovar pomona and grippotyphosa. The most common isolates associated with equine disease in North America belong to the serogroup pomona (Sillerud et al. 1987; Poonacha et al. 1993; Dwyer et al. 1995). All pomona isolates from Kentucky over a 10 year period belonged to the type or serovar kennewicki (Frellstedt et al. 2008) and a single genetic variant was responsible for most abortion in mares in one study (Timoney et al. 2011). The characterization of leptospiral isolates in our study was performed using a cross agglutination absorption protocol, which does not allow identification of the genomospecies but only the serogroup.

Direct detection of leptospire has been attempted in ocular fluid and tissue with limited success. Darkfield microscopy requires a minimum of  $10^4$  leptospire/mL for one organism per field to be visible. Recently, electron microscopy was performed on vitreous samples from horses with ERU. Although all samples were positive by PCR and 9/11 samples tested were positive by culture, leptospire were observed by EM in only 4/17 samples. In 3 of those 4 samples, only one bacterium was observed, confirming that the number of bacteria present in ocular fluid during chronic ERU is likely very low (Brandes et al. 2007).

Aerobic culture was positive in one control horse and one uveitis horse. All isolates (*Bacillus* spp., *Micrococcus* spp., and *Streptococcus* spp.) are part of the normal conjunctival flora (Sellon et al. 2007) and were therefore thought to be contaminants. This hypothesis was supported by Gram staining of the fixed eye, where no bacteria could be identified. Although, *Streptococcus equi* has been reported to cause uveitis during outbreaks of Strangles, it is not likely that the organism would still be isolated an end stage eye.

Leptospire could be isolated from eyes both with early, chronic and end-stage disease, in contrast to a previous report from the Midwestern United States (Pearce et al. 2007). This finding has been reported previously in Germany, where 30% of horses with a positive culture result had disease for more than a year (Wollanke et al. 2001). In sera, the protective capacities correlates well with the level of agglutinating, anti-LPS antibodies. In the presence of those antibodies, leptospire are readily phagocytized by macrophages and neutrophils. Prolonged ocular survival of leptospire in the eye in the face of antibody response indicates that the immune response induced by *Leptospira* bacteria that enter the eye is ineffective in clearing the infection. This might be due to the anterior chamber associated immune deviation, a phenomenon resulting in an inability of the host to display delayed hypersensitivity reactivity to *Leptospira* antigens (Verma et al. 2005). Brandes et al. observed that leptospire in the vitreous humor of horses with ERU were surrounded by an osmophilic protein coat, which could result from leptospiral masking by host proteins (Brandes et al. 2007), impairing recognition of the bacteria by phagocytes. The prevalence of the serogroup grippotyphosa in horses with ERU in Europe led to the hypothesis that although many pathogenic serovars may penetrate the eye and induce antibody production and disease, only few are able to evade immune response and persist (Hartskeerl et al. 2004).

It was interesting that although all horses with positive culture had bilateral disease, only one eye was positive. This could be due to a lack of sensitivity of culture or alternatively, the bacteria was no longer present in the other eye. In the 4 horses with positive culture in which both eyes were sampled, one horse had both eyes negative by qPCR, 2 horses had both eyes positive by qPCR and in one horse the contralateral eye was negative by qPCR. In the two cases with a positive qPCR bilaterally, it is likely that the organism was present in both eyes but too rare to be detected by culture. However, in the horse with a negative qPCR and culture in the contralateral eye, it is possible that *Leptospira*-associated uveitis in one eye induced inflammation in the other eye, a phenomenon known in human medicine as sympathetic ophthalmia. In this disease, penetrating injury to one eye in which the uveal tissue is traumatized, results in the release of ocular autoantigens in the lymphatic system and development of autoimmunity. Enucleation of the traumatized eye is required to preserve vision in the contralateral eye. It is possible that the invasion of one eye by leptospire result in a similar phenomenon, inducing an autoimmune uveitis in the contralateral eye. This phenomenon was observed two centuries ago by Wardrop: “It is known among some farriers that if the eye first affected with this disease suppurates and sinks into the orbit, the disease does not attack the other eye, or subsides if it has commenced in it. Thus, they have adopted a practice of altogether destroying the diseased eye, in order to save the other which is crudely done by putting lime between the eyelids, or thrusting a nail into the cavity of the eyeball, so as to excite violent inflammation and suppuration.”(Wardrop 1819). Additional studies are needed to determine if select cases in which the disease starts in one eye and progress to the other eye could benefit from enucleation of the affected eye to preserve vision in the contralateral eye.

There was no correlation between serology and ocular test results, or between the qPCR and MAT results on ocular fluid. This finding has been reported before, both in human and horse uveitis cases (Wollanke et al. 2004; Pappachan et al. 2007). Three horses had no detectable ocular antibody by MAT but had positive qPCR results. One hypothesis is that the antibody concentrations have declined below the threshold of positivity when testing is performed because ERU often develops months to years after acute infection. Alternatively, the leptospiral isolate could belong to a serovar not tested by our panel of antigens for MAT. Seventy percent of ERU horses with positive ocular MAT had a negative qPCR. Similar findings have been reported in the diagnosis of infectious uveitis in people (De Groot-Mijnes et al. 2006; Fekkar et al. 2008). One study showed that determination of the GWC and PCR were complementary in the diagnosis of uveitis: with PCR only, a correct diagnosis would have been missed in nearly half of the cases. Although cases with a negative PCR but evidence of local synthesis of antibodies may have been false positive, the presumptive diagnosis made on these cases was later substantiated by a positive response to specific treatments (De Groot-Mijnes et al. 2006).

The exact role of leptospire in the pathogenesis of ERU has not yet been elucidated. One hypothesis is that the bacterium has a direct effect on the uvea through the release of enzymes and toxins. During systemic disease, leptospire cause vasculitis: a glycoprotein toxin disrupts the

endothelial cell membranes of small vessels, allowing further migration of leptospire in the tissues, localized ischemia and capillary leakage (Sellon et al. 2007). Other toxins that could have a role in ERU include LPS and a hemolysin produced by several serovars, including pomona and hardjo. Another theory is that the presence of *Leptospira* in the eye activates an inflammatory response, and that cross-reaction between infectious agent and autoantigens results in an autoimmune disease. Cross-reactivity between leptospire and equine ocular tissue has been demonstrated in many studies (Parma et al. 1992; Lucchesi et al. 1999; Verma et al. 2005). Wollanke et al. argued that the continued presence of the organism is likely necessary to maintain disease, because vitrectomy can be curative. During vitrectomy, the content of the posterior chamber is removed and replaced with a gentamicin solution removing bacteria, antibodies and effector cells but not ocular proteins. If an autoimmune response to normal ocular antigen was responsible for the perpetuation of the disease, uveitis would recur following surgery. One recent study further substantiates this theory, because horses with no evidence of leptospiral infection had a poor response to vitrectomy (Tömördy 2010).

There is no report of the sensitivity or specificity of ocular PCR, MAT, and leptospiral culture in the diagnosis of leptospire-associated uveitis in horses. Leptospiral uveitis in people is diagnosed by use of a positive serology, but exposure is not as common in people as it is in horses. During MAT, the patient serum is reacted with live antigen suspensions of leptospiral serovars and observed for agglutination. The test can be challenging to implement and interpret, and is somewhat subjective. There is also a risk of cross-contamination of the antigen cultures if strict quality control is not implemented. High degree of cross-reaction can occur between different serogroups, especially in acute phase samples and “Paradoxical” reactions in which the highest titer is not to the infecting serogroup have been reported (Levett 2001). There are no reports of studies using other indirect methods of antibody detection, such as an ELISA test, on ocular fluid in horses. These tests are easier to standardize than MAT and should therefore be considered in further studies on ERU. Several recent studies compared ELISA to MAT in the serodiagnosis of bovine leptospirosis and found good sensitivity and specificity (Sakhaee et al. 2010; Sankar et al. 2010). PCR, when performed on ocular fluid, could lack sensitivity because of the small amount of ocular fluid that can be safely sampled and the low number of leptospire present.

We cannot at this time recommend a single diagnostic test: MAT and PCR should be performed together on ocular fluid in order to increase the detection of *Leptospira*-associated uveitis. Because of the lack of sensitivity and length of culture, it is not a practical test for clinical cases, although it remains important for epidemiologic purposes. Combining GWC and PCR in the diagnosis of ocular toxoplasmosis increased the sensitivity to 93% (Fekkar et al. 2008). It is also noteworthy that no studies in horses to date have compared results obtained from analyzing vitreous samples or aqueous samples. In most studies from Germany, samples are obtained during vitrectomy whereas in most studies from North America, aqueous humor was used for testing. It has been reported that in the diagnosis of ocular toxoplasmosis in people, PCR on vitreous might yield better results than on aqueous humor (De Groot-Mijnes et al. 2006). Positive



results have been obtained from both types of samples, both in our study and in others (Faber et al. 2000; Wollanke et al. 2001).

Breed distribution was similar between uveitis and control horses. However, when comparing the uveitis group to the horses that were admitted to the VTH in the same time period, Appaloosa and Quarter Horses were overrepresented; Thoroughbreds were underrepresented. This finding is consistent with the observation of other authors (Dwyer et al. 1995; Spiess 2010). It has been suggested that Thoroughbred horses may be underrepresented because they have less exposure to leptospirosis due to their decrease exposure to the outdoors as racehorses. This is not applicable to our hospital population where a large proportion of Thoroughbreds seen are broodmares. Therefore, the difference could be due to a breed resistance to the disease. However, broodmares may also be less likely to be presented to a referral institution than performance horses, in which intact vision is essential. Only four patients had both no detectable antibodies and DNA in ocular fluid. It is interesting to note that 2 of these patients were Appaloosa horses. Although, the sample size is too small to reach significance, none of the Appaloosas in this study had *Leptospira*-associated uveitis: two had positive serum titers but negative aqueous titer and PCR, the other had a negative PCR result, which was the only test performed. This finding is different from the study by Dwyer, that showed that Appaloosa horses that were seropositive had more severe disease than Appaloosa horses that were seronegative, implicating leptospirosis in the pathogenesis of the disease in this breed as well (Dwyer et al. 1995). It is possible that the genetic predisposition of Appaloosas to uveitis does not require exposure to *Leptospira* for the development of the disease and their disease could be an auto-immune phenomenon only.

Ophthalmic examinations showed that all stages of uveitis were present in this study. As previously reported, phthisis bulbi was much more common than glaucoma. Cataracts were very prevalent in this study, because many cases had advanced disease and blindness. In many cases, observation of the posterior chamber was not possible due to obstruction by cataracts or extensive synechiae, this possibly accounting for the low number of posterior chamber pathology reported in the table. No attempt was made in donated horses to further identify these pathologies, but in client owned animals, ultrasonographic examination of the posterior chamber and retina or electroretinography was performed when indicated.

Histologic examination allowed confirmation of the diagnosis of ERU in all cases by identification of the three pathognomonic features: infiltration of lymphocytes and plasma cells into the nonpigmented ciliary epithelium, a thick acellular hyaline membrane adherent to the inner aspect of the nonpigmented epithelium and eosinophilic linear inclusions in the nonpigmented ciliary epithelium. In addition to these classic findings, linear inclusions of eosinophilic material were also identified in the retina and optic nerve head of many affected horses. These inclusions were less numerous and appeared more faint than those observed in the ciliary body and were therefore often difficult to identify. This was an interesting finding, as it has not been reported before. However, as with the inclusions in the ciliary body, the nature or significance of these inclusions is unknown as this time.

The results of this study suggest that leptospire contribute to the pathogenesis of ERU in the southern US to a greater extent than previously thought. Further studies from larger areas using a standardized technique could provide valuable information on the geographical differences found in previous studies.

## CONCLUSION

In summary, we have shown leptospirosis is associated with Equine Recurrent Uveitis in the Southern United States. Our findings contradict the results of a recent study from the southeastern United States (Gilger et al. 2008).

Although horses with uveitis were more likely to be seropositive to multiple serovars and had higher titers to serovars pomona and grippotyphosa, serology was of limited value for the diagnosis of *Leptospira*-associated uveitis and more invasive diagnosis techniques were required.

Leptospire could be isolated by culture from the ocular fluid of 21% of horses with ERU, confirming a diagnosis of leptospirosis-associated ERU. However, because of the fastidious nature of the organism, culture lacks practicality and sensitivity for the diagnosis of clinical cases. Forty five percent of horses with ERU had a positive *Leptospira* Real-Time PCR on ocular fluid and 70% of horses had *Leptospira* antibodies in ocular fluids. Ocular production of antibodies confirming infection was found in 59% of horses with uveitis. There was no significant association between qPCR results and ocular antibody production. Our results indicate that PCR and MAT are complementary in the diagnosis of leptospirosis-associated ERU and should both be performed if the sample volume allows it.

Serotyping of isolates confirmed that pomona is the most common serovar causing uveitis in North America and implicated serovar grippotyphosa in ERU outside of Europe for the first time.

Efforts should be made to standardize diagnosis techniques in the future to allow comparison between studies. To facilitate standardization, techniques such as ELISA should be tested on ocular fluids of horses with ERU. Further studies should also be performed to determinate the difference between results obtained on aqueous humor and results obtained on vitreous fluid.

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APPENDIX 1. MEDICAL TREATMENT OF ERU

Mediations	Dose	Indication	Caution
Topical medication			
Prednisolone 1%	q 1-6 hours	Potent anti-inflammatory medication with excellent ocular penetration	Predispose to corneal fungal infection
Dexamethasone 0.1%	q 1-6 hours		
Flurbiprofen 0.03%	q 1-6 hours	Anti-inflammatory medication with good intraocular penetration	Decrease corneal epithelialization
Profenal 1%	q 1-6 hours		
Diclofenac 0.1%	q 1-6 hours		
Suprofen 1%	q 1-6 hours		
Indomethacin 1-2%	q 1-6 hours		
Cyclosporine A 0.02%-2%	q 6-12 hours	Strong immunosuppressant	Poor eye penetration, weak anti-inflammatory effect
Atropine HCl 1%	q 6-48 hours	Cycloplegic, mydriatic	May decrease gut motility and predispose to colic
Systemic medications			
Flunixin meglumine	0.25mg/kg PO or IV q 12 to 24 hours	Potent ocular anti-inflammatory medication	Long-term use may predispose to GI and renal toxicity
Firocoxib	0.1mg/kg PO or IV q 24 hours	COX-2 Selective anti-inflammatory medication Decreased renal and GI toxicity	

Table continued

Ketoprofen	2.2mg/kg IV q 24 hours	Anti-inflammatory medication	Long-term use may predispose to GI and renal toxicity
Phenylbutazone	2.2-4.4 mg/kg PO or IV q 12 to 24 hours		
Aspirin	25-30mg/kg q 24-48 hours		
Prednisolone	0.5-1 mg/kg PO or IM q 24 hours	Potent anti-inflammatory medication	Risk of laminitis, must taper dose
Dexamethasone	0.025-0.1 mg/kg PO or IM q 24 hours		
Sub-conjunctival			
Prednisolone acetate	5-10mg	Short term	Predispose to infectious keratitis, cannot remove therapy once given
Dexamethasone	15mg	Short term	
Betamethasone	15mg	Depot	
Triamcinolone	20-40mg	Depot	
Methylprednisolone acetate	20-40mg	Depot	

APPENDIX 2. DATA FOR CONTROL AND UVEITIS HORSES

Thb: Thoroughbred, QH: Quarter Horse, TWH: Tennessee Walking Horse, G: Gelding, F: Female, M: Stallion, OS: left eye, OD: right eye, OU: both eyes, B: bratislava, C: canicola, G: grippotyphosa, H: hardjo, I: icterohaemorrhagiae, P: pomona, Neg: all test performed negative, NP: not performed, PCR: positive PCR test. Bolded serovar in serology column indicates the dominant serovar in serum. Bolded serovar in the left eye and right eye column indicate the serovars with higher ocular titers than serum titers.

Horse	Group	Breed	Sex	Age	State	Eye Affected	Blind	Serology	Left eye	Right eye
294A	Control	QH	G	7	La			<b>B</b>	Neg	Neg
289G	Control	Arabian	G	17	La			<b>B, C, G, I</b>	Neg	Neg
117	Control	Thb	F	17	La			<b>B, C, H, I, P</b>	Neg	Neg
500A	Control	QH	G	6	La			<b>B, C</b>	Neg	Neg
462	Control	Thb	F	5	La			<b>B, H</b>	Neg	Neg
404	Control	QH	F	10	La			<b>B, H, I, P</b>	Neg	Neg
200I	Control	Thb	M	3	La			<b>C, H</b>	Neg	NP
200j	Control	Thb	M	1,5	La			<b>H</b>	Neg	Neg
357C	Control	TWH	G	9	La			<b>B</b>	Neg	Neg
289H	Control	QH	F	14	La			<b>H</b>	Neg	Neg
644485	Control	Paint	G	7	La			Neg	Neg	Neg



Table continued

Horse	Group	Breed	Sex	Age	State	Eye Affected	Blind	Serology	Left eye	Right eye
103	Control	QH	F	13	La			<b>B</b>	Neg	Neg
108	Control	QH	F	19	La			<b>B, C</b>	Neg	NP
2i	Control	Arabian	F	9	La			Neg	Neg	<b>H</b>
289i	Control	QH	G	5	Ms			Neg	Neg	Neg
465	Control	Thb	G	4	La			Neg	Neg	Neg
289J	Control	QH	M	2	La			Neg	Neg	Aerobic culture
339	Control	Paint	G	11	La			<b>B</b>	Neg	Neg
464	Control	Thb	G	5	La			<b>B</b>	Neg	Neg
183	Control	QH	F	21	La			<b>B</b>	Neg	Neg
68	Control	Paint	F	9	La			<b>B</b>	Neg	Neg
645061	Uveitis Client	QH	G	10	La	OU	OD	<b>B, C</b>	Neg	NP
644441	Uveitis Client	QH	F	5	La	OD	visual	<b>G, H</b>	NP	Neg
633933	Uveitis Client	Morgan	G	15	La	OU	visual	NP	B, I, P	NP
643102	Uveitis Client	Appaloosa	G	11	Ar	OU	OS	NP	NP	Neg

Table continued

Horse	Group	Breed	Sex	Age	State	Eye Affected	Blind	Serology	Left eye	Right eye
643131	Uveitis Client	QH	F	16	La	OU	OS	<b>B, C, I, P</b>	Culture pomona, PCR	NP
642596	Uveitis Client	Paint	F	13	Ks	OU	visual	Neg	Culture grippotyphosa, PCR	PCR
642145	Uveitis Client	Shire	G	20	La	OU	OD	<b>B, I, P</b>	PCR, B, P	NP
641649	Uveitis Client	QH	G	12	Ar	OS	OS	<b>B, I, P</b>	Neg	NP
640807	Uveitis Client	QH	F	6	La	OU	OS	<b>B</b>	B	NP
640134	Uveitis Client	QH	F	4	Ms	OS	visual	Neg	Neg	NP
640021	Uveitis Client	QH	G	5	La	OD	visual	Neg	NP	Neg
640997	Uveitis Client	QH	F	6	La	OU	visual	<b>B, C, I, P</b>	PCR	PCR
641537	Uveitis Client	QH	G	7	La	OU	visual	NP	PCR	NP
638944	Uveitis Client	Dutch	F	13	Tx	OU	OS	<b>B, C, G, I, P</b>	NP	Culture pomona, PCR
643645	Uveitis Client	Thb	M	7	La	OU	visual	<b>G, I</b>	PCR	NP
644117	Uveitis Client	QH	F	2	La	OU	visual	<b>B, P</b>	Culture pomona, PCR, B, G	PCR, B, G

Table continued

Horse	Group	Breed	Sex	Age	State	Eye Affected	Blind	Serology	Left eye	Right eye
646057	Uveitis Client	QH	G	18	La	OU	visual	Neg	<b>B, I, P</b>	<b>B, P</b>
357	Uveitis Donation	QH	F	17	La	OU	OU	<b>B, C, H, I, P</b>	<b>B, H, I, P</b>	<b>B, C, H, P</b>
357B	Uveitis Donation	QH	F	21	La	OU	OU	<b>G, H</b>	Neg	Culture grippotyphosa, <b>G</b>
294B	Uveitis Donation	Thb	F	15	La	OD	OD	<b>B, H</b>	Neg/normal	<b>B, C, G, H, I</b>
351	Uveitis Donation	QH	F	9	La	OU	OU	<b>B, G, H</b>	PCR, <b>B, G, H</b>	PCR, <b>B, G, H</b>
333	Uveitis Donation	Arabian	F	7	Ms	OU	OU	<b>B, G</b>	PCR, <b>G, H</b>	Aerobic culture, PCR, <b>B, G, H, P</b>
322	Uveitis Donation	Appaloosa	F	14	Tx	OU	OU	<b>B</b>	Neg	Neg
477	Uveitis Donation	QH	M	26	Tx	OS	OS	<b>G</b>	PCR, <b>G, H</b>	Neg/normal
4409211	Uveitis Donation	QH	G	6	Mo	OU	OS	NP	PCR, <b>H</b>	PCR, <b>H</b>

Table continued

Horse	Group	Breed	Sex	Age	State	Eye Affected	Blind	Serology	Left eye	Right eye
298	Uveitis Donation	Appaloosa	F	20	La	OU	visual	<b>B, H, P</b>	Neg	Neg
298b	Uveitis Donation	QH	F	17	Tx	OS	OS	<b>B</b>	<b>B, H</b>	NP
404b	Uveitis Donation	Thb	G	9	La	OU	OU	<b>B, P</b>	<b>B, H, I, P</b>	<b>H, P</b>
333c	Uveitis Donation	QH	M	18	Ms	OU	OU	<b>B, P</b>	<b>B, H, P</b>	<b>B, H, I</b>
203	Uveitis Donation	QH	F	20	Ms	OU	OD	<b>B, P</b>	<b>PCR, B, H, I, P</b>	<b>PCR, B, H, I, P</b>
479a	Uveitis Donation	QH	F	25	La	OU	OS	<b>B, P</b>	<b>B, H, P</b>	Culture pomona, <b>PCR, B, G, P</b>

## VITA

Florence Polle was born in March 1984 in Chatenay Malabry next to Paris, France. She moved to the south of France at age 7, where she started riding horses and competing in show jumping. She began her veterinary education at the Ecole Nationale Vétérinaire de Toulouse, France in 2002. After receiving her veterinary degree in 2007, Dr. Polle completed a six months veterinary internship at La Clinique Vétérinaire du Lys, followed by a one year internship in equine medicine and surgery at the Ontario Veterinary College in Canada.

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