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Archives of Veterinary Science
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A histopathologic and immunohistochemical comparison of corneal and conjunctival tissue from horses that were seropositive and seronegative for leptospirosis

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Submitted: *13/05/22*
Accepted: *14/07/23*

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Abstract: Equine recurrent uveitis (ERU) is the most common cause of blindness in horses. Leptospirosis has long been cited as a cause of ERU, particularly Leptospira interrogans serovar Pomona. Horses infected with the bacterium present several disorders, including uveitis, which alters the composition of the aqueous humor and impedes the nutrition of ocular structures, resulting in sequelae, such as iris atrophy, synechiae, and corneal changes. Previous studies have demonstrated that the opacity in both the cornea and lens is a consequence of the antigenic relationship between the bacteria and components of the ocular tissues and does not require the presence of living bacteria. In this study, blood samples, aqueous humor, and vitreous were obtained for microscopic agglutination test - MAT (SANTA ROSA, 1970) for leptospirosis from 29 horses (58 eyeballs). Histological and immunohistochemistry analysis of corneal and conjunctival tissue samples were performed and was noted that horses seropositive for leptospirosis exhibited corneal thickness significantly higher than the seronegative animals (P= 0.0347). This increase in thickness is probably due to the presence of inflammation. Evidence of inflammation, such as corneal blood vessels and inflammatory cell infiltrate was observed in a higher number of tissues from seropositive compared to seronegative horses. During immunohistochemistry, the anti-metalloproteinase 1 (anti-MMP1) of seropositive animals exhibited statistically significant results when compared to the area of immunohistochemistry reaction in seronegative animals (P=0008). Seropositive individuals had altered corneas (increased thickness and evidence of inflammation); therefore, inhibitory mechanisms of tissue metalloproteinases (TIMPs) in the cornea may be activated to prevent the degradation of corneal tissue during inflammation, resulting in the smallest reaction area of MMP1 in seropositive horses and a greater area in the seronegative individuals.
Keywords: horses; immunohistochemistry; leptospirosis; uveitis

1. Introduction

Equine recurrent uveitis (ERU) is a spontaneous disease that affects up to 15% of horses and is described as a model for human autoimmune uveitis Fingerhut et al. (2022). The disease is characterized by repeated episodes of intraocular inflammation, and many corresponding clinical and pathologic features are similar to recurrent uveitis in humans. ERU is the most common cause of blindness in horses, but the underlying causes of pathogenesis remain unknown in many cases Lucchesi et al. (2002), Hartskeerl et al. (2004), Rohrbach et al (2005), Fingerhut et al. (2022). Leptospirosis has long been cited as a cause of ERU, particularly *Leptospira interrogans* serovar Pomona. One theory proposes that after an initial injury or infection and a subsequent delayed hypersensitivity reaction, "memory" T-lymphocytes remain in the uveal tract Deeg et al (1999). The incidence of ERU in Europe is reported to be 7-10% and up to 25% in the USA Szemes P and Gerhards H (2000), Gilger B and Hollingsworth S (2017). The prevalence of ERU in the UK is believe to be much lower, about 0,3% Slater J (2014). Naturally occurring leptospirosis in equine species was reported first by Lubashenko and Novikova (1947) in Russia Lubashenko S and Novikova L (1947). An association between ERU and *Leptospira*l infection was initially suggested by Rimpau (1947).

A relationship between breed and risk for the development of ERU exists in Appaloosas and German Warmbloods Deeg et al. (2004), Fritz et al. (2014). In addition, differences in the *Leptospira* serovar also play a role in the disease. Lower environmental levels of the L. pomona and L. grippotyphosa serovars are associate with a lower prevalence of ERU Lowe RC (2010).

Medical treatment for ERU has the objetives of controlling inflammation, decrease pain, minimize chronic changes and prolong vision. Generally, it consists of topical anti-inflammatory agents (corticosteroids and/ or nosteroidal anti-inflammatory drugs), topical mydriatic-cycloplegics (most commonly atropine), combine with systemic anti-inflammatory therapy (such as flumexin meglumine, phenylbutazone, aspirin, dexamethasone and prednisolone) Systemic corticosteroids are necessary when facing more severe cases Frellstedt L (2009).

Inflammation has been shown to result from a direct bacterial effect or a local immune response to bacteria in the eye Rohrbach et al. 2005). Acute clinical signs include blepharospasm, photophobia, lacrimation, chemosis, miosis, hypotonia oculi, iritis and aquous flare. Chronically, and increased aqueous flare, hypopyon corneal edema, corneal vascularization,

conjunctival and circumcorneal congestion and vitreal haze. Sequelae include synechiae, vitritis, cataract formation, iris atrophy, chorioretinitis, retinal detachment and glaucoma Wada et al. (2003), Frellstedt L (2009). Blindness is caused by cataract formation, glaucoma or retinal detachment Cook C and Harling D (1983). Previous studies have demonstrated that the opacity in both the cornea and lens is a consequence of the antigenic relationship between *Leptospira* spp. and components of the ocular tissues and does not require the presence of living bacteria Parma et al. (1997). These results, in addition to the observed infiltration of the cornea with neutrophils and lymphocytes, indicate the mechanism of tissue damage Parma et al. (1997).

The objective of this investigation was to analyze the changes observed in the corneal and conjunctival tissues from horses that were seropositive and seronegative for L. *interrogans*, using histopathology and immunohistochemistry techniques.

2. Materials and Methods

This study included 58 eyeballs from 29 horses, which were randomly selected from the King Meat Abattoir, in the municipality of Apucarana, State of Parana, south of Brazil. The company kindly allowed the researchers to collect the globes. The origin of this particular set of horses slaughtered was the State of Paraná, Santa Catarina and Minas Gerais, Brazil. Blood samples were obtained by jugular venipuncture from 29 horses and the eyeballs, aqueous humor, and vitreous body were collected after the animals were slaughtered.

Blood samples, aqueous humor, and vitreous were obtained for microscopic agglutination test - MAT (SANTA ROSA, 1970) for leptospirosis. Seropositive horses were defined as those individuals with a sample titer ≥ 80 and 26 serovars from L. *interrogans* were studied: andamana, australis, autumnalis, batavie, brasiliensis, bratislava, butembo, canicola, castellonis, copenhageni, cynopteri, fronn, grippotyphosa, hardjo, hebdomadis, icterohaemorrhagiae, javanica, pyrogenes, panama, pomona, patoc, shermani, sentot, tarassovi, wolffi and whitcombi.

The eye tissue from the 58 samples was fixed in 10% buffered formalin for 24 h. For adequate fixation, 5 ml of aqueous humor was replaced by a fixative with a syringe. A 10 mm strip was cut sagittally from the anterior to the posterior segment and the cut again into a smaller segment containing the cornea, limbus, parts of the sclera and bulbar conjunctiva. These strips were embedded in paraffin and mounted in slides, and histological examinations were performed after staining with hematoxylin and eosin (HE).

Histopathological changes were then characterized, such as corneal thickness and conjunctival epithelium thickness, and histomorphometric measurements were taken using the Image Pro Plus 6.0 Image Analysis Software (Media Cybernetic Inc., Rockville, MD, USA). The presence of blood vessels and cellular infiltrate, conjunctival congestion, and ciliary process congestion also was indicated.

Additional slides were prepared from paraffin blocks by a tissue microarray technique Wan et al. (1987) for immunohistochemical staining procedures. For the immunohistochemical study, tissue sections were cut at 5 µm, mounted on 3-aminopropyltriethoxy-silanecoated slides, deparaffinized, and rehydrated. Endogenous peroxidase activity was blocked by incubation with 5 ml hydrogen peroxide in 95 ml methanol for 15 min. at room temperature. After washing three times with a phosphate-buffered saline (PBS) solution for 5 min. each, an antigen retrieval using Imuno Retriever (Agilent Technologies-Dako, Santa Clara, California) was performed at 99º C for 40 min. Tissue sections (TMAs) were incubated with the primary antibodies (anti-MMP1 e anti-MMP9) overnight at 4º C. The slides were then incubated three times in a phosphate-buffered saline bath for 15 min., allowed to dry, and rinsed in Envision kit solution (Agilent Technologies-Dako, Santa Clara, California) for 30 min. The slides were washed with a phosphate-buffered saline solution and incubated with the chromogen 3,3'-diaminobenzidine tetrahydrochloride (DAB) (1:1) until a brown reaction was visible. Slides were then rinsed in tap water, counterstained with Harry's Hematoxylin, and mounted.

Immunohistochemistry utilized a panel of two antibodies: anti-MMP1 and anti-MMP9 (anti-metalloproteinases 1 and 9). The area and density of immunohistochemistry reaction were evaluated using the Image Pro Plus 6.0 Image Analysis Software (Media Cybernetic Inc., Rockville, MD, USA).

Statistical analysis was performed using one-way analysis of variance (ANOVA) for continuous numeric data from histomorphometry and Fisher`s exact test to compare proportions (vessels and cellular infiltrate, conjunctival congestion, and ciliary process congestion). Student`s t-tests were performed to analyze corneal thickness values as well as the area and density of anti-MMP1 and anti-MMP9 reactivity, comparing seropositive and seronegative horses. A P-value of < 0.05 was required for statistical significance. For these calculations the software MedCalc® Statistical Software version 20.027 (MedCalc Software Ltd, Ostend, Belgium) was used.

2.1. Results

The microscopic agglutination test (MAT) identified 14 positive animals, and 15 that were negative. One seropositive animal demonstrated positivity in both the aqueous humor and vitreous body, while a seronegative horse was found to be positive in the vitreous body. In total, 5 serovars were identified from the samples. L. *interrogans*: icterohaemorrhagiae, autumnalis, patoc, sentot, and hebdomadis (Table 1).

Animal	Serovar Leptospira interrogans	Serum Titer	Aqueous Humor Titer	Vitreous Body Titer	Histopathologial changes
1	-----	0	0	0	
2	-----	0	0	0	
3	Icterohaemorrhagiae	160	80	160	Discrete corneal blood vessels and inflammatory cell infiltrate
4	-----	0	0	0	Bulbar conjunctival congestion
5	-----	0	0	0	
6	Icterohaemorrhagiae	40	0	0	
7	-----	0	0	0	
8	Autumnalis	320	0	0	
9	Icterohaemorrhagiae	40	0	0	
10	Icterohaemorrhagiae	40	0	0	
11	Icterohaemorrhagiae	40	0	0	
12	Icterohaemorrhagiae and Castellonis	40/40	0	80/0	
13	Icterohaemorrhagiae	640	0	0	Discrete corneal blood vessels and inflammatory cell infiltrate + bulbar conjunctival congestion
14	Autumnalis	80	0	0	
15	Icterohaemorrhagiae	320	0	0	
16	-----	0	0	0	Bulbar conjunctival congestion
17	Icterohaemorrhagiae	40	0	0	
18	Icterohaemorrhagiae and Patoc	80/160	0	0	Discrete corneal blood vessels and inflammatory cell infiltrate + bulbar conjunctival congestion
19	Icterohaemorrhagiae	80	0	0	
20	-----	0	0	0	
21	Icterohaemorrhagiae and Patoc	80/160	0	0	
22	Icterohaemorrhagiae and Autumnalis	80/80	0	0	
23	Icterohaemorrhagiae and Patoc	80/80	0	0	
24	Icterohaemorrhagiae and Australis	80/80	0	0	
25	Icterohaemorrhagiae	80	0	0	
26	-----	0	0	0	Discrete corneal blood vessels and inflammatory cell infiltrate + bulbar conjunctival congestion
27	Hebdomadis	160	0	0	
28	-----	0	0	0	
29	Shermani	80	0	0	Discrete corneal blood vessels and inflammatory cell infiltrate + bulbar conjunctival congestion

Table 1 – Microscopic agglutination test (MAT) serovars and titers detected in serum, aqueous humor and vitreous body and histopathological changes in horses randomly selected from a meat abattoir in Jaboticabal, São Paulo, Brazil, in 2007.

The histopathological changes observed on HE-stained sections were: Presence of blood vessels and a mixed-cell inflammatory cell infiltrate (with lymphocytes predominately), in the cornea in 17.25% of the samples (5/29, being 1 in seronegative and 4 in seropositive animals). This difference was not statistically significant, P= 0.8. Bulbar conjunctival congestion was present in 20.69% of the samples (6/29, being 3 seronegative and 3 seropositive animals). This difference was not statistically significant, P= 0.32. Total corneal thickness was significantly increased (P=0.035) in seropositive horses for leptospirosis compared to seronegative horses. (Figure 1).

The MMP1 (Figure 2) exhibited statistically significant results (P= 0.008) between the area of immunohistochemistry reaction and seronegative horses for leptospirosis (Figure 3). There was a significantly smaller area of reaction in the cornea of seropositive animals. However, the antibody anti-MMP9 did not demonstrate statistically significant results in the analyzed

parameters. For the density parameter of the two antibodies, there was no significant differences observed when comparing seropositive and seronegative individuals ($p>0.05$).

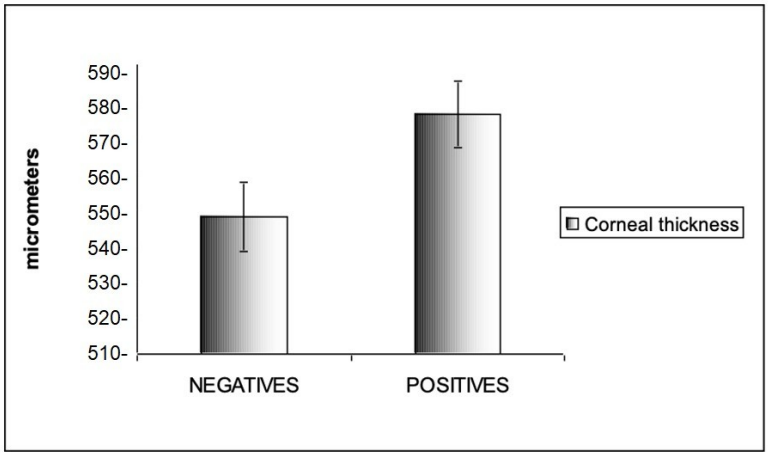

Figure 1 – Histogram demonstrating the mean and the standard deviation (error bars) of corneal thickness (µm) from seropositive and seronegative horses for leptospirosis.

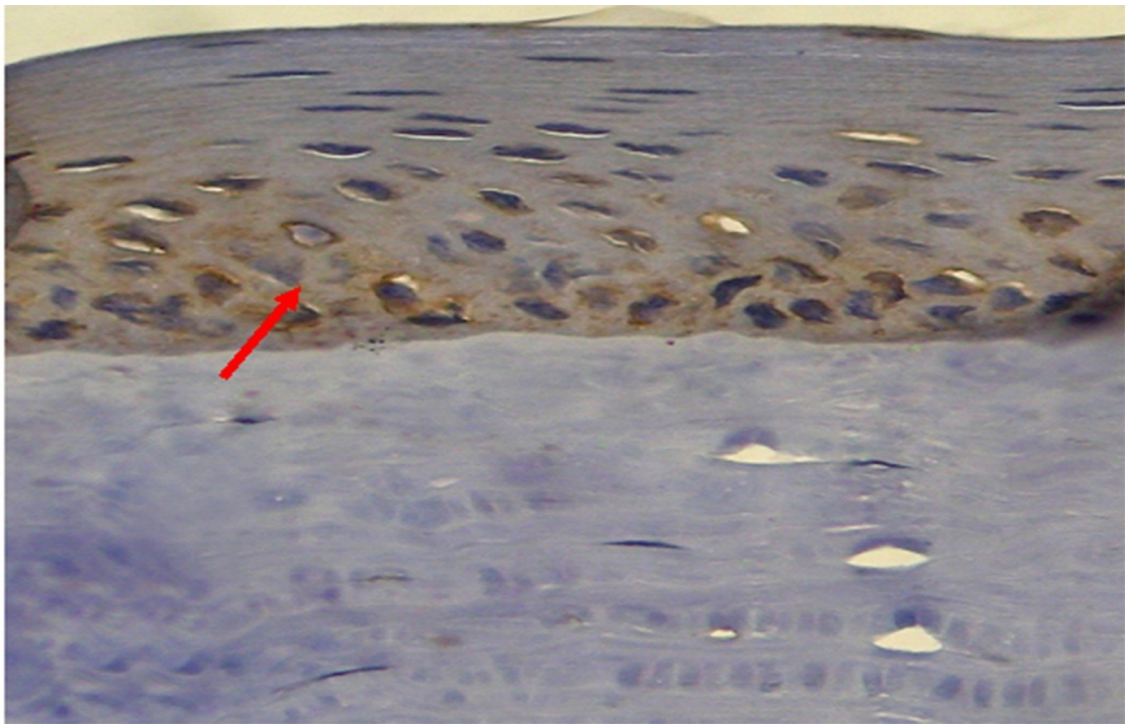

Figure 2 – Immunopathology of corneal segment from the left eye from a seronegative horse for leptospirosis. Immunohistochemistry reaction to antibody anti-MMP1, brown staining (arrow). Avidin-biotin peroxidase staining, Harry's Hematoxylin counterstain 40x

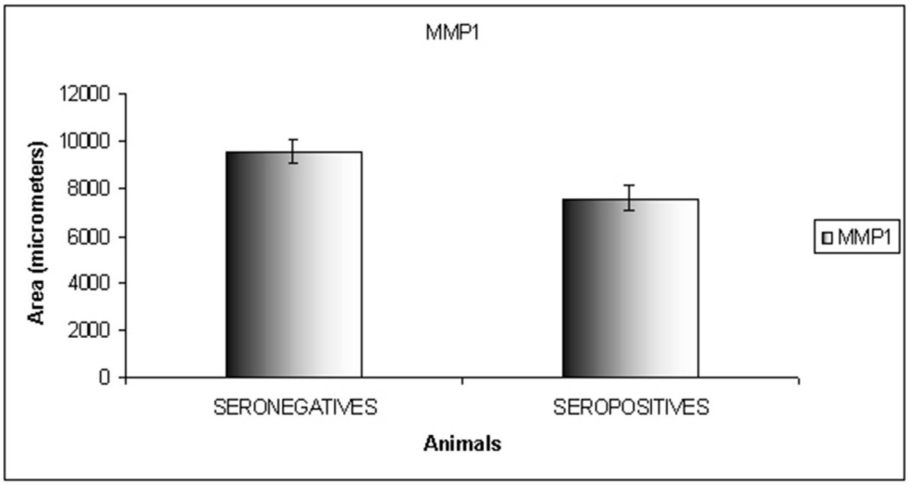

Figure 3 – Histogram demonstrating the mean and standard deviation (error bars) of the corneal immunohistochemistry reaction area (µm) for antibody anti-MMP1 from seropositive and seronegative horses for leptospirosis.

3. Discussion

In the present study, leptospirosis seropositive horses, with discrete or even without ocular signs, exhibited corneal thickness that was significantly higher than for negative animals (P=0,0347). Such findings are discussed in the literature, indicating antigenic activity between the bacterium and eye tissues, especially the cornea. Based on these findings, ERU is considered an organ-specific autoimmune disease Parma et al. (1997), Lucchesi et al. (2002). Furthermore, the occurrence of organ-specific disease was also found to be related to a positive reaction in the vitreous body of a patient that was seronegative for leptospirosis. An antigenic relationship between this bacterium and the equine cornea has been described in previous studies Parma et al. (1985), Parma et al. (1987), Parma et al. (1992) (1), Parma et al. (1992) (2), Parma et al. (1997), Lucchesi and Parma (1999). Our findings suggest that an immune response to 90 kDa protein participates in the pathogenesis of equine uveitis (Lucchesi and Parma (1999). *Leptospira*l antibody prevalence is high in horses with ERU in Switzerland (Voelter et al. (2020), but *Leptospira*-associated ERU is uncommon in the UK. Serology alone may not help to definitively diagnose *Leptospira*-associated uveitis in this country (Malalana et al. (2012).

In this study, the anti-MMP1 exhibited statistically significant results (P= 0.008) between the area of the immunohistochemistry reaction and seronegative horses for leptospirosis. These results demonstrated that there was a smaller area of reaction in the cornea of seropositive animals. The seropositive horses investigated did display increased corneal thickness that might be a reflection of subclinical corneal edema in the majority of the animals. Corneal inflammation may produce inhibitory mechanisms for tissue metalloproteinases (TIMPs), which may have been activated to prevent the degradation of corneal tissue during inflammation. This possibly resulted in the smallest area of MMP1 reaction in seropositive animals. This study also substantiates previous investigations indicating that in cases of corneal inflammation, some TIMPs are activated, which prevents excessive degradation of normal healthy tissue (Brooks and Ollivier (2004), Ollivier et al. (2007). Healing of corneal wounds is an exceptionally complex process, which involves the integrated actions of multiple proteinases, growth factors, and cytokines produced by epithelial cells, stromal keratocytes, inflammatory cells, and lachrymal glands. Matrix Metalloproteinases (MMP) play an important role in both normal and diseased corneal metabolism for animals as well as humans. This activity is highly regulated by the control of transcription and proenzyme (Pro-MMP) activation and by inhibition of the active enzyme by tissue inhibitors of metalloproteinases (TIMPs). Metalloproteinase 1 (MMP1) is an interstitial collagenase and Metalloproteinase 9 (MMP9) is a gelatinase B (Ye and Azar (1998), Brooks and Ollivier (2004), Ollivier et al. (2007).

Five, 25% (5/20) of the seropositive animals also showed some evidence of inflammation such as blood vessels and inflammatory cell infiltrate and 15% (3/20) showed bulbar congestion as well. An immune-mediated attack on the cornea may explain the presence of inflammatory cells in the corneal tissue that may have been recruited by circulating leptopiral antibodies produced in more infected organs (Wada et al. (2003).

Corneal edema and conjunctival congestion also are classic clinical signs of ophthalmic changes in horses with leptospirosis (Braga et al. (2011).

The authors suggest the observed corneal and conjunctival changes observed here are a reflection of subclinical keratitis and/or keratouveitis in the latent or chronic stages of *Leptospira*l infection in horses.

4. Conclusions

Corneas sampled from horses that were seropositive for L. *interrogans* (serovars icterohaemorrhagiae, autumnalis, patoc, sentot, and hebdomadi) are significantly increased in thickness, present a higher prevalence of blood vessels and inflammatory cell infiltrates and smaller area of reactivity against MMP1, compared to samples from (normal) seronegative horses. The association of these findings gives support to the theory of an antigenic relationship between *Leptospira* sp. and components of the ocular tissues as a potential cause of subsequent delayed hypersensitivity reaction.

Acknowledgments: FAPESP – Fundação de Amparo à Pesquisa do Estado de São Paulo – Brazil (Foundation for the Support of Research of São Paulo State). Laboratory of Animal Leptospirosis Diagnosis, Department of Preventive Veterinary Medicine and Animal Reproduction of FCAV/UNESP, Jaboticabal, Brazil. Laboratory of Experimental Pathology, Department of Pathology, PUC / PR – Curitiba, Brazil.

Animal Welfare Approval: The present study was approved by the Ethical and Animal Welfare Commission (CEBEA) – FCAV/UNESP-Jaboticabal-SP, Brazil. (Process # 014312-07).

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