# Improving the laboratory diagnosis of leptospiral uveitis in horses by using an indirect ELISA for the detection of antibodies against Leptospira spp. in intraocular samples

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**Summary**: Equine recurrent uveitis (ERU) is a frequently occurring disease, at least in horses in Germany. A vitrectomy is predominantly the course of action in cases where a chronic intraocular leptospiral infection is present. In some cases, the clinical and ophthalmological diagnosis is not conclusive and in these cases a preoperative laboratory test using anterior chamber fluid is indicated. However, false negative results occur when only MAT is used. The aim of this study was to investigate intraocular samples from horses with recurrent uveitis by using ELISA to detect anti-leptospiral antibodies and to compare the results to those of more established laboratory tests, i. e. MAT and PCR. Preliminary investigations indicated a high sensitivity of ELISA in the detection of leptospiral uveitis in horses. 80 eyes of 72 diseased horses were included in the study. The patients' history was taken and a thorough ophthalmologic examination was performed. Intraocular fluids were obtained by diagnostic anterior chamber paracentesis (n = 29) or therapeutic vitrectomy (n = 80) and submitted for MAT, ELISA and PCR to detect leptospiral antibodies and/or DNA. The ELISA allowed the separate determination of the immunoglobulin classes M, G and A. For statistical evaluation Cohen's kappa and the chi-square tests were applied and a p-value of 0.05 or less was considered to be significant. 22 healthy horses (n = 42 eyes) served as controls. 78% of vitreous samples from horses with ERU and 10% of healthy controls reacted positively in the MAT, whereas in the ELISA it was 85% and 0%, and in the PCR it was 61% and 0%, respectively. Specific IgA was detected in 84% of the vitreous samples from horses with ERU, and in 11% of cases, IgA was solely detectable. Differences were highly significant (p<0.001). None of the aqueous samples in the control group contained leptospiral antibodies. Up to 64% of aqueous humour specimens that were obtained for diagnostic purposes yielded a positive result in the MAT. For the ELISA, it was even 89%. Anti-leptospiral IgA was the most frequently detected immunoglobulin class. There was a high level of agreement in the MAT and ELISA results. However, ELISA provided a greater number of positive results. The detection of IgA against leptospires proved to be especially sensitive for an intraocular leptospiral infection and, thus, important for the laboratory diagnosis of leptospiral uveitis in horses. Looking at the cost involved in laboratory tests, it would be reasonable to start with an IgA ELISA. Only if there are negative results, additional tests could be performed. In single IgA negative cases there might be positive reactions using MAT, PCR or ELISA for IgG or IgM.

**Keywords**: horse, ophthalmology, uveitis, diagnostics, ELISA, leptospires, ERU

## Introduction

With a prevalence of 8% (*Szemes* and *Gerhards* 2000) equine recurrent uveitis (ERU) is the most frequently occurring ocular disease in horses worldwide. Moreover, it is the main reason for visual impairment and loss of vision in horses (*Kalsow* and *Dwyer* 1997, *Gerhards* and *Wollanke* 2001, *Sandmeyer* et al. 2017). Thus, together with the laborious and costly treatment of the disease as well as its possibly negative impact on the horses' performance, ERU constitutes a great economic problem for horse owners and the equine industry. Furthermore, medical treatment that is essential during acute uveitic attacks, includes substances which are listed as prohibited by the FEI (Fédération Equestre Internationale) and other sport horse federations, meaning that these horses cannot take part in competitions.

ERU is characterized by acute, recurring, and painful inflammatory episodes alternating with quiescent stages of variable duration (*Morter* et al. 1969). The intraocular fluids of affected horses can be easily obtained from a therapeutic vitrectomy (*Gerhards* et al. 1999) or an anterior chamber paracen-

tesis, and are therefore available for further analysis (*Wollanke* 2002, *Gesell* et al. 2006). These analyses have made a valuable contribution to the understanding and diagnosis of ERU. 90% of the vitreous samples from horses with ERU contained antibodies against leptospires in the microscopic agglutination test (MAT). Leptospiral DNA was detected in a polymerase chain reaction (PCR) in 70% of the specimens, and culture was successful in 50% of cases (*Wollanke* et al. 2004 a). Consequently, ERU can primarily be considered as a consequence of a chronic intraocular leptospiral infection. Moreover, autoimmune reactions can also be observed (*Gilger* et al. 1999).

Studies on autoimmune reactions, carried out by the research group of Deeg (*Deeg* et al. 2001, *Deeg* et al. 2002, *Deeg* et al. 2004, *Deeg* et al. 2006, *Deeg* 2008), were performed on identical intraocular samples from the same equine eyes used for the detection of an intraocular leptospiral infection mentioned above (*Wollanke* et al. 1998a,b, *Brem* et al. 1999a, *Wollanke* et al. 1999, *Wollanke* et al. 2000b, *Gerhards* and *Wollanke* 2001, *Wollanke* et al. 2001a,b, *Wollanke* 2002, *Hartskeerl* et al. 2004, *Wollanke* et al. 2004 a, *Niedermaier* et al.

2006, *Brandes* et al. 2007, *Roczek* 2008, *Loibl* 2009, *Wiehen* 2012, *Popp* et al. 2013, *Roth* et al. 2014, *Schinagl* 2017).

Recurrences of equine uveitis can only be observed during a persistent leptospiral infection. A vitrectomy, however, removes not only the vitreous cloudiness that is caused by the intraocular inflammation and impairs vision, but also the leptospiral infection itself (*Wollanke* et al. 2004a) and, therefore, successfully prevents further inflammatory episodes (*Winterberg* and *Gerhards* 1997, *Wollanke* 2002, *Wollanke* et al. 2004a, *Gerhards* and *Wollanke* 2005, *von Borstel* et al. 2005, *Gerhards* and *Wollanke* 2006, *Tömördy* et al. 2010, *Schinagl* 2017). Consequently, in addition to the removal of vitreous opacities, the presence of an intraocular leptospiral infection is the primary indication for a therapeutic vitrectomy in affected horses (*Gerhards* et al. 1999, *Wollanke* 2002, *Wollanke* et al. 2004a, *Gerhards* und *Wollanke* 2005, *von Borstel* et al. 2005, *Gerhards* und *Wollanke* 2006, *Tömördy* et al. 2010, *von Borstel* et al. 2010, *Schinagl* 2017).

In most cases, a careful ophthalmological examination can differentiate between leptospiral uveitis and other forms of uveitis, e.g. phacogenic uveitis, traumatic uveitis, septic uveitis, leopard coat pattern uveitis or a chronic iritis (*Wollanke* 2002, *Wollanke* et al. 2004a, *Gerhards* and *Wollanke* 2006). However, in some cases the ophthalmological findings are questionable (either no abnormal findings during quiet intervals or cases of possible phacogenic or leopard coat pattern uveitis). In these cases, the indication for surgery can be verified by sampling anterior chamber fluid and testing for leptospiral antibodies and leptospiral DNA prior to the vitrectomy (*Gerhards* et al. 1999, *Wollanke* 2002, *Wollanke* et al. 2004a, *Gerhards* and *Wollanke* 2005, *Gerhards* and *Wollanke* 2006, *Tömördy* et al. 2010, *Schinagl* 2017).

As has been shown in earlier investigations, there is an intraocular antibody production in ERU (*Wollanke* 2002, *Wollanke* et al. 2004 a). MAT results with paired samples (serum and intraocular fluid) from the same horses showed that they were independent. Intraocular titres are usually much higher than serum titres and intraocular fluids and serum from the same horse often react with different serovars, meaning that the titres in intraocular fluids cannot result from serum leakage.

Intraocular titres are usually much higher than serum titres and are often directed against different serovars (*Wollanke* et al. 1998a, *Wollanke* et al. 2001b, *Wollanke* et al. 2004a). There is hardly any albumine in inflamed eyes, which would be expected from the serum leakage, because albumines are smaller than globulines (*Wollanke* 2005). Furthermore, antibodies against leptospires were found in the intraocular fluids, but none were found against other infectious agents such as Borrelia, Toxoplasma and Herpes virus, which were detectable in the serum of the horses (*Wollanke* 1995, *Wollanke* et al. 1998a,b,c, *Wollanke* et al. 2004a, *Wollanke* et al. 2017a). Finally, in horses with intraocular bleeding there are no antibodies detectable within a very short amount of time in the intraocular fluids. In eyes suffering from glaucoma and a severe breakdown of the blood-ocular barrier, there are no antibodies detectable in the intraocular fluids either (*Wollanke* 2002, *Wollanke* et al. 2004 a). Thus, leptospiral antibodies in intraocular fluids are not a consequence of the blood-ocular barrier breaking down and leakage of serum proteins

into the eye during acute attacks, but rather proof of an intraocular leptospiral infection.

Only a few reports on the application of an enzyme-linked immunosorbent assay (ELISA) for the intraocular detection of specific, anti-leptospiral antibodies in horses with ERU have been published to date (*Halliwell* et al. 1985, *Parma* et al. 1987, *Brem* et al. 1999a, *Verma* et al. 2005, *Loibl* 2009). Preliminary investigations, however, foreshadowed that the ELISA-technique, and especially the intraocular detection of IgA against leptospires, was very sensitive for the diagnosis of leptospiral uveitis in horses (*Brem* et al. 2005).

The primary purpose of this study was to investigate intraocular specimens (vitreous and aqueous humour) of horses with ERU using an indirect ELISA-technique to detect specific, anti-leptospiral immunoglobulins M, G and A. A further aim of the study was to analyse and evaluate the suitability of the ELISA for the detection of an intraocular leptospiral infection and to compare its results to those of more established laboratory tests, i.e. MAT and PCR. An optimization of the approach to laboratory diagnosis in the context of diagnostic paracenteses in horses without a definite clinical diagnosis in terms of ERU was hence an integral part of the study.

## Materials and methods

### Horses

72 diseased horses were included in the study. The patient population comprised cases that were presented or referred to the equine clinic of the Ludwig-Maximilians-University (LMU) Munich, Germany, for vitrectomy either following a clinical diagnosis of recurrent uveitis or a positive test result regarding leptospiral antibodies and/or DNA in an aqueous humour analysis. 22 horses without ocular complaints that had to be euthanized for ethical reasons served as controls. The age of the horses with ERU ranged between 2 and 15 years (average 7.8 years). The control horses were between 2 and 32 years old (average 13.0 years). 23 mares (control group: 9), 43 geldings (control group: 11) and 6 stallions (control group: 2) of different colour and breed were represented. The horses mainly came from Germany, although some came from Austria, Switzerland and the Czech Republic.

### History

The history of the patient group focused on the total duration of the disease, the number of uveitic attacks observed and the period of time that had passed since the last inflammatory episode. For the control horses a prior ocular disease or irritation was anamnestically excluded as far as was possible.

### Ophthalmologic examination

Every horse was subjected to a complete ophthalmoloical examination. This examination particularly focused on the unambiguity of clinical signs in terms of ERU, the severity of ocular changes as well as the manifestation of the disease predominantly as anterior uveitis, posterior uveitis or panuveitis. In the control

**Table 1**     Overview of intraocular specimens analysed in the course of the study.   |   Übersicht über die intraokularen Proben, die in der vorliegenden Studie untersucht wurden.

| Type of sample | Total | Number of samples in groups | Groups | Abbreviations |
|---|---|---|---|---|
| Vitreous material | 122 | 80 | ERU | VM1 |
| | | 42 | Healthy controls | VM0 |
| Aqueous humour | 71 | 16 | Healthy eye of horses with unilateral ERU | AH1a |
| | | 2 | ERU | AH1b |
| | | 11 | ERU in question | AH1c |
| | | 42 | Healthy controls | AH0 |

group a careful ophthalmic examination was performed while the horse was alive, if possible, otherwise straight after euthanasia. Therein, any ocular changes that are known to be symptoms of ERU led to an exclusion of the horse from the study.

*Intraocular specimens*

122 vitreous specimens were analysed in the course of this study (Table 1). Thereof, 80 vitreous samples were obtained during therapeutic vitrectomies (VM1). These surgeries were performed as described by *Gerhards* et al. (1999). A three-way port attached to the vitrectomy instrument allowed the sterile withdrawal of 3–5 ml undiluted vitreous material with a 5 ml-syringe at the beginning of the surgery before the dripping line for the lavage fluid was opened. 42 vitreous specimens of healthy controls were obtained directly after euthanasia using a sterile intravenous catheter (VM0).

Furthermore, 71 aqueous specimens were analysed (Table 1). Thereof, 42 belonged to healthy horses in the control group (AH0). In 16 cases an aqueous sample was withdrawn from the clinically healthy eye of horses that underwent a vitrectomy (AH1a). This was done at the request of the horses' owners, in order to rule out a potentially unapparent intraocular infection of the other eye.

Two samples were obtained during synechiolysis which was necessary before a vitrectomy could be performed (AH1b). Another 11 horses lacking unambiguous clinical signs of recurrent uveitis underwent an anterior chamber paracentesis to indicate whether a vitrectomy was to be carried out (AH1c). (Note: Far more horses were subjected to a diagnostic anterior chamber paracentesis during the study, but only cases with a laboratory diagnosis of leptospiral uveitis that subsequently underwent a vitrectomy were included in the study). In horses with ocular complaints, 0.5–1.0 ml of aqueous humour was extracted by limbal paracentesis with a 2 ml-syringe and a 27G hypodermic needle under a short general anaesthesia. In the control horses the sample was obtained the same way immediately after euthanasia.

*Microbiological analyses*

Intraocular samples were submitted for the following laboratory tests: MAT and ELISA for the detection of leptospiral antibodies, and PCR for the detection of leptospiral DNA. The tests were conducted by the leptospirosis laboratory, which is part of the Bavarian Health and Food Safety Authority (LGL), in Oberschleißheim, Germany.

The microscopic agglutination test was performed in accordance with the guidelines of the O.I.E. (Office International des Epizooties, The World Organisation for Animal Health, OIE 2014). The following leptospiral serovars were used as live antigens: Bratislava, Canicola, Copenhageni, Grippotyphosa, Hardjo, Icterohaemorrhagiae, Javanica, Pomona, Pyrogenes, Saxkoebing, Sejroe and Tarassovi. Titres higher than 1:100 were assessed as being specific. The highest titre measured was included in the results. To simplify the statistical evaluation, the respective serovar was not taken into account any further.

The in-house ELISA-technique initially assumed an antigen extraction and was developed by *Kettner* (1997). This indirect technique, which utilized a complete leptospiral antigen, allowed both the qualitative and quantitative determination of the immune response as single immunoglobulin classes, i.e. IgM, IgG and IgA, were detected separately using anti-horse IgA, anti-horse IgG and anti-horse IgM and could reach different values. Thus, a sample could deliver a negative (-), borderline (+/-), weakly positive (+), positive (++) or highly positive result (+++) depending on its optical density, determined using photometry (wave length 405 nm) (Table 2). For statistical evaluation a borderline result was assessed as being negative.

The serovars for the ELISA were chosen according to the positive results in the MAT and the geographical origin of the horse. Each specimen was analysed applying between one and four different serovars. In the case of a negative test result in the MAT, the ELISA was conducted with a Grippotyphosa and a Bratislava antigen. The following serovars were used: Bratislava, Canicola, Copenhageni, Grippotyphosa, Icterohaemorrhagiae and Pomona. To simplify the statistical evaluation and the comparison of results from different horses, the ELISA results for the respective leptospiral serovars were not differentiated. Only the highest extinction measured for each immunoglobulin class accounted for the evaluation.

**Table 4**     Agreement of test results (MAT, ELISA and PCR) as to leptospiral antibodies and DNA in aqueous and vitreous specimens of the same eye in horses with recurrent uveitis.  /  *Übereinstimmung derErgebnisse von Kammerwasser- und Glaskörperproben aus denselben Augen bei Verwendung von MAR, ELISA und PCR.*

| Laboratory test | Total | $N_{AH+VM+}$ | $N_{AH-VM+}$ | $N_{AH+VM-}$ | $N_{AH-VM-}$ |
|---|---|---|---|---|---|
| MAT | 14 | 8 | 4 | 2 | 0 |
| ELISA | 12 | 8 | 2 | 2 | 0 |
| PCR | 11 | 0 | 4 | 6 | 1 |

79 vitreous samples from horses with ERU, the 42 vitreous specimens of the control group and all aqueous samples were analysed by quantitative real time PCR targeting the lipL32 gene present only in pathogenic Leptospira spp. This technique was established by *Roczek* and *others* (2008).

*Statistics*

For statistical evaluation Cohen's kappa and the chi-square tests were applied (SPSS for Windows version 16.0). A p-value of 0.05 or less was considered significant.

## Results

*Ophthalmologic examination:*

In 66 (82.5%) out of 80 equine eyes in the patient group typical ocular findings in terms of ERU allowed a definite clinical diagnosis. In 14 cases (17.5%) the findings were uncertain in terms of ERU, but an intraocular leptospiral infection was confirmed by aqueous humour analysis prior to surgery.

*Laboratory findings:*

78% (62/80) of the vitreous samples from horses with ERU (VM1) – but only 10% (4/42) of those in the control group (VM0) – contained leptospiral antibodies that were detectable by MAT. The difference was highly significant (Fisher's Exact Test, $p < 0.001$) (Table 3). The titre found most often among horses with ERU was 1:800. The median was 1:400 and the highest titre was 1:102,400. The four positive specimens in the control group all yielded a titre of 1:100 and belonged to three different horses. Anti-leptospiral antibodies were detected in 85% (68/80) of the vitreous samples from horses with ERU (VM1) using ELISA, but none were detected in the control group (VM0) (Table 3). The difference, again, was highly significant (Fisher's Exact Test, $p < 0.001$).

IgM antibodies against leptospires were detected in 34% (27/80) of the vitreous samples from horses with ERU. For IgG it was 74% (59/80) and for IgA it was 84% (67/80). In 11% of the cases, the detection of IgA – compared to IgM or IgG – was most frequently solely successful (9/80). IgG was once solely detected. 39% (31/80) of the vitreous specimens contained detectable immunoglobulins of both classes, G and A, and 34% (27/80) had all three classes. Leptospiral DNA was detected in 61% (48/79) of the vitreous samples obtained from horses with recurrent uveitis (VM1), whereas

none of the specimens in the control group (VM0) showed a positive reaction (Table 3). The difference was highly significant (Fisher's Exact Test, $p < 0.001$).

None of the aqueous samples in the control group (AH0) contained leptospiral antibodies when tested with MAT or ELISA (Table 4). 3 out of 16 (19%) specimens that had been taken from horses' clinically healthy eyes during general anaesthesia for vitrectomy on the respectively other eye demonstrated a positive result in MAT as well as in ELISA (AH1a).

Titres of 1:100, 1:200 and 1:400 were determined by MAT. Specific IgM was not detectable with ELISA whereas IgG against leptospires was detected twice, and IgA was detected three times, i.e. in all cases. All three horses underwent a vitrectomy on their owners' request within the following week. One of the two samples from horses with obvious clinical signs of recurrent uveitis (synechiolysis prior to surgery because of miotic pupils and impaired vision into the vitreous) reacted positively when both MAT and ELISA were applied (AH1b). The titre was 1:200, and specific immunoglobulins of classes G and A were detectable in the same sample. The second sample, however, remained negative. 64% (7/11) of the aqueous samples from equine eyes without definite clinical signs in terms of ERU – but in the end at least one positive test result – contained leptospiral antibodies detectable by MAT (AH1c). When ELISA was applied, it was 89% (8/9). Titres up to 1:1,600 were determined by MAT.

ELISA delivered the following percentages of positive results considering the single immunoglobulin classes of anti-leptospiral antibodies: IgM 22% (2/9), IgG 56% (5/9) and IgA 89% (8/9). Thus, IgA was detected in every sample that was assessed as positive by ELISA. Differences between horses with ERU and those in the control group were highly significant with regards to MAT and ELISA results (Fisher's Exact Test, $p < 0.001$).

Leptospiral DNA could not be detected by PCR in any of the aqueous samples of the control group (AH0). One specimen in the group of horses that suffered from unilateral recurrent

**Table 4** Agreement of test results (MAT, ELISA and PCR) as to leptospiral antibodies and DNA in aqueous and vitreous specimens of the same eye in horses with recurrent uveitis. / *Übereinstimmung derErgebnisse von Kammerwasser- und Glaskörperproben aus denselben Augen bei Verwendung von MAR, ELISA und PCR.*

| Laboratory test | Total | $N_{AH+VM+}$ | $N_{AH-VM+}$ | $N_{AH+VM-}$ | $N_{AH-VM-}$ |
|---|---|---|---|---|---|
| MAT | 14 | 8 | 4 | 2 | 0 |
| ELISA | 12 | 8 | 2 | 2 | 0 |
| PCR | 11 | 0 | 4 | 6 | 1 |

**Table 3** Differences between horses with ERU and controls concerning positive test results as to leptospiral antibodies or DNA in vitreous material. | *Vorkommen von Antikörpern gegen Leptospiren und Nachweis von Leptospiren-DNA in Glaskörperproben*

| Laboratory test | ERU | Control | p-value |
|---|---|---|---|
| | Percentage of positive results | | |
| MAT | 78 % | 10 % | $p < 0.001$ |
| ELISA | 85 % | 0 % | $p < 0.001$ |
| PCR | 61 % | 0 % | $p < 0.001$ |

**Table 5** Agreement of MAT and ELISA as to positive or negative test results in the detection of leptospiral antibodies in intraocular samples of horses with ERU. | *Vergleich der Ergebnisse beiden Antikörpertests (MAR und ELISA) in intraokularen Proben von an ERU erkrankten Pferden.*

| Specimen | Total | $n_{both+}$ | $n_{both-}$ | $n_{MAT+ELISA-}$ | $n_{MAT-}$ |
|---|---|---|---|---|---|
| Vitreous | 80 | 61 | 11 | 1 | 7 |
| Aqueous humour | 14 | 8 | 1 | 1 | 4 |

uveitis and underwent paracentesis on the other eye reacted positively in PCR (AH1a). The specimen belonged to one of the three horses whose aqueous samples also contained antibodies against Leptospira spp. PCR was applied in only one of the two cases with a definite clinical diagnosis of ERU (AH1b). Thus, it delivered a positive test result. An investigation of the aqueous humour by PCR was initiated in 9 out of 11 cases without definite clinical signs of recurrent uveitis (AH1c). Therein, 5 (45%) samples were assessed as positive. The difference regarding PCR results of horses with ERU and those in the control group was highly significant (Fisher's Exact Test, $p < 0.001$).

In the context of this study samples from both ocular compartments of the same equine eye, i.e. aqueous and vitreous fluids, were taken for further analysis and then compared to the laboratory results from 14 (MAT), 12 (ELISA), and 11 (PCR) eyes with recurrent uveitis. Table 5 gives an abridgement of the test results (MAT, ELISA and PCR) for both ocular compartments.

In eight cases specific antibodies were detectable in both samples by MAT (57%). Four times the MAT result was negative for both compartments (29%). Only twice did the aqueous humour contain detectable antibodies against leptospires (14%), whereas the vitreous of the same eyes was MAT negative. Thus, the qualitative information of the MAT regarding the two ocular compartments agreed in 86% of the cases.

Eight paired samples contained anti-leptospiral antibodies that could be detected by ELISA (67%). Two pairs agreed in their negative result (17%). Specific antibodies were only detectable in the aqueous humour twice (17%). The same combination of negative and positive test results for the distinct immunoglobulin classes could be observed for nine paired samples (75%). Three pairs differed in their ELISA profile (25%). In four cases aqueous and vitreous samples agreed in their negative PCR result (36%). Leptospiral DNA was only detected in the aqueous humour six times (55%) and only once in the vitreous specimen (9%).

80 vitreous samples from horses with ERU were analysed for leptospiral antibodies using MAT and ELISA. Therein, 61 (76%) showed a positive result in both tests. 11 (14%) did not contain any detectable anti-leptospiral antibodies. Specific antibodies were solely detected by MAT once (1%), and in seven cases (9%) they were solely detected by ELISA (Table 5). A high level of agreement was observed between MAT and ELISA tests ( = 0.675; $p < 0.001$). None of the seven vitreous

samples that reacted positively when only ELISA was applied contained specific IgM antibodies. In four cases IgG (57%) and in six cases IgA (86%) antibodies against leptospires were detected. IgA was solely detectable three times, and IgG was solely detectable in one case.

14 aqueous samples from horses with ERU were analysed for leptospiral antibodies using MAT and ELISA. In eight cases (57%) anti-leptospiral antibodies were detectable in both laboratory techniques. One specimen did not react positively in any of the two tests (7%). Four times solely the ELISA result was positive (29%). A further sample showed a positive result when only MAT was applied (7%) (Table 6). There was no significant level of agreement calculated.

Only one of the four aqueous samples with anti-leptospiral antibodies solely detectable with ELISA contained specific antibodies of all three immunoglobulin classes tested (25%). One contained IgG as well as IgA (25%) and two specimens contained solely IgA (50%). Thus, IgA against leptospires was detectable in all samples with a negative test result in the MAT.

The sensitivity and specificity of the ELISA technique in analysing vitreous samples from horses with ERU (including those with a positive test result in the aqueous humour analysis for leptospiral DNA and/or antibodies beforehand) was calculated setting the MAT as the basing point, as is often described in literature (gold standard). The sensitivity was 98% and the specificity 61%.

The diagnostic sensitivity and specificity of the MAT, ELISA and PCR based on definite ocular findings in terms of ERU were only calculated for cases without a laboratory confirmation of an intraocular leptospiral infection by aqueous humour analysis beforehand. Thus, the calculation was made for 66 (MAT and ELISA) and 65 (PCR) vitreous samples from horses with clinical ERU, respectively, and 42 specimens of control horses (Table 7).

## Discussion

Although some authors have already used the MAT to investigate the local humoral immune response to leptospires in horses with ERU and others have concentrated on the analysis of the systemic antibody production of affected horses, information on the detection of intraocular antibodies against leptospires in horses with ERU using ELISA is very rare.

**Table 6** Sensitivity, specificity, positive and negative predictive values for MAT, ELISA and PCR using vitreous samples from horses showing definite ocular findings in terms of ERU but without laboratory confirmation prior to vitrectomy. | *Sensitivität, Spezifität, positiver und negativer prädiktiver Wert für MAR, ELISA und PCR bei Verwendung von Glaskörperproben von Pferden, bei denen präoperativ ausschließlich anhand klinisch-ophthalmologischer Untersuchung die Diagnose ERU gestellt wurde und präoperativ keine Labordiagnostik durchgeführt wurde.*

| Laboratory Test | Sensitivity | Specificity | Positive Predictive Value | Negative Predictive Value |
|---|---|---|---|---|
| MAT | 82 % | 91 % | 93 % | 76 % |
| ELISA | 86 % | 100 % | 100 % | 82 % |
| PCR | 72 % | 100 % | 100 % | 70 % |
| IgM-ELISA | 39 % | 100 % | 100 % | 51 % |
| IgG-ELISA | 80 % | 100 % | 100 % | 79 % |
| IgA-ELISA | 86 % | 100 % | 100 % | 82 % |

As described previously (*Wollanke* et al. 2004a, *Loibl* 2009), significant differences in the detection of intraocular antibodies against leptospires using MAT (as well as ELISA) were observed in horses with recurrent uveitis and healthy controls. The percentage of positive results in the MAT among clinically healthy horses regarding vitreous samples (10%), however, was slightly higher than in other studies (0–6%) (*Wollanke* et al. 1998a, *Wollanke* et al. 2001a,b, *Gesell* 2004, *Wollanke* et al. 2004 a). *Wollanke* and others (2001b) hypothesized that in such cases, too, recurrent uveitis could develop eventually. However, a follow-up examination of the affected equine eyes was not possible as the samples had been taken after the control horses had been euthanised.

In the present study specific IgA was the immunoglobulin class most often intraocularly detected by ELISA (in aqueous as well as vitreous humour). Moreover, in some cases it was solely detectable. This suggests that the detection of IgA against leptospires is in fact very sensitive for an intraocular leptospiral infection. According to the results of this study, a false positive result of the IgA ELISA seems very unlikely because in intraocular samples from the controls – unlike MAT – no anti-leptospiral antibodies were detectable with ELISA, and no bacterial DNA was detected with PCR. This argues for the reliability of the ELISA technique in the diagnosis of an intraocular leptospiral infection. The intraocular appearance of IgA can probably be attributed to local production and secretion, as IgG – which was detected more frequently in serum than IgA (*Loibl* 2009) – despite its lower molecular weight (IgG = 180 kDa compared to the dimeric IgA (150 kDa as monomer)) (*Tizard* 2004) was less frequently found in intraocular samples. *Wagner* and others (1997) also observed a selective and considerable relative increase of the IgA content in vitreous samples compared to the autogenic serum in horses with ERU. This observation was not made in healthy horses. The authors assumed an increased local synthesis as an expression of a local immune reaction to an intraocular antigen. Moreover, *Verma* and co-workers (2005) detected IgA antibodies against leptospiral lipoproteins, LruA and LruB, using ELISA solely in uveitic equine eyes. The results of our study together with those found in literature point to the crucial role of (specific) IgA in the pathogenesis of ERU and the value of an IgA ELISA for the diagnosis of an intraocular leptospiral infection in horses.

In 14% of the vitreous specimens of horses with suspected leptospiral uveitis by ophthalmological examination leptospiral antibodies could not be detected neither with MAT nor ELISA. This observation is consistent with studies of other authors who also failed to detect specific antibodies in all of the intraocular samples from horses suffering from recurrent uveitis (*Wollanke* et al. 1998a,b, *Wollanke* et al. 2001a,b, *Wollanke* et al. 2004a, *Brandes* et al. 2007, *Tömördy* et al. 2010, *Wiehen* 2012, *Kulbrock* et al. 2013, *Baake* et al. 2016, *Dorrego Keiter* et al. 2016, *Dorrego Keiter* et al. 2017). The different percentages of Leptospira positive and negative samples might be attributable to different methods in case selection and sampling techniques.

Biofilm formation plays an important role in the survival of bacteria and evasion from the immune system and their protection from environmental threats, and has been described for apathogenic and pathogenic Leptospira spp. (*Ristow* et al.

2008, *Brihuega* et al. 2012, *Kumar* et al. 2015, *Thibeaux* et al. 2017). This phenomenon might contribute to false negative test results.

Furthermore, *Wollanke* and others (1998a) hypothesized that other pathogens besides Leptospira spp. and further factors could have an influence on the development of ERU, but until today no other infectious agents, e.g. herpes virus, Toxoplasma spp., or Borrelia spp., could be linked to ERU (*Wollanke* 1995, *Wollanke* et al. 1998b, *Wollanke* et al. 2000b). Only Micronema (= Halicephalobus) and Borrelia spp. seem to induce some kind of severe uveitis, but clinically different from the typical ERU (*Rames* et al. 1995, *Burgess* et al. 1986, *Barnett* et al. 1995, *Hahn* et al. 1996, *Isaza* et al. 2000, *Kinde* et al. 2000, *Wollanke* et al. 2000a, *Boswinkel* et al. 2006, *Wagner* et al. 2011, *Priest* et al. 2012, *Wollanke* et al. 2017 a).

Finally, ERU could be a purely autoimmune-mediated process in some cases, an approach that is favoured by others (*Deeg* et al. 2001, *Deeg* et al. 2002, *Deeg* et al. 2004, *Deeg* et al. 2006, *Deeg* 2008, *Gilger* et al. 2008). At this point, however, it seems to be important to keep in mind that Deeg and coworkers performed their studies on vitreous material which had been tested positive for leptospiral infections in most cases, as mentioned in the introduction. Besides, other ocular samples used for investigations in autoimmune reactions have not been tested for a leptospiral infection. It is plausible that a persistent intraocular leptospiral infection is accompanied by certain autoimmune reactions (*Wollanke* 2002, *Wollanke* et al. 2004 a).

Regional differences might play a role in the prevalence of leptospiral uveitis because humidity and vectors (e.g. mice and rats) are important both for the survival of the bacteria in the environment and for the infection of horses (*Faine* et al. 2000, *Wollanke* et al. 2004 a,b). If leptospires are present in the surroundings of equids, most horses have detectable antibodies in the serum and some of them may develop uveitis (*Wollanke* et al. 1998a, *Szemes* and *Gerhards* 2000). There is not a single serovar which causes recurrent uveitis in horses. To the contrary, different serovars are able to infect the equine eyes and cause recurrent uveitis (*Wollanke* et al. 2001b, *Wollanke* et al. 2004 a). Which particular serovar can be detected in the eyes depends on the region in which the horse grew up or lives, which vectors live there, and thus, which serovars are attendant. Leptospiral uveitis is a common problem not only in horses in central Europe, but in other countries and continents, too – though in different prevalence (*Witmer* 1954, *Williams* 1968, *Morter* et al. 1969, *Bernard* 1993, *Faber* et al. 2000). In leopard coat pattern horses, uveitis often is less painful, initially shows a cataract formation, tends to develop glaucoma in many cases, and is not a leptospiral uveitis (*Baumgart* and *Gerhards* 2014).

While not a single aqueous sample of the control horses without a history or clinical signs of recurrent uveitis reacted positively in neither MAT nor ELISA, 19% of the aqueous specimens of the clinically healthy eye of horses with unilateral ERU, however, contained anti-leptospiral antibodies that were detectable in both MAT and ELISA. Consequently, a preventive screening of equine eyes without a history or symptoms of ocular disease does not seem advisable due to anaesthetic

risks and the danger of an ocular infection following paracentesis. Performing an anterior chamber paracentesis in the clinically healthy eye of horses with unilateral recurrent uveitis, however, can be taken into consideration during general anaesthesia for a vitrectomy of the affected eye. Consequences of a positive test result regarding an intraocular leptospiral infection, i.e. performing a vitrectomy or awaiting definitive clinical symptoms, need to be carefully pondered.

Leptospiral DNA could be detected in 61% of the vitreous samples from horses with ERU, whereas in the control group none showed a positive reaction. Other authors reported on 57% to 100% of positive test results in intraocular samples from horses with ERU (*Faber* et al. 2000, *Wollanke* 2002, *Gesell* 2004, *Wollanke* et al. 2004a, *Brandes* et al. 2007, *Wiehen* 2012, *Baake* et al. 2016, *Dorrego Keiter* et al. 2016, *Schingl* 2017). The frequent detection of leptospiral DNA in intraocular material of equine eyes with recurrent uveitis confirmed that leptospires play a decisive role in the aetiology and pathogenesis of the disease.

In this study, the detection of specific antibodies seemed more sensitive for an intraocular leptospiral infection than the application of PCR. No additional information was gained by the use of PCR when aqueous samples were investigated following diagnostic paracentesis. A different study based on a larger patient population, however, revealed that, especially in cases without a definite clinical diagnosis of ERU, PCR serves as a valuable supplementation to MAT or ELISA in the analysis of aqueous samples for determining the aetiology of ocular changes (*Gesell* et al. 2006). The different results of this study compared to others can possibly be ascribed to differences in the patient population, the smaller number of aqueous samples analysed or deviant criteria in the patient selection process. In both studies the PCR was carried out by the same laboratory. Therefore, different laboratory techniques do not account for the different results.

It is remarkable that the detection of leptospiral DNA in this study was nearly as successful in the aqueous humour (in total) as in the vitreous material of horses with (suspected) recurrent uveitis. This suggests that at the beginning of the disease, leptospires disperse more equally in the equine eye (and are therefore more easily detected in the aqueous humor). However, as the disease progresses, the leptospires persist mainly in the avascular vitreous body, having been eliminated from the uvea and the anterior chamber (*Brandes* et al. 2007).

In 90% of the vitreous and 64% of the aqueous specimens of horses with ERU, the test results of MAT and ELISA for leptospiral antibodies were consistent, i.e. in each case negative or positive. Otherwise, anti-leptospiral antibodies were mostly detected solely by ELISA.

The observation that none (vitreous), or only one (aqueous) of the samples, respectively, that showed a positive test result solely in the ELISA contained detectable IgM antibodies can presumably be ascribed to the fact that IgM is primarily responsible for agglutination and therefore, of all the immunoglobulin classes is best detected by MAT (*Hanson* 1973, *Priya* et al. 2003). The samples only reacted positively in the ELISA as they seemingly did not contain any detectable

amounts of specific IgM. However, one needs to be aware that IgM against leptospires could be least frequently detected intraocularly in this study. Besides, it was noticeable that the majority of samples with a negative result in the MAT contained specific IgA, and some of them in turn contained solely IgA. This observation is consistent with the retrospective evaluation of ELISA results of intraocular samples from horses with ERU where anti-leptospiral IgA was the most frequently detected immunoglobulin class (99%), and was solely detectable in 63% of specimens (*Loibl* 2009). Thus, compared to the other two immunoglobulin classes tested, especially the detection of IgA by ELISA, it seems suitable to complement the MAT in the analysis of intraocular specimens for anti-leptospiral antibodies.

Although in this study aqueous and vitreous humour of the identical equine eye were obtained and analysed from only very few cases of recurrent uveitis, it seemed justified to compare the laboratory results of the two compartments as other studies had achieved similar results (*Wollanke* 2002, *Gesell* et al. 2005, *Gesell* et al. 2006). Furthermore, in this study, too, MAT and ELISA results largely agreed in the paired samples. There is no considerable barrier between the aqueous humour and the vitreous body meaning that there is a diffusion of soluble agents between the two compartments (*Gum* 1991).

Due to the aqueous humour dynamics, the distribution of antibodies within the eye is relatively equal. Thus, one can act on the assumption that findings in the aqueous chamber correlate well with the situation in the posterior eye segment (*Thurau* 2003). Therefore, the suitability of a diagnostic anterior chamber paracentesis to verify an indication for a vitrectomy in cases missing definite clinical signs in terms of ERU could be confirmed. Furthermore, unnecessary, invasive and expensive intraocular surgeries can be avoided.

This equine clinic has years of experience and knowledge in the examination of equine uveitic eyes and diagnosing recurrent uveitis based on clinical findings. Furthermore, studies have proved the high agreement of the clinical diagnosis "leptospiral uveitis" with a successful laboratory investigation of intraocular samples from the respective eye for leptospiral antibodies by MAT (*Wollanke* et al. 2004 a). Thus, it seemed justified to judge the sensitivity and specificity of the applied laboratory tests in this study by comparing their results to existing or missing ocular changes in terms of ERU.

The highest level of agreement with the definite clinical diagnosis "ERU" was calculated for the ELISA technique and especially for the detection of specific IgA. It was also ascribed the highest sensitivity (86%) and specificity (100%) in assessing vitreous samples from clinically sick and healthy equine eyes. The most reliable way of evaluating laboratory tests, though, would be to assess them with samples from known carriers. In the diagnosis of leptospirosis, however, culture is time consuming and insensitive (*Faine* 1982) and therefore, seems inappropriate.

In the literature the MAT is often described as gold standard (*Vinetz* 1997) and newly developed ELISAs are often evaluated with the MAT being considered as the basing point. Bearing in mind that these two serological tests seemingly detect

different antigen-antibody-systems (*Adler* et al. 1980, *Adler* et al. 1982, *Ballard* et al. 1984), doubts arise regarding the actual comparability of the two methods and the evaluation of the ELISA-technique on the basis of its agreement with the MAT results. *Woodward* and others (1997) also advised caution when interpreting ELISA results compared to MAT, as MAT itself would not deliver definitely correct results. *Yan* and others (1999) also criticized that the main source of errors in assessing the ELISA technique was a comparison of results from the MAT because the serological status, as determined by MAT, often would not mirror the actual infection status of the animal (cattle). *Ribotta* and others (2000) even assumed that the ELISA had a higher sensitivity than the MAT because it was able to detect both agglutinating and non-agglutinating antibodies. Thus, the actual specificity of the ELISA would be underestimated in an evaluation with the MAT set as gold standard. Further, a titre of 1:100 is often set as the cut-off in the MAT (OIE 2014). Considering a lower titre, e.g. 1:50, as being positive, a higher level of agreement might be achieved between MAT and ELISA. Numerous authors from both veterinary and human medicine have suggested that the ELISA had a higher sensitivity than the MAT in detecting anti-leptospiral antibodies (*Adler* et al. 1980, *Brem* et al. 1999b, *Cumberland* et al. 1999, *Ribotta* et al. 2000, *Dutta* and *Christopher* 2005, *Loibl* 2009). Retrospective evaluation of case records on suspected leptospiral uveitis in horses with a negative result in the MAT revealed that 37% of the very same intraocular samples tested positive for leptospiral antibodies with ELISA (*Loibl* 2009). Moreover, an increasing number of leptospiral infections in humans have been reported worldwide in both developing and developed nations. However, only a few laboratories possess the expertise and the equipment necessary for accomplishing a MAT in the diagnosis of leptospirosis. Therefore, the ELISA technique and the development of new indirect laboratory tests are of great importance for the routine diagnosis of leptospiral infections. This is proved by the increasing application of the ELISA both in veterinary and humane medicine.

In this study it was shown that the ELISA technique is appropriate to complement or even replace the MAT in detecting intraocular antibodies against Leptospira spp. in horses suffering from recurrent uveitis. This conclusion is especially valuable for aqueous humour analyses following diagnostic paracenteses in cases without definitive clinical signs in terms of ERU.

## Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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