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ORIGINAL REPORT

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Immunohistochemical staining of immunoglobulin G in healthy equine, canine, and feline corneas

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Abstract

Objective: Establishing an immunohistochemical approach for semi-quantitative assessment of the presence of immunoglobulin G (IgG) in equine, canine, and feline corneas.

Procedures: Healthy corneas of horses, dogs, and cats, euthanized because of a fatal disease or an unrecoverable trauma unrelated to and without a history of ophthalmic disease were formalin-fixed, paraffin-embedded, and determined to be pathomorphologically healthy by light microscopy. Automated immunohistochemistry was performed using primary antibodies against IgG, biotin-conjugated secondary antibodies, and streptavidin-peroxidase, as well as diaminobenzidine for visualization. After counterstaining with hematoxylin, epithelium, stroma, Descemet´s membrane (DM), and endothelium were semi-quantitatively scored for the presence of IgG on a 4-grade scale (0 = no, 1 = faint, 2 = medium, 3 = strong staining) by light microscopy.

Results: Corneal specimens of 20 horses (40 eyes) with a median age of 15.5 years (range 2–31 years), 12 dogs (21 eyes) with a median age of 10.0 years (range 4–16), and 13 cats (24 eyes) with a median age of 10.0 years (range 2–18) were included in the study. Different sexes and breeds were represented. In all corneas (100%), significant medium signal intensity in the stroma was observed. Variable immunosignal was obtained in epithelium, DM, and endothelium.

Conclusion: This method reproducibly allows for the detection of IgG in healthy equine, canine, and feline corneas, particularly stroma. Semi-quantitative results evidence medium presence of IgG in the corneal stroma. Further research is needed to evaluate IgG presence in diseased corneas.

KEYWORDS

canine, cornea, equine, feline, immunoglobulin G, immunohistochemistry

1 | INTRODUCTION

The cornea normally is a clear and avascular tissue containing only few antigen presenting cells.[1,2] Since the corneal stroma contains abundant extracellular matrix suitable for diffusion of small soluble proteins,[3,4] it is conceivable that the humoral immune system might play a role in initiating immune-mediated processes. Yet, the question whether immunoglobulins are constitutively present in the corneas of our domestic animal patients has been a neglected topic in veterinary basic research so far.

The presence of blood proteins including albumin and globulins was discovered by Krause within normal ox corneas as early as 1932, and subsequent protein analyses of fluid extract from the healthy corneal stroma of ox, pig, and man corroborated his findings.[5–7] This led to thorough characterization of the soluble protein content of healthy rabbit and human corneas, confirming immunoglobulins as a normal constituent of the corneal stroma by quantitative and immunohistochemical approaches.[8–11] Although all 5 immunoglobulin classes are present in the stroma, immunoglobulin G (IgG) makes up the largest share, being proportionally overrepresented to the other immunoglobulins compared with serum and other ocular tissues.[9–11] In fact, the lapine and human cornea contain the highest IgG levels of the entire eye including tears and aqueous humor under non-pathological circumstances.[12]

Diffusion experiments in rabbit corneas identified the limbal blood vessels as the most likely source of IgG.[8] With a molecular weight of 150 000 daltons,[13–15] it slowly diffuses centripetally with negligible losses across the epithelium and endothelium,[9,16] reaching an equilibrium at about half of the serum concentration after 50–70 days.[10,17] Its uniform distribution throughout the stroma from Bowman's membrane to Descemet's membrane barely differs in the central versus peripheral region,[9,11] with a linear immunohistochemistry staining pattern, parallel to the epithelium and along the stromal collagen fibers.[9] The variance of corneal IgG content under pathological circumstances is not well understood. The fixed negative charges represented by proteoglycans (PG) and glycosaminoglycans (GAG) have been suggested to play a role,[18] as well as permeability of limbal vasculature and local IgG production under inflammatory conditions.[19,20]

Concerning the clinical significance of corneal IgG presence, a variety of studies have shown antibody-antigen-reactions[21–27] as well as possible local antibody production in the stroma or limbus.[28] Furthermore, corneal immune-complex deposition has been reported as a manifestation of systemic[29–33] and corneal disease[34–37] or after therapeutic intravenous immunoglobulin infusions.[38]

In veterinary ophthalmic research, isolated efforts have been made to identify a correlation between the manifestation of corneal immune-mediated disease and the presence of antibodies within lamellar keratectomy samples of canine and equine corneal tissues. The immunohistochemical studies on canine chronic superficial keratitis (CSK) and equine superficial stromal immune-mediated keratitis (IMMK) did detect antibody presence in diseased corneas. However, the few healthy canine and equine corneal specimen included in the studies as control samples did not reveal any immunoglobulins.[39,40] This is in contradiction with the evidence on rabbit and human corneas and warrants targeted studies of immunoglobulins within the healthy corneas of companion animals.

Thus, we investigated the presence and distribution of IgG within healthy equine, canine, and feline corneas using an immunohistochemical approach under standardized conditions. We hypothesized that IgG would be detectable by chromogenic immunohistochemistry and be evenly distributed throughout the corneal stroma in a similar manner in horses, dogs, and cats. The goal of this study was to provide baseline immunohistochemical staining patterns for future comparison with diseased corneas.

2 | MATERIALS AND METHODS

2.1 | Sample collection

Corneal specimens were obtained at the small animal surgery and equine hospitals of the Veterinary University of Vienna during a period ranging from January 2018 until May 2020. Inclusion criteria defined horses, dogs, and cats without a history of or macroscopically evident ophthalmic disease, euthanized because of a terminal disease or an unrecoverable trauma, to be eligible. Globes were collected by enucleation within 2 h of humane euthanasia and immediately immersed in 4% buffered formaldehyde.

2.2 | Processing and immunohistochemistry

Standardized fixation took place overnight at room temperature for no shorter than 12 and no longer than 24 h to prevent over-fixation. Globes were then bisected in a sagittal plane to obtain a strip of equine cornea including the dorsal and ventral limbus or a sagittal ring of canine and feline cornea and sclera. Paraffin wax-embedded and hematoxylin-stained sections were used to confirm the absence of pathological changes by light microscopy. For

TABLE 1 Breed distribution

Equine breeds	Number of animals	Canine breeds	Number of animals	Feline breeds	Number of animals
Warmblood	8	Mixed breed	4	Domestic Shorthair	9
Haflinger	3	Pomeranian	2	Maine Coon	2
Icelandic	3	Standard Poodle	1	Domestic Longhair	1
Arabian	2	Fox Terrier	1	British Shorthair	1
Tinker	1	Hovawart	1		
Andalusian	1	Border Terrier	1		
Connemara	1	Chihuahua	1		
Noriker	1	American Staff. Bullterrier	1		

Both eyes of all horses were included in the study, whereas in 3 dogs and 2 cats only one eye met the inclusion criteria.

immunohistochemistry, 3–4 μm sections were placed on coated slides and dried to enhance tissue adherence.

The avidin-biotin-complex method was performed automatically on an Autostainer [Lab Vision AS 360, Thermo Scientific]. First, antigen retrieval was performed on deparaffinized and rehydrated sections by heating in citrate buffer (pH 6) [Thermo Scientific/AP-9003–125]. Incubation in H_2O_2 [UltraVision Hydrogen Peroxide Block; Thermo Scientific/AP-9003–125] served to block endogenous peroxidase, and a protein-blocking agent [UltraVision Protein Block; Thermo Scientific/AP-9003–125] was applied in order to reduce unspecific antibody binding. After application of the primary antibodies [Anti-Horse IgG F(c) goat antibody 608–1103 (dilution 1:30 000), Rockland Immunochemicals, Inc.; Anti-Dog IgG F(c) goat antibody 604–1103 (dilution 1:30 000), Rockland Immunochemicals, Inc.; Anti-Cat IgG goat F(c) antibody 602–1103 (dilution 1:50 000), Rockland Immunochemicals, Inc.] the appropriate biotinylated secondary antibody [Biotinylated anti-goat-IgG (dilution 1:300), Vector Laboratories/BA-5000] was used, followed by incubation with streptavidin–peroxidase [Streptavidin Peroxidase; Thermo Scientific/TS-060-HR] and visualization with diaminobenzidine [DAB Quanto; Thermo Scientific/TS-125-QHDX]. Subsequently, all sections were counterstained with hematoxylin, dehydrated, and mounted.

Signal specificity was proven by using equine, canine, and feline lymph node tissue as positive control, and proper negative controls by omitting the primary antibody as well as by using peptide blocking [Horse IgG F(c) fragment 008–0103 (dilution 1:30 000), Rockland Immunochemicals, Inc.; Dog IgG F(c) fragment 004–0103 (dilution 1:30 000), Rockland Immunochemicals, Inc.; Cat IgG F(c) fragment 002–0103 (dilution 1:50 000), Rockland Immunochemicals, Inc.] for every corneal and lymph node specimen undergoing immunohistochemical staining. If residual signal was present in peptide blocking, it was subtracted from the signal intensity shown by the immunohistochemically stained slides to obtain a final score.

2.3 | Semi-quantitative assessment

All corneal specimens were assessed by light microscopy. The authors developed a semi-quantitative scoring system which one observer (VMH) applied to grade immunohistochemical signal intensity distinctly in corneal epithelium, stroma, Descemet's membrane (DM), and endothelium on a scale from 0 to 3 (0 = no signal, 1 = low, 2 = medium or 3 = high signal intensity). Additionally, non-uniform patterns of signal uptake within each corneal layer were recorded.

2.4 | Statistics

Descriptive statistics were used for animal-related and processing-associated data as well as signal intensity scores. Differences in signal intensity between species, eye laterality, age, or sex were performed using Chi-Square tests and Mann–Whitney U tests. The impact of age on the signal intensity was modeled by logistic regression analysis. For all statistical analyses, a p-value below 5% ($p < .05$) was seen as significant.

3 | RESULTS

3.1 | Material

Forty eyes (20 OD and 20 OS) were collected from 20 horses with a median age of 15.45 years (range: 2–31 years). The animals comprised 12 geldings (60%), 7 mares (35%), and one stallion (5%), and of the 8 breeds in total, warmbloods were overrepresented (40%; Table 1).

Twenty-one eyes (11 OD and 10 OS) were collected from 12 dogs with a median age of 10.00 years (range: 4–16 years). Neutered females ($n = 5$) were the most frequent sex, followed by neutered males ($n = 3$), intact females and intact males ($n = 2$ each). There were 8 breeds represented, with one third of the eyes stemming from mixed breed dogs (Table 1).

Twenty-four eyes (12 OD and 12 OS) were collected from 13 cats with a median age of 10.00 years (range: 2–18 years). Neutered females ($n = 5$) were the most frequent sex, followed by neutered males and intact females ($n = 4$ each). There were 4 breeds represented, with domestic short hair cats being overrepresented (9 cats; Table 1).

All corneal specimens were adequately assessable, however, on 6 equine (15%) and 2 canine (9.52%) slides there was less than one third of the corneal length (limbus to limbus) retained. In 15% of equine ($n = 6$ eyes) and 12.50% of feline slides, the endothelium was lost during processing. The endothelium was detached from DM but conserved enough to assess its signal intensity in 25% of equine ($n = 10$ eyes), 85.71% of canine ($n = 18$ eyes), and 58.33% of feline ($n = 14$ eyes) slides.

3.2 | Immunohistochemical results

Lymph node tissue (Figure 1) and peptide control samples (Figures 2 and 3) served as adequate positive and negative controls, respectively. The different corneal layers had a similar staining pattern in all species and no differences between the central versus the peripheral regions were noted. Within the stroma, signal was observed in regular, linear streaks parallel to the epithelium and reaching from the epithelial basement membrane to Descemet's membrane (Figures 4, 5 and 6). While no signal could be observed within Descemet's membrane of horses (Figure 4), in over one third of dogs and over 90% of cats it did show a low signal expression, which in all but a single cat cornea was confined to half of the layer thickness adjacent to the stroma (Figure 5). The epithelium differed between species in the percentage of samples either showing a low signal intensity in a diffuse pattern or none. In 45% of horse, 28.57% of dog, and 41.67% of cat eyes, there was a low signal expression within the basal epithelial cell layers observed in peptide control slides, which in 42.5% of equine, 38.09% of canine, and 25% of feline was also present in the immunohistochemically stained slides and interpreted as an artifact (basal artifact). In 75% of equine, 100% of canine and 87.50% of feline stained slides a thin layer of immunosignaling covering superficial epithelial cells was interpreted to be another artifact (superficial artifact; Figure 5), with differing signal intensity between individuals. The endothelium generally showed some intracellular signal already in the peptide control slides in all species, therefore in every specimen a definite score thought to represent the true IgG signal was calculated by subtracting the intracellular signal intensity of the stained slides from that of the control slides.

Table 2 summarizes the semi-quantitative scores of the immunohistochemical signal for IgG in each corneal layer of horses, dogs, and cats.

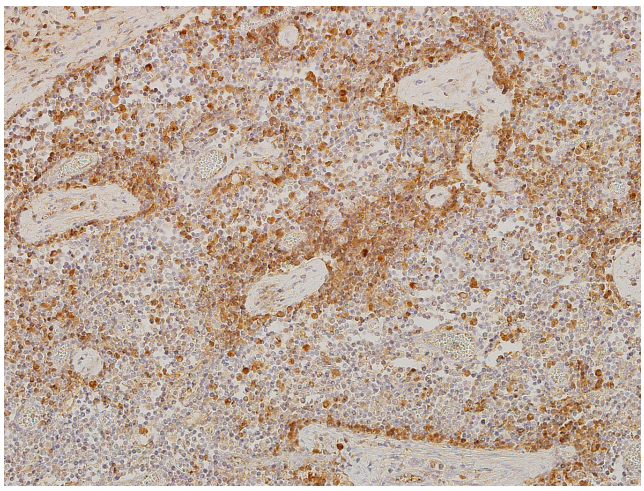

FIGURE 1 Immunohistochemically stained lymph node, serving as adequate positive control. The signal intensity represented the highest score, 3

3.3 | Comparative statistics

There was no statistically significant correlation of immunohistochemical signal results with eye laterality, sex, or age in any of the species ($p > .05$).

4 | DISCUSSION

This is the first study to purposefully investigate the presence of immunoglobulins in healthy corneas of horses, dogs, and cats. Immunoglobulins can generally be found in any ocular tissue that has enough extracellular space to accommodate them.[12]

Comparability of previous studies among themselves as well as with our results is limited due to different investigative approaches as well as differing tissue processing requirements for antigen detection. Some studies quantified IgG by extraction from different corneal sections to measure its concentration,[5,10,11] whereas others detected IgG within the tissue by immunohistochemistry.[8,18,39,40]

Limitations of this study include the small sample size and the inability to perform an ophthalmic examination

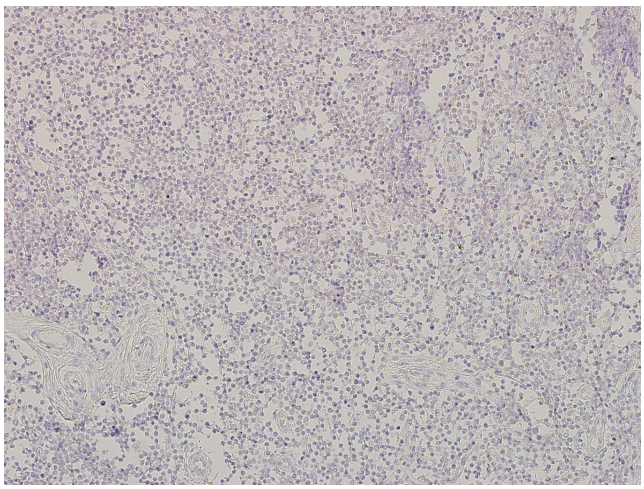

FIGURE 2 Peptide control sample of a lymph node, serving as adequate negative control. The signal intensity score was 0

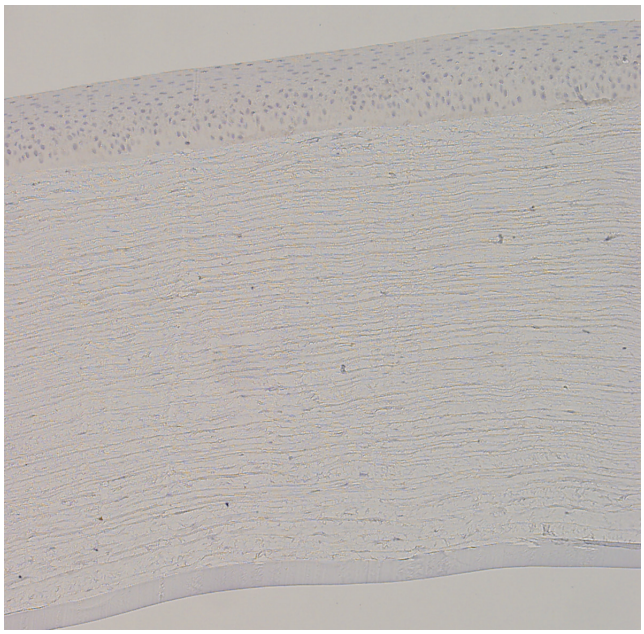

FIGURE 3 Peptide control sample of an equine cornea, serving as adequate negative control. The signal intensity scores were epithelium: 0, stroma: 0, and DM: 0. The endothelium of this specimen was lost in processing

FIGURE 4 Immunohistochemically stained equine cornea. The signal intensity scores were epithelium: 0, stroma: 2, DM: 0, and endothelium: 2

FIGURE 5 Immunohistochemically stained canine cornea. The signal intensity scores were epithelium: 0 with superficial artifact: 2, stroma: 2, and DM: 0. The endothelium was detached and could not be assessed in this specimen

before globe collection. Nevertheless, we consider histopathological examination a reliable tool to confirm corneal health.

Our findings of IgG being invariably present in the stroma are in line with existing literature evidencing the constitutive IgG content of bovine, porcine, lapine, and human corneas.[5,8,11] The regular, linear staining pattern we observed for IgG throughout the stroma has also been described by other studies and might be due to immunoglobulin adherence to and movement along the stromal collagen fibers.[8,9]

All previous studies described DM of healthy lapine and human corneas to be consistently devoid of IgG signal,[8,9] which is in line with the results of our investigated equine corneas. Since it is a basement membrane with tight collagen arrangement, immunoglobulins are not expected

to be present within it, although antibody adsorption to its edge has been described.[8,41] The fact that the half of DM adjacent to the stroma showed a signal in over one third of our canine and in over 90% of our feline specimen might have an anatomical explanation. Histologically, in humans, an anterior banded fetal layer, laid down during embryogenesis, and a posterior non-banded post-natal layer, representing the progressively thickening basement membrane of the endothelium throughout life, can be distinguished and are known to have differing collagen compositions.[42] It is unknown whether this also holds true for horses, dogs, or cats, and a light microscopical study failed to distinguish the two layers in feline corneas.[43] Another explanation could be the thickness of Descemet's membrane that might have permitted partial IgG diffusion from the stroma. However, layer thickness in dogs (10–15 μm)[44] and cats (9.43 μm)[43] lies between that of the other species which did not display any signal (horse: 30 μm[44]; rabbit: 7–10 μm[45]; human: 5–7 μm[46]). Animal-related variables such as age or sex might have played a role, but statistical analysis failed to detect a correlation within our results.

FIGURE 6 Immunohistochemically stained feline cornea; epithelium not included in the picture. The signal intensity scores were stroma: 2, DM: 1, and endothelium: 3. Note that the signal expression in DM is concentrated on the half adjacent to the stroma

Previous studies differed widely in their results of the immunoglobulin content within epithelium and endothelium.[5,8,9] The scattered positive epithelial cells reported by Allansmith and colleagues (1978) in human corneas differ from our signal, which appeared rather diffuse. Comparable with our observation of a superficial artifact, the same study also described a positive signal along the anterior edge of the epithelium and the posterior edge of the endothelium.[9]

The endothelial layer provided the most variable results between species and individuals in our study. The fact that in all specimen it already showed some signal of varying intensity in the peptide blocking could possibly be attributed to a so-called "edge effect" caused by lifting of tissue borders from the slide with subsequent overexposure to the immunoperoxidase reaction.[47] Direct immunofluorescence studies on the endothelium reported no evidence of IgG signal in lapine endothelium[8] and occasionally a positive cytoplasmic staining in human endothelial cells. The latter study also described a frequent loss of endothelium during processing.[9] It remains unclear whether our variable results of epithelial and endothelial layers represent physiological inter-individual and interspecies variance or simply artifacts.

Previous research on the antibody content did not detect IgG within any layer of healthy canine and equine control corneas.[39,40] In the case of Eichenbaum and colleagues' (1986) chromogenic immunohistochemistry on 14 corneoconjunctival biopsies of CSK-affected dogs and 4 healthy control biopsies, fixation took place in 4% formaldehyde and 1% glutaraldehyde for a minimum of 12 h.[39] Glutaraldehyde possesses an increased fixation rate and enhanced fixation strength compared with formaldehyde, resulting in a greater risk for over-fixation and loss of immunoreactivity in the tissue,[48,49] and it has been found to decrease antibody penetration into fixed tissue.[50] Since Stradleigh and Ishida (2016)[49] recommend a 10-fold lower

TABLE 2 Signal intensity of IgG in immunohistochemically stained corneas

	Dogs $n = 21$				Cats $n = 24$				Horses $n = 40$			
Signal intensity	**0**	**1**	**2**	**3**	**0**	**1**	**2**	**3**	**0**	**1**	**2**	**3**
Epithelium	80.95%	19.05%			41.67%	58.33%			95%	5%		
Stroma			100%				100%				100%	
DM	61.90%	38.09%			8.33%	91.67%			100%			
Endothelium	14.29%	71.43%	14.29%		37.50%	58.33%	4.17%		27.50%	27.50%	25%	5%

concentration of glutaraldehyde (0.1% in combination with 4% formaldehyde) to prevent deleterious effects on immunogenicity, the concentration and fixation time Eichenbaum and colleagues (1986) used might be partly responsible for their negative results of immunoglobulin in normal corneas and inconsistent staining in diseased specimen.[39,51] We suspect their 1:1000 dilution of the primary antibody, being substantially lower than ours, and greater section thickness of 5 µm as opposed to 3–4 µm as potentially contributing factors to the differing results. The stromal exposure of their lamellar keratectomy samples might have caused a wash-out effect on the soluble protein content of the stroma, the same possibly being the case in Pate and colleagues' study (2012) on keratectomy samples of 10 horses with superficial stromal IMMK and one unaffected control horse. That study used a protocol of direct immunofluorescence on paraffin-embedded sections different from our chromogenic detection method and is therefore difficult to compare other than an increased section thickness of 6 µm.[40] Their negative control sample did not stain for IgG, IgM, nor IgA, while all diseased samples stained positively for all antibodies in both epithelium and stroma. Since all IMMK-specimens showed cellular infiltrate and edema, and half of them neovascularization,[40] an enhanced antibody concentration within the corneas due to vascular permeability and possible local antibody production is plausible.[17,52,53] An insufficient sensitivity of the method to detect immunoglobulins in their normal corneal concentration would also be a possible explanation.[47] Pate and colleagues (2012)[40] described a linear stromal staining pattern for IgG, IgM, and IgA within IMMK-affected corneas that matches our own observations regarding IgG. Further studies are warranted to examine whether the corneal IgG content is changed under pathological conditions.

We conclude that IgG is constitutively present throughout the healthy equine, canine, and feline corneal stroma. Further research is needed to determine the reasons for the variable presence or intensity of signal in the corneal epithelium, DM, and the endothelium.

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CONFLICT OF INTERESTS

None.

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