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Equine keratitis and the possible involvement of equine adenovirus type 1 (EAdV1) and type 2 (EAdV2)

Inaugural Dissertation
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Abbreviations

CAV	Canine adenovirus (CAV-1 & CAV-2)
CEN	Cauda equine neuritis
CPE	Cytopathogenic effect
CR	Chronic-recurrent
DNA	Deoxyribonucleic acid
dtr.	Dioptre
EAdV	Equine adenovirus (EAdV1 & EAdV2)
EHV	Equine herpesvirus (EHV-2 & EHV-5)
EKC	Epidemic keratoconjunctivitis
ERU	Equine recurrent uveitis
HAdV	Human adenovirus
HSV	Herpes simplex virus keratitis
mRNA	Messenger ribonucleic acid
nPCR	Nested polymerase chain reaction
OD	Oculus dexter
OS	Oculus sinister
OU	Oculus uterque
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PLR	Pupillary light response
PTF	Precorneal tear film
RNA	Ribonucleic acid
RT PCR	Reverse transcriptase polymerase chain reaction
SAC	Subacute-chronic
SCC	Squamous cell carcinoma
SCID	Severe combined immunodeficiency disease
SPK	Superficial punctate keratitis
STT	Schirmer tear test
VK	Viral keratitis

1 INTRODUCTION

At present, the etiology of superficial punctate keratitis (SPK) in the horse remains a mystery. Viruses (LAVACH 1990) as well as fungal organisms (BROOKS et al. 2000) have been accused of causing the manifestation of corneal disease historically referred to as 'viral keratitis'.

Certain adenoviruses are known to cause corneal disease in humans. Most commonly, adenoviruses cause an epidemic keratoconjunctivitis, but they can also cause conjunctivitis without corneal involvement that resembles the clinical findings associated with herpes simplex virus keratitis (HSV).

Certain forms of equine keratitis – or keratoconjunctivitis, are suspected of having viral etiology. Although equine herpesviruses (EHV) are the viruses most commonly associated with equine keratoconjunctivitis, their role in the pathogenesis of such ocular disease has not been conclusively defined.

The purpose of this study is to determine if equine adenoviruses (EAdV) can be isolated from the eyes of horses suffering from keratitis, and if so, can they be associated with any specific clinical manifestation.

2 LITERATURE REVIEW

2.1 Corneal and Conjunctival Anatomy

The mucous membrane lining the upper and lower eyelids and the third eyelid (palpebral conjunctivae) as well as the anterior sclera (bulbar conjunctivae) is referred to as the conjunctiva (LAVACH 1990; SEVERIN 1996; SLATTER 2001). With the exception of its insertion at the limbus, the bulbar conjunctiva is freely moveable. Pigmentation near the limbus, although variable, tends to be most prominent in the temporal bulbar conjunctiva. A limbal zone of pigmentation is present in all normally colored horses. The palpebral conjunctiva tightly adheres to the underlying tarsi forming the innermost layer of the eyelids. The conjunctival sac refers to the potential space in the lower fornix lined by the conjunctiva. To allow unrestricted ocular movements, the conjunctiva lining the upper and lower fornices is loosely folded (LAVACH 1990).

The cornea (Fig. 1) makes up the anterior fifth of the fibrous tunic of the globe and has several functions. In addition to supporting the intraocular contents the cornea both refracts and transmits light due to its curvature and transparency, respectively. The cornea is avascular and normally clear but is abundantly innervated with non-myelinated nerve fibers. Both aqueous humor and tears provide nourishment and cleansing, while the eyelids and membrane nictitans protect the cornea from environmental hazards (SAMUELSON 1999).
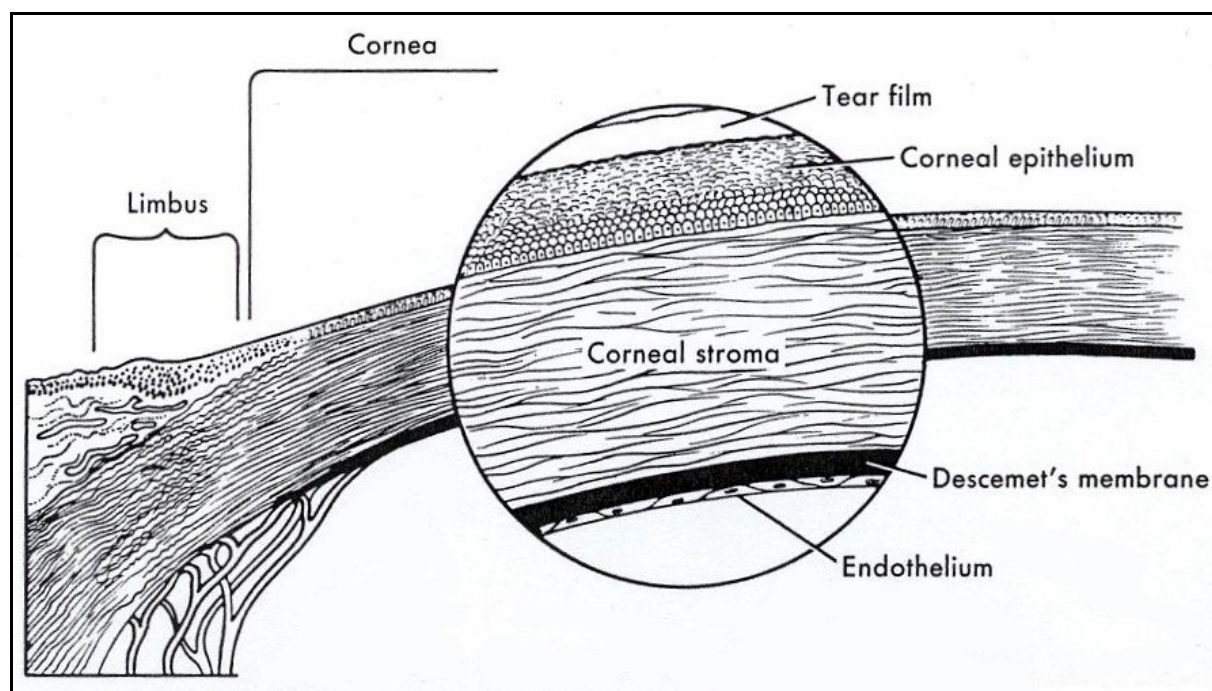
Figure 1: Histology of the normal cornea (From LAVACH 1990)

The cornea is made up of the following five layers:

Precorneal Tear Film

The precorneal tear film (PTF) (Fig. 2) consists of an outer lipid layer primarily secreted by the tarsal (meibomian) glands and the glands of Zeiss; a middle aqueous layer which is secreted by both the lacrimal gland and the gland of the third eyelid; and an inner mucoid layer derived from conjunctival goblet cells that serves as a bonding agent between the lipophobic aqueous tear layer and the lipophilic corneal epithelium (SEVERIN 1996; SLATTER 2001).
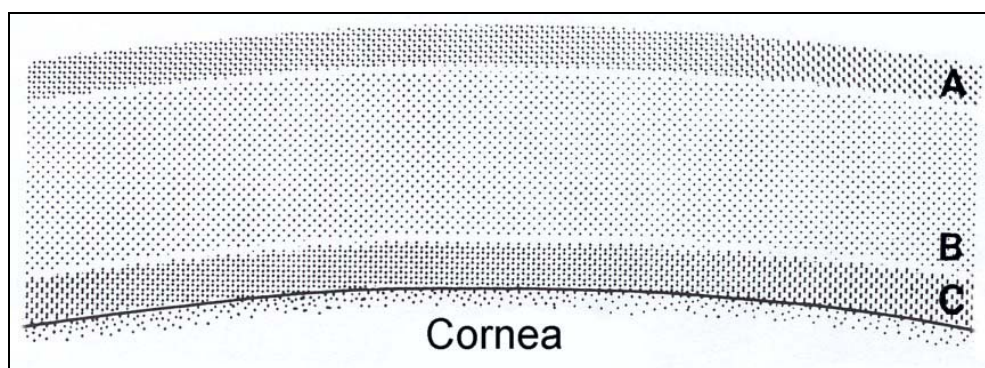
Figure 2: Precorneal tear film (PTF). A, Superficial lipid layer. B, Aqueous layer. C, Inner mucoid layer. (Modified from SLATTER 2001)

Epithelium and its Basement Membrane

The epithelium is of variable thickness, is non-keratinized, and is made up of simple squamous cells. The surface cells of this layer possess villous projections that attach to the deep mucoid layer of the PTF (SLATTER 2001).

Stroma

The stroma is made up of fibrocytes, keratocytes, collagen, and ground substance and comprises the bulk of the cornea (SLATTER 2001). The collagen fibrils are arranged in lamellae in which the fibrils run parallel to one another. Successive layering of the lamellae lends a lattice pattern to the stroma. By eliminating scatter from transmitted light, normal transparency is ensured. Occasional lymphocytes, macrophages, and neutrophils are distributed amongst the collagen fibrils (BARNETT et al. 1995; SLATTER 2001).

Descemet's Membrane

Descemet's membrane serves as the basement membrane of the corneal endothelium and is laid down throughout life, increasing in thickness with age (SLATTER 2001).

Endothelium

The endothelium is made up of a single layer of squamous cells and forms the posterior layer of the cornea. It is important in maintaining the normal state of corneal detergescence (SLATTER 2001).

Because blood vessels and lymphatics are lacking in the cornea, nutrients and oxygen must be provided by the PTF, aqueous, and perilimbal capillaries (BARNETT et al. 1995).

Horses have elliptically shaped corneas that are much greater in diameter horizontally than vertically. This, along with the lateral positioning of their orbits, provides them with an exceptional horizontal field of view (SAMUELSON 1999). Corneal thickness varies in all domestic species, but is usually less than 1.0 mm. The equine cornea is thinnest at the center and reaches maximum thickness at the limbus (SLATTER 2001).

2.2 Corneal Reaction to Insult

There are many different specific keratopathies, however, in lesions of clinical importance one of the following reactions is typically observed: edema; vascularization; scar formation; pigmentation; cellular infiltration or accumulation of an abnormal substance within the cornea (SLATTER 2001).

Edema

Edema is the result of excess fluid accumulation within the stroma which forces the collagen laminae apart thereby causing a loss of transparency. The accumulation of fluid occurs as a result of disturbances in either epithelial or endothelial regulating function. Clinically, corneal edema appears hazy-blue and is either localized around a specific lesion or is seen throughout the entire surface. Once the underlying cause has been removed and the fluid-balance reestablished, corneal edema is usually reversible. However, in cases of chronic corneal edema, vascularization, and occasionally, bullous keratopathy may occur. Bullous keratopathy is recognized as fluid-filled vesicles forming beneath and within the corneal epithelium. Recurring ulceration may occur as a result of their presence (SLATTER 2001).

Vascularization

Blood vessels are lacking in the normal cornea. Vascularization of the corneal stroma occurs in response to pathologic processes and to aid in stromal healing. Corneal vascularization is either superficial or deep and provides insight as to the duration of the corneal inflammation. Superficial vessels are contained within the anterior third of the stroma and form an extension of the conjunctival circulation originating at the limbus. These vessels are bright red in color and tend to branch extensively. Deep vessels can usually be identified by their dark red, straight appearance, and lack of branching. Because they are continuous with the ciliary circulation, they disappear at the limbus. The depth at which the vessels appear are some indication to the depth of the initiating corneal lesion (SLATTER 2001).

Scar Formation

Scar formation results from reparation carried out by fixed keratocytes and invading fibroblasts and macrophages after corneal stromal destruction has occurred. Collagen fibrils are not laid down in regular lattice patterns, thus, they interfere with light transmission. Scars may clear optically with time, but rarely do they disappear completely. Corneal scars in the horse tend to become pigmented. Injuries involving the deep corneal layers are often associated with denser and more permanent scarring, and are less apt to regain transparency. Nebula, macula, and leukoma are the terms used to describe corneal opacities of increasing size, respectively (SLATTER 2001).

Pigmentation

Pigment can be deposited in the stroma or in the epithelium and is a non-specific response to corneal inflammation. Stromal pigment is produced by normal limbal melanoblasts which enter the stroma during inflammation (SLATTER 2001). Pigment found in the epithelial layer

of the cornea migrates from the basal layer, which shares embryologic origin with the layer of the conjunctiva that naturally contains pigment. In chronic corneal diseases, especially those involving continual exposure, irritation, or xerosis (dryness), pigmentation is more common (SLATTER 2001).

2.3 Conjunctivitis and Keratoconjunctivitis

Conjunctivitis is inflammation of the conjunctiva. There are several classifications for conjunctivitis based on duration, type of discharge, appearance and etiology. When possible, an attempt should be made to obtain an etiological diagnosis in order to implement appropriate therapy (SLATTER 2001). It is also of utmost importance to differentiate primary conjunctivitis (caused by a local irritant which may pave the way for opportune infection) from secondary conjunctivitis resulting from concurrent systemic disease (SEVERIN 1996). According to BROOKS (1999) conjunctivitis occurs in the presence of both infectious and non-infectious diseases of the lids, external eye, anterior uvea as well as in diseases of the nasolacrimal system and the orbit. The signs that occur with conjunctivitis, regardless of cause, are conjunctival hyperemia and ocular discharge; the nature of which is variable (BARNETT et al. 1995).

Keratitis and conjunctivitis often occur simultaneously. Nevertheless, the term keratoconjunctivitis is inconsistently used by clinicians to describe diseases of the cornea with conjunctival involvement. REBHUN (1999) describes several forms of conjunctivitis having bacterial, viral, and parasitic etiology. However, he uses the term keratoconjunctivitis only to describe the clinical form of ocular disease caused by Onchocerca microfilariae. Other authors (THEIN & BÖHM 1976; SPURLOCK et al. 1989; RAMSEY et al. 1994; COLLINSON et al. 1994; YAMAGATA et al. 1996) also use the term keratoconjunctivitis when referring to

specific ocular diseases involving both the conjunctiva and the cornea.

2.4 Keratitis

2.4.1 Classification of Keratitis

In general, keratitis can be classified according to etiology, anatomical location, or character of the corneal lesions. Because the cornea has only a limited spectrum of reaction, and each layer of the cornea may react differently to an individual etiological agent it is quite possible that similar clinical manifestations result from different types of inflammation (SLATTER 2001). A combination of topographic and etiologic classification seems to be appropriate for general clinical use (BARNETT et al. 1995; SEVERIN 1996; SLATTER 2001).

SLATTER (2001) classifies keratitis according to anatomical location as follows: superficial; interstitial; deep; and ulcerative (considered an independent group due to the frequent involvement of all corneal layers). For didactical purposes, this anatomical classification has been adhered to. Specific etiological agents allow further definition of individual corneal diseases. BARNETT et al. (1995) use three categories based on depth to classify keratitis: superficial; deep; and interstitial. However, in contrast to Slatter, each group is precisely defined. SEVERIN (1996) also classifies keratitis according to anatomical location, but has also included keratoconjunctivitis as an independent group.

BROOKS (1999) classifies corneal disease according to the character of the lesion: Corneal ulceration; corneal lacerations/perforations; equine ulcerative keratomycosis; iris prolapse in

the horse; corneal stromal abscess; and other keratopathies (including nonulcerative keratouveitis; eosinophilic keratitis; herpes keratitis; burdock pappus bristle keratopathy, calcific band keratopathy; limbal keratopathy; and superficial corneal erosions with anterior stromal sequestration).

2.5 Equine Viral Keratoconjunctivitis

2.5.1 Clinical Forms

Superficial punctate or linear corneal opacities with or without concomitant conjunctivitis are the clinically manifest forms of keratitis most often associated with viral etiology (WILKIE 1998; BROOKS 1999; MAGGS 2003). BARNETT et al. (1995) describe three distinct forms of viral keratopathies: Superficial punctate keratitis (SPK), ulcerative viral keratitis, and macular keratitis. However, it has been suggested that each form merely represents a different stage of the same disease (MOORE 1987; MAGGS 2003).

2.5.2 Viral Etiology

Equine herpesviruses have been repeatedly isolated from eyes of horses suffering from certain forms of keratitis or keratoconjunctivitis (THEIN & BÖHM 1976; COLLINSON et al. 1994; BROOKS 1999; VON OPPEN 2000; BESTHORN 2002; MAGGS 2003). Although they are the viruses most often considered as being the causative agent of corneal disease; adenoviruses, and other viruses have also been incriminated. Equine adenoviruses (EAdV) have been isolated from ocular samples from several eyes of foals showing signs of keratoconjunctivitis (ENGLAND et al. 1973).

2.6 Adenoviruses

The family Adenoviridae is comprised of two official genera (Mastadenovirus and Aviadenovirus) and one proposed genus (Atadenovirus). The pathogens of each genus are as

follows: Genus Mastadenovirus: human adenoviruses 1-49, equine adenoviruses 1 and 2, canine adenovirus 1 (infectious canine hepatitis virus), canine adenovirus 2, bovine adenoviruses 1, 2, 3, and 10, and ovine adenovirus 10. Genus Aviadenovirus: hemorrhagic enteritis virus (fowl, turkeys), marble spleen disease virus (fowl), unnamed viruses that cause pneumonitis and edema. Genus (proposed) Atadenovirus: ovine adenovirus 287, bovine. adenoviruses 4, 5, 6, 7, and 8, and egg drop syndrome virus (MURPHY et al.1999).

Adenovirus virions are nonenveloped, have a hexagonal appearance, show icosahedral symmetry, and range from 80-100 nm in diameter. A single linear molecule of double-stranded DNA, consisting of 36 to 44 kbp, makes up the genome. Virus replication occurs in the nucleus and is facilitated by extensive modulation of the host immune response. The host range for adenoviruses is narrow. Several adenoviruses cause persistent infection which may be reactivated by immunosuppression. Equine adenoviruses have been shown to cause severe disease in immunocompromised hosts. When inoculated into newborn hamsters, some of the adenoviruses of humans, cattle, and chickens have caused tumors. These viruses have been used in experimental studies on oncogenesis, but have failed to cause tumors in its natural host (MURPHY et al.1999).

Adenoviruses appear to be highly host specific and have been isolated from several mammals, including humans, as well as from birds. Most of the viruses have been isolated from the upper respiratory tract, but some have been found in feces. The majority of mammalian adenoviruses produce subclinical infections, occasionally causing upper respiratory disease. Avian adenoviruses, however, are the cause of many important clinical syndromes (MURPHY et al.1999).

2.6.1 Equine Adenoviruses

To date, two equine adenoviruses have been identified: Equine adenovirus 1 (EAdV1) and EAdV2. EAdV1 was first isolated in 1969 from an Arab foal with respiratory disease in Pennsylvania, U.S.A. (TODD 1969). Several studies followed in order to assess the degree of involvement of adenoviruses in equine respiratory disease. Adenoviral infection was recognized and associated with a progressive pneumonia in Arab foals (MCCHESNEY et al. 1970; MCCHESNEY et al. 1973; ROBERTS et al. 1974; WHITLOCK et al. 1975). Ocular

and nasal discharge are common clinical findings observed in foals suffering from adenoviral respiratory infection (MCCHESNEY et al. 1970; MCCHESNEY et al. 1973; ROBERTS et al. 1974). Although adenoviruses contributed greatly to the death of these foals, evidence began to emerge that an underlying defect was the primary cause of their moribund conditions. A combined immunodeficiency was identified by MCGUIRE and POPPIE (1973) and was determined by THOMPSON et al. (1976) to be inherited as a simple, recessive, autosomal gene. Equine adenovirus 1 has also been isolated from horses suffering from cauda equine neuritis (CEN) (EDINGTON et al. 1984).

In a study conducted in Newmarket, UK, in 1972 and 1973, antibodies against adenoviruses were found in 35% of the Thoroughbred horses under surveillance (ROSE et al. 1974). Respiratory disease had not been reported in the majority of these horses (ROSE et al. 1974).

An adenovirus antigenically distinct from EAdV1 was isolated in the late 1970´s from the feces of two foals with diarrhea. Neither the pathogenicity nor the distribution of EAdV2 among horses has been established (STUDDERT & BLACKNEY 1982).

2.6.2 Adenoviruses and Their Role in Ocular Disease

Although adenovirus keratoconjunctivitis is the most common eye disease in humans (SUNDMACHER et al. 2001), their involvement in ocular disease of animals has not been well documented. Human adenoviruses (HAdV) 1-5, 7, and 10 are known to cause conjunctivitis and have also been associated with community outbreaks of follicular conjunctivitis or pharyngoconjunctival fever (COOPER et al. 1999). Epidemic adenovirus keratoconjunctivitis involving HAdV 8, 19, and 37 have been known to occur in eye clinics (COOPER et al. 1999). Aside from the keratoconjunctivitis epidemica form, adenoviruses can also cause conjunctivitis with or without corneal involvement. If the cornea is involved, very small epithelial punctate opacities (viral centers of destruction) can be observed and may further progress to larger star-like opacities with subepithelial infiltrates until finally, the typical nummulare which occupy the anterior stoma result. These nummulare can either be small and isolated, or they may be large and run together (SUNDMACHER et al. 2001). It is this form of adenoviral keratoconjunctivitis that may mimic herpesvirus keratitis (CHODOSH et al. 1995).

In a study conducted in Queensland, Australia, adenoviruses were isolated from cattle suffering from conjunctivitis and keratoconjunctivitis (WILCOX 1969). Corneal edema (blue eye) has also been associated with canine adenovirus 1 proliferation in the eye, especially in the uvea and corneal epithelium (BLOGG 1983). Blue eye has also occurred in dogs after vaccination with a live canine adenovirus 2 (CAV-2) (BLOGG 1983).

Although equine adenoviruses have repeatedly been isolated from conjunctival swab samples in foals (the majority being Arab foals with severe combined immunodeficiency disease [SCID]) suffering from respiratory disease (TODD 1969; THOMPSON et al. 1976), and Thoroughbred racehorses in training (ROSE et al. 1974), as well as from clinically healthy foals (WILKS & STUDDERT 1972) there has been no published evidence of primary adenoviral involvement in equine ocular disease (keratoconjunctivitis/keratitis).

2.7 Diagnosis

2.7.1 Equine Ocular Samples

Several methods for obtaining samples for further diagnostic evaluation are available as an adjunct to ophthalmoscopy examination. The least invasive means of obtaining ocular samples for antigen isolation is the removal of a small amount of tear fluid from the inferior fornix (conjunctival sac). A sterile cotton swab applicator is introduced into the conjunctival sac between the nictitating membrane and the lower eyelid after causing protrusion of the third eyelid with slight digital pressure applied to the dorsal globe. Using the hand corresponding to the eye from which a sample is to be taken, the examiner opens the eye by placing his or her index finger in the fold of the upper eyelid created by the dorsal globe and bony orbit. Gently moving the lower lid downward with his or her thumb, the examiner simultaneously raises the upper eyelid while slightly applying pressure to the dorsal globe. This causes the nictitans to protrude, thus protecting the cornea from inadvertent injury from the cotton swab applicator during its insertion into the conjunctival sac. This procedure generally does not require local anesthesia and is well tolerated by most horses (BARNETT et al. 1995).

Corneal swabbing may also be performed in order to obtain a sample directly from the area of

interest (i.e. peripheral edge of an ulcer). Local anesthesia is required for this procedure, and it is often necessary to use manual or chemical restraint. After local anesthesia has been instilled, the eye is manually opened and a sample is taken from the area of interest by gently swabbing the cornea with a sterile cotton applicator (BARNETT et al. 1995).

Corneal scrapings may also be obtained, especially in cases of chronic keratitis resistant to antibacterial therapy or if fungal etiology is suspected (BROOKS 1999). For this procedure, the horse must either be placed under general anesthesia or local nerve blocks (auriculopalpebral nerve and frontal nerve) and sedation can be performed to facilitate the procedure in the standing animal. The edge of the area of interest is then scraped with the blunt end of a scalpel or Kimura spatula. The sample is then placed in an appropriate transport medium and sent in for further diagnostic evaluation (BARNETT et al. 1995).

2.7.2 Virus Isolation and Identification

Specific antibody detection from serum samples is an indirect means of identifying adenoviral infection. It is important to note that the mere presence of antibodies does not necessarily represent a clinically manifest infection. The disadvantages associated with this method are that it is time consuming and requires a large quantity of specific antigens (WEITGASSER et al. 2002). In a Newmarket, UK, study, 35% of all horses tested had antibodies against adenoviruses, although the majority of them failed to show signs of respiratory disease (ROSE et al. 1974).

Virus can also be isolated in cell cultures from ocular swab specimens. This method is time consuming and the incubation period may take as long as 21 days (VAN RIJ et al. 1982). Additionally, intranuclear inclusion bodies may be identified in cells infected by adenoviruses or slow herpesviruses (ROBERTS et al. 1974). However, although the adenovirus inclusions are larger, they are similar in appearance to those associated with slow herpesviruses. Therefore, diagnosis of adenovirus infection based on intranuclear inclusion bodies may be inadequate unless concomitant virus isolation is carried out.

Polymerase chain reaction (PCR) has proven to be a very sensitive and specific means of detecting viral DNA (RATY et al. 1999). This method is effective in identifying target sequences even when only minute specimen samples are available. Before a nucleic acid can

be identified, it must first undergo in vitro amplification. Electrophoresis is then carried out and the bands are made visible with ethidium bromide staining or by using a Southern-blot (BESTHORN 2002). The specimen samples can be evaluated directly, without having to undergo prior cell culturing.

Adenoviral isolation from ocular conjunctival swab specimens using the PCR method of amplification has been used extensively in human medicine (KINCHINGTON et al. 1994; MORRIS et al. 1995; HUSSAIN et al. 1996; KAJIWARA et al. 1999; COOPER et al. 1999; WEITGASSER et al. 2002). Epidemic keratoconjunctivitis (EKC) in humans requires rapid diagnosis in order to control the spread of disease, and adenovirus PCR has proven to be faster and more sensitive than the diagnostic methods routinely used (cell culture and antigen detection, respectively) (KINCHINGTON et al. 1994; COOPER et al. 1999).

A multiplex PCR developed at the Centre for Equine Virology, The University of Melbourne, Australia (DYNON et al. 2001) to rapidly identify viruses causing equine respiratory disease includes single round PCRs for both EAdV1 and EAdV2, among others. The primers were specifically designed to conserved regions of the hexon genes of EAdV1 and EAdV2 (REUBEL & STUDDERT 1997; REUBEL & STUDDERT 1998), and were shown to be highly specific and sensitive (DYNON et al. 2001).

Polymerase chain reaction has several advantages over other diagnostic methods of testing. It offers greater sensitivity for infectious organisms while requiring only an extremely small sample volume. Results can be obtained within hours and multiple organisms can be tested for in the same clinical sample (ADLEBERG & WITTWER 1995). There are, however, some limitations associated with PCR diagnosis of which one must be aware. The greatest advantage of the PCR, its ability to selectively amplify extremely small numbers of viral DNA or RNA molecules, is generally also considered to be its greatest drawback. Cross-contamination between specimens may negate the advantage of detecting very small quantities of target viral nucleic acid (REUBEL & STUDDERT 1998).

2.7.3 Corneal Staining Using Rose Bengal Vital Dye

The use of rose bengal dye in cases of keratitis suspected of having viral etiology has been referred to in the literature (MILLER 1993; SLATTER 2001; MAGGS 2003). Until recently, it has been thought that rose bengal only stains dead or degenerated cells along with mucous strands (NORN 1967; NORN 1970). FEENSTRA and TSENG (1992b), however, were able to demonstrate that rose bengal actually stains healthy, intact corneal epithelial cells. To reach the corneal epithelial surface, rose bengal must first penetrate the pre ocular tear film which serves as a blocking mechanism to protect the anterior surface of the eye. The innermost layer of the pre ocular tear film contains mucin, which serves as a diffusion barrier preventing rose bengal from gaining access to the corneal epithelium (FEENSTRA & TSENG 1992a). Therefore, corneal uptake of rose bengal stain (Fig. 3) provides information regarding the integrity of the PTF/corneal epithelial interface (FEENSTRA & TSENG 1992b).
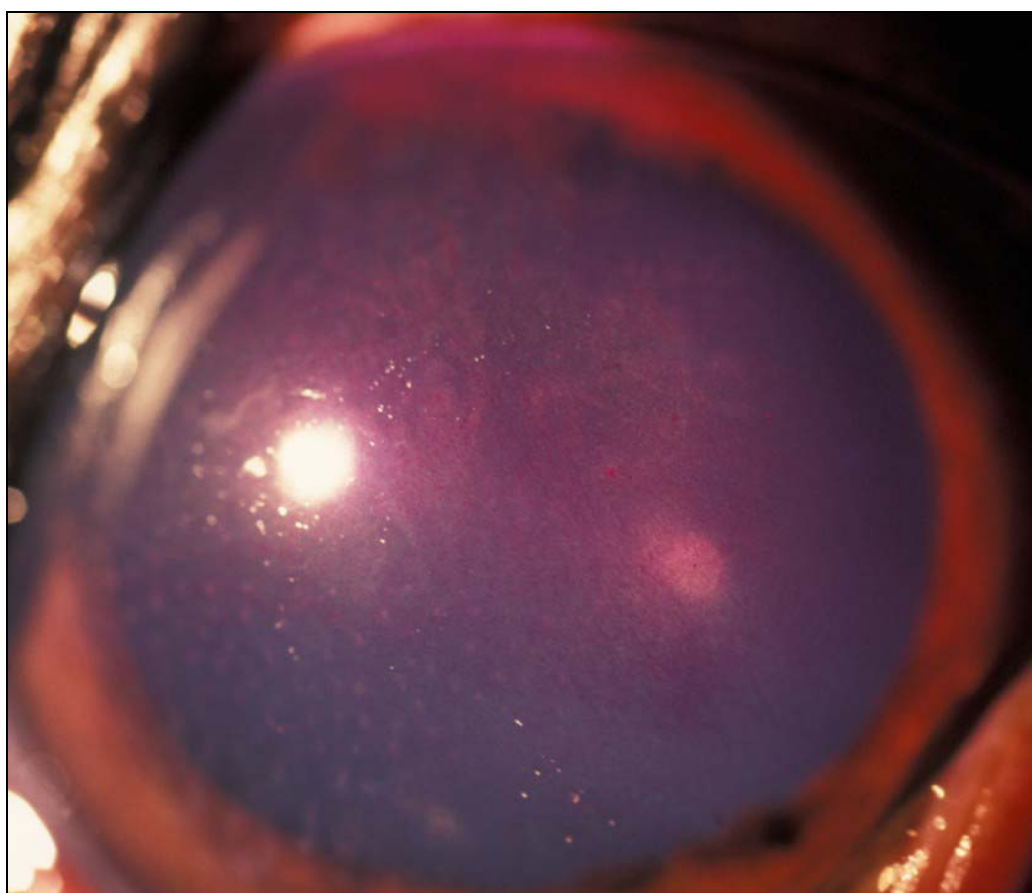

Figure 3: Corneal staining with rose bengal dye.

Rose bengal dye uptake was observed at the edges of dendritic or geographic corneal

epithelial defects, closely resembling the lesions seen in HSV keratitis, in four human patients with confirmed adenovirus etiology (CHODOSH et al. 1995). With viral infection of the corneal epithelium, rose bengal stains the virus-infected cells at the leading edge of epithelial ulceration, probably because these cells are at a stage of cytopathogenic effect (CPE) when they lose their microvilli (HOLLENBERG et al. 1976) and can no longer bind surface mucins, which normally block rose bengal access to the cells (CHODOSH et al. 1992; FEENSTRA & TSENG 1992b).

Rose bengal has also been used to help identify epithelial micro erosions that had only resulted in faint or negative fluorescein staining associated with equine keratomycosis (BROOKS et al. 2000). These six cases presented with multifocal, punctate, superficial corneal opacities and were presumed to be manifestations of 'viral keratitides'. The question remains, however, if the fungi had caused changes in the mucin layer of the PTF allowing the rose bengal stain uptake in the corneal epithelium or if the defects were present from other causes (BROOKS et al. 2000). The ability to cause damage to the mucin layer interface with the microvilli of the corneal epithelium has also been associated with herpetic and adenoviral keratitis in human medicine (HOLLENBERG et al. 1976; CHODOSH et al. 1992; FEENSTRA & TSENG 1992b).

2.8 Treatment of Viral Keratitis

Specific treatment protocols for adenoviral keratitis do not exist for the horse. In fact, no standard treatment protocols have been established for any forms of equine keratitis having presumed viral etiopathogenesis. In general, the current treatment regime consists of symptomatic treatment of the signs associated with 'viral keratitis' (conjunctivitis, chemosis, corneal opacification, neovascularization, etc.) using topically applied ophthalmic ointments or solutions containing steroids, with or without concomitant antiviral therapy.

There are several topical antiviral drugs available for the treatment of herpetic keratitis in humans such as vidarabine, idoxuridine, trifluridine, and acyclovir, several of which have been used with varying degrees of success in the horse. To date, an effective ophthalmic antiviral agent against human adenoviruses is unavailable (REINHARD & SUNDMACHER 1997). Persistent or recurrent corneal opacities (nummulare) remaining after epidemic keratoconjunctivitis (EKC) are believed to be the result of an immune reaction of the host

against adenovirus replication in keratocytes (REINHARD & SUNDMACHER 1997). Although local steroid mono-therapy is often implemented in both the acute and nummular stages of EKC, it is known to cause severe side-effects, and its use should be discouraged (SUNDMACHER et al. 2001).

In humans, the use of topical corticosteroids to treat an acute infection may activate the true underlying cause of disease in cases of misdiagnosis of herpesvirus and fungal infections while also promoting the replication of adenoviruses thereby increasing the antigen load that induces subepithelial corneal infiltrates (TULLO 1985). Corticosteroid use during the chronic phase (subepithelial immune infiltrates are present) of adenoviral infection may prolong a normally self-limited disease by months or even years (TULLO 1985).

THEIN and BÖHM (1976) were the first to attempt treatment of presumed viral keratitis in the horse. A small group of seven horses were treated with a topical ophthalmic preparation containing deoxyuridine. They reported complete corneal clearing in all cases by the fourth week of treatment. Additionally, they claimed that those horses also treated with dexamethasone were more likely to have recurring signs of keratitis superficialis (THEIN & BÖHM 1976). This observation has also been reported by others (MILLER 1993; WILKIE 1998).

Topical cyclosporine A (CsA), a powerful immunosuppressive drug which selectively inhibits T-lymphocytes, has been successfully implemented to treat a horse with a multifocal punctate keratopathy in addition to other forms of equine keratitis (READ et al. 1995; GRATZEK et al. 1995). At therapeutic concentrations, CsA inhibits T-cell proliferation, but is non-cytotoxic (GILGER & ALLEN 1998).

3 MATERIALS AND METHODS

3.1 Patient Groups

All horses included in the disease or control groups used in this study were either admitted as stationary patients or were seen on an out-patient basis in the Clinic for Equine Surgery and Internal Medicine at the Ludwig-Maximilians-University, Munich, Germany within the time period of November, 2002 through July, 2003.

A total of 47 horses (92 eyes) diagnosed with various keratopathies (43/92), uveitis (11/92), glaucoma (2/92), squamous cell carcinoma (1/92), and hyphema (1/92) were included in the study group. Additionally, the clinically inapparent contralateral eyes (34/92) of the horses described above were included in this group as well. Clinical findings observed during direct ophthalmoscopy resulted in a horse being placed within the study group. Horses with uveitis or glaucoma may present with similar signs to those seen in some cases of keratitis, e.g., blepharospasm, photophobia, epiphora, corneal opacities, and have therefore been included as possible differential diagnoses; as were the cases of hyphema and squamous cell carcinoma.

The 36 horses (72 eyes) comprising the control group were subjected to identical ophthalmoscopy examinations to those carried out on the horses of the disease group. Only those horses free from signs of ocular disease were included in the control group.

3.2 Examination

3.2.1 Patient History and Clinical Examination

A complete history was obtained upon presentation in the clinic. Emphasis was placed on clinical signs observed; course and duration of the presenting complaint; and prior treatment. Upon completion of the general examination a bilateral ophthalmologic examination was performed.

3.2.2 Ophthalmologic Examination

3.2.2.1 Examination in well-lighted area

Prior to direct ophthalmologic examination each horse was observed from a distance in a well lighted examining room. The bony orbita and palpebral fissures were examined for symmetry. Eyelid position was evaluated, and any deviation from normal or presence of palpebral edema was noted. Assessment of any ocular and/or nasal discharge was made according to its appearance and viscosity.

The menace response was measured bilaterally in each patient by rapidly moving a hand within the horse's visual field. Care was taken to avoid creating wind currents or contact with the eyelashes which could lead to false positive reactions. The menace response was tested from several different angles.

During the direct ophthalmologic examination the palpebral margins and the conjunctiva were examined for signs of inflammation, and the position of the third eyelid was evaluated. Opening of the palpebral fissure is readily achieved by using the thumb and forefinger from the same hand while approaching from the temporal aspect of the eye. The conjunctival surface appearance, color, and consistency were then evaluated. Prolapse of the third eyelid using digital pressure placed indirectly on the dorsal globe through the upper eyelid facilitated examination of the outer surface.

3.2.2.2 Examination in a darkened examining room

Direct and consensual pupillary light responses (PLR) were performed using a hand-held focal light source (Carl Zeiss, Jena, Germany). The menace response was also tested before the ophthalmoscopic examination began. A systematic eye examination beginning with observation of the adnexa was then carried out. Examination of the conjunctiva and cornea was performed using a hand-held light source and head mounted magnifying lens.

A distant retroilluminisense examination was done in order to highlight opacities of the light

refracting media of the eye. This part of the examination was performed using a direct ophthalmoscope (Carl Zeiss, Jena, Germany) with the diopter wheel set at 0 dioptre (dpt.). Starting at a distance approximately one arm length from the horse's eye, the cornea; anterior lens capsule; posterior lens capsule; and the posterior segment of the eye were examined, respectively, by gradually reducing the distance between the examiner and patient while maintaining a fundus reflex through the viewing slot of the ophthalmoscope. By examining the eye in this manner subtle changes in the refracting surfaces of the eye, which may have been otherwise missed were easily and readily recognized.

Direct ophthalmoscopy was carried out with a direct ophthalmoscope (Carl Zeiss, Jena, Germany). The examination was initiated at the corneal surface with the ophthalmoscope set at 6 dpt. The eye is divided into quadrants to ease localization and documentation of lesions. Once all four quadrants have been examined, the dpt. wheel is stepped down one dpt. setting and the examination of each of the quadrants is continued in a sequential manner. This process is repeated until examination of the fundus is completed at -2 dpt. ($\pm$ 1 dpt.).

3.2.3 Ancillary Diagnostic Tests

A Schirmer tear test (STT) was performed on each eye that was to be subjected to staining with rose bengal dye. Sterile cotton swab applicators with transport medium (Amies, Switzerland) were used to obtain samples from the conjunctival sacs of both eyes (OU) and submitted to Vet Med Lab (Ludwigsburg, Germany) for bacteriological examination. A second conjunctival sac sample was taken OU using a dry (no transport medium), sterile cotton swab applicator and submitted for viral DNA analysis using the polymerase chain reaction method of amplification.

Slit lamp (Carl Zeiss, Jena, Germany) evaluation was carried out on three horses. All horses presenting with superficial punctate corneal opacities as well as those with pitted corneal surfaces observed during retroilluminisense examination were subjected to vital staining of the external eye using fluorescein-natrium minims (Fluoreszein SE Thilo®, Alcon Thilo, Freiburg, Germany), and rose bengal strips (Chauvin, Essex, England) to further evaluate corneal integrity and the corneal – precorneal tear film interface, respectively. Each eye was rinsed with 5 ml of 0.9% natrium-chloride solution (Soluco NaCl 0.9%, B. Braun, Melsungen,

Germany) before being evaluated.

3.2.4 Documentation

All ophthalmoscopy findings were recorded on a modified eye examination chart (Figs. 4 &
5) from the Clinic for Equine Surgery and Internal Medicine, Ludwig-Maximilians-
University, Munich, Germany. Special emphasis was placed on corneal changes (e.g.
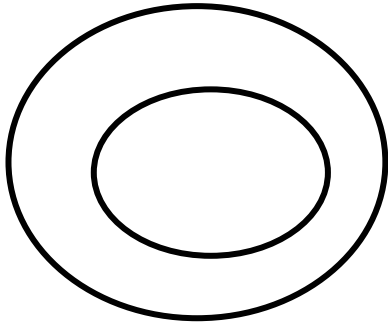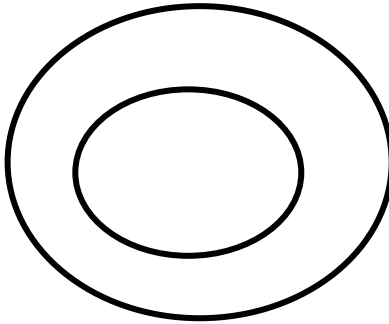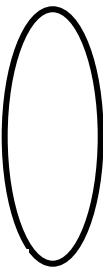opacities, neovascularization, color, and surface appearance).

Cornea

OD **OS**
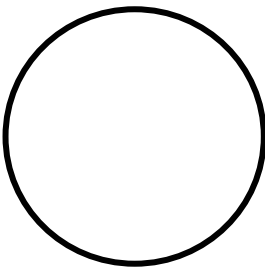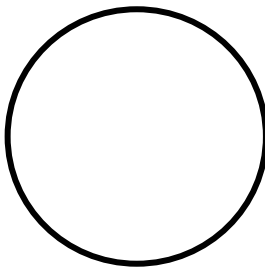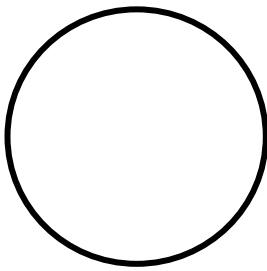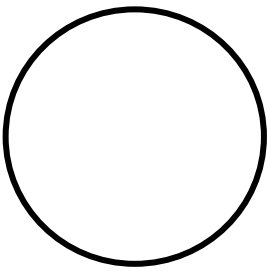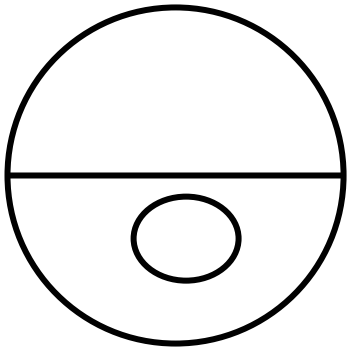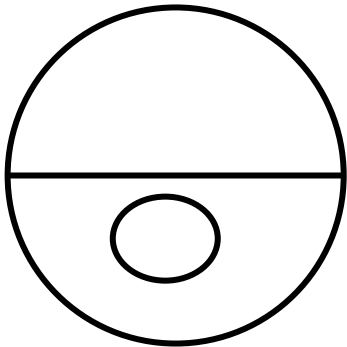
Lens

a.   p.   a.   p.

Vitreous

Fundus

Owner/Horse_____

Date:_____Diag.:_____

Figure 4: Modified eye examination chart, LMU Munich (front)

Right Eye (OD)		Left Eye (OS)
	Adnexa	
	Eyelids	
	Conjunctiva / Nictitating Membrane / Sclera	
	Cornea	
	Anterior Chamber	
	Iris	
	Pupillary Light Response (direct / consensual)	
	Lens	
	Vitreous Body	
	Optic Papilla	
	Retinal Vessels	
	Tapetal Fundus (Tapetum lucidum)	
	Non-tapetal Fundus (Tapetum nigrum)	
	Intraocular Pressure (IOP) (mmHg)	
	Other Changes	

Figure 5: Modified eye examination chart, LMU, Munich (back)

3.2.5 Clinical Diagnosis

A definitive clinical diagnosis was made upon completion of the ocular examination. Most of the horses included in this study suffered from various forms of keratitis, however, a small number of horses with other ocular diseases (equine recurrent uveitis, glaucoma, squamous cell carcinoma, and hyphema) were included due to similar presenting signs (e.g. punctate corneal opacities, corneal edema, epiphora, blepharospasm). Inflammatory diseases of the cornea were classified according to anatomical location and etiology as described by BARNETT et al. (1995).The following clinical manifestations of equine keratitis comprise the keratopathies diagnosed in the horses included in this study.

Keratopathies

Superficial keratitis (Keratitis superficialis)
- Epithelial opacities
- With or without corneal vascularization
- Fluorescein / rose bengal staining may be positive

Superficial punctate keratitis (Keratitis punctata seu maculosa)
- Superficial punctate/linear epithelial opacities
- With or without corneal vascularization
- Variable degree of epithelial /stromal involvement
- Fluorescein staining may be positive/ rose bengal staining usually positive

Interstitial keratitis (Keratitis parenchymatosa)
- Cloudy, stromal opacity
- With or without corneal vascularization
- Fluorescein negative / rose bengal usually positive

Eosinophilic keratoconjunctivitis (Keratitis eosinophilica)
- Originates at limbus, may infiltrate stroma
- Yellow-to-white caseous corneal plaques
- Stromal edema and corneal vascularization

Keratoconjunctivitis / Conjunctivitis

- Conjunctival hyperemia / chemosis

- With or without corneal involvement (opacities, vascularization, edema)

- May stain with fluorescein and / or rose bengal

Corneal Ulcers

- Break in corneal epithelium with varying depth of penetration

- With or without corneal vascularization

- Fluorescein positive in ulcers superficial to Descemet's membrane

Group of ocular diseases affecting inner structures of the eye:

Equine recurrent uveitis (ERU)

Glaucoma

Hyphema

Endophthalmitis

3.3 Sample Processing

3.3.1 Sample Collection

Samples for both the bacteriological examination and the viral DNA analysis were collected from the conjunctival sacs of both eyes. Local anesthesia was unnecessary, as the nictitating membrane shielded the cornea from inadvertant contact. The eyelids were manually opened using the hand corresponding with the eye from which the sample was to be taken (left hand used to open left eye; right hand used to open right eye) in order to insure a rostroventral insertion of the sterile cotton swab applicator into the conjunctival sac. Slight digital pressure was placed on the dorsal aspect of the globe in order to expose the nictitating membrane. The tip of the cotton swab applicator (COPAN®, Brescia, Italy) for the PCR samples was placed into the conjunctival sac and rotated several times before being removed and placed into its sterile transport sleeve. The above procedure was repeated on the same eye using a second sterile cotton swab applicator (Amies, Switzerland) containing a transport medium to obtain a

separate sample for bacteriological examination. The sample collection was repeated on the contralateral eye. In this manner both cells and tear fluid could be obtained for evaluation. The samples were then labeled for transport.

3.3.2 Sample Preparation

All of the samples were transported to Vet Med Lab, Ludwigsburg, Germany via auto-courier. The samples to be tested for equine adenoviruses were stored at a temperature of -20°C prior to preparation. The cotton swab applicator was placed in 300μl-500μl of phosphate buffered saline (PBS). DNA-extraction was achieved from 200μl of the lysat using the QIAmp® DNA Mini Kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions. Upon completion of the extraction process the initial volume was reduced to 50μl. The samples to be tested for bacterial involvement were evaluated in the bacteriology department, Vet Med Lab.

3.3.3 Polymerase Chain Reaction (PCR)

3.3.3.1 Equine adenovirus isolation and identification

The PCR used for EAdV isolation and identification at the Vet Med Lab is based on the single round PCR developed at the University of Melbourne, Australia (DYNON et al. 2001). A fraction (10%) of the sample nucleic acid (5μl) was placed in a test tube containing 45μl of the reaction solution. The PCR was then performed in a Thermocycler Perkin Elmer GeneAmp® PCR System 9700. The amplified DNA was analyzed by gel electrophoresis on a 2% agarose gel. The bands specifically labeled with ethidium-bromide were compared with the positive control bands. Any matching bands were considered to represent a positive PCR-result. If, however, no band was visible and the negative control was also negative, this represented a negative result. Every positive PCR result was reconfirmed by sequence analysis of the PCR fragment.

It was not possible to determine the actual quantity of the virus DNA present, even if detected. The conjunctival swab sampling technique used in this study cannot provide consistently equal sample volumes, nor is the testing system capable of determining the quantity of DNA isolated.

In order to detect EAdV-1 or EAdV-2 DNA at the Vet Med Lab, at least 10 genome copies must be present per PCR. Only 4-7% of the original sample volume is finally used for the actual PCR evaluation. Therefore, a positive result requires that the conjunctival swab sample initially contained at least 150-250 genome copies. The amount of DNA required for a positive PCR result is anticipated to be found in the conjunctival sack of horses suffering from ocular disease caused by this causative agent.

3.3.3.2 Equine herpesvirus type 2 and type 5 isolation and identification

Conjunctival swab samples were also taken from several horses diagnosed with "viral keratitis" and were sent to the Vet Med Lab for evaluation. The nested polymerase chain reaction (nPCR) used to isolate EHV-2 and EHV-5 DNA was previously described by BESTHORN (2002).

3.4 Categorization of Horses with Ocular Disease

3.4.1 Clinical Diagnosis of Eyes Included in the Study Group

None of the horses undergoing ophthalmoscopy required sedation or other means of manual restraint in order to complete the examination. To facilitate ocular examination tropicamide eye drops were instilled to provide mydriasis in those eyes not previously treated with atropine.

Although the contralateral eyes in the majority of the horses included in the disease group were free from clinical disease, they were not included in the control group. These clinically inapparent eyes were considered to be at a greater risk of harboring a viral causative agent than the eyes of horses without ocular disease. Therefore, each single horse, as opposed to each individual eye, in the disease group (Table 1) was considered to be a potential

adenovirus-carrier. Sterile conjunctival swab samples, identical to the ones used on the horses in the disease group, were collected from 72 eyes from 36 horses which made up the control group. Admission to the control group necessitated that a horse be free from signs of ocular disease.

Table 1: Clinical diagnoses of the eyes of the horses presenting with signs of ocular disease

DIAGNOSIS	TOTAL NUMBER OF EYES
Superficial Keratitis	7
Superficial Punctate Keratitis (SPK)	20
Interstitial Keratitis	2
Eosinophilic Keratitis	2
Keratoconjunctivitis/Conjunctivitis	7
Corneal Ulcer	4
Endotheliitis	1
Equine Recurrent Uveitis (ERU)	11
Glaucoma	2
Hyphema	1
Squamous Cell Carcinoma (SCC)	1
Clinically Inapparent Contralateral Eyes	34
TOTAL	**92**

4 RESULTS

4.1 Conjunctival Swab Samples

4.1.1 Results of Bacteriological Examination

Bacteria were isolated from 15/72 (21%) of the eyes comprising the control group, and from 24/92 (26%) of the eyes included in the study group. A heterogenous assortment of bacteria was isolated from both groups; however, a direct correlation between a positive bacterial result and clinical disease was not established in this study.

4.1.2 DNA Isolation of EAdV-1

EAdV-1 could not be isolated from any of the eyes in the study group (0/92; 0%), and was only successfully detected in both conjunctival swab samples (2/72; 3%) from a single horse in the control group.

4.1.3 DNA Isolation of EAdV-2

EAdV-2 was not isolated from any of the conjunctival swabs tested, regardless from which group they were taken (0/92; and 0/72; 0%).

4.2 Adenovirus Isolation in Specific Ocular Diseases

No correlation between specific ocular diseases and adenovirus isolation could be made. No adenovirus DNA was isolated from the conjunctival sacs from horses suffering from ocular disease, regardless of the clinical presentation.

4.3 DNA Isolation of Equine Herpesvirus Types 2 and 5

Several of the horses (n=23) in the study group presenting with signs associated with "viral keratitis" (VK) were subjected to PCR evaluation (n=46 eyes) to detect the presence of equine herpesvirus DNA. Viral DNA was isolated from twenty-eight (28/46; 61%) of the eyes tested: EHV-2 (14/46; 30%), EHV-5 (14/46; 30%), or both (7/46; 15%).

4.4 Corneal Staining with Fluorescein-Sodium and Rose Bengal Dyes

The horses (n=23) described in section 4.3 were also subjected to corneal staining with fluorescein-sodium and rose bengal dyes. Prior to instillation of the dyes, each eye was evaluated for adequate precorneal tear film production using the Schirmer tear test (STT). None of the eyes revealed values lower than the minimum reference value (10 mm/min).

Corneal staining with fluorescein-sodium and rose bengal dyes was conducted on 28/46 eyes from the disease group described in section 4.3. From the 28 eyes stained, 16/28 (57.14%) retained rose bengal, and 5/28 (17.86%) retained fluorescein-sodium. Rose bengal and fluorescein-sodium stain uptake was observed together in 3/28 (10.71%) of the eyes tested.

5 DISCUSSION

5.1 Clinical Diagnosis and Presentation of Viral Keratitis (VK)

Findings accumulated during direct ophthalmoscopy led to a diagnosis of ocular disease in 58/92 (63%) eyes from the horses comprising the study group. The classification of ocular diseases used in this study adhered to the one published by BARNETT et al. (1995). Because of the cornea's limited spectrum of reaction to injury, the combination of topographic and etiologic classification of keratitis/keratopathies seems to be the most advantageous (BARNETT et al. 1995; SEVERIN 1996; SLATTER 2001).

Categorization of corneal disease based on the depth of a lesion (superficial; interstitial; and deep) allows for simple but accurate anatomical subdivisions. Eosinophilic keratoconjunctivitis is included in the group of deep keratopathies (BESTHORN 2002). Corneal ulcers maintain a unique status in that they do not consistently damage all layers of the cornea. The four eyes diagnosed with corneal ulcers in this study were included in the group of deep keratopathies due to posterior stromal involvement.

In this study, special emphasis was placed on the cases of VK. The most universally accepted form of keratitis associated with a viral causative agent is superficial punctate keratitis (SPK). The most common clinical presentation is dominated by diffuse to disseminated, punctate corneal opacities with or without corneal vascularization. In humans, these opacities are assumed to represent an immune response to adenovirus antigens found in the corneal stroma following adenovirus infection of the corneal epithelium (CHODOSH et al. 1995). A definitive etiological agent has yet to be identified in the horse, although several studies have been carried out suggesting that equine herpesviruses play an important role in the pathogenesis of SPK (THEIN & BÖHM 1976; COLLINSON et al. 1994; MATTHEWS 1994; KERSHAW et al. 2001; KERSHAW et al. 2001). Adenovirus keratoconjunctivitis is the most common eye disease in humans (SUNDMACHER et al. 2001), however the role of adenoviruses in ocular disease of animals has not been well documented. Aside from keratoconjunctivitis epidemica, adenoviruses also cause a distinct form of conjunctivitis with or without corneal involvement, in humans. It is this form, most notably when the cornea is involved, which most closely resembles the manifestation of SPK observed in horses.

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Horses presenting with SPK can be generally divided into two categories: subacute-chronic (SAC) and chronic-recurrent (CR). The corneas of the horses suffering from SAC SPK generally present with a diffuse distribution of punctate corneal opacities, varying both in size and intensity. Several of these horses also presented with simultaneous superficial corneal vascularization. Horses with CR SPK revealed more extensive corneal involvement with disseminated distribution of larger, denser punctate opacities. These opacities were often diamond or star-shaped and arranged in a grid-iron pattern with fine linear appendages extending towards the neighboring opacities from each apex. The punctate corneal opacities tend to coalesce and form leukomata within the central cornea as the disease progresses. In humans, this clinical presentation is associated with adenoviral infection (SUNDMACHER et al. 2001), and may mimic herpesvirus keratitis (CHODOSH et al. 1995). These CR cases were most often accompanied by varying degrees of corneal vascularization.

According to THEIN and BÖHM (1976), changes in the corneal epithelium due to herpesvirus infection may result in dendritic-shaped lesions similar to those seen in cases of herpes simplex virus keratitis in humans. These dendritic lesions have also been documented in cats with feline herpesvirus-1 keratitis (NASISSE 1990). Three horses included in the study group showed rose bengal/fluorescein-positive, dendritic/geographic epithelial lesions.

In this study, 38 eyes were diagnosed with keratopathies that may be associated with a viral causative agent. Corneal staining with fluorescein and rose bengal dyes was carried out on 28 of these eyes. Rose bengal stain uptake was recorded in 16/28 (57%) and fluorescein in 5/28 (18%) of the eyes. Three of the fluorescein positive eyes were simultaneously positive for rose bengal stain uptake. Dendritic/Geographic epithelial lesions, as described above, were seen in three eyes. In humans, these lesions are produced by rapid replication of HSV-1 within the corneal epithelium (MADER & STULTING 2002). In two of the eyes (2/3, 66.6%) the lesions stained concomitantly with both rose bengal and fluorescein. In the third eye only rose bengal stain uptake could be observed. Several of the eyes presenting with SPK revealed very subtle corneal opacities requiring meticulous corneal examination incorporating retroillumination (Figs. 6 & 7) to accurately visualize them.
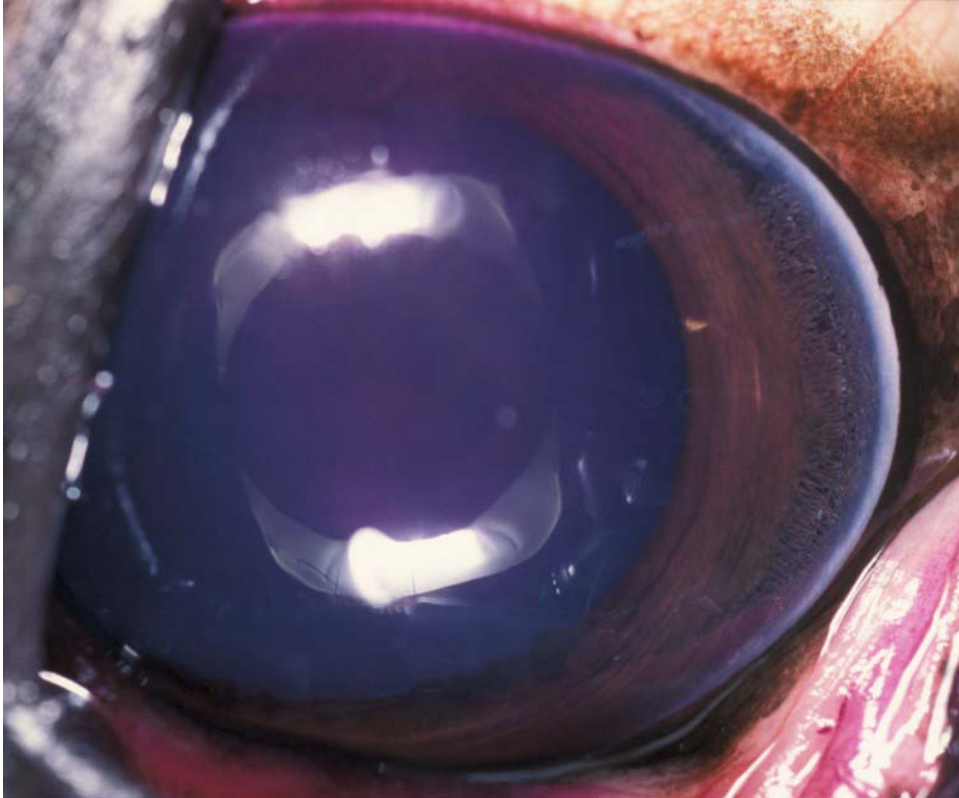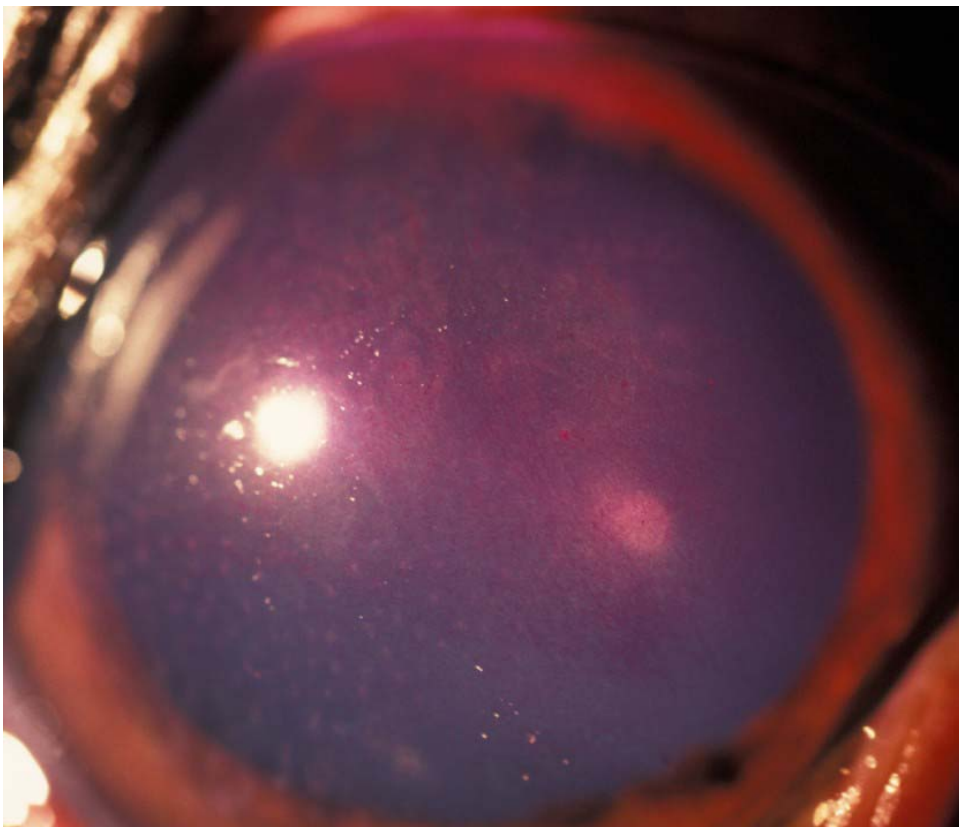
Figure 6: View of the cornea using oblique lighting

Figure 7: Same eye as in Fig. 6 using retroillumination technique

5.2 Bacteriological Conjunctival Swab Samples

Although several different species of bacteria were isolated from both groups (15/72, 21% control group; 24/92, 26% study group), they were considered to have little to no clinical relevance as pathogens within this study. From the 24 eyes in the study group from which bacteria were isolated only 11 (46%) were from the eyes affected with clinical disease. Of these, only 5 (21%) eyes revealed clinical changes that could be associated with secondary bacterial infection, i.e. a break in the corneal epithelium. The bacteria detected in both the study and the control groups were heterogenic and predominantly gram positive in origin, consistent with the reports in the literature (MCLAUGHLIN et al. 1983; WHITLEY & MOORE 1984; MOORE et al. 1988).

5.3 DNA Isolation of EHV (Types 2 and 5)

In the past, SPK has been most often associated with herpesvirus infection (THEIN & BÖHM 1976; MAGGS 2003); however, it has also been linked to fungal infection (BROOKS et al. 2000). The attempts at identifying EHV as the primary etiological agent associated with SPK have been confusing, at best. A study conducted by BESTHORN (2002) revealed that no definitive correlation between equine keratitis and EHV could be proven. These results contradict the results obtained by VON OPPEN (2000), who was able to detect EHV-2 DNA in horses with ocular disease significantly more often than in control horses. However, the number of horses included in the study was relatively small (n=29 in the study group), and included only one horse with SPK. Another study (KRÜDEWAGEN et al. 2001) compared EHV-DNA and inclusion body isolation from 15 horses suffering from keratoconjunctivitis or conjunctivitis to 15 controls and was also unable to demonstrate a significant difference between the two groups.

Conjunctival swab samples from 23 horses (46 eyes) from the study group suspected of having viral etiology were submitted for EHV2 and EHV5 evaluation by PCR. Nevertheless, an attempt was not made to establish a relationship between EHV2 and/or EHV5 to equine keratitis in this study.

5.4 DNA Isolation of EAdV (Types 1 and 2)

Equine adenovirus 1 is predominantly associated with secondary respiratory disease in foals suffering from Severe Combined Immunodeficiency Disease (SCID) (STUDDERT 1978; PERRYMAN et al. 1978), but have also been isolated from thoroughbred horses, with respiratory disease, in training (BURRELL et al. 1996); and horses suffering from cauda equina neuritis (CEN) (EDINGTON et al. 1984). Equine adenovirus 2 was initially isolated from diarrheic foal feces in 1978 (STUDDERT & BLACKNEY 1982), but has not been linked to any specific disease. Its role as a pathogen in equine disease remains unclear (REUBEL & STUDDERT 1997).

In humans, adenoviruses cause a form of keratitis, in addition to EKC, which may be misdiagnosed as HSV-1 keratitis (CHODOSH et al. 1995). This manifestation of adenoviral keratitis presents with epithelial to anterior stromal punctate corneal opacities (nummuli) with or without concomitant conjunctivitis (SUNDMACHER et al. 2001), and may be clinically inapparent or go undiagnosed (SUNDMACHER et al. 2001). The similar clinical presentation of this form of adenoviral keratitis and herpesvirus keratitis in humans, in addition to the fact that adenoviruses have been isolated from the conjunctival sacs of foals suffering respiratory infection (MCCHESNEY et al. 1973; ROBERTS et al. 1974) led to the postulation that adenovirus keratitis could exist in the horse. An attempt was made to isolate EAdV1 & EAdV2 from the conjunctival sacs of horses suffering from ocular disease using the PCR method of amplification.

Viruses are generally accepted as the cause of certain forms of equine keratitis, and are referred to as such in the major veterinary ophthalmology textbooks (LAVACH 1990; SEVERIN 1996; BROOKS 1999; SLATTER 2001). However, conclusive evidence as to which viruses actually lead to the development of clinical disease has yet to be presented.

Several eyes from the study group (46 eyes from 23 horses) suspected of having viral etiology, were concomitantly tested for the presence of herpesvirus DNA. It was possible to isolate equine herpesvirus DNA from 28/46 eyes, which confirmed that the conjunctival swab samples taken throughout this study contained adequate sample material for PCR testing.

5.5 Corneal Staining With Fluorescein and Rose Bengal Dyes

Fluorescein staining of the external eye is used routinely in veterinary ophthalmology. Any eye suspected of having corneal involvement should include fluorescein staining to evaluate corneal integrity. Intact epithelium of the cornea and conjunctiva does not permit penetration of fluorescein. Staining techniques and interpretation has been described elsewhere (SEVERIN 1996; SLATTER 2001). Corneal stain uptake with fluorescein was generally slight or negative in cases of VK in this study.

CHODOSH et al. (1995) published a retrospective study documenting the staining of the corneal epithelium with rose bengal in cases of adenoviral keratitis. The clinical manifestation of the cases presented was very similar to HSV keratitis, for which they possibly could be misdiagnosed.

Reference to the use of rose bengal dye in cases of presumed VK is relatively common in the veterinary literature (MILLER 1993; BROOKS 1999; MAGGS 2003); however, very little evidence on interpretation and indications for its use is available (BROOKS et al. 2000). In the past, it was thought that rose bengal stained only dead and degenerated cells along with mucus strands (NORN 1967); but not intact, vital corneal epithelial cells. Two studies conducted by FEENSTRA and TSENG (1992a; 1992b) demonstrated that rose bengal staining of intact corneal epithelial cells occurs when the PTF fails to provide adequate surface protection. Rose bengal staining of live cells can be blocked by such tear components as mucin and albumin.

5.6 Conclusions and Future Relevance

The results of this study provide no evidence of adenoviral involvement in the pathogenesis of keratitis in the horse. Indeed, adenoviruses could not be isolated from any of the eyes suffering from ocular disease. One of the main difficulties in discussing VK is the lack of uniformity in the description of their clinical manifestations (VON OPPEN 2000; BESTHORN 2002). Although distinct forms of viral keratopathies have been described (BARNETT et al. 1995), it has been suggested that these forms merely represent different stages of the same disease (MOORE 1987; MAGGS 2003). The clinical manifestation seen most commonly during this study was that of SPK with variable degrees of corneal

opacification (arranged in a gridiron pattern) and vascularization (Fig. 8). The corneal opacities were usually small, whitish, epithelial to sub-epithelial, and arranged in a gridiron pattern. The corneal surface area containing these opacities varied in size from eye to eye. There are, however, several other keratopathies (e.g. interstitial keratitis, superficial keratitis, etc.) with unclear etiology that may also be caused by viruses.

Figure 8: Superficial punctate keratitis (SPK) with distinct corneal opacification and vascularization.

Although EAdV1 & 2 DNA could not be isolated from any of the eyes in the study group a small step towards identifying the causative agent in equine SPK has been taken. The lack of adenoviral DNA from the conjunctival sacs of horses suffering from keratopathies associated with viral pathogens; however, does not prove that they are not involved. In order to completely rule out adenoviral involvement in the disease process of the manifestations of disease described in this study, more intricate diagnostic testing must be carried out. This

would entail corneal scrapings and/or biopsies; which given their invasive nature, present certain problems of their own.

In human ophthalmology, PCR is a fast and sensitive means of detecting adenoviral DNA (KINCHINGTON et al. 1994; MORRIS et al. 1995; COOPER et al. 1999) from conjunctival swab samples. One of the greatest advantages of PCR; the ability to selectively amplify extremely small quantities of viral DNA or RNA molecules from clinical specimens, thus making their detection by simple methods much easier; is also considered one of its greatest disadvantages (REUBEL & STUDDERT 1998). Not only can contamination of the samples lead to amplification of erroneous DNA, but it is also possible to detect and amplify very small amounts of DNA, due to its stability, in cases where an active infection does not exist (ADLEBERG & WITTWER 1995). This can lead to difficulties when interpreting the results. Also, it is generally not possible to differentiate between an active and latent infection. With reverse transcriptase (RT) PCR, this problem can be overcome. Due to the relative instability and rapid tissue degradation of messenger RNA (mRNA), it may be used to differentiate active from latent or prior infection (ADLEBERG & WITTWER 1995).

Due to the sensitivity and specificity of adenoviral PCR (COOPER et al. 1999), it is assumed that their detection from the conjunctival sacs of horses would be relatively unproblematic. Therefore, it is unlikely that EAdV1 or EAdV2 are pathogens associated with keratopathies in the horse. The EADV-PCR used at the Vet Med Lab was based on the PCR established by DYNON et al. (2001) and utilized 297/73 equine adenovirus as its control.

In the future, emphasis should be placed on accurate ophthalmological diagnosis, and on the response to therapy. It is imperative that the corneal lesions present be precisely described and documented in order to adequately describe the various clinical manifestations. The use of rose bengal dye as an additional diagnostic tool is extremely helpful, especially in cases with very subtle corneal opacities. Rose bengal stain uptake occurs when the protective barrier between the PTF and corneal epithelium has been interrupted. Additionally, rose bengal actually makes viewing subtle corneal opacities much easier. Both adenoviruses and herpesviruses have been associated with rose bengal corneal staining in humans (MARSH et al. 1976; CHODOSH et al. 1995).

While there are still many questions that need to be answered before the pathogenesis of VK

is completely understood, this study has provided data that has lead to a further narrowing of the possible viral pathogens involved in this enigmatic corneal disease complex of the horse.

6 SUMMARY

Equine keratitis and the possible involvement of equine adenovirus type 1 (EAdV1) and type 2 (EAdV2)

Richard McMullen

Introduction

The etiology of keratitis, specifically, superficial punctate keratitis (SPK) in the horse remains enigmatic in many cases. Although equine herpesviruses are commonly associated with SPK, they cannot consistently be isolated from affected eyes. Adenoviruses, aside from causing epidemic keratoconjunctivitis, may also cause independent conjunctivitis and/or keratitis resembling the clinical manifestation of herpes simplex virus keratitis (HSV) in humans. A viral causative agent is suspected in SPK in horses based on the clinical response to antiviral medications as well as the recrudescent nature of the disease after treatment with topical glucocorticoids.

Objective

The objective of this study was to attempt to isolate equine adenoviruses (EAdV) from the conjunctival sacs of horses suffering from keratitis using the polymerase chain reaction (PCR) method of amplification for detection. If detection is possible, an attempt would be made to determine if their presence could be associated with any specific clinical manifestation of equine keratitis.

Materials and Methods

Eyes from 83 horses were divided into the study group (47 horses; 92 eyes) and the control group (36 horses, 72 eyes). The study group consisted of horses with primary keratopathies or keratitis (43/92), other ocular diseases [ERU (11/92); glaucoma (2/92); hyphema (1/92); squamous cell carcinoma (1/92)], and the remaining unaffected contralateral eyes (34/92). Inclusion in the control group was limited to horses determined to be free from ocular disease. Conjunctival swab samples were taken and submitted for single round PCR screening of equine adenoviral DNA.

Results

Neither equine adenovirus type 1 (EAdV1) DNA nor equine adenovirus type 2 (EAdV2) DNA could be isolated from any of the eyes included in the study group (0/92; 0%). Equine adenovirus type 1 (EAdV1) DNA was isolated from both eyes from a single horse in the control group (2/72; 2.8%)

Conclusion

According to the data obtained in this study, no association between EAdV1 or EAdV2 and ocular disease in the horse could be made.

7 ZUSAMMENFASSUNG

Equine Keratitis und die mögliche Beteiligung equiner Adenoviren Typ 1 (EAdV1) und Typ 2 (EAdV2)

Richard McMullen

Einleitung

Die Ätiologie vieler Keratitiden beim Pferd, vor allem die der Keratitis punctata, ist immer noch ungeklärt. Equine Herpesviren, die oft in Zusammenhang mit Keratitis punctata gebracht werden, können nicht regelmäßig und verlässlich aus jedem betroffenem Auge isoliert werden. Adenoviren verursachen beim Menschen eine epidemische Keratokonjunktivitis, aber auch Herpes Simplex Keratitis-ähnliche Konjunktivitis und/oder Keratitis. Da bei der Keratitis punctata des Pferdes nach antiviraler Therapie eine Verbesserung der Symptomatik eintritt und bei lokaler Glukokortikoidbehandlung Rezidive auftreten, wird ein Virus als Erreger vermutet.

Ziel

Es wurde versucht, equine Adenoviren-DNA mittels PCR aus Konjunktivalsacktupferproben von Pferden nachzuweisen. Sollte dies möglich sein, würde sich die Frage stellen, ob das Auftreten einer bestimmten Form der Keratitis mit positiven Adenovirus-DNA-Nachweisen korreliert.

Material und Methoden

Die Augen von 83 Pferden wurden in eine Untersuchungs- (47 Pferde; 92 Augen) und eine Kontrollgruppe (36 Pferde; 72 Augen) unterteilt. Sowohl Augen, die an primären Keratopathien oder Keratitiden (43/92) erkrankt waren, als auch Augen von Pferden mit anderen Augenerkrankungen [ERU (11/92), Glaukom (2/92), Hyphäma (1/92), Plattenepithelkarzinom (1/92)] bildeten die Untersuchungsgruppe. Die Kontrollgruppe beinhaltete ophthalmologisch gesunde Pferde. Es wurden Konjunktivaltupferproben entnommen und mittels single round PCR auf equine Adenoviren-DNA getestet.

Resultate

Aus Augen der Untersuchungsgruppe konnten weder equine Adenoviren Typ 1-DNA (EAdV1) noch equine Adenoviren Typ 2-DNA (EAdV2) isoliert werden (0/92; 0%). Ein Pferd der Kontrollgruppe wies in beiden Augen equine Adenoviren Typ 1-DNA auf (2/72; 2,8%).

Schlussfolgerung

Aus den Ergebnissen der vorliegenden Studie ergeben sich keine Hinweise auf einen Zusammenhang zwischen EAdV1 oder EAdV2 und Augenerkrankungen beim Pferd.

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