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Investigation of the prevalence and the pathogenic role of Equine Herpesvirus 5 in wild roaming Przewalski horses

Diplomathesis

University of Veterinary Medicine Vienna

Submitted by

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Abbreviations

AHV/AsHV	asinine Herpesvirus
BALF	Bronchio-alveolar lavage fluid
BLAST	Basic Local Alignment Search Tool
CT	computed tomography
EBV	Epstein-Barr-Virus
EDTA	ethylene diamine tetraacetic acid
EGUS	equine gastric ulcer syndrome
EHM	Equine Herpesvirus Myeloencephalopathy
EHV	Equine Herpesvirus
EMPF	equine multinodular pulmonary fibrosis
F	female
gB	Glycoprotein B
gH	Glycoprotein H
gL	Glycoprotein L
HHV	Human Herpesvirus
IAD	Inflammatory airway disease
IDT	Integrated DNA Technologies
IL-10	Interleukine 10
IPF	Idiopathic pulmonary fibrosis
Kbp	kilo base pair
LT	Lung tissue
M	male
MHC II	Major histocompatibility complex II
NCBI	National Centre for Biotechnology Information

Neg.	negative
NS	Nasal swab
PBLC	Peripheral blood leukocytes
PBS	phosphate buffered saline
PCR	Polymerase chain reaction
Pos.	positive
RAO	Recurrent Airway obstruction
SD	standard deviation
TTL	trans-tracheal lavage

1 Introduction

1.1. Family Herpesviridae

The Family *Herpesviridae* belongs to the enveloped double-stranded DNA-viruses. The Herpesvirus can be further categorized into α-, β- and γ- herpesvirus based on their genetic, biological and morphological characteristics. The α-herpesvirus replicate fast, but β- and γ-herpesvirus have quite a slow replication cycle. The α-herpesvirus targeted cells are lymphocytes, sensory neurons and the trigeminal ganglion. β-herpesvirus seem to be latent in secretory glands, kidney and lymphoreticular tissue, like the spleen or lymph nodes (MacLachlan 2017). The γ-herpesvirus typically target B- and T-Lymphocytes (Scheurer et al 2021). Epidemiology of α- and γ-virus infections are markedly different. It is known, that Herpesvirus can cause a latent infection. α- and β-herpesvirus primarily support lytic replication, while γ-herpesvirus favour the establishment of latency (Ackermann et al 2016). Herpesvirus can infect humans, other mammals, fish, birds and reptiles. Herpesviridae are host-specific and adapt well to their host, which means that not all infected individual show symptoms.

	Alpha	**Beta**	**Gamma**
replication cycle	short	long	long
Latency in	trigeminal ganglion	sectretory glands	B- lymphocytes
	sensory neurons	kidney	T-lymphocytes
	lymphocytes	lymphoreticular tissue	
disease patterns	Equine Herpesvirus Myeloencephalopathy (EHM)	fever	poor performance syndrome
	respiratory disease	lung disease	respiratory disease
	abortion	Encepahlitis	EMPF (Equine Multinodular Pulmonary Fibrosis)

Table 1.: Differences between α-, β- and γ- herpesvirus

1.2. Equine Herpesvirus

Classification:

Until now there are nine known equine herpesvirus that can be further categorized α-, β- and γ- herpesvirus.

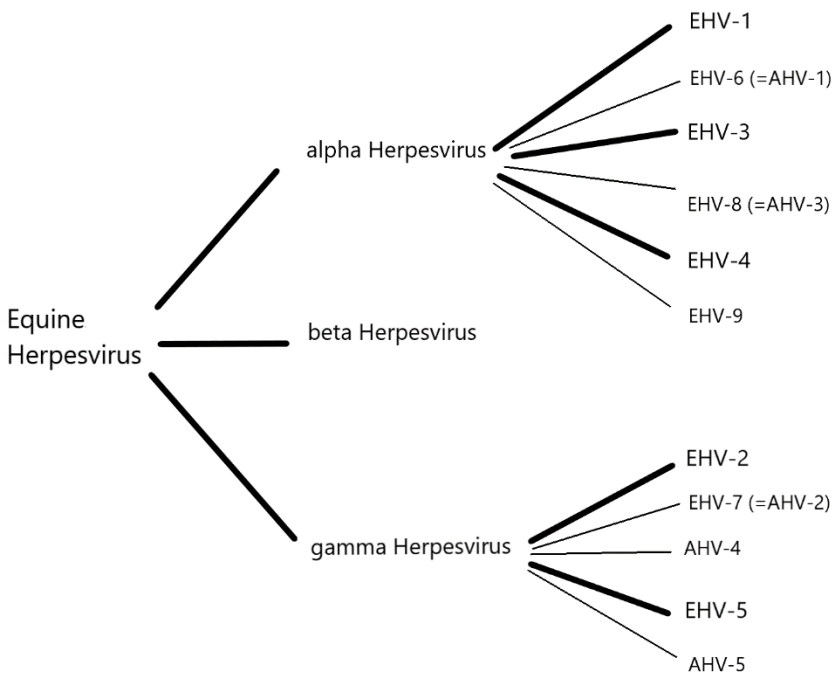


Figure 1: Classification of Equine Herpesvirus

EHV-1, EHV-3, EHV-4, AHV-1 (=EHV-6) and AHV-3 (= EHV-8), EHV-9 belong to the subfamily of α-herpesvirus. EHV-2, EHV-5, AHV-2 (= EHV-7), AHV-4 and AHV-5 were classified as γ-herpesvirus. The *Equine Herpesvirus* can be categorized in these subfamilies, but they can also be categorized according to their main hosts. Only five herpesvirus-species can cause disease in horses, the others mainly affect equids as donkeys, half-donkeys and zebra, but have also been detected in horses. These are known as asinine herpesviruses.

Epidemiology

Herpesviruses are a common and important viral infection in horses. The herpesviruses are spread worldwide. It is estimated that up to 60 % or more horses worldwide are latently infected with EHV-1 and/or EHV-4 (Lunn et al. 2009). Infection with equine herpes viruses is age-independent but possible at any time. However, most horses probably become infected during the first weeks or months of life (https://www.msd-tiergesundheit.de/fokusthemen/equines-herpesvirus-ehv/allgemeines-zu-equinen-herpesviren-ehv/ 06.07.22).

1.2.1. α-Herpesvirus

1.2.1.1. Equine Herpesvirus 1

EHV-1 is ubiquitous in the horse population with many latent infected animals. However, because of the latent infection, clinical outbreak often happens many years after primary infection (Marenzoni et al 2011). When horses immune system is weakened secondary to stress or other diseases. This virus targets T-lymphocytes and sensory neurons in the trigeminal ganglion for latent infection. After reactivation the virus spreads through infected secretion of the respiratory tract or lochial fluids via direct or indirect contact. It takes about 2-10 days to cause subclinical or clinical manifestation. EHV-1 can cause three different forms of disease. First, the respiratory form causes mild nasal discharge with cough, fever and sometimes even mucosal petechiae. Second, the abortion form happens 2 weeks to 4 months after infection. The mare usually shows no prior symptoms and the abortion happens soon after the virus reaches the foetus. This form is frequently seen in winter or spring, cause in this seasons most mares are in late pregnancy stage. Sometimes weak or premature foals are born. The third and most severe form, the neurological form, also referred to as Equine Herpesvirus Myeloencephalopathy (EHM). Mild symptoms as fatigue, anorexia and fever are frequent, but also hindlimb ataxia and paralysis up to tetraplegia can occur. Therapy and prognosis depend on the severity of the disease. In EHV-1 cases antiviral therapy with acyclovir or valacyclovir is often used during treatment. Vaccination for EHV-1 is available in combination with EHV-4 and are quite common among sport horses (Gerber et al 2016). EHV-1 and EHV-4 are also present in horse herds in Austria. In spring 2021, outbreaks with a neurological course occurred in some herds (https://www.ages.at/mensch/krankheit/krankheitserreger-von-a-bis-z/equine-herpesviren 13.01.22).

1.2.1.2. Equine Herpesvirus 3

EHV-3 also belongs to the α-herpesvirus and can cause coital exanthem. The virus replicates strictly in epithelial cells of the mucosa and the genital tract. It can cause pathognomonic gross lesions on the mucosa and the perineum of the mare or on the penis, but normally do not affect the general condition of the patient. These lesions are described as pustulae and later appear as depigmented skin. Transmission happens during mating, so diseased horses should not be used for breeding and also recovered horses should be further observed, to recognise recurring symptoms at an early stage. The infection is mostly self-limited, but an anti-inflammatory ointment can be used locally on the lesions (Gerber et al 2016).

1.2.1.3. Equine Herpesvirus 4

This is also an α-herpesvirus and replicates in the upper airways and causes necrosis in the epithelial cells of the respiratory tract. The target cells are the same as for EHV-1. The infection is typically seen in young adult horses and mainly causes respiratory symptoms as bronchopneumonia and pharyngitis, often with nasal discharge but can lead to abortion. EHV-

4 has a high seroprevalence. Stress can cause a reactivation of the virus, but is mainly asymptomatic in older horses. The disease normally shows a mild progression, has no severe impact on the general condition and is self-limiting. A combined vaccination for EHV-1 and EHV-4 is available (Gerber et al 2016).

1.2.2. γ- Herpesvirus

The γ-herpesviruses are much less known even though they are much more common than α-viruses. γ-herpesvirus forgo lytic infection upon cell entry and establish a latent infection, which is why they do not result in clinical sign at primary infection (Williams et al 2014). γ-herpesviruses are thought to establish latency in the lymphatic system only (Scheurer et al 2021).

1.2.2.1. Equine Herpesvirus 2

EHV-2 belongs to the subfamily γ-herpesvirus, is the most commonly detected herpesvirus and can be found in many healthy horses. This was proven by many studies were 90 % of the tested horses were seropositive for EHV-2, but did not show any symptoms (Torfason et al 2008). The infection happens quite early in life, often shortly after birth. One month old foals typically shed strains of EHV-2 that are identical to those infecting their dams, whereas older foals often shed different virus strains (Bell et al 2006). The odds of shedding EHV-2 decrease with age (Scheurer et al 2021). Rushton et al 2013 found that horses younger than eight years of age, housed in free stalls with high population densities have a significantly increased risk for infection with EHV-2 and EHV-5, but this findings had not been proven by others.

The pathogenicity is still unknown. If symptoms occur, the most common are keratoconjunctivitis, respiratory disease with pneumonia and pharyngitis, fever, enlarged lymph nodes, inappetence/anorexia, general malaise, and poor performance (Nordengrahn et al 2002). EHV-2 causes a latent infection and could be a predisposing factor for Rhodococcus equi- infection in foals (Nordengrahn et. Al 2002).

1.2.2.2. Equine Herpesvirus 5

EHV-5 belongs to the subfamily γ-herpesvirus and was first detected in Australia in horses with upper respiratory tract disease (Turner and Studdert 1970; Bell et al 2006). EHV-5 is a large DNA virus with the size of 182 kilobase pair (kbp). Because of its biological characteristics, compared to other γ-herpesvirus, EHV-5 was originally classified as β-herpesvirus, but after studying its genomic patterns, it was reclassified. EHV-5 can be further categorized in genotypes. Based on previous studies genotypes are not correlated to their geographic origin, which means, that different genotypes could be found in the same area. It is also possible for one horse to have different EHV-5 types at the same time. It was described, that there was no correlation between a specific genotype and clinical signs (Stasiak et al 2021).

Epidemiology

After EHV-2, EHV-5 is the second most commonly detected herpesvirus in the horse population. EHV-5 is ubiquitous around the world and can be detected in diseased as well as in healthy horses. Epidemiological data for the prevalence of EHV-5 are scanty (Patel et al 2005).

Even if EHV-2 is more often detected, horses showing different respiratory symptoms, had a two times higher chance of being EHV-5 positive (El Hage et al 2021). The specific age of the most frequent EHV-5 detection differs between studies. Marezoni et al (2012) searched for an age dependent prevalence of viral detection and discovered that there is a difference between the age group 1-3 years and 4-17 years. In the group of 1-3 year old patients the probability of an infection with EHV-5 was significantly higher. The different results between age groups may be related to the different stages of viral infection.

One of the main characteristics of herpesvirus is the latent infection (Sellon and Long 2014). Although the viral genome is present, its expression is limited and no infectious virions are formed. EHV-5 is capable of producing the homologous gene encoding interleukin-10 (IL-10) and of producing viral-derived IL-10 protein. The latter causes host immunosuppression by inhibiting the production of major histocompatibility complex II (MHC II). Asymptomatic carrier horses are of great importance, because of the periodic shedding of virus in their respiratory secretions, which plays a key role in the spread of the pathogen. Although the virus has a strong tropism towards the respiratory tract, it has also been detected it many other samples as bone marrow, gastric mucosa and liver.

Viral infection by EHV-5

The exact pathogenesis is still unknown. Van Cleemput et al (2019) established a hypothetical pathogenesis: The primary infection happens in foals around weaning and normally does not result in any clinical signs. The virus enters the host via the nose and migrate to the nasopharynx, where EHV-5 infects lymphocytes residing in lymphoid follicles. These lymphocytes spread via bloodstream or via lymphatic vessels. In the lymphatic tissues the virus spreads via cell-cell transfer. Via bloodstream EHV-5 reaches the lung. EHV-5 infected lymphocytes might infect epithelial cells, which could amplify the infection and high viral load in respiratory secretions. EHV-5 does not replicate but induces a lytic infection in equine T and B lymphocytes in vitro and this lytic infection causes nuclear fragmentation and apoptosis. The virus seems to establish latency in peripheral blood leukocytes (PBLC) (Van Cleemput et al 2019). It also seems to have an affinity for macrophages (Poth et al 2009). Older horses have a decreased amount of virus, with a probable latent infection, while younger horses are mainly acutely infected with a high viral load, which shows that the viral phase has a huge impact on the amount of detectable virus (Marenzoni et al 2010).

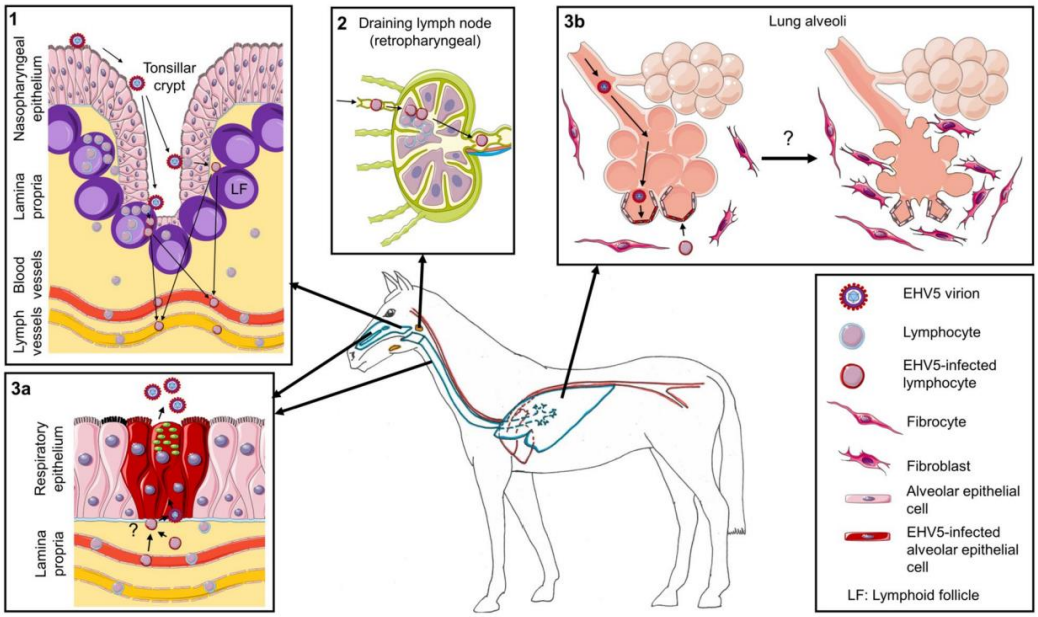


Figure 6 Hypothetical model of EHV5 pathogenesis in the horse. Drawings are based on SMART servier medical art templates. The horse's respiratory tract is designated in blue, the circulatory system in red and the upper airway lymph nodes in orange. (1) EHV5 virions are propelled by the mucociliary escalator towards the tonsillar crypts, embedded in the nasopharynx. Here, EHV5 directly infects lymphocytes residing in lymphoid follicles (LF). Infected lymphocytes then transport the virus either directly to the bloodstream or via the lymph vessels and (2) the draining lymph nodes (especially the retropharyngeal lymph nodes) to the bloodstream. In the lymphoid follicles or draining lymph nodes, EHV5 spreads to neighbouring lymphocytes via cell–cell transfer. EHV5-infected lymphocytes might either succumb due to apoptosis or survive and function as a life-long reservoir for EHV5. Via the bloodstream or via lymphocyte-homing, EHV5-infected lymphocytes (re)route to different parts of the respiratory tract, e.g. the nasal cavities or the trachea (3a) or the lungs (3b). (3a) EHV5-infected lymphocytes might transfer infection to epithelial cells, which could amplify the infection and shed a high viral load in respiratory secretions. (3b) EHV5 infects alveolar cells and spreads to neighbouring cells via cell–cell transfer. Viral replication, together with host-specific predisposing factors might eventually trigger the onset of fibrosis and EMPF due to yet unknown reasons.

Figure 2: Hypothetical pathogenesis of EHV-5 infections (Van Cleemput et al 2019)

Viral replication

To deliver the viral genome across the cellular bilayer, enveloped viruses catalyse a membrane-fusion reaction in which the viral envelope merges with the cellular membrane to enter the cell. This results in a release of viral nucleocapsid or nucleoprotein core into the cytoplasm. For this entry mechanism specific viral envelope proteins are essential for the attachment to cells and the membrane fusion (Mettenleiter et al 2004).

Viral glycoproteins

The virus carries many different envelope glycoproteins. Three of these glycoproteins essential for entry (gB, gH and gL) are conserved throughout the herpesvirus family. The glycoprotein B is essential for herpesvirus cell entry, as well as spread between cells and has been suggested to be a major target for neutralizing antibodies of the host (Back et al 2016). For detection of EHV-5 via PCR gB is often used, cause it is suggested to be quite stable in its structure. It has been commonly used in phylogenetic analysis of herpesvirus genomes. However, a considerable variability was observed within the central region around the furin cleavage site, which was also found in other studies (Stasiak et al 2021). This variability

reduces its suitability as an analytical tool to investigate phylogenetic or disease-causing factors.

Detection of viral infection

EHV-5 DNA can be detected in nasal swab secretion, Bronchoalveolar lavage fluid (BALF), lung tissue as well as PBLC. The analysis is done via Polymerase-chain reaction (PCR) preferably quantitative PCR. In some studies even a virus-specific PCR assay comes to use. Most used PCR cannot discriminate between latent, non-replicating or lytic state of the detected virus (Fortier et al 2009). This differentiation could give a better insight on the pathogenic effects of each state. However it is speculated that the lytic state causes the disease.

What kind of diseases are related to EHV5 infections?

EHV-5 can be found in many healthy horses, but could also be detected in relation to diseases and symptom complexes. For instance, EHV-5 has been found in relation to poor performance syndrome (Fortier et al 2009), gastric ulcer (Pennington et al 2017), ulcerative keratopathy and oral ulcers, bone marrow pathologies, leukaemia and lymphoma. This induction of lymphoproliferative disease by EHV-5 in horses can be resembled to Eppstein-Barr-virus (EBV) in humans (Schwarz et al 2013).

EHV-5 seems to play a prominent role in manifesting Equine Multinodular Pulmonary Fibrosis (EMPF) in horses (Williams et al 2007). This correlation has been shown to be highly significant in many studies. EHV-5 was detected in all known EMPF cases (Williams et al 2007).

Since the symptom complexes and diseases show several similarities, EHV-5 is often compared with other γ-herpesviruses, such as AHV and EBV and their role in different pathogenesis.

1.3. Equine Multinodular Pulmonary Fibrosis (EMPF)

EMPF is a multifactorial and chronic progressive lung disease in horses, most likely caused by EHV-5, which results in fibrosing nodular lesions in the lungs.

Epidemiology

In 2007 Williams et al were the first to document an association between viral infection and lung fibrosis in any veterinary species and the first to describe this disease. In 2009 Poth et al were the first to report the occurrence of EMPF in Europe. Equines that suffer from EMPF are typically adult-aged horses, even though the infection happens earlier in life. It seems to be a distinct disease of the domestic horse (Bell et al 2007). In a study from Williams et al 2007 the average age of EMPF horses was 14,5 years old.

Clinical disease description

However, the symptoms for EMPF are not pathognomonic, the typical clinical signs include mainly respiratory signs as increased respiratory rate and bronchovesicular sounds with wheezes, respiratory distress, as well as intermittent, low-grade fever, poor performance or progressive exercise intolerance, weight loss and fatigue. Also typical for EMPF is, that the clinical sings are therapy resistant. Anamnesis may provide a clue, as horses had treatment but did not show any clinical improvement (Easton-Jones et al 2020).

Figure 3: EMPF positive horse with typical weight loss (picture belongs to Dr. Korbacska-Kutasi)

Additional clinical signs can be keratokonjunctivitis, oral cavity ulcerations and lymphadenopathy. Secondary to the extensive pulmonary fibrosis, patients can develop heart failure (Easton-Jones et al 2019).

Most common laboratory changes are hypoxaemia, leukocytosis due to mature neutrophilia, lymphopenia, anaemia and hyperfibrinogenaemia (Schwarz et al 2013).

Association between viral infection and EMPF

In all horses diagnosed with EMPF, EHV-5 was detected in various samples, the NS seemed to be the most frequent. In the lungs of horses suffering from EMPF, EHV5 antigens were localized in alveolar pneumocytes and interstitial fibroblasts (Van Cleemput et al 2019).

In an experiment six healthy horses were infected with EHV-5 by placing virus-containing fluids in the accessory lobe of the lung. In four out of five blood samples EHV-5 was detected between the 4th and 42nd day *post infectionem*. All but one horse had neutralizing antibody titers. The gross pathology of the lungs showed similarities to horses with spontaneous EMPF. The severity of the histological lesions grew over time and alveolar collagen content was significantly increased in infected horses. This *in vivo* study provides evidence that a γ-herpesvirus alone can induce lung fibrosis (Williams et al 2013).

Nasal swab (NS), Bronchoalveolar lavage fluid (BALF), transtracheal lavage (TTL), lung tissue (LT) and PBLC can be used for the detection of EHV-5. Lung and lung related tissue as lymph nodes, BALF and TTL have a higher viral load than other tissues and most severely affected tissues had a higher viral burden (Marenzoni et al 2011). The easiest and most sensitive method to detect an infection is the nasal swab, but for reaching an EMPF diagnosis BALF and lung biopsy are the best clinical tools. Transcutaneous lung biopsy is the gold standard, but there is a risk for side effects as nose bleed and damage to the lung tissue. The detection of EHV-5 in blood is highly associated with EMPF, because EHV-5 seems to be latent in leukocytes (Pusterla et al 2015; Van Cleemput et al 2019).

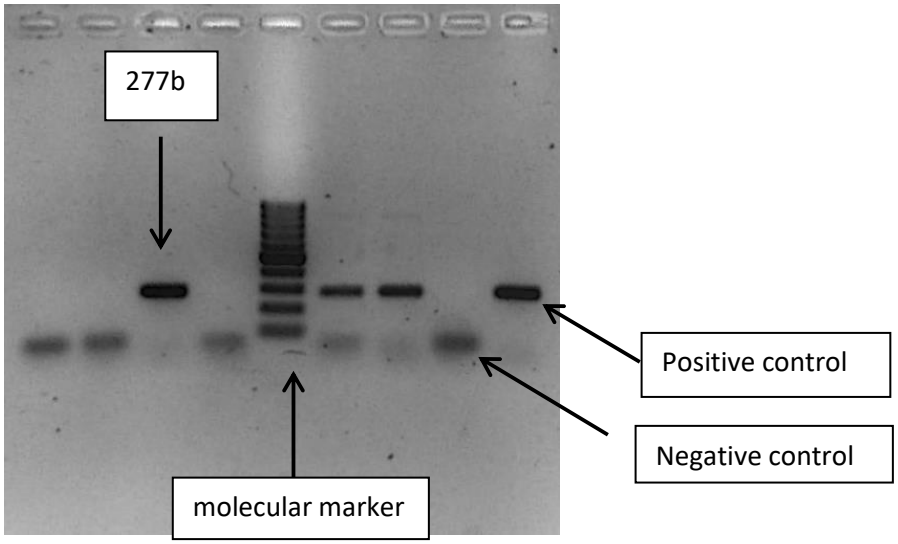


Figure 4: PCR with two positive samples (picture belongs to Dr. Korbacska-Kutasi)

Diagnosis

The anamnesis is a very important part of the diagnosis. A typical history would be a horse, that has been showing poor performance and signs of lung disease for a long time and has not shown any improvement after various therapy attempts.

A complete clinical examination with particular focus on the respiratory tract should be performed. The most common symptoms have been described above. Endoscopic examination of the respiratory tract can reveal enlargement of the pharyngeal lymphoid formations and mucoid secretions in the trachea and bronchi. The BALF can give an insight in pulmonary cellularity and can be used for EHV-5 detection with PCR. Characteristic findings are neutrophilic inflammation with marked mucous accumulation.

Thoracic radiographs reveal moderate to severe interstitial pulmonary pattern with nodules, so if the disease is already severe, you can use it as an diagnostic tool. The severity of radiographic findings is not associated with EHV-5 viral loads (Easton-Jones et al 2019).

The ultrasound of the lung can also be a good tool for finding fibrosis, sometimes even peripheral nodules can be visualized as subpleural anechogenicities. However, the ultrasound can only identify superficial changes. The depth of the ultrasound is not sufficient to see the

entire lung. Thoracic ultrasound can show pleural irregularity and thickening with wide comet tail echoes.

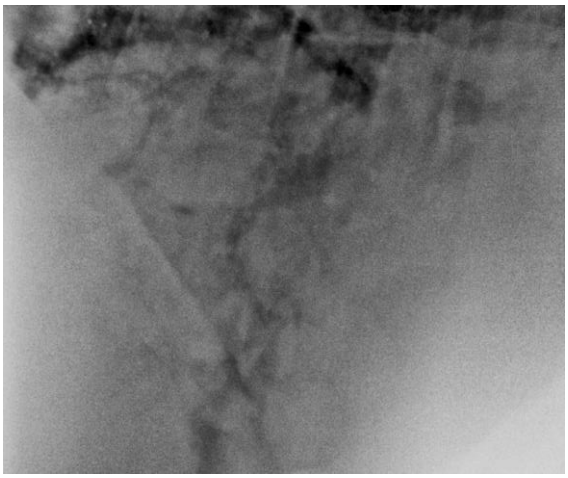


Figure 5: X-ray of common EMPF lungs; multiple nodules can be seen (pictures belong to Dr. Korbacska-Kutasi)

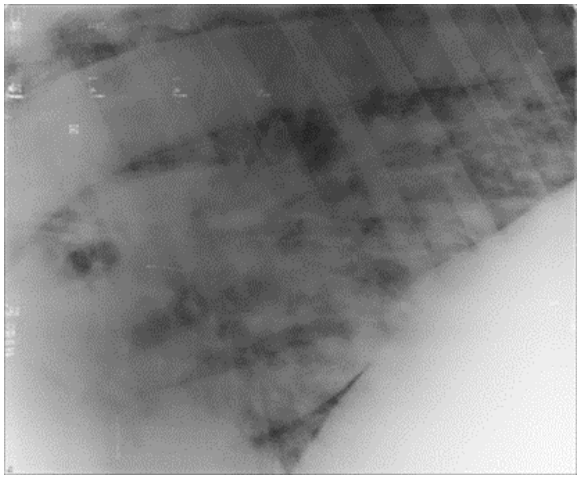
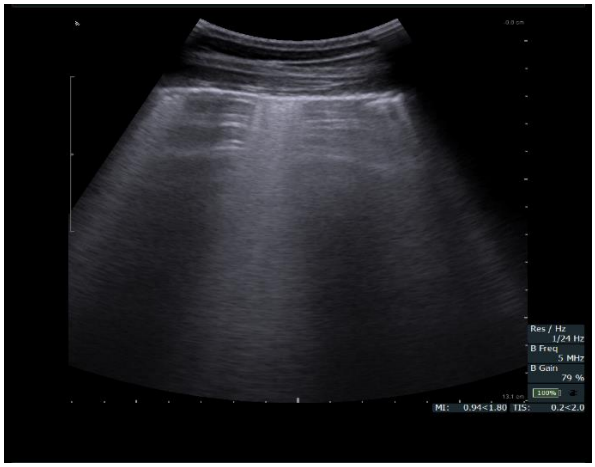


Figure 6: X-ray of EMPF with a single big nodule

Thoracic ultrasound can show pleural irregularity and thickening. Sometimes even peripheral nodules can be visualized.



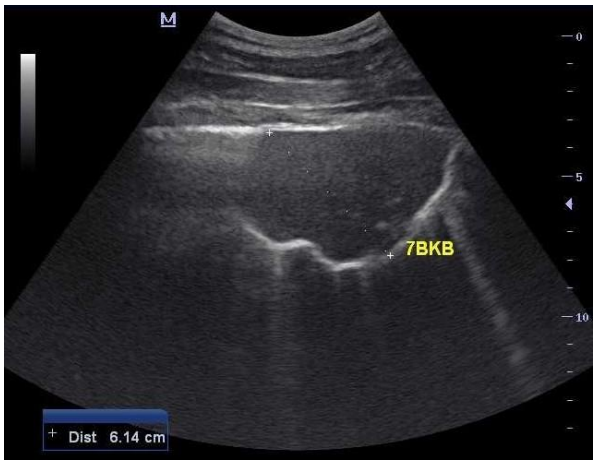


Figure 7 (left): Ultrasound of an EMPF horse with mild lesions, pleural roughness, and comet tail echoes can be seen. (picture belongs to Dr. Korbacska-Kutasi)

Figure 8 (right): Ultrasound of an EMPF horse, a nodule can be displayed (picture belongs to Dr. Korbacska-Kutasi)

The BALF can give an insight in pulmonary cellularity and can be used for EHV-5 detection with PCR. Characteristic findings are neutrophilic inflammation with marked mucous accumulation. Transcutaneous lung biopsy is the only method to really confirm the diagnosis

of EMPF in a living patient, even if this method is highly invasive. Biopsy and histopathology on lung tissue is considered the gold standard for EMPF diagnosis (Pusterla et al 2015; Easton-Jones et al 2019).

The necropsy is characterized by enlarged lungs that fail to collapse and diffuse pleural thickening. There are two known forms, first, the more common one, is the diffuse nodular form. The nodules are discrete, have a sharp demarcation and appear tan-white and firm. These form shows numerous fibrous nodules up to 5 cm that are restricted to and spread over the whole lung with just small, about 10-40 %, parts of healthy lung in between (Easton-Jones et al 2019). Second, less common, the discrete nodular form shows discrete disseminated up to 10 cm in diameter large nodules with discrete borders and large areas of normal parenchyma often resembled to a tumour (Poth et al 2009).

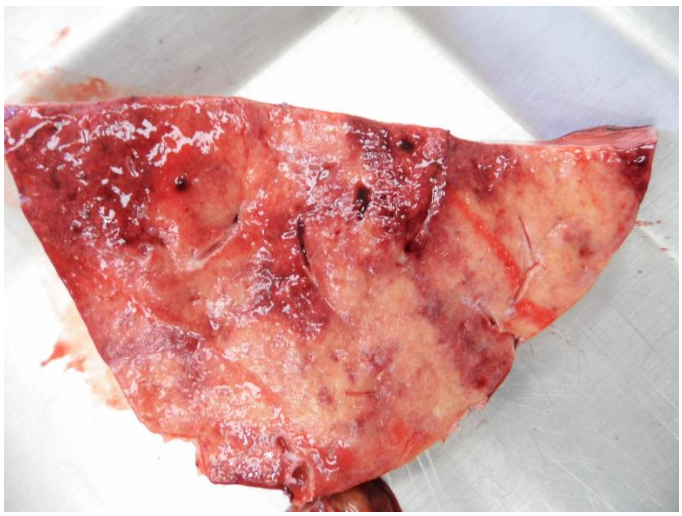


Figure 9: A piece of lung from an EMPF horse: the pale, whitish areas are the fibrotic lesions. (picture belongs to Dr. Korbacska-Kutasi)

Histological examinations show marked interstitial fibrosis with type two pneumocyte hyperplasia, “alveolar-like” structure, honeycombing, lymphocytes, neutrophils, alveolar macrophages and multinuclear giant cells with eosinophilic intranuclear inclusion bodies. The formation of inclusion bodies, which can be found in EHV-5 infected hosts, is normally a main feature of the α- and β- herpesvirus. These inclusion bodies resemble the Cowdry bodies type A, which are eosinophilic or basophilic nuclear inclusions composed of protein and nucleic acid, seen in *Herpes simplex* or *Cytomegalovirus*. In the effected lung areas seems to be a complete remodelling of the lung architecture (Poth et al 2009).

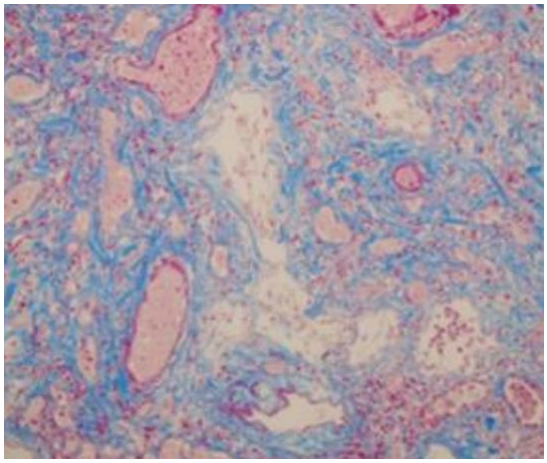


Figure 10: histopathology: lung of an EMPF horse (picture belongs to Dr. Korbacska-Kutasi)

Differential diagnosis

Inflammatory airway disease (IAD), Recurrent Airway Obstruction (RAO), lung metastases, fungal granuloma can be cited as a differential diagnosis. Other agents causing interstitial pneumonia are for example equine influenza, thermal and chemical injuries, toxic gases, ingested toxins, endotoxins, chronic left heart failure, uraemia and pneumoconiosis (Poth et al 2009).

Treatment

Treatment is mainly supportive with broad-spectrum antimicrobials against secondary infections, anti-inflammatories including dexamethasone (0,2 mg/kg i. m. every 24h for 7d, then a daily 10 % decrease in dose) NSAIDs (non-steroidal anti-inflammatory drugs) like flunixin meglumine (1,1 mg/kg i. v. q. 24 h) and in hypoxic patients oxygen therapy. Bronchodilation with clenbuterol (0,8 mg/kg p. o. 2x/d every 12 hours) or mucolytic mediation with bromhexidine (0,025 mg/kg p. o., q. 12 h) can be provided. Antiviral therapy with acyclovir (30 mg/kg orally every 12h) or valacyclovir (30 mg/kg p. o. 3x/day) is recommended. Acyclovir can be administered intravenous or orally. Studies have shown that the plasma concentration with oral administration was insufficient and showed poor bioavailability. Intravenous administration showed significantly better bioavailability. When administered orally, valacyclovir, at the same concentration as acyclovir, showed a significantly higher plasma concentration and significantly greater bioavailability. Therefore, valacyclovir is an effective agent for the treatment of horses (Garré et al 2007).

Even with treatment the prognosis is infaust and most patients need to be euthanised due to lack of response to the therapy (Reed et al 2018). There is not much information about the survival span of horses with EMPF. Antiviral treatment seemed to have no effect on the survival span, whereas patients with corticosteroid treatment had a lower short-term mortality. Short term survival is approximately 18-27.5 days. Only a few long term survivor are known (Easton-Jones et al 2019).

1.4. Asinine Herpesvirus (AHV/AsHV)

The asinine Herpesvirus, a γ-herpesvirus, typically infects donkeys, half-donkeys or zebras, but can also be detected in horses (Scheurer et al 2021). AHV-5 seems to be correlated to respiratory disease, interstitial fibrosing pneumonia and pyogranulomatous pneumonia. Asinine γ-herpesviruses have been shown to be closely related to EHV-2 and EHV-5 (Maboni et al 2021). In experimentally infected donkeys mild depression, fever, nasal and ocular discharge, conjunctivitis and dependent oedema were reported as the most seen symptoms (Reed et al 2018).

Maboni et al (2022) detected AHV-4 and -5 but no other AHV in a group of free-ranging donkeys. Other pathogens were found in the lung and gastrointestinal system, which may have weakened the immune system of the host and caused the reactivation of the virus (Maboni et al 2022).

1.5. Epstein Barr Virus

The EBV is also known as the Human Herpesvirus 4 (HHV-4), is one of the most common viruses in humans and can occur in all age groups. EBV is a γ-herpesvirus. It gets spread via saliva, blood or genital secretion. The target cells are memory B-lymphocytes and epithelial cells. It can cause immunosuppression, glandular fever, lung fibrosis and is linked to many diseases as multiple sclerosis and cancers as Hodgkin´s or Burkitt´s lymphoma and nasopharyngeal carcinoma (Back et al 2016). EBV is associated with Idiopathic Pulmonary Fibrosis (IPF). The current prevailing view is that IPF is not primarily an inflammatory disease but instead is the result of abnormal wound healing in the lung in response to alveolar epithelial injury from a variety of potential causes (Williams et al 2014). This disease has a high mortality, unknown cause and poorly understood pathogenesis (Williams et al 2013). The average survival time after diagnosis is five years. The progression of the disease cannot be controlled even with aggressive anti-inflammatory-therapy (Williams et al 2014). Since IPF and EMPF are both caused by γ-herpesviruses and their clinical manifestations are very similar, EMPF is often used as a model for IPF. Horses with EMPF are used in studies primarily aimed at investigating IPF.

1.6. Przewalski horses

Przewalski horses are the last surviving subspecies of wild horse. The Przewalski horse has 66 chromosomes, domestic horses have 64. Przewalski horses are smaller than most domesticated horses with a short body and legs. They have a height of 1.3 to 1.5 meters and weigh around 300 kg. They have a beige to reddish-brown coat, an erect mane and a shorter tail than most domestic horses. Some people mistake Przewalski horses for Fjord ponies, because of the resemblance in colour and short manes. Females, or mares with foal live in family groups with a dominant stallion and about three to five mares, while younger males live in bachelor groups. Although this breed was said to go extinct, because of cooperative

breeding programs between different countries and captivities to prevent inbreeding, research shows that the yearly population growth is 9 %.

Figure 11: Przewalski horses in Hortobagy, Hungary (picture belongs to Dr. Korbacska-Kutasi)

2 Material and Methods

2.1. Origins of the samples

A herd of free-ranging Przewalski horses was used for this study. The horses all came from different zoos, mainly from Germany. The closed herd consisted of a total of 309 horses at the time of the experiment in 2015 and lived in a reserve in Hortobágy in Hungary. This reserve is the first and biggest National park in Hungary. In 1997 Przewalski horses were brought into the park in an area about 2.400 ha of steppe. The herd consisted of several small groups that roamed free in these area and without human or other equine contact in the reserve. Since the population grew steadily and fast a decision of selection was made. From this herd, eleven horses were selected due to chronic diseases such as lameness, dental abnormalities or aggressive behaviour. Of the selected horses, eight were male and three were female.

The animals were shot in the head by an experienced and licensed shooter. The animals were killed because of their poor health and not specifically for the experiment. The experiment lasted two days from shooting to complete sample collection. On the first day, seven horses were shot, examined and the findings documented. On the second day, the remaining four horses were shot. The horses were assigned ID numbers and their name, passport number, age, herd position and medical history were documented in order to be able to trace back any findings and diseases. The average age was 9.9 years with a standard deviation (SD) of six years.

ID	Passport ID	sex	Date of birth	Position in herd
1	4415	m	23.07.2004	harem stallion
2	5454	m	05.10.2009	bachelor
3	4730	m	08.05.2006	harem stallion
4	2652	m	30.05.1994	bachelor
5	5211	f	08.08.2008	member of harem, no foal
6	2300	f	05.05.1992	member of harem, no foal
7	3903	m	28.06.2003	harem stallion
8	4995	m	24.05.2007	harem stallion
9	5324	m	20.07.2006	harem stallion
10	4360	f	30.05.2004	member of harem, no foal
11	6073	m	13.08.2012	bachelor

Table 2 : Details about the horses; m= male, f= female

2.2. Collecting and processing of samples

As the horses were not used to human touch, no clinical examination could be performed. However, the horses were observed from a distance to detect apparent injuries and diseases. Body condition and general health were recorded and compared with the medical history. All samples were taken post mortem, immediately after death. For this study, nasal swabs, blood samples and lung tissue were collected and a pathological and histo-

pathological examinations were performed. The necropsy lasted approximately 30 minutes per patient. All samples were taken by the same person.

2.2.1. Nasal swab

A 15 cm sterile rayon-tipped swab (Heinz Herenz, Hamburg Germany) was advanced into the left or right ventral part of the nostril in the direction of the ventral meatus and allowed to soak for 5-10 seconds while rotating carefully. After the collection the swabs were placed into a sterile tube filled with 3 ml sterile 0.9 % NaCl solution and stored at -20 °C until processing for nucleic acid extraction.

2.2.2. PBLC

Whole blood was drawn from the left vena jugularis directly after the shooting. The area was cleaned and the samples were taken via Vacutainer and EDTA tube.

As a first step, the EDTA-inhibited blood samples were centrifuged at 1860 xg for 10 minutes. As a result of the centrifugation, three clearly separable fractions were obtained. The top layer, the serum, was removed. The thin layer formed over the erythrocyte fraction was aspirated with a pipette. This layer, also called buffy coat layer, was immediately pipetted into Milli-Q water and mixed carefully. After 40 seconds, the solution was mixed with 10x phosphate buffer (phosphate-buffered saline, PBS). A further 10-minute centrifugation at 1860 xg followed. The supernatant was carefully poured off. The PBS was scanned for white blood cells that had settled to the bottom of the test tube. After further mixing, a repeated centrifugation was performed. The supernatant was poured off. The leucocytes were then washed in PBS. After homogenisation, the samples were stored at -70 °C until further processing.

2.2.3. Lung tissue

Complete necropsy was carried out on the spot in all horses, but detailed results are not part of this study. Two times ca. 1 cm^3 of lung tissue was taken from the lungs. One sample was sent to the Department of Microbiology and Infectious Diseases for PCR testing and the other to the Department of Pathology for histopathology.

Figure 12: gross pathology performance on one of the przewalski horses (picture belongs to Dr. Korbacska-Kutasi)

For histopathology, samples were subsequently embedded in paraffin using an automatic tissue processor (Shandon Citadel 2000; Thermo Scientific). Paraffin blocks were cut at 4 µm and were stained with hematoxylin and eosin in an automatic staining device (Shandon Varistain 24-4; Thermo Scientific).Slides were evaluated by an experienced histopathologist with -2, -19 and 20x magnification.

2.2.4. PCR

PCR with virus specific primers were performed on all samples (NS, PBLC, lung tissue) for detection of EHV-5.

The reference sequence used for the alignment was >NC_026421.1 Equid herpesvirus 5 strain 2-141/67, complete genome of the GenBank. The NC_0262 Equid herpesvirus 5 b gene complete was used specifically for the gB search because all of our gB sequences were included in this sequence.

2.2.5. DNA extraction

The lung biopsy was homogenized in TE-buffer (10 mM Tris, 1 mM EDTA, pH 8.0). Viral DNA was extracted by adding 10 µL proteinase K (Sigma Aldrich) to 90 µL of the buffers. Thereafter nucleic acid extraction was performed in a Magnatrix 8000+ robot (NorDiag AB), using the NorDiag Vet Viral NA extraction kit according to the manufacturer´s instructions.

2.2.5.1. Amplicon primers

Based on all available sequences in NCBI GenBank for EHV-5 glycoprotein B (gB) a highly variable region flanked by conserved regions that could serve as primer binding sites was selected. The primers were designed to be specific for EHV-5 and not to amplify EHV-2.

Primer sequences and their positions in the gB gene of EHV-5 (NC_026421) are as follows: F 5'-AAGCACGAGAAAAGCTACCAT-3' (1090-1110) and R 5'-ACAGCTGCTCCAAGACCC-3' (1572-1589). The rationale for this design was to amplify a region that could serve as a suitable fingerprint for different strains. The variable region contains a putative protease cleavage site (Sorem & Longnecker, 2009) and an analogous region has been used for genotyping of cytomegalovirus (Chou & Dennison, 1991). Primers were designed in the PrimerQuest® design tool on the webpage of Integrated DNA Technologies (IDT) and purchased from Eurofins MWG Operon.

2.2.5.2. Amplicon PCR and library preparation

The amplicon PCR and library preparation were performed according to the Illumina protocol "16S Metagenomic Sequencing Library Preparation" with minor modifications, and are briefly described below.

Amplicon PCR was performed using KAPA HiFi Hot Start ReadyMix (KAPA Biosystems, Wobum, MA), with primer concentrations of 200 nM. Thermal cycling started with an initial activation step at 95 ºC for 3 min and proceeded with 35 cycles three-step cycling at 98 ºC for 20 s, 55 ºC for 30 s and 72 ºC for 30 s, thereafter 72 ºC for 5 min and then hold at 4 ºC. The presence of amplification products of the expected size, about 500 base pairs (bp), was confirmed by gel electrophoresis. The amplicons were thereafter purified from free primers and primer dimers by a clean-up step using AMPure XP beads. Dual indices and Illumina sequencing adapters were added in an eight-cycle PCR reaction and a second clean-up was performed according to the manufacturer´s instructions. Quantification of the libraries was performed using a Bioanalyzer for verification of the size and a Qubit dsDNA high-sensitivity assay kit (Life Technologies) to determine the concentration of each library. Libraries were diluted with Tris pH 8.5 to reach a concentration of 4 nM each and were then pooled.

2.2.5.3. Sequencing

The sequencing was done using the same primers, 100 µl of PCR product was spiked, gel purified (QiaQuick Gel Extraction Kit), concentrated to 30 µl and finally dried. Sequencing was performed on two primers (one on one strand and one on the other). Sequences were aligned, sequencing errors were corrected using the SECentral. Finally, the sequences were identified by BLAST analysis (whether EHV-5 or not) and used for phylogenetic analysis.

2.3. Analysis of the collected sequences

The sequences obtained were compared using 'Clonemanager Suite 9'. The alignment of the sequences was determined. The programme showed the match in percent as well as the number of matching and non-matching base pairs. Furthermore, a family tree of the sequences was created.

The DNA sequences were also transmitted into proteinsequences and were aligned. The proteinsequences were used, because they are more compact and reliable than the DNA sequences.

For an better overview we included sequences of Hungarian sport horses, which never had contact to ours. Our eleven sequences were aligned with eight other sequences. This sampling was done in 2013 and were all NS or PBLC samples. The horses had either asthma or EMPF, with different stages of disease. Horse MP42 was diagnosed with EMPF, horse MP94 had either a mild form of EMPF or asthma and the number 86-2020L was a suspected EMPF healed horse. The others had asthma in different stages. In MP42 genotype I. was the only detected type. In MP94 only genotype XI. and in 86-2020L only genotype III. could be detected.

3. Results

3.1. PCR Results

NS, PBLC and LT was taken of each horse, in total 33 samples. Three of these 33 (9 %) samples could not be used for PCR, due to lack of sample quality, two of eleven (18 %) of the PBLC sample (horse 7 and 8) and one of eleven (9 %) of all LT sample (horse 3). This results in 30 samples, which could be used for PCR, eleven NS samples, nine PBLC samples and ten LT samples.

If we divide our patients in two age groups: group 1 (3-10 years) and group 2 (10- >20 years), the patients positive on all samples are in the younger group and the patients negative on all samples are all in the older group.

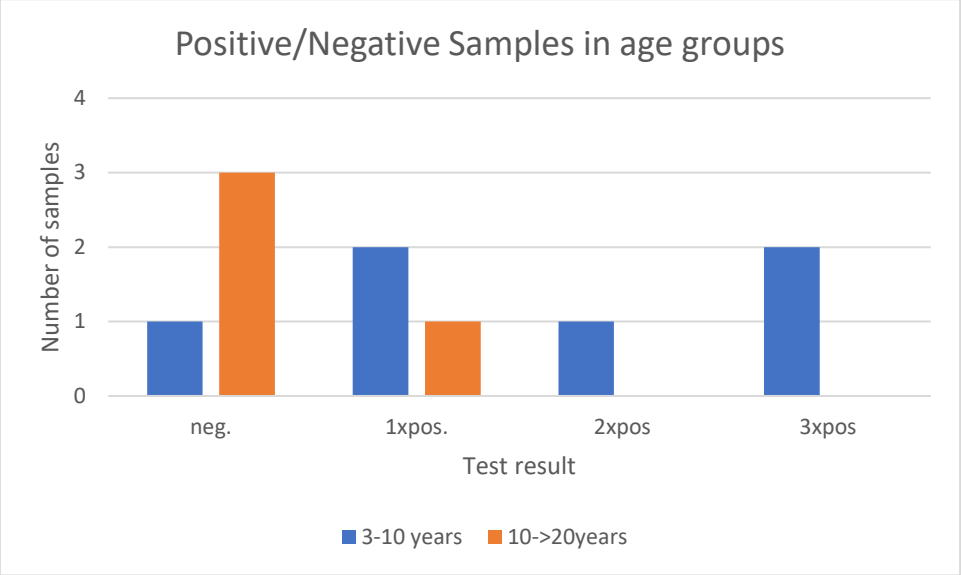


Figure 13: All results divided in age groups, pos. = positive, neg. = negative

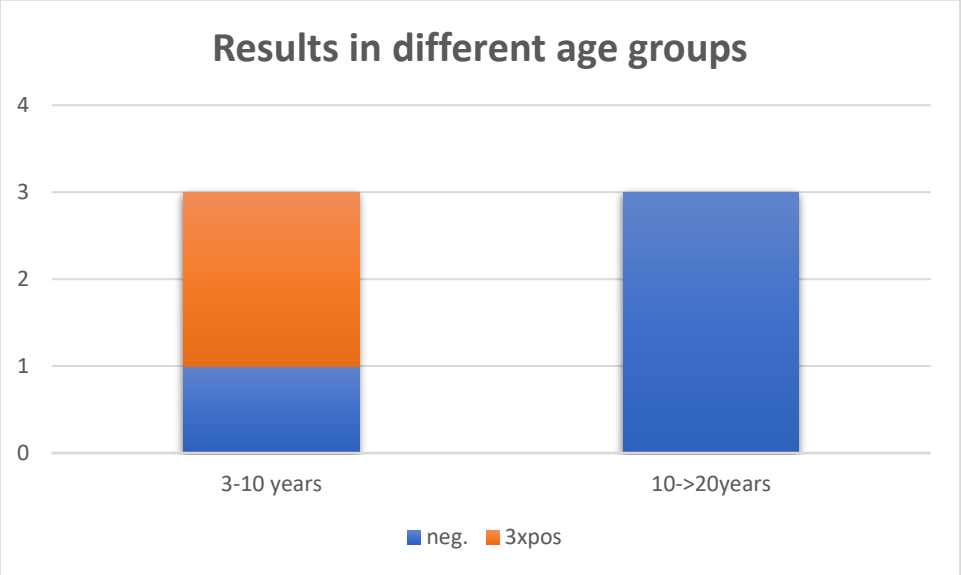


Figure 14: Results of horses that were positive/negative on all samples divided in age groups

In total, in six out of eleven (54,5 %) horses EHV-5 was detected in at least one of the samples. Five out of eleven (45,5 %) NS samples were positive for EHV-5. In three out of nine (33,3 %) PBLC samples detection for EHV-5 was positive. In lung tissue of three out of ten (30 %) tested horses EHV-5 could have been detected.

This means that the most EHV-5 detection could be achieved with the nasal swab. All horses positive on LT were also positive on NS.

ID	Anamnesis	NS	PBLC	LT
1	right front leg lameness	neg.	neg.	neg.
2	tumour on the right cheek	neg.	pos.	neg.
3	domestic horse phenotype	pos.	neg.	X
4	behavioural changes: aggression towards stallions and foals	pos.	neg.	neg.
5	poor condition	pos.	pos.	pos.
6	lameness and bone proliferation on the left knee	neg.	neg.	neg.
7	surplus	neg.	X	neg.
8	domestic horse phenotype	neg.	X	neg.
9	infertile, severely inbred	pos.	pos.	pos.
10	pathological lesion	neg.	neg.	neg.
11	infected wound in the left gluteal region	pos.	neg.	pos.

Table 3: Anamnesis of horses and Results of PCR: NS = nasal swab, PBLC = peripheral blood leukocytes, LT = lung tissue, neg. = negative, pos. = positive

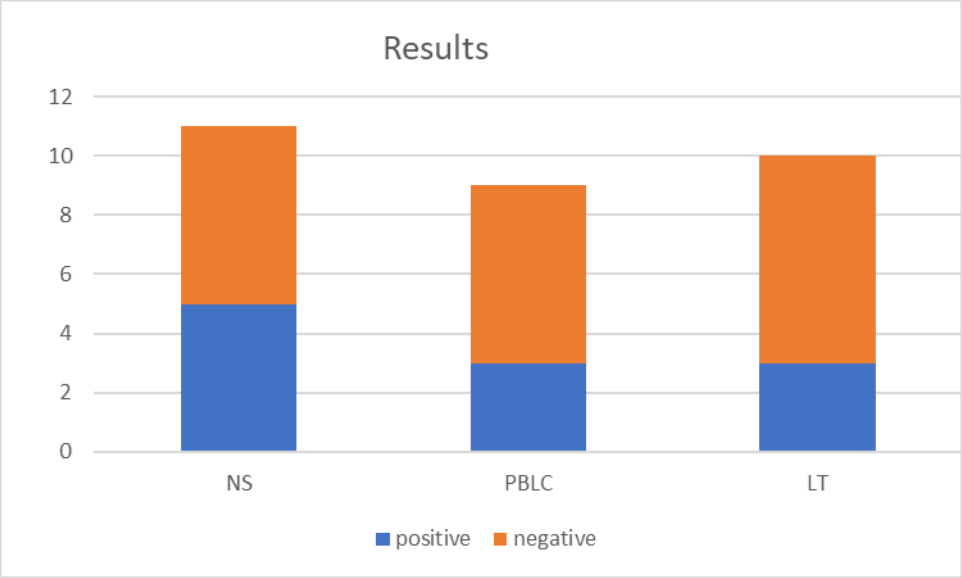


Figure 15: EHV-5 positive vs negative in different samples NS = nasal swab, PBLC = peripheral blood leukocytes, LT = lung tissue

3.2. Pathology

At gross pathology, none of the horses showed pathognomonic lesions indicating EHV-5. Histopathology performed on lung tissue showed fibrosis in three horses (5, 9,11) and two of these three horses were also positive for EHV-5 (5,9). However, these lesions were not the typical nodular lesions of EHV-5 related pulmonary fibrosis.

ID	Histopathology
1	mild alveolar emphysema
2	severe alveolar emphysema
3	severe alveolar emphysema, fresh-skeleton haemaspiration, chronic fibrinous pleuritis
4	severe alveolar emphysema
5	mild alveolar emphysema, fresh haemaspiration, minimal peribronchiolar mixed cell (lymphoplasmacytic + eosinophilic granulocytic) interstitial pneumonia with solitary nodular fibrosis
6	chronic nodular fibrinous pleuritis and minimal peribronchiolar/perivascular lymphoplasmacytic interstitial pneumonia
7	severe alveolar emphysema, fresh-skeleton haemaspiration, chronic fibrinous pleuritis
8	mild alveolar emphysema, fresh haemaspiration and minimal peribronchiolar lymphoplasmacytic interstitial pneumonia
9	Severe alveolar emphysema, mild fibrosis

10	mild alveolar emphysema, fresh haemaspiration, minimal peribronchiolar lymphoplasmacytic interstitial pneumonia
11	Mild peribronchiolar/perivascular lymphoplasmacytic interstitial pneumonia with very mild multifocal fibrosis and eosinophilic granulocytes

Table 4: histopathological findings, bold are the horses where fibrosis of any kind could be found

Three (27,3 %) of the horses just had a mild alveolar emphysema, four (36,4 %) had a minimal peribronchiolar lymphoplasmacytic interstitial pneumonia and three (27,3 %) had a chronic fibrinous pleuritis. None of these findings could be described in relation to EHV-5 infection. However, three (27,3 %) of the horses did show fibrosis in the lung and one (9 %) of them had a single fibrotic nodule, but still not the pathognomonic lesions of EMPF.

3.3. Sequencing

PCR was performed on all samples. Sequencing should have been done on all samples, but only four samples of different horses (Nr. 2, 4, 9, 11) could be due to lack of sample quality of the other samples. The sequencing was done on the PBLC sample of horse 2, on NS in horse 4, NS/PBLC/LT in horse 9 and on NS/LT in horse 11.

ID	Symptoms	genotype	reference sequence	reference sequence acc no
2	severe alveolar emphysema	VII., IV.	isolate EHV5.2-141, complete genome (AUS1967)	KU315429.1
4	severe alveolar emphysema	VII.	isolate EHV5_VII_56 glycoprotein B gene, partial cds	MW526324.1
9	severe alveolar emphysema	XII., XI., VIII., VII., V., IV., III.	isolate EHV5_XII_92 glycoprotein B gene, partial cds	MW526356.1
11	mild fibrosis	IV., II., I.	isolate EHV5_IV_38 glycoprotein B gene, partial cds	MW526306.1

Table 5: Sequencing results and reference sequence. When several genotypes were found in one horse, the genotype that was most present was listed first.

The reference sequences were chosen from the National Library of medicine – National Centre for Biotechnology Information (NCBI) GenBank. A Basic Local Alignment Search Tool (BLAST) with our sequences was run and the sequences of the GenBank which most aligned with our sequences were chosen as reference.

They all had different genotypes of EHV-5. Also, different types could be detected in the same horse at the same time. Genotype VII. and IV. could be detected most. Horse 9 had the most genotypes and in horse 4 only one genotype could be detected.

The obtained sequences were aligned. PW2 was a complete sequence, the others were just the gB part of the sequences, so PW2 was cut to the length of the other sequences to get more accurate results.

Sequence	Start	End	#Match	NonMatch	%Match
PW4	1	396	261	138	65
PW2L	1	361	289	93	75
PW11	1	295	278	91	75
PW9	1	309	268	101	72

Table 6: Protein sequence aligned, showing how they match; PW4 = sequence of horse 4, PW9 = sequence of horse 9, PW2L = sequence of horse 2, PW11 = sequence of horse 11

The alignment showed that the sequences of horse no. 2, 9 and 11 were closer related than the sequence of horse no. 4. The sequences of horse 2, 9 and 11 were over 70 % matching The sequence of horse 4 was just 65 % overlapping with the other sequences and was the longest sequence.

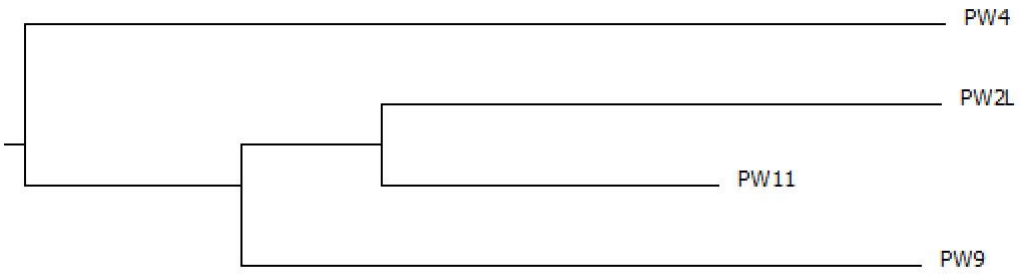


Figure 16: Phylogenetic tree of the DNA sequences PW = Przewalski, Numbers equal the horses ID

This tree shows that the sequences of horses 2, 9 and 11 are more closely related than those of horse 4.

In this study, we also tried to gain more information about the prevalence of EMPF. Three horses had low grade fibrotic changes in the lungs, but these lesions did not look like the pathognomonic nodular fibrosis of EMPF. The lesions could have been a very early form of

EMPF or of a different origin. Thus, no horse could be diagnosed with EMPF. Due to the lack of EMPF diagnosis, no conclusions could be drawn about the general prevalence of EMPF.

To compare our sequences with horses non-related, we took sequences of another project. These eight horses were sport horses from Hungary, which never had contact to the Przewalskis tested. The sampling was done in 2013 and were all NS or PBLC samples. The horses had either asthma or EMPF, with different stages of disease. Horse MP42 was diagnosed with EMPF, horse MP94 had either a mild form of EMPF or asthma and the number 86-2020L was a suspected EMPF healed horse. The others had asthma in different stages. In MP42 genotype I. was the only detected type. In MP94 only genotype XI. and in 86-2020L only genotype III. could be detected.

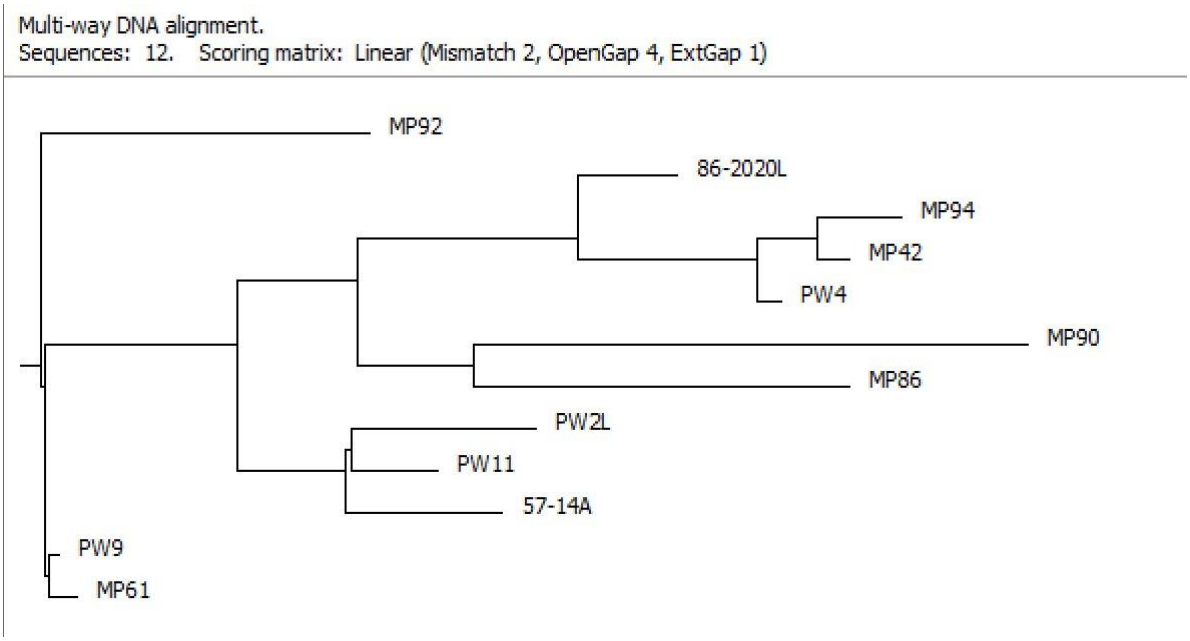


Figure 17: Phylogenetic tree of the samples of sport horses in Hungary (MP) with no contact to the Przewalski (PW).

This figure shows that even though the Przewalski and the other, domesticated, horses have never had contact with each other, the Sequences still are mixed. This also shows, like other studies, that the genotype of the EHV-5 does not equal with a specific pathological pattern.

4. Discussion

4.1. Connection to EHV5 epidemiology of our and other studies

For this experiment, Przewalski horses were used that had no contact with other domesticated horses for at least ten years. Despite the lack of contact, EHV-5 was still detected. The origin of this infection is unknown and could not be further clarified by this experiment. This could be due to the small number of subjects, but, as has been shown in other experiments, tracing the origin of the virus is difficult. Although the virus could be categorised into different genotypes, it was shown that animals living in the same region do not necessarily have the same genotypes. Thus, even the genotype of EHV-5 cannot be used to trace the course of infection and predict spread.

Although the virus was first detected in 1970 (Turner and Studdert 1970), it seems to have been around for much longer. This is shown by a study from Iceland (Torfason et al 2008). In this study, the population of Icelandic horses located in Iceland was used. In order to prevent the entry of diseases, there is a law in Iceland that once Icelandic horses have left the country, they cannot return to Iceland. Therefore, the population has been isolated from other horses for thousands of years and are therefore immunologically naive to many diseases. Despite this isolation, EHV-2 and EHV-5 could be detected in the experiment. This shows that the virus has existed for a very long time and must have arrived there with the first settlement of Iceland.

In our study, we could not draw any correlations to the prevalence with regard to gender or other environmental characteristics. All lived in the same environmental conditions and we only had three females, which had quite different results. One was positive on all samples and two were negative on all samples. No correlation between sex and virus detection was observed. In other studies, age prevalence was found, but not for sex or housing type. If divided in two age groups, we found that all horses that were positive on more than two samples were in the younger group (3-10 years) and horses negative on all samples, were all in the older group, but due to the small number of patients, we could not find a significance. The correlation between younger horses and a higher detection of EHV-5 can also be seen in other studies (Marenzoi et al 2010).

To get a better overview of the correlation between EHV-5 and EMPF we used the data of another experiment to compare with our findings. In the other study of Hungarian sport horses one was diagnosed with EMPF, one seemed to have a mild form or early stage of EMPF and one was supposed to be a maybe healed EMPF case. The alignment of our sequences and the sport horses did show a similar result as other studies. Meaning that no pattern between a specific genotype and clinical symptoms could be drawn. The EMPF horse had a similar genotype with one of our horses with no signs of EMPF. Even though this correlation is suspected, further research needs to be done.

Most of the studies regarding EMPF are mainly about EHV-5 detection. There was no coincidental findings of EMPF. If studies are aiming directly at EMPF, the horses were diagnosed previous to the experiment. The average age of EMPF cases in different

studies varies between 13 (Wong et al 2008) and 17,2 years (Poth et al 2009). In one experiment with an age range of 4-28 years of EMPF diseased horses the average age was 14,5 years (Williams et al 2007). In another study the average age was 16 years with a SD of 6.1 years (Pusterla et al 2015). Although EHV-5 infection is found more often in younger horses, EMPF tends to occur later in life. The reason for this could be, that the EMPF horses could not fight or get rid of the infection at a young age and the virus is therefore a permanent trigger for the immune system.

However, in a study (Rushton et al 2013) from Austria, in which Lipizzaners were tested in different types of housing, it was found that mares kept with their foals in a free stall with high stocking density, showed more EHV-5 positive animals, than the stallions in individual stalls. However, it should be noted, that a significantly larger number of mares were present for testing and in high stocking density the spread of diseases is much easier and faster.

So far, no geographical prevalence has been proven, as EHV-5 has been detected with similar frequency in different populations worldwide. 75-83 % (Bell et al 2006), 79 % in one group (Dunowska et al 2002), 35 % (El Hage et al 2021), 73-80 % in younger, 20-40 % in older horses (Marenzoni et al 2010), 47 % (Pennington et al) and 63 % (Torfason et al 2008) are the prevalence of EHV-5 in other studies from different countries and different age groups.

Maboni et al (2022) were searching for AsHV 4 and 5 in deceased free-ranging donkeys with prior respiratory symptoms. Ten of 13 of these donkeys showed moderate-to-severe interstitial fibrosing pneumonia, and one had pyogranulomatous pneumonia at necropsy. In all the diseased donkeys AsHV could be detected and in donkeys without the previously mentioned lesions no herpesvirus could be found. This showed a direct link between detection of γ-herpesvirus and fibrosis of the lung.

4.2. Did we gain any EHV5 correlation with EMPF

In this study EHV-5 could be detected in six out of eleven patients in at least one of the samples. In three horses, fibrosis was found in the lungs and two of these horses were also positive at PCR on all samples. However, the fibrotic lesions did not show the typical nodular formation of fibrosis of EMPF, which means that the lesions were either a quite early form of EMPF, where the pathognomonic lesions were not formed yet, or the fibrosis had another origin. This experiment also shows, that the infection alone only triggers a disease in rare cases, due to many infected horses with lack of symptoms. There is a hypothesis that a certain genotype could be responsible for triggering the disease (Back et al 2016). For this purpose, the genotype of horses suffering from EMPF was analysed and compared with healthy horses. However, since affected horses often have different genotypes, or a horse can have several genotypes at the same time,

research is difficult. Due to these difficulties, the hypothesis could not be proven until now. In our study, too, no connection could be established between a certain genotype and symptoms.

The exact pathogenesis is not clear. It is suspected, that the disease is multifactorial and may be related to a weakened immune system. However, there is a study by Williams et al (2013) in which healthy horses were infected with EHV-5. In some of the infected horses, EMPF was later detected. This showed, that EHV-5 alone can cause these pulmonary fibroses. However, the circumstances needed to tell exactly when an animal becomes ill are still unclear and needs further investigation.

The detection of EHV-5 is rather easy. The fastest, easiest and cheapest methods is the nasal swab. For detecting a EHV-5 infection, the NS is the best tool, but most of the horses positive only on NS mainly have a subclinical infection. Previously described in the introduction, is a hypothetical pathogenesis (Figure 3), which shows, that the infection happens in the upper airways and does not always wander into the lower airways, where it can cause the disease. In order to detect the virus by PCR, the glycoprotein B is often searched for. Several studies use it as a tool for EHV-5 detection, as it appears to be relatively stable in its structure. Recent studies have shown, that there is some variability in the gB. This variability reduces its suitability as an analytical tool and its informative value about the infection status (Back et al 2016; Stasiak et al 2021). Furthermore, the tests cannot distinguish between latent and lytic infection. However, the detection of EHV-5 in NS alone has no significance for a possible EMPF disease. The probability of EMPF is higher in horses, where EHV-5 was detected in PBLC or lung tissue, than in NS. A quantitative determination of the viral load does not help, as there is no correlation between an increased viral load and a higher probability of an EMPF disease. As already mentioned, the lung biopsy and following histopathology is the only way to diagnose EMPF in a living animal. However, this biopsy may be associated with consequences such as lung haemorrhage, following nose bleeds, coughing and respiratory distress (Pusterla et al 2015). Unfortunately, there is still no viable alternative. A CT scan, as in humans, might be an option, but a horse is simply too big to fit in the CT scan. Even if they would fit in, the cost-benefit ratio is questionable, due to the expense of general anaesthesia, equipment and the significance of this examination.

Up until now, there is no knowledge about the prevention of the disease. Since many virus carriers are asymptomatic and the trigger of the disease is not yet fully understood, it cannot be prevented by targeted measures. It will probably also take more time, as other uncertainties about EHV-5 and its diseases have to be resolved beforehand.

Just one of our horses was negative on NS but positive on the other samples, which is quite uncommon. If EHV-5 can be detected in the lower airways, it can usually be found in any other samples, which leads to the suspicion, that there must be a higher viral activity in these horses. Whether this higher activity is correlated to the emergence of EMPF needs further research. Another hypothesis, that needs additional investigations is, that immunosuppression leads to increased viral activity and the link between these and EMPF.

4.3. Connection between γ-herpesvirus and lung fibrosis

Not only in horses with EMPF could pulmonary fibrosis be detected. Humans and other animals, like donkeys or mice, also showed infection with a γ-herpesvirus in combination with fibrosis in the lungs. Even if the fibrosis does not occur in a nodular form as in EMPF, it still shows up in connection with the virus. There is a study were EHV-5 was experimentally inducted directly in the lung and the virus alone caused EMPF (Williams et al 2013).
In humans, fibrosis of the lungs has also been associated with the presence of a γ-herpesvirus. IPF is a highly researched disease for which the EMPF affected horse also serves as a model (Williams et al 2007). The pathogenesis, as the one for EMPF, is not completely known. It seems to have a correlation with immunosuppression and can have a long latency. As EHV-5 it could also be associated with other diseases of the immune system.
Interestingly, pulmonary fibrosis is also found very frequently in geriatric donkeys and horses, but these changes are mostly age-related (Gerber et al 2016). Our horses with mild fibrosis were 3-9 years of age. This age group of domesticated horses does not belong to the geriatric class. On the other hand feral horses possibly have more diseases and pathogens which can lead to age related changes already in the younger animals. It is not yet clear, whether pulmonary fibroses other than EMPF can also be associated with EHV-5, or whether they are of a different origin or simply age-related.

The correlation between EHV-5 and other diseases has also not yet been proven. There is one study (Pennington et al 2017), that links EHV-5 to gastric ulcers. During gross pathology of our study the stomach was also inspected for gastric ulcers. In three (27,3%) of the horses (3, 7 and 11) gastric ulcerations could be found. Horse 3 was positive in one sample, 7 was negative in all samples and 11 was positive in two samples. However, we were not looking for EHV-5 in the gastric mucosal, which is why no real correlation could be drawn. It would have been interesting, if EHV-5 could have been detected in the gastric mucosal of any of the horses, especially horse 11, because in this horse a lung fibrosis was found as well as gastric ulcers. The correlation between EHV-5 and gastric ulcers still needs further research.
The involvement of EHV-5 in lymphoproliferative diseases (Schwarz et al 2013) is also not based on any proven findings. Here again, a parallel can be drawn with EBV, since the influence of the herpes virus on the disease has not yet been fully clarified. EHV-5 could be detected in several tissues without causing an apparently related disease in these tissues. Whether EHV-5 also causes changes or diseases in tissues other than the lungs, requires further research.

4.4. Problems

In our study, we used the gB for virus detection. At that time, gB seemed to be quite stable and good for the usage for virus detection. But, as seen in other studies, some parts are quite variable. This means that some of the present EHV-5 cannot be detected or some PCR is false positive. Our findings about this can also be proven by other studies. (Stasiak et al 2012; Marenzoni et al 2010; Back et al 2016)

gB general

Later studies revealed a considerable variability was around the putative furin cleavage site, a horizontal transfer and/or evolution of EHV-5 within individual hosts is suggested. Furin cleavage plays a role in facilitation of the viral infection (Stasiak et al 2021). gB also seems to be a major target for neutralizing antibodies of the host. gB seems to evolve during time, so that a horse with 2 different genotypes, can have a different proportion of these genotypes and even loose or gain other genotypes over time. The goal of many studies regarding the gB, are to find a link between a specific genotype and clinical signs. However, no evidence of such relation could be found yet (Back et al 2016). Ours, as well as many other studies, could not proof this relation. The problem is, that the gB itself seems to change constantly. Even though two gB have the same genotype, they can differ from another. Not just this instability of the gB, makes assumption difficult, but also the correlation to a clinical outcome. EMPF horses can have different genotypes, but completely healthy horses can carry the same genotype as a diseased one. This lack of pattern not only complicates finding a cause, but also makes it difficult to predict an clinical outbreak.

In this study due to the patients not being used to human touch, no physical examination could be performed, which could have given an insight on lack of performance or clinical signs typical for EMPF. Due to the small number of patients no significant results could be gained. Also prevalence of sex and breed could not be shown in this study. One horse showed fibrosis in the lung, but not pathognomonic lesions for EMPF could be seen. Due to the lack of EMPF diseased horses, no prevalence for EMPF could be established.

Although EHV-5 is quite common, a lot of details are still unknown. EHV-5 seems to be present since thousands of years (Torfason et al 2008) and was first discovered in 1970 (Turner and Studdert 1970). However, the origin of the virus and the first appearance could not be dated. The differentiation of the different genotypes could not give an insight on tracking the origin of the virus. Different genotypes could be found in the same population and same genotypes appeared at different locations, which do not seem to be correlated.

The correlation between EHV-5 detection and EMPF has been significantly proven. However, when EHV-5 causes the disease is not known. There had been speculations of a specific genotype causing the disease, but this theory could not have been proven till now.

Up until now no other way than PCR is known for detection. AG/AK test for fast detection would be helpful for scientific purpose, but not for clinical use, because detection alone, does not mean that the horse is also sick.

5. Summary

Dieses Experiment wurde durchgeführt, um die Prävalenz von EHV-5 beim frei lebenden Przewalski-Pferden und dessen pathogene Folgen festzustellen. Elf Tiere wurden aus diversen Gründen ausgemerzt. Von diesen Tieren wurde eine komplette pathologische Untersuchung durchgeführt und Nasentupfer, Vollblut und Lungengewebe entnommen. Diese Proben wurden mittels PCR auf EHV-5 untersucht. Bei den Proben wurde versucht, die DNA zu sequenzieren und diese miteinander zu vergleichen. In fünf Tieren, konnte kein EHV-5 nachgewiesen werden, in zwei Tieren jedoch in allen Proben. 3 Tiere zeigten Fibrosen in der Lunge, jedoch keine typischen EHV-5 Läsionen. Von vier Tieren konnte die virale DNA sequenziert werden. Diese wurden mit weiteren EHV-5 Proben verglichen. Es konnte keine Relation zwischen dem Genotyp von EHV-5 und klinischen Symptomen festgestellt werden. Was jedoch festgestellt werden konnte, ist, dass der Nachweis von EHV-5 bei jüngeren Tieren höher war. Zusammenfassend hat dieses Experiment gezeigt, dass weitere Untersuchungen durchgeführt werden müssen, um EHV-5 und die mit ihm einhergehenden Erkrankungen besser zu verstehen.

This experiment was conducted to determine the prevalence of EHV-5 in free-living Przewalski horses and its pathogenic consequences. Eleven animals were culled for various reasons. A complete pathological examination was performed on these animals. Nasal swabs, whole blood and lung tissue were collected. These samples were tested for EHV-5 by PCR. An attempt was made, to sequence the DNA of the samples and compare them with each other. No EHV-5 could be detected in five animals, but in two animals in all samples. Three animals showed fibrosis in the lungs, but no typical EHV-5 lesions. The viral DNA of four animals could be sequenced. These were compared with other EHV-5 samples. No relation between the genotype of EHV-5 and clinical symptoms could be established. What could be determined, however, is that the detection of EHV-5 was higher in younger animals. In conclusion, this experiment has shown that further research needs to be done to understand EHV-5 better and the diseases associated with it.

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7. List of figures and tables