Low Virulent Respiratory Viruses in Standardbred Trotters

Relationship to Health and Athletic Performance

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Abstract

Respiratory viral infections are known to affect equine health worldwide and are commonly associated with clinical signs such as fever, cough, and nasal discharge. While clinical respiratory signs will prevent horses from training and racing, it is also known that horses can be infected by viruses and yet not exhibit any clinical signs (i.e. subclinically infected). However, it is not clear whether these subclinical infections may have any effect on the horse´s performance and health. In this thesis well-known equine respiratory viruses such as equine influenza (EIV) and herpesvirus (EHV) type 1 and 4, as well as the less explored equine rhinitis A and B virus (ERAV and ERBV) and EHV type 2 and 5, were investigated on monthly basis in a longitudinal study over a year in actively racing Standardbred trotters. The presence and amount of the viruses and their antibodies were identified by diagnostic methods such as serology and PCR assays. Genetic information was obtained by sequencing. The health of the horses was also monitored and their performance determined by both objective and subjective methods. A high seroprevalence of ERAV and ERBV in the samples suggested that these viruses were widespread in the study cohort of well-managed horses. EHV-5 and EHV-2 were detected in about three-quarters and almost one third of the nasal secretions samples respectively. We could however not find any associations between these subclinical infections and poor performance. This work also includes a case report of equine multinodular pulmonary fibrosis (EMPF) in a horse, which was identified with co-infection by EHV-5 and the asinine herpesvirus type 5. Genetic analysis of a segment of the gB gene of EHV-5 in healthy horses with two samples taken one year apart and from the EMPF case study horse pre and post mortem allowed to classify EHV-5 strains into four different genotypes. While viral strains appeared stable over time, horses could be infected with multiple strains that varied in detection qualitatively and quantitatively over time. The single strain detected in the EMPF case was also found in the clinically normal horses.

The work in this thesis provides knowledge of subclinical presence of respiratory viruses in elite Standardbred trotters and further genetic information of EHV-5 showed a range of interactions between EHV-5, the host and the environment over time.

Keywords: Equine, poor performance, ERAV, ERBV, EHV-2, EHV-5, EMPF, phylogenetic analysis, viral load.

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To my family

When you are on a great horse, you have the best seat you will ever have.
Sir Winston Churchill

Kaffe och bullar gör mig glad!
Tage Danielsson

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Back H, Kendall A, Grandón R, Ullman K, Treiberg Berndtsson L, Ståhl K & Pringle J (2012). Equine Multinodular Pulmonary Fibrosis in association with asinine herpesvirus type 5 and equine herpesvirus type 5: a case report. *Acta Veterinaria Scandinavica* 54:57.

II Back H, Penell J, Pringle J, Isaksson M, Ronéus N, Treiberg Berndtsson L & Ståhl K (2015). A longitudinal study of poor performance and subclinical respiratory viral activity in Standardbred trotters. *Veterinary Record Open* 2:e000107.

III Back H, Ullman K, Treiberg Berndtsson L, Penell J, Ståhl K, Valarcher J-F & Pringle J (2015). Viral load in nasal secretion of equine herpesviruses 2 and 5 in actively racing Standardbred trotters: temporal relationship of shedding to clinical findings and poor performance. *Veterinary Microbiology* 179(3-4), 142-148.

IV Back H*, Ullman K*, Leijon M, Söderlund R, Penell J, Ståhl K, Pringle J & Valarcher J-F (2015). Genetic variation and dynamics of infections of equine herpesvirus type 5 (EHV-5) in individual horses (Submitted manuscript).

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*shared first authorship

The contribution of Helena Back to the papers included in this thesis was as follows:

I Main author, participant in laboratory work and analysis of results.

II Main author, participating in study design and planning, sampling of all horses included in the study and participant in statistical analysis of the results.

III Main author, participating in study design and planning, sampling of all the horses included the study and execution of the endoscopic examinations. Primary contributor to laboratory work and participant in statistical analysis of the results.

IV Main author, sampling of the included horses. Participant in planning the study. Shared laboratory work and participant in the data analysis and interpretations of the results.

Abbreviations

aa	Amino acids
AHV-5	Asinine herpesvirus type 5
bp	Base pair
BAL	Bronchoalveolar lavage
CF	Complement fixation
CMV	Cytomegalovirus
CPE	Cytopathic effect
DNA	Deoxyribonucleic acid
EBV	Epstein-Barr virus
EAV	Equine arteritis virus
EHV-1	Equine herpesvirus type 1
EHV-2	Equine herpesvirus type 2
EHV-4	Equine herpesvirus type 4
EHV-5	Equine herpesvirus type 5
EIV	Equine influenza virus
ELISA	Enzyme-linked immunosorbent assay
EMPF	Equine multinodular pulmonary fibrosis
ERAV	Equine rhinitis A virus
ERBV	Equine rhinitis B virus
gB	Glycoprotein B
HA	Haemagglutinin
HR	Heart rate
IAD	Inflammatory airway disease
NS	Nasal swab
NGS	Next generation sequencing
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
SAA	Serum amyloid A

SD	Standard deviation
SFE	Standardized field exercise test
SLU	Swedish University of Agricultural Sciences
SVA	National Veterinary Institute (Sweden)
TY	Training yard
TW	Tracheal wash
VN	Virus neutralization
WBC	White blood cell

1 Introduction

Respiratory viral infections affect equine health worldwide and are commonly associated with clinical signs such as fever, cough, and nasal discharge. Outbreaks of respiratory infections, especially caused by equine influenza virus (EIV), continue to have major impact on both equine health and welfare and causes of financial losses to the industry due to cancelled events or temporarily close down of race tracks (Legrand et al., 2013).

Consequently considerable research has been performed on equine respiratory viral infections, preferably those that cause severe clinical disease, and the understanding of equine respiratory viral pathogens is continuously growing, accompanied by the development of commercial vaccines for EIV and equine herpesvirus (EHV) type 1 and 4 infections. Vaccination, together with biosecurity, plays an important role in preventing infectious diseases. However, it can be challenging to design a vaccine with satisfactory level of protection and therefore fully effective vaccines are not always available. Vaccines both serve the purpose of protecting the individual horse from infection and that of preventing spread from already infected animals. Since animals vaccinated against EIV (Elton & Cullinane, 2013; Bryant *et al.*, 2010) as well as EHV-1 (Goehring *et al.*, 2010) have been identified to shed lower amount of virus compared to unvaccinated horses, vaccination thereby limiting the spread of the virus and protecting the wider population of horses from infection.

Optimal function of the respiratory tract is crucial for the horse and its athletic performance. Subclinical respiratory abnormalities have been associated with exercise intolerance that often appears only at maximal athletic exertion (Fraipont et al., 2011; Fogarty & Buckley, 1991). Furthermore, viral infections, especially subclinical, have been associated with the poor performance syndrome for decades (Mumford & Rossdale, 1980). On many occasions, respiratory viruses have been blamed when a horse has performed below its expected level of performance. For example, during the last 20 years

blood samples from approximately 13 000 Swedish horses have been analysed at the National Veterinary Institute (SVA) in Uppsala Sweden for presence of antibodies to equine rhinitis viruses (unpublished data) due to suspicion that subclinical infections were causing poor performance, despite lack of scientific evidence. Equine respiratory viruses in general and herpesviruses in particular (due to their high prevalence and capability of latency), have frequently been incriminated as reasons for poor performance in athletic horses (Wood et al., 2005). Supporting the link between viral infection and the poor performance syndrome, horses with impaired performance and respiratory problems were shown to have significantly higher lactate concentrations in the blood during treadmill exercise tests compared to well performing horses (Courouce-Malblanc *et al.*, 2002). Furthermore, together with lameness, respiratory problems such as inflammatory airway disease (IAD) are among the most common reasons for race horses failing to race, train and perform as expected (Wilsher et al., 2006).

That a clinical respiratory viral infections can impair athletic performance is expected. Thus, the presence of clinical signs of respiratory disease due to viral or other infections is a clear indication for horses not to enter competition. It is, however, known that horses can be infected by viruses and yet not exhibit any clinical signs, widely known as being subclinically infected. The more subtle the effects of the subclinical viral infection are, if any, the more uncertain their association with the performance of the horse. Moreover, diagnostic methods to detect subclinical viral infections have been scarcely evaluated. In addition, the lack of a generally accepted definition/measurement of poor performance is lacking which prevents comparison between studies. Since poor athletic performance often occurs in the absence of obvious clinical disease, these low virulent viruses, about which we know little, have become under increased scrutiny during the last decades due to their possible association to poor athletic performance in the horse.

In the reminder of this introduction, the main equine respiratory viruses are presented, where equine rhinitis viruses and the gamma herpesviruses are examples of less known respiratory viruses, including the available evidence of their possible clinical significance and influence on poor performance, followed by a description of poor performance. The strengths and limitations of diagnostic tools we have available to diagnose these various equine respiratory viral infections, will be addressed with a final section on the relationship between subclinical respiratory viral infection and equine health and poor performance.

1.1 Equine influenza virus (EIV)

Equine Influenza Virus (EIV) is an influenza type A virus, a member of the *Orthomyxoviridae* family and is one of the most highly contagious and widespread viral respiratory diseases in horses and other equids. The clinical signs of equine influenza are characterized mainly by fever, nasal discharge and a harsh dry cough, and can predispose to secondary bacterial infections (Timoney, 1996).

Two different subtypes of EIV, H7N7 and H3N8, have been associated with disease in horses. H7N7 was first isolated in Prague in Eastern Europe in 1956 (Sovinova *et al.*, 1958), but has not been isolated from a horse since 1980 and is therefore now believed to be extinct (Webster, 1993). The H3N8 subtype of EIV was first isolated from horses in 1963 and diverged into the American and Eurasian sublineage (Daly *et al.*, 1996). However, viruses isolated in Europe have been shown to belong to the American lineage and vice versa. The American lineage has then diverged into Kentucky, South America (Argentina) and Florida (Lai *et al.*, 2001). The Florida sublineage has subsequently predominated and, based on HA sequencing, divided further into clade 1 and 2 (Bryant *et al.*, 2009). Since 2007, most EIV isolates in Sweden as well as in other European countries have been identified as Florida clade 2. In Sweden prior to 2007, vaccination was mandatory only for horses competing in equestrian sports, but not young horses nor racing trotters. However, following the outbreak of EIV in 2007 (unpublished data) mandatory vaccination in the Swedish trotting industry was imposed.

EIV vaccination was introduced in Europe and North America in the late 1960s (Cullinane *et al.*, 2010). The majority of the EIV vaccines used today are adjuvant inactivated or subunit vaccines, that induce antibodies against the haemagglutinin (HA) of the virus which can be measured and correlated with protection (Daly *et al.*, 2004; Newton *et al.*, 2000). Since dissemination of EIV to wider population of horses after race meetings or other events has been clearly documented (Cullinane & Newton, 2013), mandatory vaccination among racing and equestrian horses has played an important role in the control of EIV. However, due to the continuous antigenic evolution of EIV optimal protection requires that the available vaccines are continuously updated with the currently circulating strains (Elton & Cullinane, 2013; Gildea *et al.*, 2013).

1.2 Equine herpesviruses

Herpesviruses are large double stranded DNA-viruses that are characterized by their persistent lifelong latency (Roizmann *et al.*, 1992). The family *Herpesviridae* and are classified in three subfamilies: *Alpha*, *Beta* and *Gammaherpesvirinae*. Of the three subfamilies two are relevant for equine health.

1.2.1 Equine alpha herpesviruses (EHV-1 and EHV-4)

Equine herpesvirus (EHV) type 1 and 4 are closely related alpha herpesviruses included in the genus *Varicellovirus* with genome sizes of approximately 150 kbp for EHV-1 (Telford *et al.*, 1992) and 146 kbp for EHV-4 (Telford *et al.*, 1998). Despite the close relationship between EHV-1 and EHV-4, the existing genetic differences are sufficient to give rise to major biological divergences where separate affinity for replication sites and cells determine the clinical signs. EHV-1 is associated with respiratory disease, abortion in late gestation and neurologic disease, whereas EHV-4 mainly results in upper respiratory tract disease such as pyrexia, cough, nasal discharge and depression. But the infection may also pass asymptomatic in older horses (Allen *et al.*, 2004; Crabb & Studdert, 1995).

Clinical disease associated with both EHV-1&4 has been a problem for both the equine health and the equine industry for a long period of time. Already in early 1960 attempts was made to immunise mares and young horses against alpha herpesviruses to protect them against clinical disease with abortion and respiratory signs (Doll & Bryans, 1963b; Doll & Bryans, 1963a). Today vaccines are available on the market, providing some protection from the alpha herpesvirus EHV-1&4. However, since herpesviruses activate the immune response via both induction of antibodies (short-lived mucosal antibodies), and cytotoxic T cells and cytokines (interferons) on a cellular level (Kydd *et al.*, 2006), manufacturing of highly protective vaccine is challenging. Protection against clinical and virological disease requires both mucosal antibodies to reduce viral shedding and to combat free viral particles whereas the cytotoxic T cells are essential to lyse infected cells (Allen *et al.*, 1999). Despite extensive research over the last decades, no fully protective and long-term vaccine against the alpha herpesviruses is yet available, as a vaccine that can stimulate both humoral and cell-mediated immunity is required for optimal protection (Kydd *et al.*, 2012) which provides a challenge.

1.2.2 Equine gamma herpesviruses (EHV-2 and EHV-5)

Equine herpesvirus (EHV) type 2 and 5 are gamma herpesviruses that belong to the genus *Percavirus*, with genome sizes of 184 kbp (Telford et al., 1993)

and 182 kbp (Wilkie et al., 2015) respectively. EHV-2 and EHV-5 are closely related viruses and prior to 1987 EHV-5 was indistinguishable from EHV-2. However, studies identifying divergent profiles of restriction fragment length polymorphism facilitated differentiation of EHV-5 from EHV-2 (Browning & Studdert, 1987). Moreover further sequencing resulted in reclassification of EHV-2 and EHV-5 from beta herpesviruses to the family of slower growing gamma herpesviruses (Telford et al., 1993). Both EHV-2 and EHV-5 have been detected in the Icelandic horse population, where the horses have been isolated for more than 1000 years (Torfason et al., 2008), suggesting that the equine gamma herpesviruses are not a recently appearing microbial agents in horses. The clinical significance of infection with equine gamma herpesviruses remains unresolved, mainly because of its worldwide distribution and the high prevalence of infection in different horse populations but with only scattered reports of possible clinical problems related to these infections.

EHV-2 and EHV-5 share many similarities with the human gamma herpesvirus Epstein-Barr (EBV) in both their biology and their genetic characteristics (Brault et al., 2011; Bell et al., 2006). The average level of amino acid sequence similarity between the two viruses and EBV has been reported to be 44% (EHV-5) to 46% (EHV-2) (Agius & Studdert, 1994). EBV commonly infects humans where >90% of the adult population in the USA are persistently infected with EBV whereas the antibody prevalence in children is around 50% (Balfour et al., 2013b). EBV is linked to subclinical infection but as well associated with mononucleosis (Balfour et al., 2013a), and, like EHV-5 it has also been associated with pulmonary fibrosis (Vannella & Moore, 2008; Mora et al., 2005). Moreover, diseases such as multiple sclerosis (Mechelli et al., 2015) and different types of cancers such as Hodgkin´s lymphoma, Burkitt´s lymphoma and nasopharyngeal carcinoma (Parkin, 2006) have been associated with infection of EBV. Diversity at the whole genome level has been identified in EBV, suggesting that genetic variations may be a possible explanation for the differences in its pathogenicity (Kwok et al., 2014). However, specifically pathogenic strains or genotypes have yet to be identified.

Gamma herpesviruses infect the horses early in life. By the age of two to four months the majority of foals are infected with EHV-2 (Dunowska et al., 2011; Bell et al., 2006; Murray et al., 1996), whereas infection of EHV-5 appears to occur some months later in life (Bell et al., 2006; Dunowska et al., 2002a; Nordengrahn et al., 2002).

Both EHV-2 and EHV-5 have commonly been observed in clinical healthy foals and adult horses (Rushton *et al.*, 2013; Torfason *et al.*, 2008; Bell *et al.*, 2006; Borchers *et al.*, 2006; Ingram *et al.*, 1978; Kemeny & Pearson, 1970). A

higher prevalence of EHV-5 than of EHV-2 has been reported (Hue et al., 2014; McBrearty et al., 2013; Pusterla et al., 2013; Wang et al., 2007).

In contrast to earlier reports of non-pathogenicity, EHV-2 has been isolated in tracheal aspirates, PBMC or NS from foals with clinical respiratory disease more frequently compared to clinical healthy foals (Wang et al., 2007; Murray et al., 1996). EHV-2 has also been suggested to have the ability to induce pharyngitis (Blakeslee et al., 1975) as well as to predispose foals for Rhodococcus equi pneumonia (Nordengrahn et al., 1996).

In adult horses, EHV-2 has been attributed to clinical respiratory disease (Borchers et al., 1997), granulomatous dermatitis (Sledge et al., 2006) and keratoconjunctivitis (Rushton et al., 2015; Kershaw et al., 2001) although an another study in Austria failed to find any association between detection of EHV-2 in conjunctival swabs and ophthalmic findings (Rushton et al., 2013). EHV-2 has also been reported to be associated with lower airway inflammation and consequently poor athletic performance (Fortier et al., 2013).

In contrast to previous assumptions of non to low pathogenicity of EHV-5 (Bell et al., 2006; Dunowska et al., 2002a), selected case reports have recently linked EHV-5 with both dermatitis diagnosed with PCR assay performed on skin biopsies (Herder et al., 2012) and lymphoma (Vander Werf & Davis, 2013). Importantly, EHV-5 has also increasingly been associated with the severe lung disease equine multinodular pulmonary fibrosis (EMPF) in adult horses (Spelta et al., 2013; Poth et al., 2009; Williams et al., 2007). Despite this association, the role of EHV-5 in the pathogenesis of EMPF remains to be determined, in particular since EHV-5 has been identified in 40-76% of the NS obtained from healthy horses (Hue et al., 2014; Marenzoni et al., 2010). It remains to be established whether chronic infections of EHV-5 at some stages at certain conditions can cause lung fibrosis, or if the disease is rather connected to particular point mutations or genotypes. Of note, one horse diagnosed with EMPF was identified with a higher load of EHV-5 in the lungs, especially within the fibrotic nodules, compared to tissues such as the spleen and kidneys (Marenzoni et al., 2011). Those findings have however not been verified by comparing the viral load in the lungs and other tissues from healthy horses shedding EHV-5. Experimental induction of EMPF has been attempted where EHV-5 from two EMPF cases was isolated and thereafter inoculated into the lungs of six horses (Williams et al., 2013). While it was difficult to detect EHV-5 the inoculated horses, lesions that shared similarities with EMPF were subsequently identified in three of the cases at post mortem examination performed on 98-108 days after inoculation.

As both viruses are latent and subject to periodic reactivation, the actual site/s of latency in the horse may be of importance in directing appropriate

sampling and for understanding possible triggers for recrudescence. It has not yet been established where the latency for EHV-5 occur, whereas EHV-2 is latent in B-lymphocytes and macrophages (Drummer et al., 1996; Dutta & Campbell, 1978), although the Langerhans cells have also been suggested as a possible site for latency of EHV-2 (Borchers et al., 2006).

Due to the frequent detection of equine gamma herpesviruses in what appears to be clinically normal animals, the question arises whether actual viral load rather than solely presence of the virus may play a role in inducing clinical alterations. At least in foals undergoing episodes of respiratory disease, no correlation was found with the viral load and the occurrence of respiratory disease. (Brault et al., 2011; Bell et al., 2006). Another possible factor related to pathogenicity is genetic diversity of viral strains, which has been reported to occur in both EHV-2 and EHV-5 (Thorsteinsdottir et al., 2013; Brault et al., 2011; Sharp et al., 2007; Bell et al., 2006). Those studies where the clinical aspects were compared to selected genetic variations of the viruses, were unable to identify any association with the genetic diversity. However, those studies mainly used older methods for sequencing, which compared to more recent next generation sequencing, have limited possibilities to detect new strains/genotypes and multiple infections. The large genetic background of EHV-2&5 also presents a challenge in detecting possible genotypes that might be associated with disease.

Despite some recent reports questioning the earlier presumed non-pathogenic nature of the equine gamma herpesviruses (Fortier et al., 2013; Williams et al., 2007) the role of these viruses in causing or predisposing to clinical abnormalities or to poor athletic performance remains unclear. Hence, more detailed knowledge regarding the biology of the viruses and their interaction with their host is needed to investigate the role of the equine gamma herpesviruses in the healthy well performing horse, as well as in horses with clinical disease and/or poor performance

1.3 Equine rhinitis viruses (ERAV and ERBV)

Picornaviridae is a large and diversified virus family of small single-stranded RNA-viruses with a genome size of about 7.2-8.8 kbp and approximately 30 nm in diameter. The family includes both human pathogens such as poliovirus, hepatitis A virus, rhinovirus and animal pathogens such as foot and mouth disease virus and rhinitis viruses.

The human rhinoviruses are significant respiratory pathogens known to cause the common cold and are divided into three strains, which in total include more than 150 serotypes (Tapparel *et al.*, 2013).

The equine rhinitis viruses were first isolated in Beckenham, England (Plummer, 1962) and were previously known as equine rhinoviruses 1-3 within the genus *Rhinovirus* (Steck *et al.*, 1978; Ditchfield & Macpherson, 1965). However, genetic studies identified considerable phylogenetic differences between the equine rhinoviruses and the other members within the genus of *Rhinovirus* (Li *et al.*, 1996; Wutz *et al.*, 1996). Equine rhinovirus 1 was found to be more closely related to foot and mouth disease and therefore renamed to equine rhinitis A (ERAV) virus and reclassified to the genus *Aphtovirus*, whereas equine rhinovirus 2 was renamed to equine rhinitis B virus (ERBV) and with its three serotypes, reclassified as the so far single member of the genus *Erbovirus* (Black *et al.*, 2005; King *et al.*, 2000). ERAV exists only as one serotype and has been reported with a high genetic stability, since it has been shown that the genome of the currently circulating ERAV strains are highly similar to the original ERAV isolate that was initially described by Plummer in 1962 (Diaz-Mendez *et al.*, 2013).

ERAV can be isolated from the blood several days post infection and from the nasopharynx and in faeces for up to a month (Plummer & Kerry, 1962). Moreover ERAV has a unique feature among the picornaviruses, since it can be shed via urine for a long period of time, up to 147 days in naturally infected horses (McCollum & Timoney, 1992) and for at least 37 days in urine from experimentally infected horses (Lynch *et al.*, 2013). Studies in the USA, Ireland and Australia detected ERAV in post-race urine samples at a frequency of 17%, 29% and 23% respectively, suggesting that the persistent presence of ERAV in urine may contribute to its maintenance in training yards (TY) (Lynch *et al.*, 2013; Quinlivan *et al.*, 2010; McCollum & Timoney, 1992).

ERAV is spread by direct or indirect contact of secretions from the upper respiratory tract or via aerosol inhalation (Burrows, 1970). The shedding via urine might also be a possibilities for the virus to spread (McCollum & Timoney, 1992). In an early study, horses seronegative to ERAV showed clinical signs such as fever and nasal discharge 4-5 days after experimental infection, whereas horses identified with antibody titres developed no signs of respiratory disease after inoculation (Plummer & Kerry, 1962). However, the clinical signs associated with natural infection of ERAV are ranging from mild or subclinical appearance (Hofer *et al.*, 1978) to fever, coughing, anorexia and nasal discharge (Carman *et al.*, 1997; Studdert & Gleeson, 1978) and were the likely cause of at least two Australian outbreaks of respiratory disease (Li *et al.*, 1997).

The clinical manifestation of ERBV is less defined than for ERAV. The spread is thought to occur by direct or indirect contact with nasal secretions and aerosols (Horsington *et al.*, 2013b). Also for ERBV subclinical infection as

well as signs associated with upper respiratory tract disease such as fever, cough and nasal discharge has been reported (Diaz-Mendez *et al.*, 2010; Carman *et al.*, 1997; Burrows & Goodridge, 1978; Steck *et al.*, 1978). Seroconversion of ERAV has been reported to occur among young horses on entry to TYs, either without detected clinical signs (Black *et al.*, 2007; Powell *et al.*, 1974) or with minor respiratory disease (Klaey *et al.*, 1998). The same association has however not been established for ERBV (Black *et al.*, 2007).

ERAV has been demonstrated as being a zoonotic infection i.e. capable to infect humans (Plummer, 1962) and there are also indications that ERBV has this capacity. However, despite working in an area with a large majority of the horses identified as seropositive to ERAV and/or ERBV (Kriegshauser *et al.*, 2005), only limited numbers of veterinarians have been identified as seropositive to ERAV and/or ERBV. The high seroprevalence of neutralizing antibodies to both ERAV (37-90%) and ERBV (71-86%) in horses foremost without clinical disease is described worldwide, which indicate that the viruses are endemic in horse population (Horsington *et al.*, 2013a; Black *et al.*, 2007; Dunowska *et al.*, 2002; Klaey *et al.*, 1998; McCollum & Timoney, 1992; de Boer *et al.*, 1979). It also appears that high levels of neutralizing antibodies to ERAV can persist in horses for years (Burrows, 1970) which might play a role in protecting horses from developing clinical signs at reinfection. These observations are supported by a Canadian study where ponies experimentally infected with ERAV developed clinical respiratory signs and one year later, remained seropositive from the initial infection challenge and failed to develop clinical signs with repeated experimental infection (Diaz-Mendez *et al.*, 2014).

In contrast to the high levels of neutralizing antibodies induced by natural infections of equine rhinitis viruses, the antibody levels induced by vaccines have been disappointingly low. So far, no commercial vaccines are available against the equine rhinitis viruses. Studies have been performed where both virion proteins of ERAV (Warner *et al.*, 2001) and plasmid-based DNA vaccines (Lynch *et al.*, 2011) have been investigated in order to reach higher antibody titres after vaccination. A patent application for an equine rhinitis virus vaccine was recently published (Diaz-Mendez *et al.*), and it may be that a vaccine will be available in the foreseeable future.

Based on these studies it is likely that the majority of infections by ERAV and ERBV are mild or subclinical. As a result it has been suspected that the equine rhinitis viruses could have an impact on the athletic performance of the young horse due to prolonged recovery as well as posing a risk for secondary bacterial infection. Despite the controversies regarding the role of subclinical rhinitis virus infection in the poor performance syndrome, no reports have actually described such infection in association with poor athletic performance.

1.4 Poor athletic performance

When determining the performance of the athletic horse, numerous methods have been described, but there is still an ongoing debate upon how to best measure or detect poor performance. For example the athletic performance of trotters has been evaluated by using an annual index calculated by the French national stud, based on the natural logarithm of average earnings per race (Leleu *et al.*, 2005). Others have used the opinion of the rider/trainer (Fraipont *et al.*, 2011; Widmer *et al.*, 2009) as an indicator for the level of the athletic performance whereas field exercise tests (van Erck *et al.*, 2006; Courouce *et al.*, 1997) or exercise tests on treadmill (Morris & Seeherman, 1991) have also been widely used to further evaluate the performance of athletic horse. The treadmill has the advantage to highly standardize the workload and allow advanced physiologic monitoring but is not feasible to use for most larger studies or in the field at TY, whereas the field exercise test has the benefit of being performed at the TY where the horse normally work and is a closer reflection of the work undertaken in actual race conditions.

Clearly, all ways of measuring the athletic performance have their advantages and limitations, but since there is no ideal method to truly measure the athletic performance of the horse, it remains highly challenging to define the presence and degree of poor performance and to determine which specific factor or combination of factors impair the performance in a particular individual. Poor athletic performance in horses is multifactorial i.e. several factors contribute to the risk of developing the condition of disease (Fraipont *et al.*, 2011; Martin *et al.*, 2000; Morris & Seeherman, 1991). Certain conditions such as lameness and respiratory disease has been reported as the most common medical reasons for poor performance and training disruption among Thoroughbred racehorses and Standardbred trotters (van Erck *et al.*, 2006; Wilsher *et al.*, 2006). Apart from virus infection and viral load, there are however other components in individual horses that are even more difficult to measure but may affect their full capacity for athletic performance, examples are the physical and psychological capacity and genetic makeup.

Important factors are for example management factors such as the actual physical fitness of the horse, whether the training program is optimized for that individual, if the racing schedules is appropriately timed to allow adequate recovery periods, and supply of feed suited to meet the needs for elite athletic work. The athletic performance can also be influenced on what level of competition the horse is participating and thereby earnings per race. In addition, it is important to acknowledge that not all horses have the biological and mental capacity for top athletic performance, and thus may fail to meet owners´ and trainers´ expectations. The important viewpoint is to gain

knowledge on relevant factors for performance and health to ensure that the talented individuals are not hindered by manageable external factors.

1.5 Diagnostics of respiratory viruses

When subclinical viral activity is being considered as a possible cause for poor athletic performance in a horse there are a number of diagnostic methods that are available. Because the respiratory pathogens are commonly associated with highly similar respiratory signs, diagnosis cannot be made on clinical basis and reliable laboratory diagnostic tools are necessary to specifically identify them. Most of the direct and indirect methods such as PCR assay, virus isolation and serology, are highly viral specific. However, to determine the actual effect of a virus in a host, i.e. the pathogenicity, the laboratory results are needed to be combined with the presented clinical signs. Blood examination identifying and quantifying the concentration of inflammatory markers and endoscopic visualization of upper respiratory tract can provide some measure of the degree of clinical disease in the horse, even if the findings are not specific to viral or other infectious agents. Accurate diagnosis of viral infections is important in order to allow adequate prognosis and to support the clinical management. To appropriate interpret the diagnostic results, it is crucial to be aware of the advantages and the disadvantages of the methods currently used in most clinics or laboratories.

1.5.1 Indirect techniques

Serology

Serology means testing serum or plasma to identify presence of antibodies or antigens which are produced as a response to infection and thus, detection precious or ongoing viral infections. A wide range of methods has been developed used over the years, for example virus neutralization test (VN), complement fixation test (CF) and the enzyme-linked immunosorbent assay (ELISA).

The VN test identifies evidence of infection by detecting mainly IgG antibodies. Serial dilutions of sera are tested to determine if sufficient levels of antibodies protecting cells from infection with added virus are present (a common assay for ERAV or ERBV). This method is highly specific and measure the protective effect of the antibodies, but is more expensive and time consuming than for example the ELISA and require maintained cell cultures in the laboratory.

In the CF test, serial dilutions of sera are used to identify antibodies, mainly IgM, by catching them in a complex together with antigen and complement,

thus used to detect presence of antibodies in serum. It is a common diagnostic tool for identifying immunological evidence of EHV-1 or EHV-4 infection. Since the main target is the IgM antibodies, which decay within 60 days (Hartley *et al.*, 2005; Allen *et al.*, 2004), the assay is established to detect more recent infections. However, one disadvantage with the CF test is that they are often non-specific and therefore the CF test is not able to differentiate for example between EHV-1 and EHV-4 since the polyclonal antibodies of EHV-1/-4 are highly cross-reactive.

The ELISA method was initially described in 1971 (Engvall & Perlmann, 1971) and is a highly sensitive method to detect antibodies or antigens. Today several variations of ELISA are available to detect either antibodies or antigen to a specific pathogen in a sample. This is performed by attach a specific antigen to a solid surface (96-well microliter plate), with which antibodies from an added sample can form a complex if the sample contains antibodies matching the antigen. The complex is detected by an added enzyme that binds to the complex. To distinguish IgG antibodies elicited by EHV-1 from those of EHV-4 obtained in CF test, a type specific commercially available ELISA has been developed (Crabb *et al.*, 1995). However, as single dilutions of sera are often used in the ELISA, differences in antibody concentrations between samples are not possible to detect. In addition, since the EHV-1/EHV-4 assay targets the IgG, caution must be taken regarding time of infection as this antibodies can remain at detectable levels long time after initial infection (Hartley *et al.*, 2005).

When using VN and CF tests, the titres can differ between laboratories. This can due to the susceptibility of various cell types used in VN assays and divergent viral strains utilized in CF and VN tests used in different laboratories, which may influence the levels of antibody titres (Hartley *et al.*, 2005), and therefore cut-off values can vary between laboratories.

Serological methods have the advantage of being inexpensive and easy to perform. However, interpretation of results can be challenging. Knowledge about the duration of the production of antibodies as a response to the specific virus infection is essential for the interpretation of a positive or negative test result. Generally, at least fourfold (two dilutions) or greater increase of antibody titres between two sample occasions are required to confirm seroconversion or significance with acute infection. Most of the serological tests are not enable to distinguish between antibodies relating to vaccination or natural infection.

1.5.2 Direct techniques

Virus isolation

The classical detection assay for viruses which has been used for over 60 years (Storch, 2000) is virus isolation in a cell culture or in embryonated eggs, where clinical specimens are inoculated into living cells. Different cell culture types are required for different viruses, which might be cumbersome for the laboratory. The virus is then identified by its cytopathic effect (CPE), a step which requires highly experienced laboratory personnel. Isolation of the agent in cell culture is the only diagnostic tool that has the advantage of only identifying isolates of viable virus, and is best performed on samples from acute diseased animals. Virus isolation is a time consuming procedure where the required time for detectable CPE varies from 3-6 days for equine alpha herpesviruses (Studdert *et al.*, 1970) to >3 weeks for slow growing viruses such as the equine gamma herpesviruses (Turner & Studdert, 1970). Another disadvantage is that contamination of bacteria or fungus can occur and thereby disturb the growth and identification of the virus.

Polymerase chain reaction (PCR)

PCR is a technique for detecting presence of specific viral RNA or DNA in a sample, providing a reliable and rapid diagnostic tool that is highly specific and sensitive. Since its inception in 1985, the advancement of the PCR technique has been tremendous and several types of PCR assays are available. The real time PCR commonly used currently in laboratory diagnostics has clear advantages, foremost its´ high specificity and sensitivity. Moreover, the PCR has a larger time frame of detection compared to virus isolation with no need of the virus to be alive when it reaches the laboratory. The PCR assay has also the benefit of taking less than one day to complete, can allow many samples to be analysed simultaneously and that the probes used can increase the specificity even further. However, due to its ability to amplify very small amounts of nucleic acid, contamination can be a problem (Mifflin, 2007). Another concern can be the presence of inhibitors, for example haemoglobin in blood samples, that can reduce or even block the amplification of the nucleic acid by capturing nucleic acid, inactivate DNA polymerase or disturb the composition of ion in the reaction (Radstrom *et al.*, 2004).

1.5.3 Molecular characterisation

Sequencing

The Sanger sequencing, first described in 1977 (Sanger *et al.*, 1977) has been the main DNA sequencing method used for the following 30 years. The

introduction of next generation sequencing (NGS) has however revolutionized the opportunities to study viral diversity and evolution and was selected as the method of the year by *Nature methods* in 2007 (Method of the Year, 2008; Schuster, 2008). The several million reads generated in each NGS run gives outstanding genetic information of each sequence and provides the possibility to detect even rare genetic variants. The development in the field of DNA sequencing has been rapid which has led to a reduction in cost per base (Pettersson *et al.*, 2009) thus increasing the likelihood of using this technique. However, the NGS is still not implemented in routine diagnostic and is mainly used for research purposes, to identify and follow strains to e.g. trace disease as well as providing possibilities to better understand the pathogenicity of microbial agents.

1.5.4 Inflammatory markers

The acute phase proteins are synthesized in the liver during different inflammatory conditions or infectious diseases (Baumann & Gauldie, 1994). In equine medicine concentrations of the proteins fibrinogen and serum amyloid A (SAA) in serum are the inflammatory markers commonly used to detect nonspecific response to inflammatory or infection.

Concentrations of SAA (a major acute phase protein) have been shown to rapidly increase due to inflammatory stimulus, being elevated in less than 48 hours in horses infected with EIV (Hulten *et al.*, 1999). Because of its short half-life time in serum, reported in laboratory mice to vary between 30 min to 2 h (KluveBeckerman *et al.*, 1997; Tape & Kisilevsky, 1990; Hoffman & Benditt, 1983), the serum concentrations of SAA decrease rapidly after resolution of disease and have been suggested to well reflect recovery or the response to therapy (Hulten & Demmers, 2002). Moreover, compared to the traditional markers (WBC count or plasma fibrinogen) SAA has been found to be a more reliable marker in horses to monitor inflammation as well as being a prognostic indicator (Belgrave *et al.*, 2013).

Fibrinogen (a minor acute phase protein) is slower reacting and peaks within 1-2 weeks after stimulus and decrease slowly during recovery (Jacobsen *et al.*, 2005; Hulten *et al.*, 2002). This makes this biomarker less useful for detection and monitoring inflammation than SAA.

Due to the nonspecific reaction, measurements of acute phase proteins can be used as a convenient complimentary tool in the clinical work. However, the analysis itself can never diagnose a specific microbial infection, let alone viral infection.

1.5.5 Endoscopy

Endoscopy is a useful tool to visualize the respiratory tract of the horse. It can be used to determine the degree of pharyngeal inflammation (Raker & Boles, 1978), accumulation of mucus in trachea (Gerber *et al.*, 2004), as well as grading the degree of pulmonary exercise induced hemorrhage (Hinchcliff *et al.*, 2005). Endoscopy is also the gold standard in detection of functional upper airway disorders, which can be visualized both at rest or in exercise (McCarrel & Woodie, 2015). The bronchoalveolar lavage (BAL) and tracheal wash (TW) are sampling methods widely used both in research and in clinical practice, which provides samples from the lower respiratory tract that can be used for cytology as well as for detection of microbial pathogens.

Despite the wide range of usefulness for the endoscopy, it cannot be used alone to diagnose a specific viral infection.

1.6 Subclinical viral infections and the equine health/athletic performance

In sport horses, viruses such as EIV, EHV-1&4 are widely known to induce clinical signs and that they can affect athletic performance in horses is logical. However, if clinical signs are only mild and thereby less clear, attention is also directed towards less known respiratory viruses such as gamma herpesviruses and rhinitis viruses, which are suggested to have lower pathogenicity, or act with mainly subclinical symptoms. Since these viruses have been presumed to be low or non-pathogenic, far less research has been performed to clarify their biology and interaction with their host. However, whether these viruses truly are as innocuous to the horse as previously thought is currently more rigorously questioned by the scientific community. Key questions that arise are whether these less known viruses are associated with mild clinical disease in elite actively racing horses and if they somehow then may impair athletic performance when the infection is active.

2 Aims of the thesis

The main aim for this thesis was to investigate the role of subclinical respiratory viral infections in actively racing Standardbred trotters. The specific objectives were:

- In study I, to describe the first identified case of EMPF associated with both EHV-5 and AHV-5 in a Swedish four-year-old horse.
- In study II, to investigate activity of common respiratory viruses over time in a cohort of Standardbred trotters, using available diagnostic test including both serology and PCR assays to evaluate whether subclinical viral infections were associated with alterations in the equine health and/or athletic performance.
- In study III, to identify the prevalence and the viral load of EHV-2 and EHV-5 in NS in a cohort of Standardbred trotters and thereby evaluate whether infections as well as the obtained viral load, were associated with alterations in equine health and/or athletic performance.
- In study IV, to investigate genetic variability in the gB gene of EHV-5 in samples from healthy horses over time and also to compare genetic results to sequences from samples from a horse diagnosed with EMPF.

3 Materials and Methods

This section provides a brief description of the material and methods used in the studies of this thesis work. More detailed information is provided within each individual paper.

3.1 Animals

The four year-old Standardbred trotter described in study I, was a clinical case referred to the Equine Hospital at the Swedish University of Agricultural Sciences in Uppsala, with a 10-day history of fever, unwillingness to move, tachypnea and mild cough.

The horses participating in studies II-IV were actively racing Standardbred trotters from four different professional training yards (TYs) in the area of Mälardalen, Sweden. All horses were training and racing according to their regular schedule during the study period and were at the time of recruitment healthy and well performing with a mean age of 3 years, (range 2-8 y, SD 1.33). The horses underwent clinical examination at each sampling occasion (performed by a single veterinarian throughout the study), where signs of respiratory disease, such as fever (>38.3^{o}C), nasal discharge and cough were recorded. The health status was also monitored on a weekly basis throughout the study period.

In study IV, the selected NS were from eight horses with a high viral load of EHV-5 identified at two sampling occasions one year apart in study III. They were all healthy and well performing at both sampling occasions. In addition, the pre-mortem (lung biopsy) and post-mortem samples (lung) from the horse with EMPF reported in study I were included.

3.2 Study design

Study I was a case report describing the first Swedish case of EMPF and the first case where both EHV-5 and AHV-5 were associated with the disease.

The study that constitutes the basis for papers II and III was designed as a prospective longitudinal study where 66 horses were followed on monthly basis from August 2010-August 2011 (13 months).

In study IV, samples from the horse described in study I and samples from eight horses participating in the longitudinal study in paper II and III were selected for further genetic analysis of EHV-5.

3.3 Sampling

Nasal swabs (NS) and serum samples were obtained from all horses at monthly intervals. In the event of clinical respiratory signs or poor performance in the interim, additional samples were also collected. The samples were transported by car at ambient temperature to the laboratory at the National Veterinary Institute (SVA) in Uppsala, where they were stored at 4ºC for 1-3 days before analysis by multiplex PCR assay on NS and serology on serum samples. The NS were then stored at -70ºC until the analysis in study III and IV were performed. The serum samples were stored at -20ºC and the analysis of SAA was performed within 1-3 weeks of sampling.

3.4 Classifications of athletic performance

The individual athletic performance of the horses was classified using both objective and subjective methods, which were registered on monthly basis at the same time as the NS and serum samples were taken.

3.4.1 Objective method

To objectively classify the performance in each individual horse, they performed a standardized field exercise test (SFE) at each sampling occasion. The SFE was performed at the TY where the horses ran a predetermined distance at a heart rate >210 beats per minute and the running times were then compared individually. At each SFE the horses were equipped with a heart rate (HR) monitor (Polar Equine CS600X, Polar Electro Sverige AB, Bromma, Sweden), to enable the driver to encourage the horse to work at a speed that induced a heart rate of 210 beats per minute (bpm). Poor performance was based on each individual horse´s running data and was defined as a higher running time than the mean of all running times+1 standard deviation (SD) for each horse. By using a crossover design (individuals serving as their own

control) when objectively measuring the performance, each horse was compared to itself which increases the potential to detect true poor performance episodes.

Figure 1. Standardized workload performed by Standardbred trotters in one training yard included in study II and III of this thesis. (Photo: Bengt Ekberg).

3.4.2 Subjective method

The opinion of the trainer was registered after each SFE or training session connected with the sampling occasions. When a trainer deemed that the athletic performance in the horse was below what they expected, it was classified as poor performance. This method relies on the trainers´ professionalism and experience to classify the performance in a qualitative (normal versus poor performance) way.

3.5 Diagnostic methods

To detect presence and degree of infection of respiratory viruses in the horses, several diagnostic methods and tools were used. Different polymerase chain reactions (PCR) were performed on NS to identify presence of viral DNA and serological methods were used to identify antibodies and to investigate levels of a marker of systemic inflammation. Furthermore next generation sequencing was utilized to investigate possible genetic variation of the glycoprotein (gB) gene of EHV-5.

3.5.1 Nested consensus PCR

The presence of herpesvirus in lung tissue and tracheal wash (TW) from the horse with multinodular pulmonary fibrosis in study I, was identified using nested consensus PCR assay. This assay was used for detection of a wide range of herpesviruses in the sample, since its target region was a segment of the DNA polymerase gene that many herpesviruses have in common. This was followed by second round amplification and sequencing in order to determine which herpesvirus was present in the sample (Ehlers *et al.*, 1999; VanDevanter *et al.*, 1996).

Phylogenetic analysis was performed using the neighbour-joining and the Kimura-two-parameter model as implemented in SplitTree4 (Huson & Bryant, 2006). By that, it was then possible to investigate the relationship between the products of EHV-5 and AHV-5 obtained from sequencing and related equine herpesviruses.

3.5.2 Multiplex real-time PCR

To identify the common respiratory viruses in NS from horses in study II, a multiplex real-time PCR assay was used, in which primers targeting the viruses EIV, EHV-1, EHV-4, ERBV and EAV were included. Extraction of RNA/DNA was performed using a Magnatrix 8000+ robot and the extraction kit NorDiag Vet Viral NA (NorDiag AB, Hägersten, Sweden) according to the manufacturer´s instructions. Thereafter TaqMan PCR using the primers and probes described in study II was performed. Amplicons detected above threshold at a quantification cycle (Cq) <40 were considered as positive results.

3.5.3 Quantitative PCR (qPCR)

Since no qPCR assay for EHV-2 and EHV-5 were available at the time when study III was initiated, we developed and validated two new qPCR assays targeting the conserved region of the DNA polymerase gene of each virus. Viral DNA was extracted from NS using a Magnatrix 8000+ robot and the extraction kit NorDiag Vet Viral NA (NorDiag AB, Hägersten, Sweden) according to the manufacturer´s instructions. Applied Biosystems 7500 Fast Real-Time PCR System (Life Technologies, Saint-Aubin, France) were used to amplify the selected regions of the nucleic acid, using the primers and the UPL-probes as are further described in study III. Cq<40 was considered as positive. The viral load within each sample, was calculated by linear regression using the ten-fold serial dilutions of known concentration of DNA copies from the validation of the qPCR assay and the given Cq values. DNA copies detected above the threshold at a quantification cycle (Cq) <40 were considered as a positive result.

The analysis of EHV-2 and EHV-5 were run in two separate assays in order to avoid the risk of failure to identify each of them if co-infection occurred. Moreover, the conserved DNA polymerase gene was used as the target gene to increase the possibility of the assay to detect the virus even if different strains were present.

3.5.4 Next generation sequencing (NGS)

In study IV a region of the EHV-5 glycoprotein B (gB) in which high variability flanked with stable nucleotides could be identified was selected to design a new PCR assay, which were followed by NGS. By using this method of "deeper" sequencing, genetic variations in that particular segment of EHV-5 would be detectable and thereby enable unmasking of any multiple infections by several strains. The amplicon PCR and library preparation was performed according to the Illumina protocol "16S Metagenomic Sequencing Library Preparation" with smaller modifications and thereafter sequenced (NGS) using MiSeq Reagent Kit v.3 600 cycles on the MiSeq instrument (Illumina) and the "Generate FASTQ" workflow. The high quality reads obtained from the NGS were trimmed, merged, and phylogenetic analysis were performed in the software program Trimmomatic, COPE 1.1.2, MUSCLE and MEGA, which are described in more detail in the materials and methods of paper IV.

The different clinical specimens included in study IV were coded with a unique letter for each horse followed by number "1" (first sampling occasion) or "2" (second sampling occasion) and the last number (1-3), illustrate the most (1) and less (3) common strain within each of the clinical specimens.

3.5.5 Complement fixation (CF) test

The CF test was used to investigate presence of mainly IgM antibodies to EHV-1 and EHV-4 in serum samples (Kriegshäuser *et al.*, 2005). The CF test determines whether there are antibodies in the serum at different dilutions, capable of forming an immune complex together with antigen and thus becomes fixed by the complement (serum from guinea pig). A fixed immune complex is not available to lyse sensitised sheep erythrocytes, resulting in a button of erythrocytes in the well. The analysis require two working days.

3.5.6 Virus neutralization (VN) test

Antibodies to ERAV and ERBV was demonstrated by VN test (Thomson *et al.*, 1976), where serum at different dilutions were incubated with virus (ERAV strain 1722/Switzerland and ERBV 5/15/95/Newmarket) on RK-13 cells. After three days of incubation at 37±1ºC the cytopathogenic effect was determined by microscopic examination.

3.5.7 Immunoturbidometric assay

The level of SAA in serum was analysed at the department of clinical chemistry at SLU using the immunoturbidometric assay (Hillstrom *et al.*, 2010), where a level of SAA>20 mg/L was considered positive for systemic inflammation (Jacobsen & Andersen, 2007).

3.5.8 Endoscopy

In study III 28 horses at the same TY underwent endoscopic examination (Endoscope pks 60914, Karl Storz, Tyttlingen, Germany), before and after each SFE from April to August 2011 to estimate the visual degree of upper respiratory inflammation. The purpose of the examination after SFE was to assess the lower airways for presence of fresh blood that could suggest exercise induced pulmonary haemorrhage. Images from each examination of the pharyngeal region and from the trachea at the level one-meter deep from the nostrils were taken and stored electronically for subsequent grading. The endoscopic findings were graded at the same occasion by an experienced equine clinician blinded to clinical and viral status of the horses. Endoscopic scoring of mucus accumulation was performed using the scale of 0-5 as previously described (Koblinger *et al.*, 2011; Gerber *et al.*, 2004). Accumulation of mucus grade 0 was considered normal whereas all grades >0 was deemed as excess accumulation of mucus. The score of pharyngeal inflammation was marked at a scale of 0-4 (Raker & Boles, 1978), where grades 0 and 1 were considered normal.

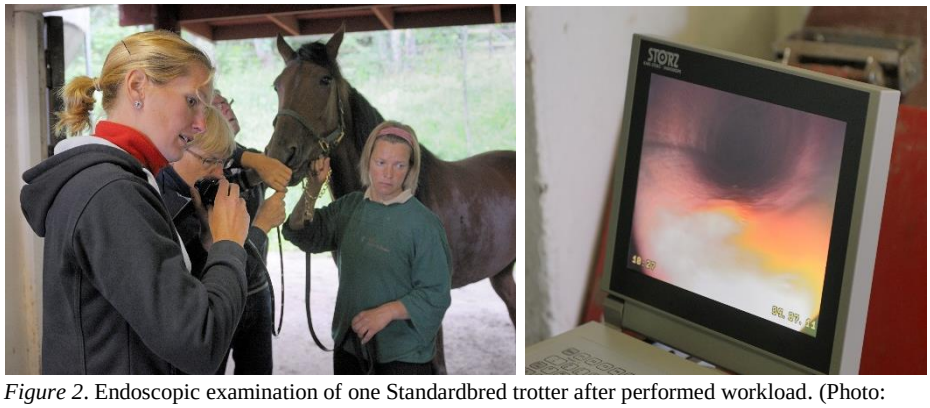


Figure 2. Endoscopic examination of one Standardbred trotter after performed workload. (Photo: Bengt Ekberg).

3.6 Statistical analysis

The statistical analyses, the graphs and the descriptive statistics were performed using the software program STATA, version 11.2 and 13.0 (StataCorp LP, Texas, USA). P-values <0.05 were considered as statistically significant.

Poor performance was classified in three ways and evaluated separately; 1) according to trainer, 2) according to deviation from normal running time or 3) either parameter classified as poor performance.

In study II associations between poor performance and viral respiratory infection were investigated by logistic regression analysis including only the outcome (assessed in three ways in separate analysis) and the different viral activity criteria evaluated in separate logistic models (one per each specific combination of exposure and outcome), generating crude odds ratios (ORs). As observations were not independent but clustered within horse (up to 13 sample occasions per horse) and TY (n=4), the standard errors from the unconditional analyses were likely underestimated hence producing overly narrow confidence intervals. Therefore, in a second step random effects logistic regression models (one per independent variable, i.e. virus infection parameter) were performed with random effects for horse and TY and adjusting for age and gender.

Due to the wide range in number of viral copies obtained from the NS of individual horses in study III, the viral load was transformed into natural logarithms (ln). The chi-squared test was used for comparing the numbers of qPCR positive samples per season, age groups and to compare the peak of viral load between EHV-2 and EHV-5 within horses. Random effects logistic regression models were used to investigate the association between the three outcomes poor performance (either subjective or objective method classified event as poor performance), clinical respiratory signs or level of SAA against the number of virus copies while adjusting for age and gender. Random effects for horse and trainer were included in the model to account for the repeated measurements within horse and clustering within trainer. Each outcome was investigated in a separate model. Random effects logistic regression model was also used for investigating associations between presence of either EHV-2 or EHV-5 and the upper respiratory inflammation grade, with random effects for horse and gender.

The maximum log likelihood estimation of the random effects logistic regression models used in both studies II and III was performed using adaptive Gaussian quadrate methods.

4 Results and discussion

In this cohort of 63 elite Standardbred trotters, 96 episodes of poor performance were identified over a year, whereas it appeared there was little activity of the common respiratory viruses.

All horses in the study were vaccinated against EIV twice a year, and thus it was not surprising that no cases of EIV were detected among them. There was minor evidence of seroconversion to EHV-1/-4, but not associated with clinical disease and with no connection to episodes of poor performance.

In contrast, a high seroprevalence of the lesser-known rhinitis viruses ERAV and ERBV in our cohort of horses suggested active and wide distribution of these viruses. However, based on observations of relatively few occasions of seroconversions, as defined by a fourfold rise in antibody titres, and limited number of PCR positive samples it was concluded that active infection by these viruses was uncommon among seropositive horses. No association of activity by either ERAV or ERBV could be associated with episodes of poor performance or clinical respiratory disease. Additionally both of the equine gamma herpesvirus EHV-2 and EHV-5 were frequently detected in most horses throughout the year, although no clinical signs or association with events of poor athletic performance were observed.

Overall, no evidence of an association between presence of low virulent viruses and poor performance was found in the studies included in this thesis.

As that the gamma herpesviruses were so commonly detected in the vast majority horses throughout the year, we examined the quantitative changes for these viruses in individuals to determine whether actual shed number of viral particles changed over time and seasons, and if so whether these changes could be related to clinical symptoms and/or impaired athletic performance. While there was a clear difference in pattern of shedding between EHV-2 and EHV-5, and a seasonal variation of viral load regardless of quantity shed, neither of

these viruses was associated with any of the clinical or performance problems observed in this group of horses.

Due to the high prevalence of EHV-5 in healthy horses coupled with its association with severe lung disease, genetic variations may explain the different clinical outcomes. By using NGS we examined a segment of an essential glycoprotein gene of EHV-5 from a subgroup of selected horses. This resulted in suggested classification of EHV-5 genotypes not previously described. We also showed stability in viral strains over time, however dynamics of the strains and genotypes were observed in individual horses over time. Since all but one of the horses in the subgroup were fully healthy and well performing, and the only horse with severe clinical disease did carry a strain of a genotype which was also found in clinically healthy horses, the question whether genetic variation have different ability to cause disease, could not be answered completely.

A major strength of these studies is that all horses were monitored with regular sampling, health checks and objective exercise testing in a longitudinal study over more than a year. On the other hand, one aspect that was a challenge in this work, similar to most research that investigates athletic performance in the horse, and as will be discussed later, is the lack of precision in measuring and quantifying actual episodes of poor athletic performance in individual horses.

4.1 Poor athletic performance

The classification of athletic performance is mainly described in study II, and 16% (96 of 584) of the events were associated with poor performance (subjective and objective classifications). The trainers classified poor performance in 52 of the events and in 45 cases the SFE showed a slower running time than normal for that horse which classified it as poor performance. The opinion of the trainer and the SFE agreed in only one horse. Since the results were obtained from high level TYs run by experienced professional trainers, including highly ranked horses based on prize money and racing results, larger numbers of poor performance would have been surprising. The opinion of the trainer was available at all sampling occasions, whereas the SFE was performed in only 327 events, due to external factors such as poor race track conditions (high amounts of ice and snow), whether the horses were racing the day after or the day before or if they were suffering from respiratory disease preventing them from training.

No association between the events of poor performance and subclinical viral activity could be identified, neither in study II nor in study III. However,

if the study had been performed in TYs with less successful and less well-managed horses, it is possible that more events of impaired performance would have occurred which in turn could have increased the probability to detect any possible associations. Another factor that limited the power of our work was the relatively low incidence of infections by EIV and EHV-1&4. To fully clarify the possible link, if any, of subclinical viral infections of these common viruses to poor performance, examinations of horses during a year that by chance had a higher incidence of actual viral infections would be required. Unfortunately, longitudinal studies focusing on actively racing horses are seldom performed mainly due to the difficulties in assessing these valuable and professionally kept animals. Nonetheless these results suggest that subclinical infections by well-known respiratory viruses are less common than expected and therefore their impact on performance, at least in well-managed horses, seems to be low.

Exercise tests can be performed either at treadmills or in the field (race tracks, TYs). One major advantage with treadmills is the possibility to accurately standardize the actual work. For example speed and angle of inclination can be fixed between tests, and the surface does not change with weather conditions. On the other hand the weight bearing effect of the sulky or the rider is absent (Gottlieb-Vedi & Lindholm, 1997). Moreover, the treadmill has also been suggested to have an impact on the locomotion of some horses (Barrey *et al.*, 1993). The field tests on the other hand have the advantage that they to a higher degree reflect the conditions encountered during races. However, parameters such as weather conditions and track surface can and will vary over a year in particular in countries such as Sweden with very different seasons which can prevent comparisons between tests performed over time.

In the studies included in this theses, it was not practically possible to use treadmills to assess the athletic performance. Therefore, we standardized the parameters for the SFE as best possible by using a set distance and a set heart rate (HR) to ensure blood lactate reached or exceeded the proposed anaerobic threshold of ≥4 mmol/l. (Courouce, 1999; Persson *et al.*, 1983). This normally correspond to a HR of 200 bpm. Furthermore, the SFE performed used a crossover design, so that the same individual was its own matched control at different time points (Maclure & Mittleman, 2000). The uncertainty of comparison of individual performance between horses can thereby be avoided, which is essential when the included horses are at different training stages and is an additional advantage with the longitudinal study design.

Regarding using the opinion of the trainer as a subjective method to classify athletic performance, a key disadvantage is that the method is highly subjective. On the other hand, horses are generally evaluated for problems of

poor performance based on the trainers' experience and this would support including and evaluating this parameter when causes of poor performance in race horses is investigated. Moreover, at sampling occasions when the SFE was not possible to perform (for example the day after a race), the opinion of the trainer could provide information about the performance of the horse. The question to the trainers was "did the horse perform as normal/as you expected", to ensure a dichotomous response based on the horse´s actual status, and not whether the horse performed as they hoped or wished it to do one day in the future.

Despite careful planning to accurately determine the athletic performance it remains a challenge. In study II and III it is possible that impaired performance of the horses occurred the week or two before or after the samples were taken and thereby was not registered. However, as most horses were examined on 13 distinct separate occasions over an entire year this work should have captured at least a portion of such events in close proximity to each other, and can thus not explain the lack of association between poor performance and subclinical respiratory viral infection.

4.2 SAA, a useful tool to detect poor performance and/or subclinical viral infection?

In the longitudinal study described in paper II and III, 10 serum samples were identified with elevated levels of SAA. In the 96 events of poor performance SAA was above the threshold in six of them. This low prevalence of elevated SAA in horses with poor performance reduced the usability of SAA as a marker for poor performance. In contrast to an earlier study on endurance horses that showed elevations of mean values of SAA in poor and intermediately performing trotters (Fraipont *et al.*, 2011), our Standardbred trotters did not on most occasions of poor performance present elevated SAA. The differences between workload of long distance endurance and the far shorter and more intensive work of the trotter race type may have an influence on our contrasting findings. However, increased levels of SAA have also been identified in endurance horses after successfully have fulfilled races at various distances (Cywinska *et al.*, 2012), suggesting a limited diagnostic value of SAA also after endurance races. In this study, levels of SAA were not elevated in horses without clinical signs shedding EHV-4/ERBV or had high antibody titers for ERAV/ERBV. Furthermore, SAA alterations could not be associated with either presence or high viral load of EHV-2 and EHV-5 in nasal secretions.

Since SAA is a non-specific marker of inflammation, elevated levels could be due to reasons not associated with infectious diseases. In study II and III, high levels of SAA were detected in horses that had wounds, recent castration, fever or fractures. This is in line with previous work where elevated levels of SAA have been identified at various diseases or conditions, including clinically apparent EIV infection (Hulten *et al.*, 1999), tissue damage (Jacobsen *et al.*, 2005) and post-surgery (Jacobsen *et al.*, 2009; Pollock *et al.*, 2005).

At the outset of this work we were interested to assess whether SAA could be used as a tool for a trainer to reveal subclinical problems of the horse that is otherwise clinically normal. However, as a low proportion of the samples in total and in horses with poor performance had elevated SAA, makes that the acute phase protein appear to not be useful for prediction of such underlying subclinical problems that could cause impaired athletic performance.

4.3 Subclinical infection of common respiratory viruses

In study II, presence of common respiratory viruses (EIV, EHV-1&4) was analyzed using a multiplex PCR assay, which also by design included ERBV and equine arteritis virus (EAV). None of the horses were identified with nucleic acid from EIV, EHV-1 or EAV in the NS, suggesting that those viruses did not commonly occur in our cohort.

Nucleic acid of ERBV and EHV-4 was identified in five respectively one NS, which were obtained from horses with no signs of clinical respiratory disease and therefore determined as detection of subclinical infection. However, only one of the ERBV positive samples was associated with poor athletic performance (according to the SFE) and the horse shedding EHV-4 had satisfactory performance based on SFE and the trainer's opinion. The acute phase protein SAA was not elevated in any of the six PCR positive samples, and thus of no predictive value in detection of these subclinical viral infections.

The CF test identified ten paired serum samples in which a fourfold rise in antibody titers to EHV-1/EHV-4 could be identified. Since eight of the pairs were associated with vaccination against alpha herpesviruses, this suggests that there were only two horses that underwent natural infection. Importantly, during the periods of seroconversion there were no detectable clinical signs of respiratory disease in either horse, which suggests subclinical infection. However, subtle and transient clinical signs could have gone undetected by the trainers and thereby not been registered since the horses were examined by veterinarian on a weekly basis. Nonetheless, there were no events of poor performance for these two horses throughout the periods of seroconversion to EHV-1/EHV-4.

The limited evidence of infection by the common respiratory viruses EIV and EHV-1&4 found, suggests a minor role of subclinical infections in our cohort of EIV vaccinated actively racing Standardbred trotters. This in turn provided an ideal study population to examine other less known viruses such as equine rhinitis viruses and equine gamma herpesviruses in relation to their role on the health and performance of the actively racing horse.

4.4 Persistence of neutralizing antibodies of ERAV and ERBV over a year

In the blood samples taken on monthly basis over a year for this cohort (584 samples) and evaluated for neutralizing antibodies to ERAV and ERBV, only 2% were completely seronegative to ERAV and only 0.2% were completely seronegative to ERBV. These findings are consistent with other studies where high seroprevalence of ERAV and ERBV have been reported worldwide and suggest the virus to be widespread in horse populations (Horsington *et al.*, 2013a; Black *et al.*, 2007; Kriegshauser *et al.*, 2005; Klaey *et al.*, 1998; de Boer *et al.*, 1979; Holmes *et al.*, 1978).

The stability of the levels of ERAV titers indicates that active infection was uncommon, and is in accordance with results from an earlier study, where high antibody titers to ERAV were maintained for years, (Burrows, 1979), suggesting that immunity following infection is long term.

Only three seroconversions to ERAV were detected during the study period, which all occurred at one occasion in one TY without accompanying clinical respiratory signs or poor performance from the horses. That these episodes of seroconversion to ERAV in the absence of clinical signs occurred in previously seropositive horses, supports earlier suggestion that persistent antibodies to ERAV are protective against clinical disease during re-infection (Diaz-Mendez *et al.*, 2014; Burrows, 1978). Of the eight seroconversions to ERBV (occurred in three TY), all animals were clinically healthy, which indicated subclinical infection, and were performing satisfactorily. As for ERAV, this also suggests that antibodies to ERBV might have a protective effect to prevent clinical respiratory disease at re-infection. Clearly, single high titers to ERBV or for that matter ERAV, as have been so commonly used in the field in Sweden (unpublished data) appear to be of little clinical value to predict recent or ongoing infection.

Figure 3 illustrates the monthly antibody titers to ERBV identified in sera from horses in one TY. The cut-off value (1:90, considered as high titer according to the SVA laboratory), is marked by a red line. Horse 10 differs from the others with its clearly higher antibody titers to ERBV without signs of

ongoing infection (fourfold increase in antibody titers or detected in NS with qPCR assays), implying that high titers can remain for at least a year without being stimulated by new infection. This horse had recently arrived at the stable and no information regarding previous respiratory disease or laboratory results was available. Since the other horses had consistently antibody titers at lower levels to ERBV with no seroconversions, active infection including viral shedding was not likely ongoing in Horse 10.

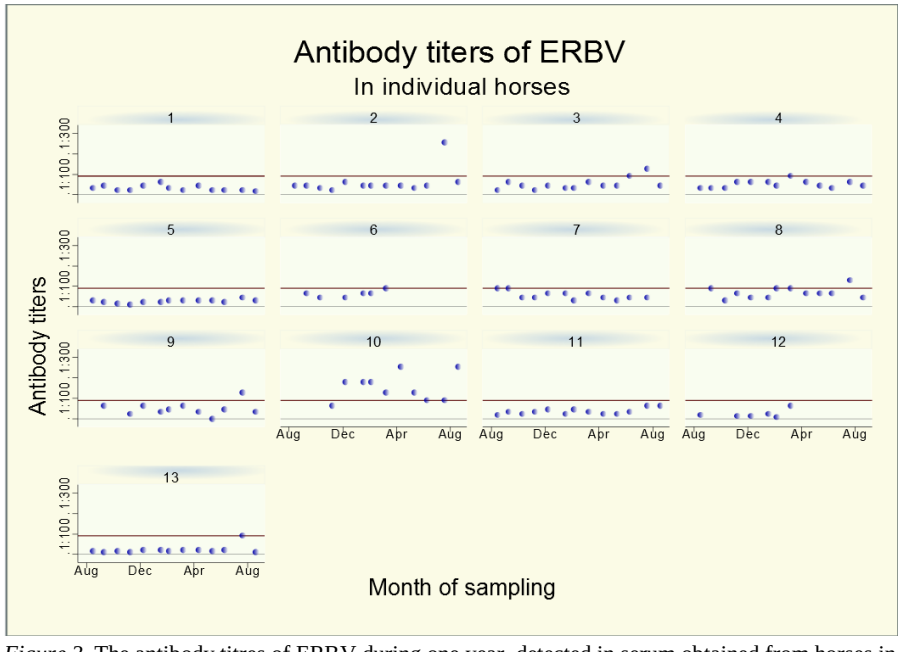


Figure 3. The antibody titres of ERBV during one year, detected in serum obtained from horses in one of the training yards described in study II. The red line indicate the threshold of the antibody titre 1:90 that is classified as "high level of antibodies" at the laboratory (SVA), where the analysis were performed.

4.5 Prevalence and viral load of EHV-2 and EHV-5 in relation to health and athletic performance

Of the monthly NS´s from the 63 racing Standardbred trotters followed over a year 74% (492/663) were positive to EHV-5 and 30% (196/663) were identified with EHV-2. Furthermore, as illustrated in table 1 of the samples contained both EHV-2 and EHV-5.

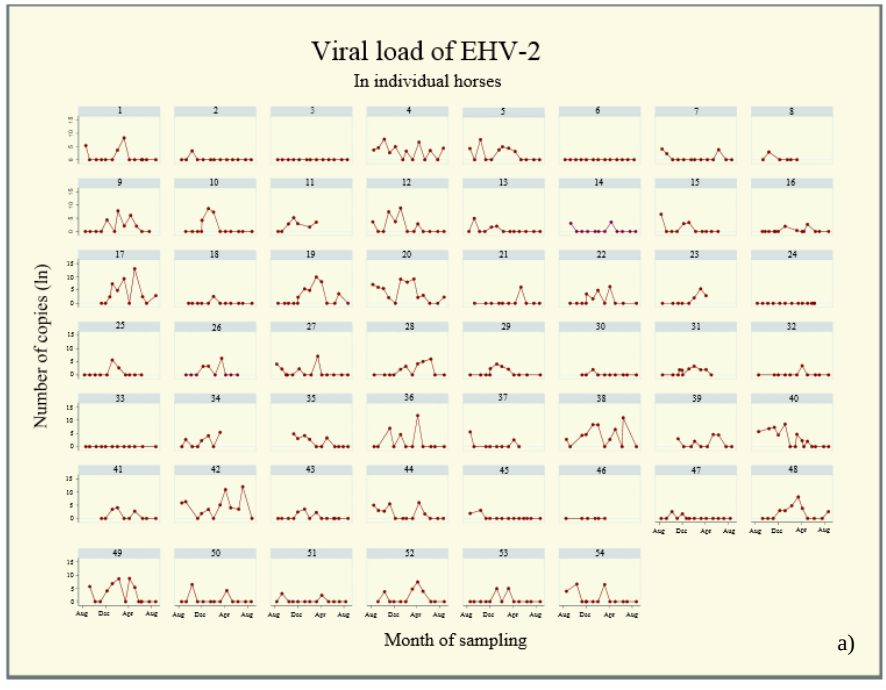
None of the horses were positive at all sampling occasions to EHV-2 whereas for EHV-5 none were completely negative. This high prevalence of both EHV-2 and EHV-5 (table 1), is in agreement with previous studies conducted elsewhere (Hue *et al.*, 2014; Wang *et al.*, 2007). Moreover the observation that EHV-5 could be detected in 100% of the horses at least once during the year illustrates the highly endemic situation of this virus in actively racing Standardbred trotters.

	Training yard 1 (N=348)	**Training yard 2 (N=124)**	**Training yard 3 (N=151)**	**Training yard 4 (N=40)**	**Total (N= 663)**
Neg samples EHV-2	238 (68%)	83 (67%)	110 (73%)	36 (90%)	467 (70%)
Pos samples EHV- 2	110 (32%)	41 (33%)	41 (27%)	4 (10%)	196 (30%)
Neg samples EHV-5	63 (18%)	49 (40%)	49 (32%)	10 (25%)	171 (26%)
Pos samples EHV-5	285 (82%)	75 (60%)	102 (68%)	30 (75%)	492 (74%)
Pos samples EHV-2 & 5 (co-detection)	100 (28%)	35 (28%)	37 (25%)	4 (10%)	176 (27%)

Table 1. The numbers and proportions of samples (N) collected on multiple occasions from 63 high performing trotters in Sweden that tested positive or negative for equine herpesvirus type 2 (EHV-2) and/or EHV-5 by the qPCR assays. Data were obtained from horses at four different training yards and from the entire study population.

The role of EHV-2 and EHV-5 in clinically detectable disease and the performance of the athletic horse is debated and information about the pattern of viral load regarding gamma herpesviruses in horse population is scanty. In this comprehensive work the presence of EHV-2 or EHV-5 could not be associated with either clinical respiratory disease or poor performance, which suggest that simply shedding of equine gamma herpesviruses has little, if any, relationship to impaired performance. Previously, based on detection of EHV-2 in association with BAL neutrophilia this gamma herpesvirus was suggested as a possible factor in poorly performing horses (Fortier *et al.*, 2013). However, that study lacked any corroborative information about the athletic performance of the horses in the study. The only other report associating EHV-2 with poor athletic performance, was a case report that lacked normal controls for comparison, describing isolations of EHV-2 from three race horses, presented with vague clinical signs and not performing on their normally high level (Studdert, 1974).

Given the above findings, the actual viral load of EHV-2 and EHV-5 in nasal secretions was analyzed individually for each sample to map viral load instead of simple presence, with the overall goal to assess whether increased viral shedding of either, or both, of these gamma herpesviruses could be related to alterations in health or performance. A wide range in the number of copies identified was observed, where between 0-54652 (mean 1528, standard deviation 19979) copies/µL in nasal filtrate were obtained for EHV-2 and for EHV-5 a range of 0-909293 (mean 14813, standard deviation 100431) copies/µL. As shown in figure 4a and b, considerable variation regarding the viral load of EHV-2 and EHV-5 was found both within and between individual horses, whereas no temporal association between peaks of viral load for EHV-2 and EHV-5 was observed. While some of the variation may have been related to the challenge to obtain representative numbers of viruses within each NS, the wide fluctuations are striking and individual immune response or stimulating factors controlling the re-excretion might be explanatory factors. Others have suggested the genetic make-up of individuals, especially the heterogeneity of the major histocompatibility complex loci, as an important factor for susceptibility to infectious diseases (Hedrick, 2002). This may be one key explanation for the high variation of viral load in horses closely housed at one TY, which were the same age and bred at the same stud farm (i.e. horses 33-38 of figure 4a and b).



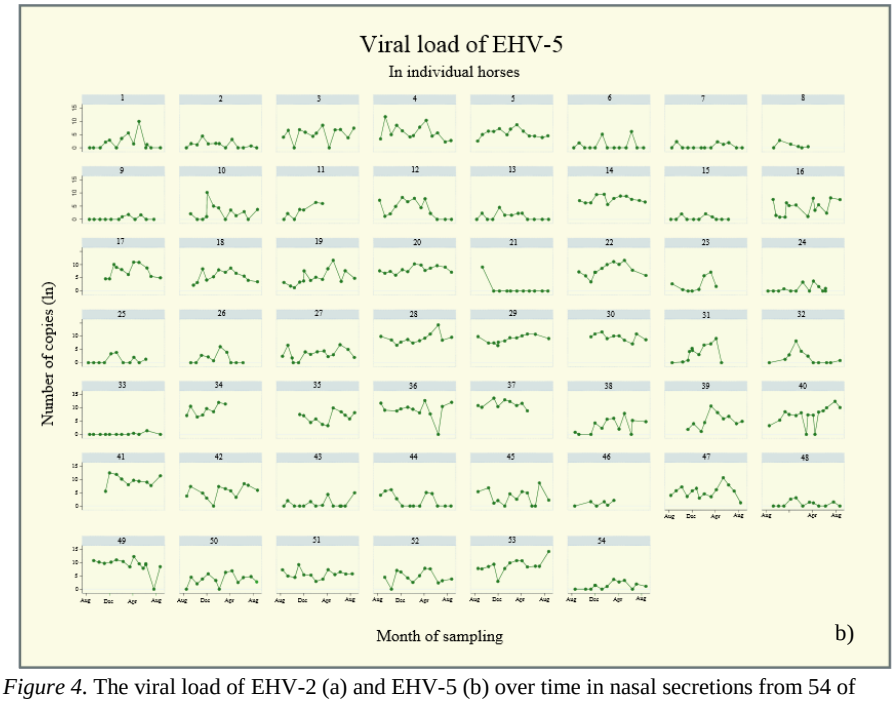


Figure 4. The viral load of EHV-2 (a) and EHV-5 (b) over time in nasal secretions from 54 of the Standardbred trotters included in study III. For descriptive purposes the copy numbers were transformed into natural logarithms to show viral load shed in nasal secretions by each individual horse over the calendar year.

Despite our finding of periods of very high and low viral shedding of EHV-2 and EHV-5 within individuals over time, there appeared to be no association of viral load of either virus alone or together, with clinical respiratory disease or episodes of poor athletic performance.

A seasonal variation in detection of the two viruses was observed. EHV-2 was most commonly identified during the winter where 44% of the samples were positive compared to only 17% of the collected samples during summer. EHV-5 on the other hand had the highest proportion of positive samples during spring (80%) and the lowest number of EHV-5 positive samples was also identified in the summer (67%).

Seasonal variation was also found quantitatively, where the mean of the shed numbers of copies were highest in spring for EHV-2 (mean 4385 copies/µL) whereas EHV-5 had its highest (24396 copies/µL) average shedding during summer. The lowest mean numbers of copies of EHV-2 was shed in summer (122 copies/µL) and in the autumn for EHV-5 (10230 copies/µL). The observed seasonal variation in viral load between EHV-2 and EHV-5 suggests that these two closely related viruses have different infection patterns, but this appeared to have no relationship to occurrence of clinical abnormalities. Previously, EHV-2 and EHV-5 have been identified at different periods of time in foals, suggesting epidemiological differences (Bell *et al.*, 2006), which is in line with our study indicating that EHV-2 and EHV-5 have different patterns of infection. These differences suggest that the two viruses interact with their host in separate ways and separate latency sites might occur.

A subgroup of the horses underwent endoscopic examination, by which a high percentage were classified with pharyngeal inflammation scores of ≥ 2 (64%). This amount of visible pharyngeal inflammation was not surprising, since 22 of the 28 horses were four years or younger. However, presence or viral load of EHV-2 or EHV-5 had no correlation to pharyngeal inflammation score or mucus accumulation. The grade of pharyngeal inflammation was actually significantly lower in horses with qPCR positive result of EHV-2 ($P=0.03$) compared to the horses that tested negative for EHV-2. Despite earlier reports that found EHV-2 to be the most prevalent herpesvirus in tracheal wash from horses with clinical respiratory disease (Fortier *et al.*, 2009), we found no association between EHV-2/-5 in nasal secretion and pharyngitis or presence of mucus, which suggests that subclinical infection of equine gamma herpesviruses is a minor risk factor for clinically unnoted pharyngitis diagnosed by endoscopy.

Due to their ubiquity in horses, presence of equine gamma herpesviruses is not sufficient to cause disease or affect performance. No evidence of association could be found between the viral load of EHV-2 and/or EHV-5 in

663 samples, and clinical disease or poor athletic performance. Therefore, additional focus on possible strain variations and genotypes of equine gamma herpesviruses is needed to further understand the pathogenicity and to possibly explain the conflicting observations of the viruses and their impact on equine health.

4.6 Genetic variation of EHV-5

Recent case reports have suggested an association between EHV-5 and EMPF. The pathogenesis of EMPF is however not fully understand and since a high prevalence of EHV-5 also has been described in healthy horses, genetic analysis could provide further understanding of the virus and its interaction with the host. EHV-5 has a large genome which over the years has been little investigated and it was only recently that the whole genome has been sequenced (Wilkie *et al.*, 2015). Against this background, NGS was used in our work to further analyse a segment of the EHV-5 gB gene over time in nine individual horses (eight healthy, one with EMPF) to explore any genetic differences in the EHV-5 detected in horses included in our studies.

In this work, 27 different nucleotide sequences (i.e. strains), representing 11 unique sequence types of the partial gB gene of EHV-5 were identified. Some individual horses were infected by up to three different strains at the same time. Phylogenetic analysis of the strains resulted in detection of four separate clusters which are here suggested as genotypes (I-IV) of the EHV-5 gB gene (figure 5). Two of the identified genotypes (I and II) did not match with any previously described strains. The diversity between strains in the phylogenetic tree within the four divergent genotypes was less than 2% (0 nucleotides (I), 2 nucleotides (II), 0-3 nucleotides (III), 0-8 nucleotides (IV)). Between the genotypes, apart from genotype I and II that diverged by only 9 nucleotides, the divergence ranged from 19-81 nucleotides, where genotype IV diverged most (by 81 nucleotides between genotype III and IV). This results revealed a homology of 83.8-95.6% between genotypes of the EHV-5 gB gene, which is highly similar to what have been described both within and between the four detected genotypes (based on the gB gene) of the beta herpesvirus, human cytomegalovirus (CMV) (Chantaraarphonkun & Bhattarakosol, 2007; Chou & Dennison, 1991).

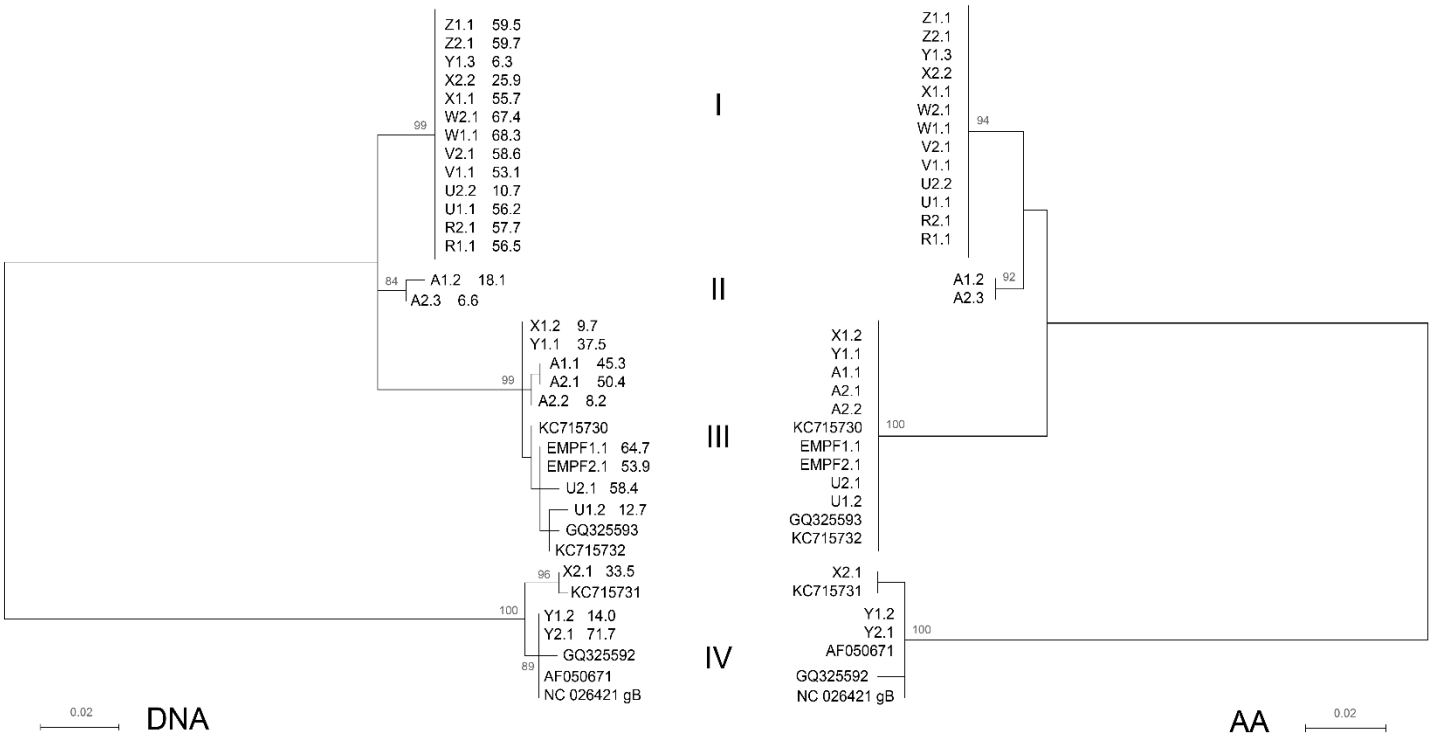


Figure 5. Phylogenetic trees based on DNA sequences (left) and translated amino acid (aa) sequences (right) showing four suggested genotypes (I-IV) of EHV-5 gB gene segment identified in nine Swedish horses. The individual strains and sequences are shown within genotypes. Only strains that included >5% of the reads in each clinical specimen were incorporated in the tree. The different clinical specimens are coded with a unique letter for each horse followed by number "1" (first sampling occasion) or "2" (second sampling occasion) and the last number (1-3), illustrate the most (1) and least (3) common strain within each of the clinical specimens. On the left side proportions of the trimmed and paired reads for the genotypes are specified in percentage. 1000 bootstrap replicates were performed for each tree and obtained values are shown where to bootstrap support is >80%. Scale bar corresponds to 2% of the total variation.

A conserved cleavage site for all strains at aa position 111-114 in our gB segment of EHV-5 was detected, which is in agreement with another study where the cleavage site of gB gene was shown to be essential in EBV for the cell-cell fusion of the virus (Sorem & Longnecker, 2009). Moreover, we observed a higher nucleotide variability closer to the cleavage site, which is supported by a study of the gB gene of EHV-5 and seven other herpesviruses (among them EHV-2), where the same cleavage site was identified and insertions and deletions mainly occurred close to the cleavage site (Holloway *et al.*, 1999).

The four genotypes were distinctly separated on nucleotide level, as well as on amino acid (aa) level (figure 5). In total five different aa sequences were identified, where genotype I-III resulted in three different aa sequences for each genotype, and the strains from genotype IV resulted in two unique aa sequences. The results shows that aa sequences within each genotype seem to be strongly conserved, but between genotypes they differ considerably. This might affect the structural or conformational constraints on this gB segment, possibly causing genotypes to interact differently with the host. Strains of gB genotypes (I and II) has not been described before and might be specifically circulating in Sweden, since differences in the geographical distribution of different genotypes has been described for EBV (Abdelhamid *et al.*, 1992). It might however also reflect the limited genetic information of EHV-5. The selected reference nucleotide sequences of the gB gene (figure 5) were strains originating from three different continents (North America, Australia and Europe). The two European isolates (genotype III and IV) originate from the horse population in Iceland, which has been isolated from direct contact with other horse populations for more than 1000 years (Torfason *et al.*, 2008). Icelandic horses are however frequently exported from Iceland to the Scandinavian countries, but are rarely in direct contact with professionally trained Standardbreds.

Dynamic of EHV-5 gB gene over time

Three different patterns of EHV-5 infection (based on the partial gB gene) over time were detected in this study. The strains referred to in this section are illustrated in figure 5. Infection of a single identical strain (genotype I) on both sampling occasions occurred in four horses (R, V, W and Z), which illustrates a high stability of the viral strains over time. Similar findings has also been described for the human herpesvirus type 6 (HHV-6, a beta herpesvirus), where the identical strain from one genotype (based on the gB gene) was detected in the same patient at two different time points (Achour *et al.*, 2008).

The second pattern was infection of two strains from different genotypes with varying proportions, which was illustrated in two horses (A and U). Horse U shed two strains, one from genotype I and the other from genotype III at both sampling occasions, but the proportions shifted over time. In horse A, also the proportions of the strains (genotype II and III) varied over time as illustrated in table 2, but not as striking as for horse U. This highlights the influence of the host-virus-environment interaction and may also be explained by the viability of different strains.

In the third pattern appearance and loss of different strains over time was observed in two of the horses (X and Y). Horse X was first identified with strains of genotype I and III, one year later an identical sequence from genotype I was detected together with a new strain classified as genotype IV, and the strain of genotype III was absent. Horse Y first harbored three strains, which were classified in three different genotypes (I, III and IV). One year later genotype IV could be detected. This third pattern feature that the number of strains and genotypes that infect adult animals can be variable, but also the high stability of strains over time. Moreover, the described changes in horse X suggests that infection with a new strain of EHV-5 occurs. Further studies are needed to investigate whether this stability is mainly related to host, virus and/or environmental factors.

The observations in our work shows a range of interactions between EHV-5 and the host over time, where the viruses persist in some horses and others have a more dynamic infection pattern including strains from different genotypes. The eight healthy horses were at the same TY and presumably under the very similar environmental infection pressure. Therefore, the different patterns of viral interaction with the host suggests that individual characteristics such as strain and the genetic background or the immune responses to EHV-5 in the host, may play important roles in the viral dynamic over time.

4.7 EMPF, associated with gamma herpesviruses?

The uncommon but severe chronic lung disease equine multinodular pulmonary fibrosis (EMPF) was described for the first time to be associated with EHV-5 in 2007 (Williams *et al.*, 2007). In study I in this thesis, the two gamma herpesviruses EHV-5 and AHV-5 were detected in lung biopsy, TW samples and in the lung lobes obtained from necropsy, from a horse where both the clinical signs and the histological changes within the lung corresponded to EMPF. At necropsy multifocal, firm and well-demarcated nodules were found to affect all lung lobes (figure 6).

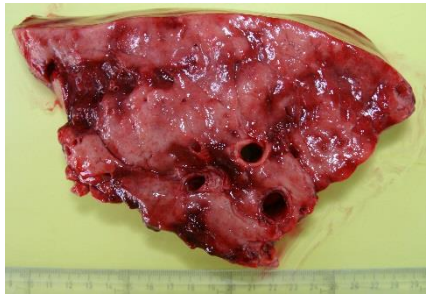


Figure 6. A section of the lung from the horse diagnosed with EMPF. The multifocal fibrotic nodules are large and distinct and only limited areas of normal parenchyma is detectable. (Photo: Rodrigo Grandón).

This horse had no history of contact with donkeys. The knowledge of AHV-5 in horses is scanty, even if the virus has been identified in healthy horses (Rushton *et al.*, 2014). The co-detection of EHV-5 and AHV-5 in an EMPF case suggests that AHV-5 may play a role as a co-pathogen, and that it deserves further attention in the attempt to understand the etiology of EMPF. On the other hand, genetic diversity of EHV-5 must also be included as a factor to consider regarding why only some horses infected with EHV-5 and/or AHV-5 develop EMPF.

As illustrated in figure 5 the sample from the EMPF horse was classified as genotype III, and that it was highly similar (one nucleotide divergence) to the nucleotide sequence (KC715730) obtained from another EMPF case diagnosed in USA (Williams *et al.*, 2013). However, other healthy horses in our study IV were also infected with strains of the same genotype. Furthermore, the EMPF case was infected with only one strain of genotype III, whereas healthy horses in which genotype III was detected were co-infected with multiple strains simultaneously, and it cannot, be excluded that genotypes or combinations of strains might be of clinical importance. In comparison, human infection by multiple strains of CMV has been suggested to cause more severe clinical manifestations in immunocompromised individuals, than infections with a

single variant (Coaquette *et al.*, 2004). Even though EBV has been associated with idiopathic pulmonary fibrosis (IPF) (Tang *et al.*, 2003) and with several types of cancer (Parkin, 2006), the pathogenesis is still unknown. To date genomic diversity of EBV has been identified, but if the pathogenicity is related to strain variability remains to be clarified (Palser *et al.*, 2015; Kwok *et al.*, 2014).

4.8 The choice of study design and its impact on the results

The study design has an impact on how reliable and robust the results are, and also influence what conclusions that can be made. Common epidemiological studies used in veterinary medicine are: Descriptive studies (case-reports and case series), experimental studies and observational studies (longitudinal, case-control and cross-sectional).

Case-report (study I) is a suitable study design to describe a clinical observation in particular if it is a new disease or a new manifestation of a previously described disease. It does not tell anything about the prevalence and does not involve any comparisons with other healthy or diseased animals. The detection of both EHV-5 and AHV-5 in a horse with EMPF, was not previously described and therefore of interest to report. However, the evidence of findings in case reports is rather low but may be case-unique.

Study II and III were based on a longitudinal study, which was characterized by a cohort (a selected number of animals) that was followed over time with health status registered during the study period. The cohort study design has the highest ability to identify an association between exposure and outcome given that the individuals are healthy at the start and that the exposure can be adequately estimated. However, if the number of cases in a certain time frame is low this study design is less useful. Instead, it is more feasible to use the case-control design where the exposure level can be compared among cases and controls to evaluate whether the exposure is related to the outcome. Another commonly used observational study is the cross-sectional study design that is characterized by a selected group of animals, where the healthy and diseased animals are studied at one single time point. In the hierarchy of cause-effect relationship the longitudinal study is known to be the most informative among the observational study types (Pfeiffer, 2010). By using the longitudinal study design instead of a cross-sectional study in paper II and III it was possible to observe how the antibody titres of ERAV and ERBV remained stable at high levels over a year. It was also possible to demonstrate that individual horses can shed EHV-2 and EHV-5 for several months, and to follow fluctuations of viral load of EHV-2 and EHV-5 over

time. It can be hypothesized that association of poor performance or clinical disease with presence of any the viruses, could have been found if a case-report or cross-sectional study design would have been used. A case report could for example have suggested association with poor performance and a high titre of ERAV/ERBV. In that case, we would not know that the titres can remain on high levels for months, and it would therefore have been easy to draw the conclusion about a strong association that does not exist. The same could happen in a cross-sectional study even if more samples are obtained. Therefore, longitudinal studies are important to verify causality between disease and a possible causative agent, given that the disease is not too rare in the population.

5 Conclusions

To investigate the role and the prevalence of subclinical viral infections and their possible association with poor athletic performance, 63 elite Standardbred trotters were followed by monthly sampling in a prospective longitudinal study over a year.

- SAA detected in serum could not be used to detect subclinical viral infection as elevated levels were very rarely found in these samples.

- The incidence of subclinical infection of common equine respiratory viruses (EIV, EHV-1 and EHV-4) in this cohort of horses was low.

- Antibody titres to ERAV and ERBV can remain on high levels in serum for at least a year without being associated with disease, poor performance or viral activity.

- A high prevalence of EHV-2 (30%) and EHV-5 (74%) was identified in nasal secretions from the horses during the study period.

- Neither the presence of EHV-2 and/or EHV-5 nor their viral load could be associated with clinical respiratory disease or poor athletic performance.

- The viral load of EHV-2 and EHV-5 were identified with divergent patterns of infection, which suggest different biological activity.

- Genetic analysis of the EHV-5 gB gene allowed classification into four genotypes (I-IV).

- Individual horses were co-infected with up to three divergent strains of different genotypes of EHV-5 gB gene, and different patterns of infection were identified. The strains showed high genetic stability over time, and some of the horses were infected with identical strains one year apart. In other horses the strains were genetically stable but the proportion or each strain shifted. Changes in strains and genotypes were also shown to occur over time.

- In a case report of a four-year-old horse EMPF was suggested to be associated with infection of both AHV-5 and EHV-5.

- The EMPF case was infected with only one strain of genotype III of EHV-5. Clinical healthy horses were also found to be infected with this strain although they were carrying other strains simultaneously.

6 Future perspectives

The field of poor performance is a large and complex topic, mainly since multiple causes are often involved and "gold standard" is lacking in determine the athletic performance. The work in this thesis have added information about low virulent infections of both well-known and less investigated equine respiratory viruses and identified them to affect the athletic performance little as long as no clinical signs occurred. However, further work in standardize the determination of athletic performance would highly benefit future research in the field of elite horses and their performance. For example standardized protocols including HR, speed and distance for each discipline, could facilitate comparison between studies.

Since horses seropositive to equine rhinitis viruses in study II developed no signs of respiratory disease when viral activity occurred, the antibodies induced by natural infection seem to be protective against clinical respiratory signs. The majority of the horses included in this work had seroconverted before the age of 2 years old. The need of a vaccine against ERAV can therefore be argued, since problems associated with both rhinitis viruses were limited in this cohort of seropositive racing Standardbred trotters. The horse population that might benefit from a vaccine is young horses seronegative to ERAV, which thereby could be protected from possible clinical disease, caused by infection of ERAV in association with entering TY.

To further understand the role of EHV-5 in the equine population, the described amplicon PCR followed by NGS (study IV), could be performed in a larger number of horses from different geographical areas. That would provide information of the geographical distribution of different EHV-5 gB genotypes and their possible clinical importance. Further genetic work is needed to clarify the interaction between EHV-5 strains in individual horses, and thereby

understand the clinical consequences of multiple or single infection. Of interest would also be to focus on the interaction between strains over time, if new infection occurs frequently and if selective latency or clearance appear as a common state for the gamma herpesviruses.

A dynamic of EHV-5 gB genotypes was in this thesis work identified in individual horses over time. To further study this dynamic, the analysis used in study IV would be of interest to perform on samples obtained from horses both more frequently (e.g. monthly samples over a year), and over a longer study period (at least two years). That kind of studies could provide further information of the interaction between the virus and its host and how the pattern of the EHV-5 strains varies over time.

The genetic analysis performed in study IV, identified only one EHV-5 gB strain classified as genotype III in the EMPF case from study I. Although conclusions based on a single case must be drawn with great caution, it would however be of interest to analyse samples from several horses diagnosed with EMPF (together with healthy control animals), by using the sequencing method described in study IV. That might reveal if single infection by strain of EHV-5 gB genotype III is associated with the severe lung disease. Moreover, the nucleotide and aa sequences of different EMPF cases could be compared and maybe similarities or characteristics could be found. That information could be one step closer to a clarification to the role of EHV-5 in the pathogenesis of EMPF.

The role of EHV-2 and ocular disease is another interesting question of the gamma herpesviruses, that is not fully understood and where genetic analysis might clarify the role of this complex virus. Eye swabs identified with EHV-2 from horses with ocular disease (and healthy controls), could be compared by using amplicon PCR assay designed for a suitable gene segment of EHV-2 followed by NGS as described for EHV-5 in study IV. This might give valuable information about possible genotypes and strains, which could be one way to support or reject causality of ocular disease and EHV-2.

7 Populärvetenskaplig sammanfattning

7.1 Bakgrund

Luftvägsvirus är allmänt kända för att orsaka kliniska symptom såsom feber, hosta, nedsatt allmäntillstånd och näsflöde hos hästar över hela världen. Luftvägsproblem anses dessutom tillsammans med hälta vara de främsta medicinska orsakerna till att tävlingshästar underpresterar, missar träningsdagar eller inte kommer till start. När ett virus orsakar tydliga sjukdomssymptom hindrar de en häst från att träna eller starta på tävling, men man vet också att virus kan infektera en häst utan att ge tydliga symptom (s.k. subklinisk infektion). Det är oklart huruvida sådana subkliniska virusinfektioner påverkar hästarnas prestation och hälsa.

Det virus som över hela världen orsakar flest utbrott med luftvägssymtom är hästinfluensa (EIV), som spelar stor roll dels för hästarnas hälsa och dels är av ekonomisk betydelse för hela hästnäringen eftersom tävlingsbanor kan tvingas stänga och många träningsdagar kan gå förlorade. Andra vanliga luftvägsvirus är herpesvirus (EHV) typ 1 och typ 4 som är kopplade till både tydliga sjukdomssymptom med också till subklinisk infektion. Det är oklart hur vanligt förekommande subklinisk infektion av dessa virus är hos tävlingshästar och om de i sådant fall har samband med nedsatt prestation. Subklinisk infektion av rhinitvirus typ A och B (ERAV och ERBV) har i Sverige däremot ofta misstänkts orsaka nedsatt prestation hos framförallt travhästar, men påståendet saknar vetenskapligt stöd.

Herpesvirus är kända för att efter infektion stanna kvar på olika platser i kroppen hos sin värd och vara vilande (s.k. latent infektion), för att sedan aktiveras vid olika typer av yttre stimuli som stress eller annan sjukdom. EHV typ 2 och 5 är två herpesvirus som har hittats hos friska individer men också sjuka hästar, t.ex. har EHV-5 associerats med fall av den allvarliga och kroniska lungsjukdomen equine multinodular pulmonary fibrosis (EMPF). Det

saknas studier om dessa herpesvirus kan försämra prestationen vid subklinisk infektion och få diagnostiska metoder finns tillgängliga för att påvisa dem.

Det övergripande syftet i den här doktorsavhandlingen var att med hjälp av olika diagnostiska metoder undersöka förekomst av dessa virus hos högpresterande travhästar på elitnivå, för att se om de har ett samband med nedsatt prestation eller ej. Vidare ville vi undersöka om den utsöndrade virusmängden av EHV-2 och EHV-5 kan relateras till sjukdom och om olika genetiska varianter av EHV-5 förekommer.

7.2 Sammanfattning av studier och resultat

Under ett års tid följde vi 63 travhästar hos fyra olika svenska proffstränare. Hästarna provtogs varje månad med både blodprov och nässvabb, samtidigt som deras prestation utvärderades med hjälp av arbetsprov och tränarnas bedömning. Hästarnas hälsostatus övervakades kontinuerligt under studietiden av veterinär. Totalt togs 663 prover under studietiden.

Majoriteten av hästarna hade förhållandevis höga antikroppsnivåer mot ERAV och ERBV i sina blodprov och många hästar hade stabilt höga nivåer under hela studien. Endast i fåtalet fall kunde en fyrfaldig stegring (tecken på akut infektion) av nivåerna ses. Detta tyder på att både ERAV och ERBV är vanligt förekommande hos tävlande travhästar och att endast ett blodprov med en hög antikroppsnivå inte betyder att hästen nyligen varit infekterad av viruset. Vidare stödjer våra resultat andra studier där hästar med höga antikroppsnivåer mot ERAV och ERBV sällan verkar uppvisa kliniska symptom. I den här studien kunde heller inget samband ses mellan subklinisk infektion av ERAV eller ERBV och nedsatt prestation hos travhästarna.

Inga fall av subklinisk infektion av EIV och EHV-1 kunde konstateras. Vidare kunde de få detekterade fallen av subklinisk infektion med EHV-4 inte korreleras till nedsatt prestation.

För att undersöka förekomsten av EHV-2 och EHV-5 hos hästarna togs en PCR analys fram som detekterar nukleinsyra för de båda virusen i nässvabbprov. Närmare tre fjärdedelar och omkring en tredjedel av alla proven visade sig innehålla EHV-5 respektive EHV-2. Mängden virus som utsöndrades vid de olika provtagningstillfällena varierade kraftigt, men varken mängden eller påvisande av de två virusen kunde relateras till nedsatt prestation eller kliniska sjukdomssymptom hos någon av hästarna.

Avhandlingen innefattar också en fallrapport från en travhäst (ej relaterad till någon av proffstränarna), som drabbats av lungsjukdomen EMPF. Hos den hästen kunde både EHV-5 och åsneherpesvirus typ 5 (AHV-5) påvisas i lungsköljprov, lungbiopsi och i lungvävnad vid obduktion.

I den fjärde studien inkluderades prover (nässvabb) tagna med ett års mellanrum från åtta travhästar hos en av proffstränarna samt lungprov från hästen diagnosticerad med EMP. En del av genen som kodar för ytproteinet glykoprotein B (gB) hos EHV-5 undersöktes med hjälp av nyutvecklade sekvenseringsmetoder. Den undersökta gB genen kodar för ett protein som är essentiellt för att viruset ska kunna ta sig in värddjurets cell. Fylogenetiska analyser visade att hästarna vid samma tillfälle kunde vara infekterade med upp till tre olika stammar. Vidare kunde de olika stammarna delas in i fyra tydligt separerade genotyper (I-IV), varav två innehöll gensekvenser som inte beskrivits tidigare. De olika stammarna visade sig vara mycket stabila över tid med få mutationer. Några av hästarna hade identiska infektioner av EHV-5 över tid medan det hos andra skedde förändringar över året där både ny infektion och reduktion av stammar och genotyper kunde ses.

7.3 Slutsatser

Endast ett blodprov där en hög antikroppsnivå mot ERAV eller ERBV har påvisats betyder inte att hästen nyligen har infekterats med viruset eller att de orsakat något problem hos hästen. Parprov med två till tre veckors mellanrum med minst en fyrfaldig stegring av antikroppstiter krävs för att konstatera aktiv/nyligen genomgången infektion av ERAV/ERBV.

Både EHV-2 och EHV-5 är vanligt förekommande hos svenska travhästar, men enbart detektion från nässvabb eller hög utsöndring av dessa virus kan dock inte kopplas till sjukdomstillstånd eller nedsatt prestation.

Både EHV-5 och AHV-5 kunde påvisas i lungan hos en häst som diagnosticerats med EMPF. Genetiska analyser kunde klassificera EHV-5 från den drabbade hästen till genotyp III. Även några av de friska hästarna bar också stammar av EHV-5 från samma genotyp

De genetiska analyserna för delar av gB genen hos EHV-5 gav nya kunskaper i hur viruset agerar med sitt värddjur. Våra studier tyder på att det finns minst fyra olika genotyper av EHV-5 som alla kodar för olika aminosyra sekvenser. Detta gör att de har förutsättningar för att interagera på olika sätt med sitt värddjur. Ytterligare studier krävs för att förstå hur förekomst och spridning av de olika genotyperna kan förklara olika sjukdomstillstånd som setts i samband med dessa infektioner. Vidare är den beskrivna metoden för sekvensering av delar av gB genen hos EHV-5 användbar vid uppföljande genetiska studier av viruset.

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